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A Collagenolytic Fungus, *Cunninghamella elegans*, for Biological Control of Plant-parasitic Nematodes[1]

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Abstract: The root-galling index of tomatoes inoculated with *Meloidogyne javanica* was decreased 70% when collagen was used as a soil amendment (0.1% w/w) and 90% when the amendment was supplemented with the collagenolytic fungus *Cunninghamella elegans*. The root-galling index was reduced 80% when the fungus was homogenized in collagen culture medium and added to soil without collagen supplement. Culture filtrates of the fungus *C. elegans*, grown on collagen as a single source of carbon and nitrogen, immobilized *M. javanica* second-stage juveniles and inhibited egg hatch. Root galling was reduced when tomato plants were inoculated with filtrate-treated juveniles. Culture filtrates reduced the motility of *Rotylenchulus reniformis* and *Xiphinema index*, but they had less effect on *Anguina tritici* and almost no effect on *Ditylenchus dipsaci*. *Cunninghamella elegans* had collagenolytic, elastolytic, keratinolytic, and nonspecific proteolytic activities when grown on collagen media, but only chitinolytic activity when grown on chitin media.

Key words: Anguina tritici, biological control, chitinase, collagen, collagenase, *Cunninghamella elegans*, *Ditylenchus dipsaci*, elastase, fungus, keratinase, *Meloidogyne javanica*, *Rotylenchulus reniformis*, *Xiphinema index*.

Biological control of plant-parasitic nematodes has been achieved through application of organic amendments (10) and fungal or bacterial parasites of juveniles, females, and eggs (12). In previous work (4), we showed that collagenase and protease suppressed infection of tomato roots by juveniles of *Meloidogyne javanica*. Also, collagen was more lethal to nematodes than were other protein amendments, probably because collagen induced collagenase in addition to other proteases.

The collagenous nature of the nematode cuticle has been reported in the animal parasite *Ascaris lumbricoides* (8), the free-living nematodes *Caenorhabditis elegans* (3) and *Panagrellus silusiae* (6), and the plant-parasitic nematode *Meloidogyne incognita* (11). Collagen was found mainly in the medium and basal cuticle layers of *C. elegans* and *M. incognita* (3,11), but it occurred in all layers of the cuticle of the adult female and second-stage juvenile of *M. incognita* (5). We suspect that the suppression of *M. javanica* by collagen amendments to soil results from the production of collagenase and the subsequent damage to the nematode cuticle.

Adding both a collagenolytic fungus and collagen to soil might accelerate the production of collagenase and thereby enhance the nematicidal effect of the amendment. The purpose of our work was to evaluate the nematicidal effect of collagen supplements to the soil when combined with a collagenolytic fungus, *Cunninghamella elegans* (Mucorales).

MATERIALS AND METHODS

Nematodes: Infective, second-stage juveniles (J2) of *Anguina tritici* were extracted from infected, seedlike wheat (*Triticum aestivum* cv. Lakhish) galls and rinsed on a 10-

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This manuscript is dedicated to the memory of the late Professor Eli Cohn, our dear colleague and friend.

μm-pore sieve to remove plant tissue. *Ditylenchus dipsaci* was propagated on garlic (*Allium sativa* cv. Lavinia) in the greenhouse. Infective juveniles, females, and males were dissected from infected plant tissues. *Meloidogyne javanica* and *Rotylenchulus reniformis* were propagated in the greenhouse on tomato (*Lycopersicon esculentum* cv. Faculta 38) and on potted seedlings of upland cotton (*Gossypium hirsutum* cv. Acala 1517-C), respectively. Juveniles were collected from infected roots in a mist chamber. Females, males, and juveniles of *Xiphinema index* were extracted from soil around fig trees (*Ficus carica* L.) grown in our screenhouse. All nematodes were washed with distilled water or phosphate buffered saline (PBS).

