PHYTONEMATODE PATHOLOGY: ULTRASTRUCTURAL STUDIES. II. PARASITISM OF *MELOIDOGYNE ARENARIA* EGGS AND LARVAE BY *PAECILOMYCES LILACINUS*[1]

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ABSTRACT

Morgan-Jones, G., J.F. White, and R. Rodriguez-Kabana. 1983. Phytonematode pathology: ultrastructural studies. II. Parasitism of *Meloidogyne arenaria* eggs and larvae by *Paecilomyces lilacinus*. Nematropica 14:57-71.

An isolate of *Paecilomyces lilacinus* (Thom) Samson, from mature cysts of *Heterodera glycines* Ichinohe, was evaluated *in vitro* for its ability to parasitize eggs of *Meloidogyne arenaria* (Neal) Chitwood. Hyphae of the fungus readily penetrated the egg shell through small pores dissolved in the vitelline layer. Invaded eggs became swollen as a result of a change in shell permeability. Once inside, a penetrating hypha enlarges, crushes the chitin and lipid shell layers in its immediate proximity, and permeates the egg content, including developing larvae whose cuticles are disrupted. Endogenous hyphae reemerge by tearing the egg shell and produce conidiophores bearing chains of conidia on the shell surface. Shells of eggs containing fungal hyphae undergo a number of ultrastructural changes. The vitelline layer splits into three discrete membranes which appear unevenly thickened, the chitin layer becomes vacuolated, and the lipid layer largely disappears. Similar disorganization of the larval cuticle occurs and larvae become necrotic.

Additional key words: soil mycoflora, Hyphomycetes, root-knot nematodes, biological control potential, population dynamics.

RESUMEN

Morgan-Jones, G., J.F. White, y Rodríguez-Kábana. 1984. Patologia de los fitonematodos: estudios sobre ultraestructura. II. Parasitismo de huevos de *Meloidogyne arenaria* por *Paecilomyces lilacinus*. Nematropica 14:57-71.

Se evaluó *in vitro* la capacidad de parasitismo con huevos de *Meloidogyne arenaria* (Neal) Chitwood de una cepa de *Paecilomyces lilacinus* (Thom) Samson obtenida de quistes maduros de *Heterodera glycines* Ichinohe. Las hifas del hongo penetraron las cascarillas de los huevos del nematodo directamente a través de poros minúsculos formados por disolución de la capa vitelina. Los huevos invadidos se hincharon como resultado de cambios en la permeabilidad de sus cascarillas. Una vez dentro del huevo, las hifas penetrantes se dilataron comprimiendo en su alrededor las capas de quitina y de lípidos de la cascarilla, extendiendose por todo el contenido del huevo inclusive de las larvas en desarrollo cuyas cutículas disolvieron. Las hifas endógenas en los huevos

reemergieron pasando por las cascarillas de los mismos en cuyas superficies produjeron conidioforos con conidios encadenados. Las cascarillas de huevos con hifas experimentaron una serie de cambios ultraestructurales. La capa vitelina se separó en tres membranas individuales cada una de grosor disparejo; también, se observó vacuolación en la capa de quitina y la de lípidos desapareció en casi su totalidad. Se observaron también cambios análogos en la cutícula de las larvas las cuales devinieron necróticas.

Palabras claves adicionales: hifomicetos del suelo, nematodos noduladores, combate biológico, dinámica poblacional.

INTRODUCTION

Among opportunistic soil Hyphomycetes consistently associated with the pathology of cyst and root-knot nematodes in some parts of the world and known to be an effective egg parasite is *Paecilomyces lilacinus* (Thom) Samson. Although cosmopolitan in its distribution, this species occurs with greatest frequency in areas with a warm climate, particularly the tropics and subtropics. Typically a soil fungus, *P. lilacinus* is also known to occur on insects. It produces a thin mycelial felt from which conidiophores arise (28). Originally classified in the genus *Penicillium* Link, as *P. lilacinum* Thom, the species was reclassified in *Paecilomyces* Bainier by Samson (28) on the basis of conidiophore morphology, whilst recognizing its similarities to the *Penicillium janthinellum* series. The fungus has also been given several other names, now considered nomenclatural synomyns, in part because of its diverse habitat range and attendant variable morphological expression. When occurring on insects, conspicuous conidiophore synnemata are formed and the names *Spicaria violacea* Petch and *S. rubidopurpurea* Akoi were established for it as a result, the latter based on a collection on silkworm in Japan, where it occurs commonly. In all circumstances the fungus produces conidial heads of a distinct, vinaceous-lilac coloration.

Jatala et al. (21) encountered *P. lilacinus* as a parasite of eggs of *Meloidogyne incognita* (Kofoid and White) Chitwood var. *acrita* Chitwood on potato roots in Peru. The eggs were heavily infected by the fungus. In inoculation experiments, *P. lilacinus* was demonstrated to have ability to invade females and eggs of *Meloidogyne* and cysts of *Globodera pallida* (Stone) Behrens. In subsequent field experiments the potential of *P. lilacinus* for controlling *M. incognita* on potatoes was assessed (22,23). Potato plants grown in plots inoculated with the fungus had a significantly lower root galling index than those grown in plots to which organic matter and nematicides had been applied. Sixty-eight percent of the egg masses from plants grown in fungus-treated soil were infected with *P. lilacinus* and over half the eggs were destroyed (22). An investigation of

the effect of multiple applications of the fungus revealed that a one-time introduction was sufficient to establish it and bring about substantial nematode population control (23). Franco et al. (14) further determined, under laboratory conditions, the efficiency of *P. lilacinus* as a biocontrol agent of *G. pallida*. The fungus was again found to readily parasitize eggs. Reduction in egg hatching, stimulated by exposure to potato root exudates, was shown to be correlated with increased infection by *P. lilacinus*.

Dunn et al. (13) demonstrated the ability of three strains of *P. lilacinus* to colonize eggs of *M. incognita in vitro*. A fourth strain, isolated from sclerotia of *Sclerotinia minor* Jagger buried in agricultural soil in Mexico, proved ineffective in this regard. Isolates were shown to enter eggs by hyphal penetration of their encasing shells, referred to as a cuticle by the authors. When a hyphal tip came into contact with an egg surface, some swelling was apparent distally. Such swelling was referred to as an appressorium. The hyphae were said to proliferate within eggs and to eventually reemerge, although no details of the process could be given in the absence of infected egg sectioning. A fungus, morphologically closely similar to *P. lilacinus*, recently described as a new species, *P. nostocioides* Dunn, has been shown capable of colonizing eggs of *Heterodera zeae* Koshy, Swarup, and Sethi *in vitro* in the same manner (12). This species, isolated from cysts of *H. zeae* in Maryland, U.S.A., was considered by Godoy et al. (17) to be a mutated form of *P. lilacinus* since it is identical to it except for the presence of occasional, swollen, aberrant conidia in otherwise normal chains.

Paecilomyces lilacinus has been frequently isolated in our laboratory from eggs of *Meloidogyne arenaria* from peanuts (17) and from eggs of *M. incognita* and cysts of *Heterodera glycines* from soybeans (15, 26), all in Alabama. We have also encountered it in cysts of *H. glycines* collected on soybeans in the vicinity of Palmira, Colombia (Morgan-Jones and Rodriguez-Kabana, unpublished data), an area where this nematode was only recently introduced (29). Preliminary greenhouse studies indicated that *P. lilacinus* is effective in reducing *M. arenaria* infestations (17). A reduction of 54% in the number of galls per gm of fresh root was recorded in soils to which *P. lilacinus* had been added, as opposed to unamended control soil. The fungus was recovered at the end of the experiments with high frequency from soil to which it had been added, indicating good competitive and colonizing ability.

Recent experiments conducted in infested fields in Malaysia, Panama, Peru, the Philippines, Puerto Rico, and the U.S.A. have indicated that *P. lilacinus* effectively controls *M. incognita* and does so to a greater extent than a number of commonly used nematicides (9,20). Encouraging

field results for biological control of *Tylenchus semipenetrans* Cobb on oranges in Peru by *P. lilacinus* have also been reported (20). The fungus reduced *T. semipenetrans* populations to lower levels than any of the three nematicides tested. Root development, plant growth, and fruit diameter were significantly increased following introduction of *P. lilacinus*.

Although the ability of *P. lilacinus* to enter eggs is known, the precise details of the process have not been investigated as was the case with *Verticillium chlamydosporium* parasitism, which formed the subject of the first paper in this series (25). Neither has the total mode of action involved in parasitism been investigated. We have previously suggested (25) that two types of activity could take place. First, enzymatic alternation of shell permeability could render eggs vulnerable to physiological disordering even before actual hyphal penetration. Second, diffusible fungal toxins might be metabolized which could deleteriously affect eggs and ultimately lead to larval demise. The latter activity could involve hyphae exogenous to the egg as well as those proliferating endogenously following colonization.

The study reported herein was undertaken to determine how a hypha enters an egg and what ultrastructural changes are brought about as a result of its occupancy. In addition, several factors relating to the pathology of eggs and hatched larvae have been investigated. Whether a fungal hypha can penetrate irrespective of the stage of egg embryonic development has been determined, as well as the effect of fungal presence on hatching and juvenile survival.

MATERIALS AND METHODS

An isolate of *P. lilacinus,* obtained from a mature cyst of *Heterodera glycines* extracted from soybean field soil from Roba, Macon County, Alabama (15), was maintained in axenic culture. Eggs of *M. arenaria* were removed from galled roots of greenhouse grown 'Rutgers' tomato plants by the procedure described previously (25). A small volume of egg suspension was placed in a sterile watch glass. Eggs at various stages of embryonic development were located using a steromicroscope at 40X magnification with transmitted light. One hundred and twenty eggs, categorized into 3 groups on the basis of approximate stage of development, were carefully removed with the aid of a sterile micropipette, one or two at a time, to 6 thinly poured water agar plates (20 eggs at a similar stage of development to each plate, two plates for each category). The categories were: 2 to 8 cell embryonic stage, intermediate stage of larval differentiation, and fully developed stage bearing viable juveniles (as evidenced by their movement within egg shells). Eggs were placed more

or less centrally on the plates, in proximity to one another but a short distance apart. Three plates (one of each category) were inoculated with a drop of *P. lilacinus* conidial suspension, prepared by adding sterile water to a young, sporulating colony of the fungus on 2% colloidal chitin agar (16). The drop was placed in the vicinity of the eggs. All 6 plates were incubated in the dark at 25C for 6 days, after which they were examined microscopically. Three parasitized eggs from each category, where hyphal penetration was evident, were removed and prepared for sectioning and transmission microscopy by the same methods used in our study of *V. chlamydosporium* parasitism (25). Three parasitized, hatched juveniles were treated similarly, as was one unparasitized egg from each uninoculated plate. The latter required forcible rupturing by means of a fine needle during fixation to make possible infiltration of the embedding medium.

RESULTS

Following the incubation period, fungal conidia had germinated to produce a mycelium in each of the 3 inoculated plates. In both inoculated and uninoculated plates of the third category, juvenile-bearing eggs, a number of juveniles had hatched. From 20 introduced eggs, 7 larvae emerged in the uninoculated plates and 11 in the inoculated plates. Larvae in the uninoculated plates were alive, evidenced by movement, whereas those exposed to proximal fungal hyphae in the inoculated plates were stationary and several had been colonized. Of the 60 eggs on inoculated plates, 80% were heavily parasitized and the majority bore fungal hyphae endogenously. There were no apparent differences in level of parasitism among the 3 egg developmental categories. Penetrating hyphae entering egg shells could be observed under the light microscope, as could emerging hyphae which gave rise to conidiophores bearing long chains of conidia (Fig. 1A, 1B) directly on the egg shell surface. Invaded eggs appeared somewhat swollen in comparison with uncolonized ones (Fig. 1A).

The presence of fungal hyphae affected egg shell permeability. Fixative, stain, and embedding medium penetrated parasitized eggs readily whereas healthy eggs remained impermeable to them.