Fungus: Cunninghamella elegans was isolated from sandy soil from Bet Dagan that had been enriched with collagen. Ten grams of moist soil was placed in a 500-ml flask containing 400 ml autoclaved water and stirred for 15 minutes. An aliquot (ca. 0.2 ml) of the suspension was spread on rose bengal medium (7) and incubated for 5 days at 28 C. Fungal colonies were transferred to a chitin-agar medium where profuse sporangiophores allowed easy identification of the species. The fungus was maintained at 10 C on chitin-agar containing per liter: 2 g colloidal chitin, 1 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4$, 0.8 g KH_2PO_4, 0.4 g KNO_3, and 20 g agar. For the soil studies, the fungus was grown in three liquid media: 1) Czapek without agar; 2) chitin medium as described above without agar, and 3) collagen medium containing per liter: 2 g collagen (Sigma), 0.8 g K_2HPO_4, 0.1 g $CaCl_2$, and 0.3 g $MgSO_4 \cdot 7H_2O$. The fungus was incubated in 100 ml of each medium in 250-ml flasks on a shaker; chitin-agar discs (1.5 cm) colonized with the fungus were used to inoculate the flasks, one disc per flask. After 5 days at 28 C, the fungus was homogenized with the culture medium or separated from the medium by centrifugation (20 minutes at 12,000 *g*) and suspended in water. The homogenate was diluted 1:1 with water before addition to soil. The culture filtrate obtained after separation of mycelium was passed through a cellulose triacetate filter (0.45-μm-pore) and used for in vitro tests.

Greenhouse tests: Four-week-old Faculta 38 tomato seedlings from surface-sterilized seeds were transplanted to 500-cm^3 plastic pots filled with untreated sandy loam soil (47% sand, 38% silt, 15% clay; pH 7.9–8.1, organic matter 0.8%). In one experiment, a mycelial suspension of *C. elegans* (0.5 g dry weight/pot), with or without 0.1% W/W collagen, was mixed into the soil at transplanting. In another experiment, the homogenized culture without additional collagen was mixed into the soil at transplanting. In both experiments, approximately 600 *M. javanica* eggs were added to the soil near the roots of each plant at transplanting. Unamended, nematode-inoculated plants served as controls. The plants were maintained in a growth chamber (28 ± 3 C). Thirty-five days after inoculation, the experiments were terminated and galling was evaluated using a subjective scale from 0 to 5, where 0 = no galling, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, and 5 = 100% of the roots covered with galls. Each treatment had eight replicates, arranged in a completely randomized block design, and the experiments were conducted three times.

In vitro studies: The effect of the fungus and its collagen culture filtrate on the motility of several plant-parasitic nematodes was studied in multiwell plates (Becton Dickinson Labware, Rutherford, NJ). *Anguina tritici, D. dipsaci, M. javanica, R. reniformis,* and *X. index* (ca. 50 vermiform nematodes/well) were incubated for 48 hours at 30 C with 1.5 ml of fungal culture filtrate or mycelial suspension (2 mg/ml dry weight). In the case of *M. javanica,* various dilutions of the culture filtrate were also tested. Deionized water and autoclaved filtrates were used as controls. Motility was the criterion used to evaluate the effect of the fungus or its culture filtrate on the nematodes. The effect on *M. javanica* egg hatch was evaluated by incubating 50 eggs per well containing 1.5 ml of the fungal culture filtrate or mycelial suspension (2

mg/ml dry weight). Deionized water and autoclaved filtrates were used as controls. The number of hatched eggs was determined after incubation at 30 C for 48 hours. All treatments were performed in eight replicates, one well per replicate, and each experiment was conducted three times.

The infectivity of *M. javanica* treated in vitro with the culture filtrate was evaluated. Four-week-old tomato seedlings were transplanted to pots with the same soil and kept under the same conditions as described for the greenhouse tests. Each pot was inoculated with 400 J2 that had been treated with the culture filtrate for 48 hours at 30 C. Juveniles were rinsed with water before inoculation. Juveniles incubated under the same conditions in water were used as controls. After 5 weeks, plants were removed and root galling was rated. The tests were performed with 10 replicates and repeated three times.