Sections of eggs from uninoculated plates at all 3 developmental stages showed shells composed of 3 distinct layers (Fig. 3A). The outer vitelline layer appeared as a more or less evenly thickened, dark, electron dense band. The chitin and lipid layers appeared as broad, largely amorphous entities of even thickness except polarly where the lipid layer was thickened. The inner limits of the lipid layer were not always clearly discernible. Sections of mature eggs containing second stage larvae ap

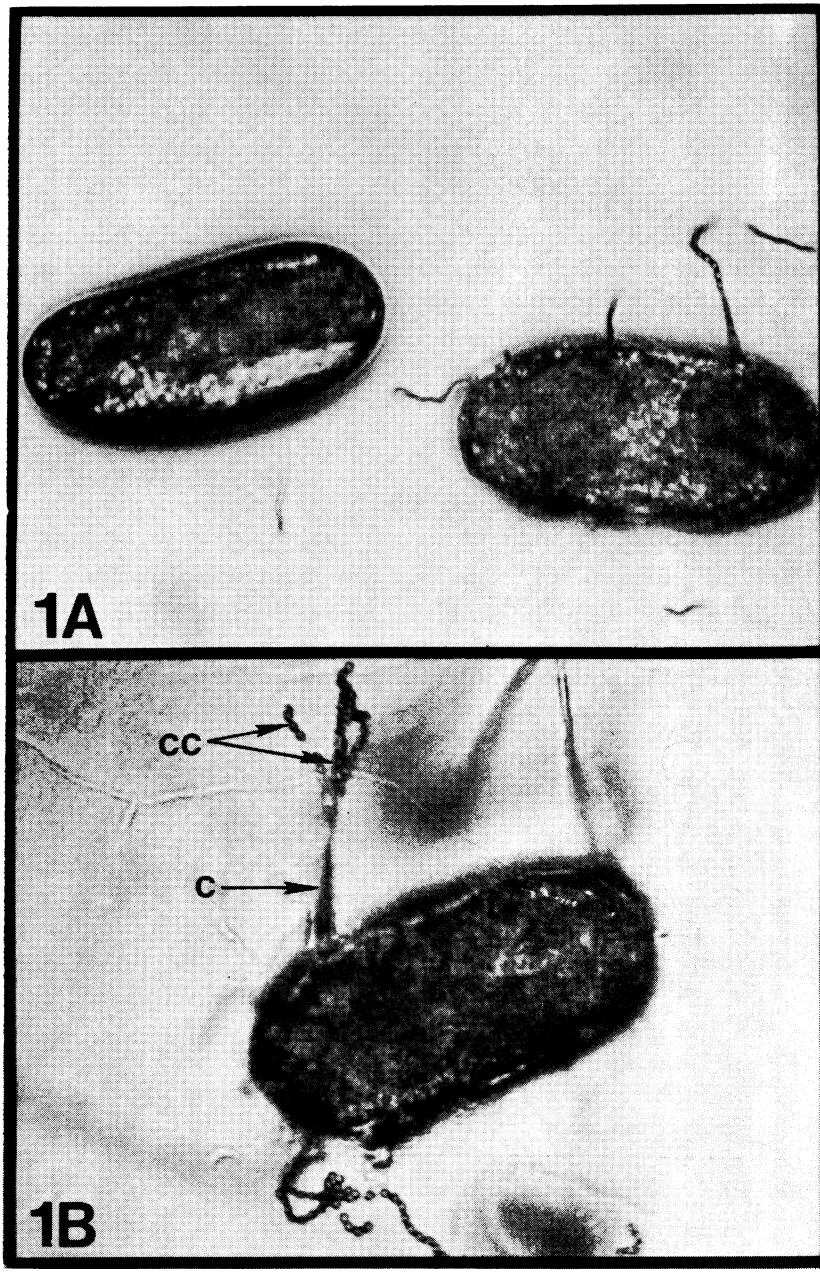
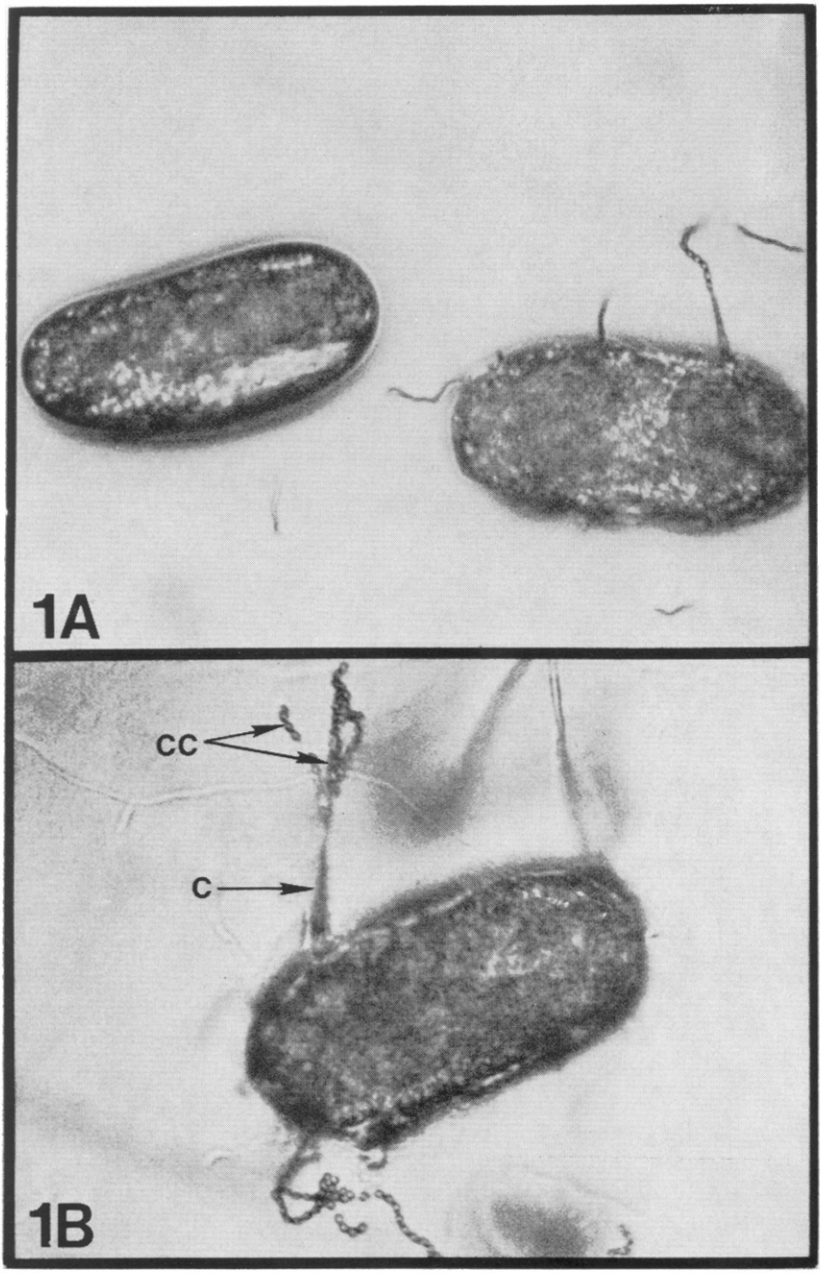


Fig. 1. A. Uninvaded (left) and invaded eggs on water agar surface. B swollen, invaded egg bearing a conidiophore (C) and chains of conidia (CC), (both X 800).



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peared similar to those illustrated previously (25), as did those of larval cuticles.

Sections of infected eggs and hatched juveniles showed hyphae present endogenously in abundance (Figs. 2B, 4A). The width of fungal hyphae varied, with some becoming considerably broader than those growing on the agar surface and heavily vacuolated. When a hyphal tip came in contact with an egg shell, two changes in configuration frequently occurred. The apical area of the hypha became inflated and some buckling of the egg shell occurred immediately peripheral to the zone of contact of the hyphal wall and the vitelline egg shell layer (Fig. 2A). This was especially the case in young eggs where embryonic development was at an early stage (category one). Following hyphal contact, the tip area became truncate and the vitelline layer was pierced at a narrow central locus, following which the hypha entered the egg by forcible rupture of the chitin and lipid layers over an area approximating in diameter the inflated hyphal tip (Fig. 2A). This occurred as a result of the penetrating hypha swelling immediately following entry through the pore formed in the vitelline layer. Endogenous fungal hyphae within larvae and eggs re-emerged, forcibly disrupting all egg shell layers in the process (Fig. 2B). In this case the vitelline layer is ruptured to a greater extent than occurs in penetration from without.

When an egg became occupied by fungal hyphae, three changes in the egg shell structure occurred. The vitelline layer split into three constituent plasmamembrane-like bands which appeared to be unevenly thickened along their length (Fig. 3B). The two inner bands were more electron dense than the outer band. A large number of more or less isodiametric, regularly spaced vacuoles appeared in the chitin layer, around each of which a very narrow electron-dense zone was evident. The inner limits of the chitin layer became indistinct and the lipid layer largely disappeared.

Juveniles colonized after hatching became engorged with fungal hyphae (Fig. 4A). In early stages of invasion some of the normal structures of larval cuticle, particularly the striated basal layer, remained evident (Fig. 4B). Following occupation by proliferating fungal hyphae, only the external cortical layer was evident (Fig. 5A) and somatic larval musculature had disintegrated. The only visible elements within some parasitized larvae were very thin flexous, branched, electron-dense strands (Fig. 5A). In extreme cases, both within eggs and without, heavily colonized larvae had a substantially contorted external cortical layer which, in some cases, had split into two narrow bands (Figs. 2B, 5B). In such instances the internal part had become totally necrotic and completely amorphous and granular in appearance.

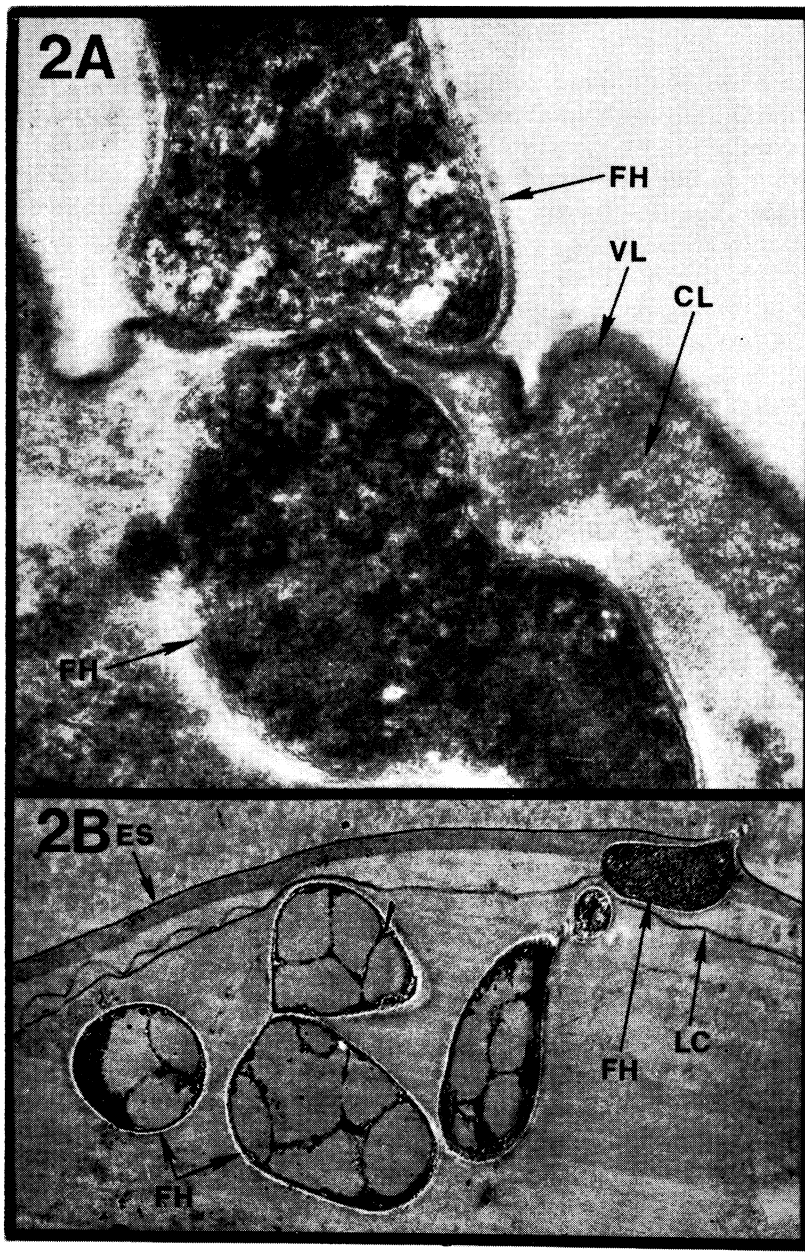
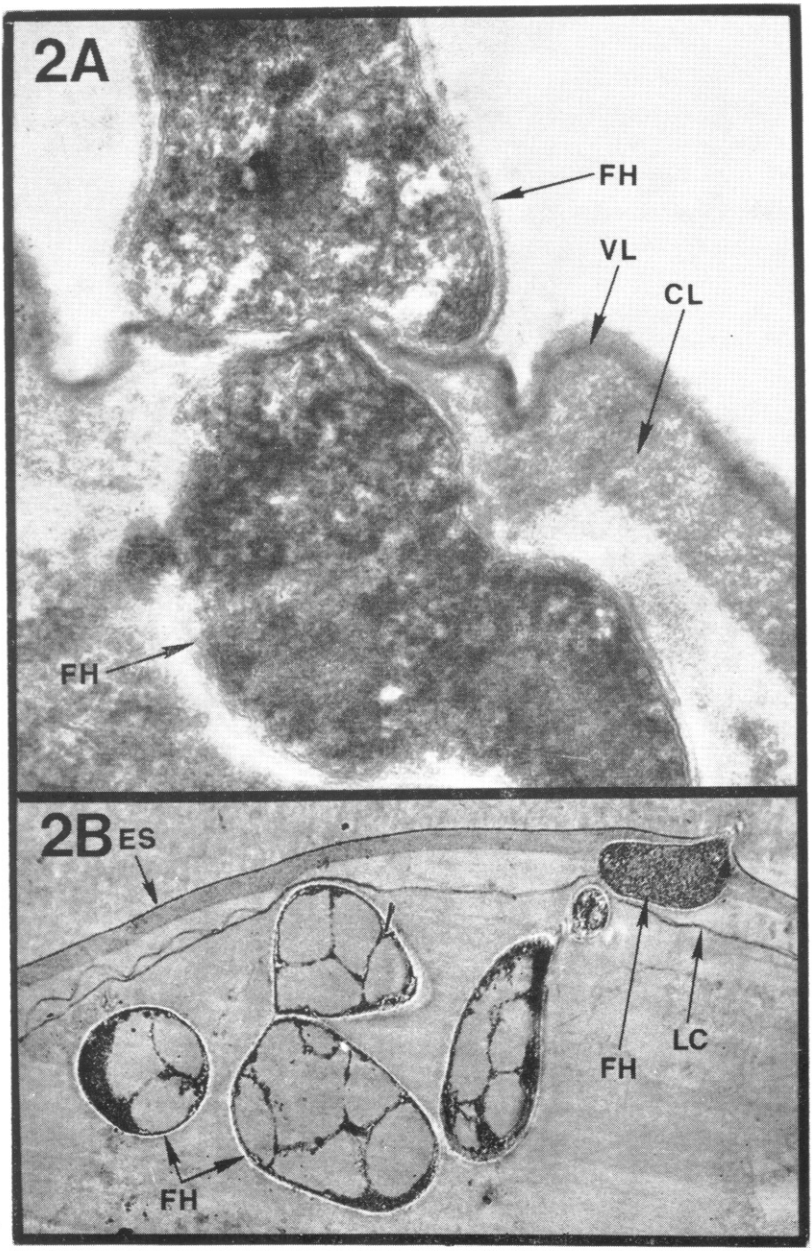


Fig. 2. A. Hypha penetrating egg shell: FH = fungal hypha, VL = vitelline layer, CL = chitin layer (X 50,000). B. fungal hyphae within egg and larva, and a hypha (right) emerging from larva and shell: ES = egg shell, LC = larval cuticle, FH = fungal hyphae (X 20,000).



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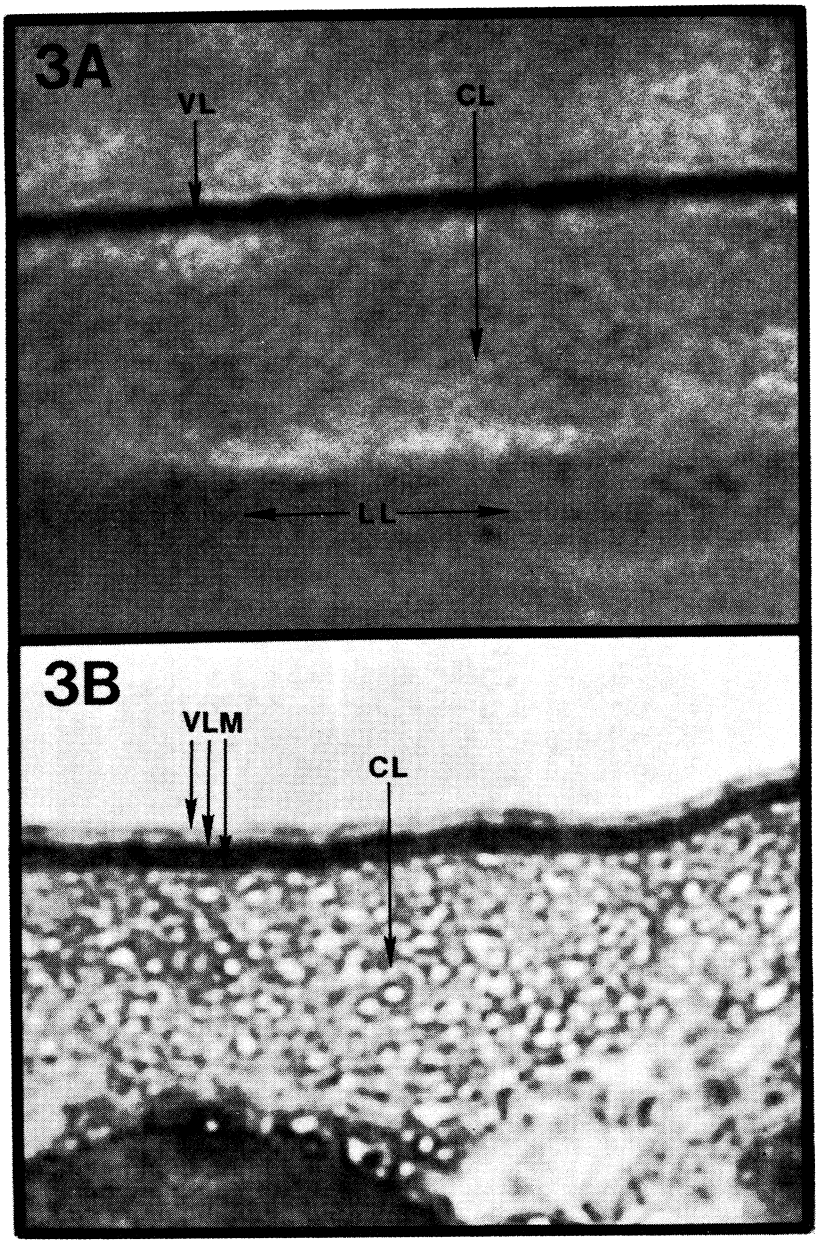
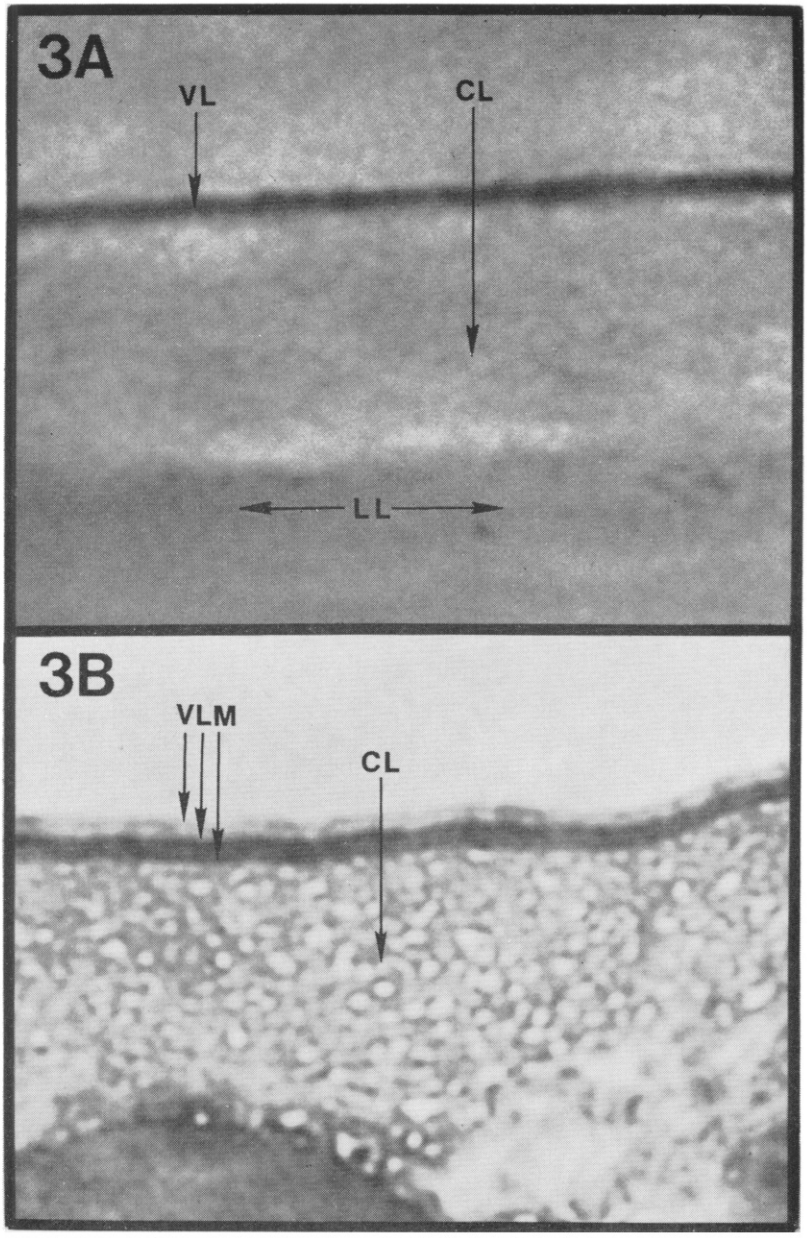


Fig. 3. A. Section of healthy egg shell: VL = vitelline layer, CL = chitin layer, LL = lipid layer. B. section of shell of diseased egg: VLM = vitelline layer membranes, CL = chitin layer with numerous vacoules (both X 120,000).



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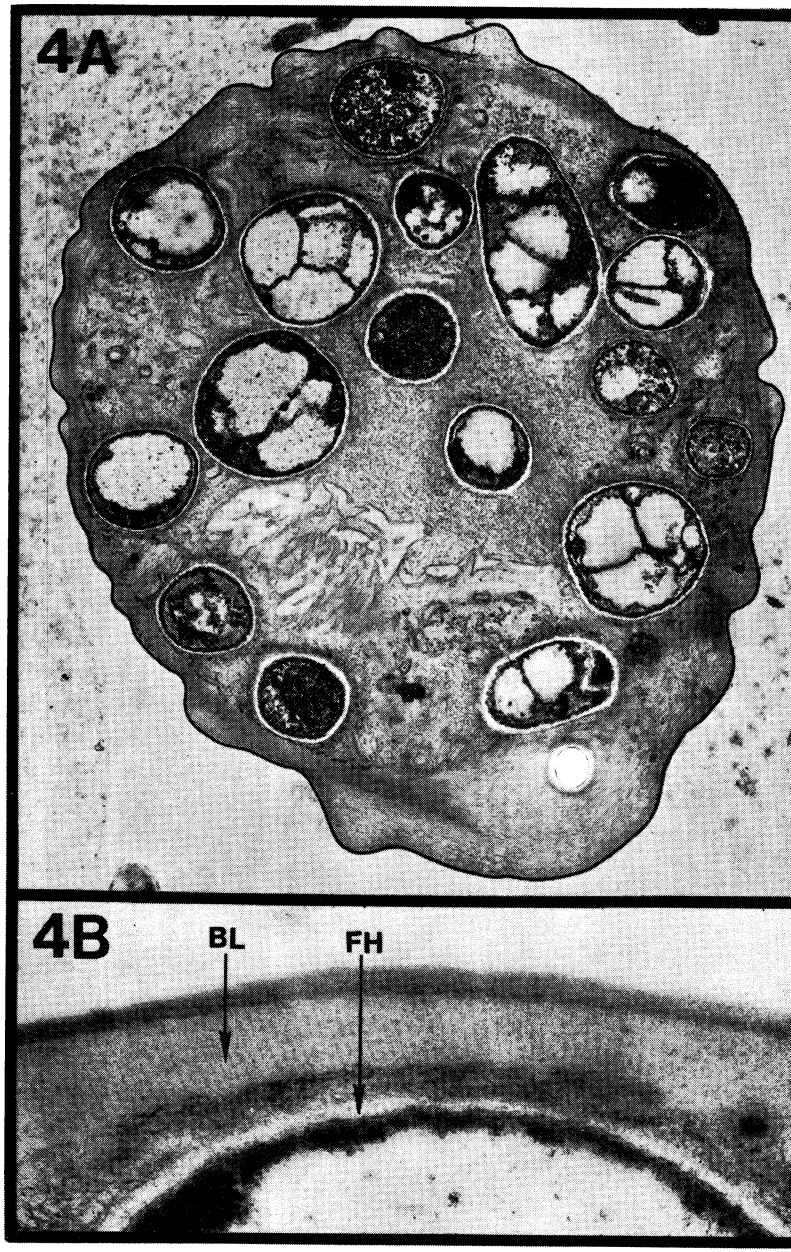
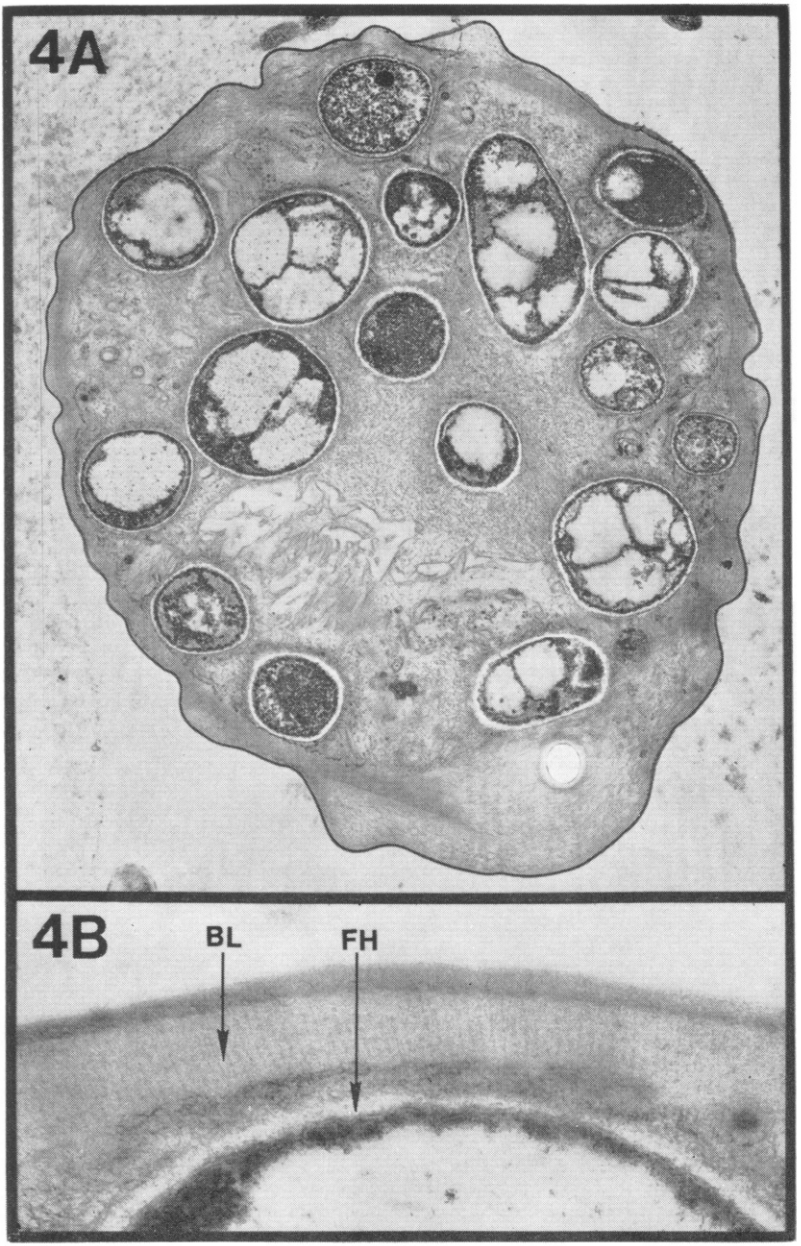


Fig. 4. A. Cross section of hatched larva with endogenous fungal hyphae ae (X 20,000). B. section of larval cuticle showing striated basal layer (BL) L) and proximal fungal hypha (FH), (X 80,000).



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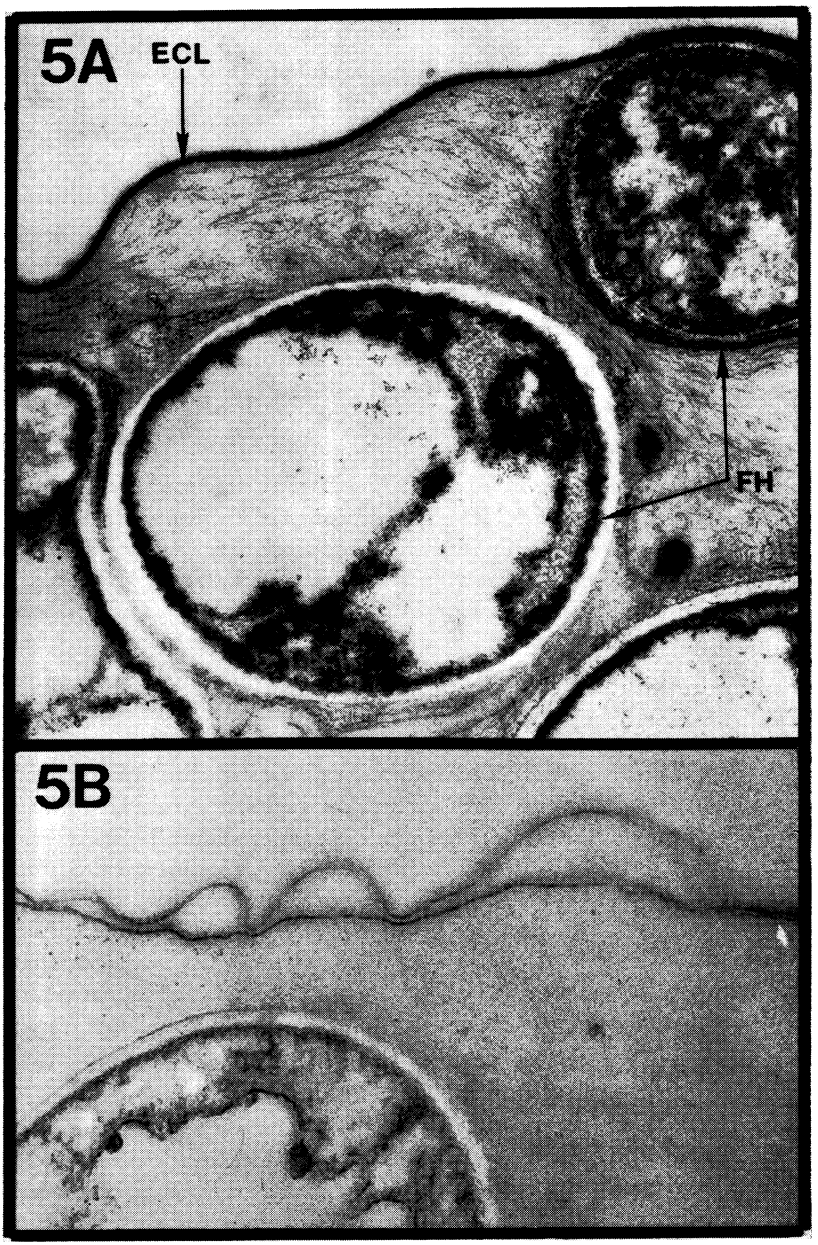

Fig. 5. A. Fungal hyphae (FH) within diseased larva with intact external cortical layer (ECL). B. fungal hypha within larva with split external cortical layer (both X 60,000).

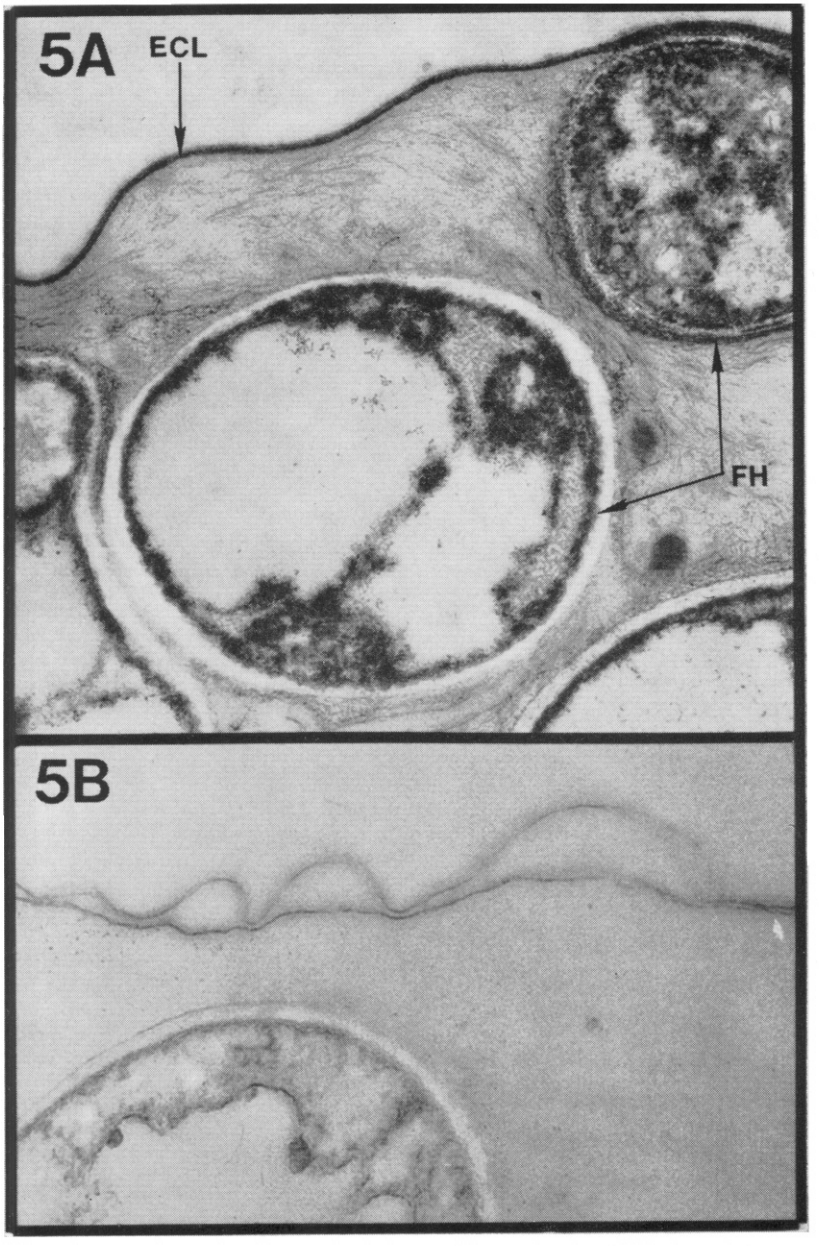

Fig. 5. A. Fungal hyphae (FH) within diseased larva with intact external cortical layer (ECL). B. fungal hypha within larva with split external cortical layer (both X 60,000).

DISCUSSION

It is evident that *P. lilacinus* is capable of parasitizing both eggs and larvae of *Meloidogyne,* leading to their destruction. The fact that hatched juveniles become incapacitated in the presence of fungal hyphae may indicate a primary, diffusible toxic effect rendering them subsequently vulnerable to colonization. In our limited sample, more larvae hatched in the presence of the fungus than without, possibly signifying a stimulatory effect. The hatching process is known to involve both prehatch movement and enzymatic hydrolysis of lipids on the part of nematodes (5). As a result of this activity, egg shell permeability is altered, increasing the likelihood of passage of hatching factors into the eggs.

The pattern of events occurring during penetration of the egg shell suggests mechanical rupturing of the vitelline layer by a narrow, tube-like hyphal extension. It is possible that some enzymatic weakening of the vitelline layer may also be involved. The entry of the fungus into an egg, irrespective of its age, results in similar changes. This indicates that the changes are brought about by the fungus rather than by egg maturation factors. The lipid layer, in particular, is known to be partially emulsified by movement of a mature larva prior to hatching. The fact that it disappears in young eggs where embryonic development is at an early stage, when fungal hyphae are present endogenously, indicates the disruptive effect of the fungus. Following dissolution of the lipid layer, the chitin layer is exposed to the fungus with the result that it is, at least in part, broken down. The presence of prominent vacuoles in this layer may indicate the hydrolysis of its chitinous inclusions, giving rise to a sieve-like appearance in section. Because the egg shell is rendered totally ineffective as a protective entity surrounding developing larvae, physiological disorders ensue, leading to a halting of growth and maturation. Ultimately *P. lilacinus* totally destroys the egg contents.

As a potential biological control agent *P. lilacinus* appears to have a number of advantages. Most reports indicate that it is a good competitor in most soils, particularly in warmer regions (11). Its optimum growth temperature is 26-30C. It readily produces abundant inoculum in the form of long chains of conidia. It has been reported to degrade chitin (27) and it is strongly proteolytic (1,7,10,19). This is of some significance since the egg shell of *Meloidogyne incognita* has been reported to be made up mostly of protein and chitin (6). *P. lilacinus* has antagonistic activity against bacteria (3,24) and fungi (4,8), a fact supporting the view that it is a strong competitor *in vivo.* Germination of conidia, however, has been shown in some instances to be inhibited by soil mycostatic factors (18). Arai et al. (2) isolated leucostatin and lilacin, two water-soluble peptide antibiotics, from a fungus determined as *Penicillium lilacinum.* Leucosta-

tin is active against gram-positive bacteria and many fungi. Of all the fungi which we have encountered to date as parasites of eggs and cysts of phytonematodes, we consider *P. lilacinus* to have the greatest potential for application as a biocontrol agent in subtropical and tropical agricultural soils.

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