Enzymatic activity of Cunninghamella elegans filtrates: The culture filtrates of *C. elegans* from the chitin, collagen, and Czapek liquid media were tested for chitinase, collagenase, elastase, keratinase, and protease activity. The enzyme assays were performed with substrates that release a dye into the medium when degraded. Proteolytic activity was tested with a substrate solution of 6 mg/ml Azocasein (Sigma) in a buffer solution of 0.05 M Tris-HCl (pH 7.6) and 5 mM $CaCl_2$. Culture filtrate (0.5 ml) was incubated with 0.5 ml of the substrate solution at 37 C for 20 minutes. The reaction was terminated by adding 0.5 ml 10% trichloroacetic acid, and the mixture was incubated for 30 minutes on ice. The mixture then was centrifuged for 15 minutes, and the supernatant was analysed in a spectrophotometer (Schimadzu UV 120-02) at 400 nm. Elastase activity was determined by using elastin congo red (Sigma) (13). Collagenase activity was tested with a synthetic peptide (p-phenylazobenzyloxycarbonyl-L-propyl-L-leucylglycyl-L-propyl-D-arginine) (Boehringer) (14). Keratinase was tested by incubating 2 mg keratin azure (Sigma) and 1 ml fungus culture filtrate at 37 C for up to 15 hours. After centrifugation, the supernatant was read at 595 nm. Chitinase activity was determined by incubation of 2 mg chitin azure (Sigma) with 0.5 ml culture filtrate and 0.5 ml 0.05 M buffer citrate, pH 5.5, at 40 C. The reaction was terminated after 18 hours by adding 0.5 ml 10% trichloroacetic acid, and the mixture was incubated for 30 minutes on ice. The mixture then was centrifuged, and the supernatant was read at 540 nm.

Data were subjected to an analysis of variance, and means were separated by Duncan's multiple-range test ($P = 0.05$). Because similar results were obtained with repeated experiments, data presented are from one representative experiment.

Results

The addition of collagen to soil reduced the root-knot galling index in tomato roots by 70% (Fig. 1). When both *Cunninghamella elegans* hyphae and collagen were added to soil, the galling index was reduced by about 90%, irrespective of the medium on which the hyphae were grown. However, fungal hyphae grown on collagen medium, as well as on the two other media (data not shown), did not significantly influence root galling in the absence of supplemental collagen (Fig. 1). When added to soil, the collagen culture homogenate reduced the galling index by 80%, the chitin culture homogenate reduced it by only 30%, and the Czapek culture homogenate did not reduce root galling (Fig. 2).

The motility (expressed as a mean ± SE percentage of the water controls) of nematodes treated with collagen culture filtrate was 0 ± 0 for *X. index*, 3 ± 0.3 for *R. reniformis*, 10 ± 3 for *M. javanica*, 62 ± 7 for *A. tritici*, and 90 ± 10 for *D. dipsaci*. The motility of *M. javanica* juveniles decreased as the concentration of the collagen culture filtrate increased (Fig. 3). About 30 ± 8% of the immobilized juveniles that had been treated with undiluted culture filtrate recovered (i.e., became motile) after 24 hours in deionized water. The root-galling index of tomato plants inoculated with untreated J2 was 4.5, and with J2 pretreated with undiluted culture filtrate it was

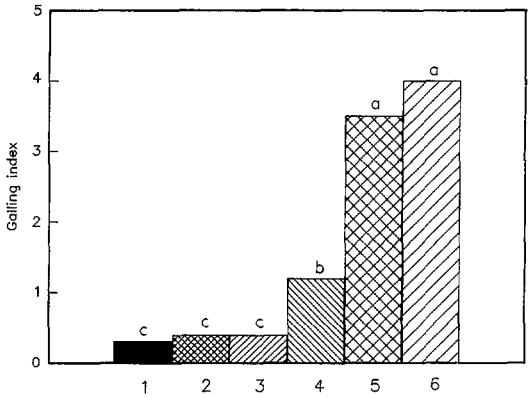

FIG. 1. Galling of tomato roots by *Meloidogyne javanica* as affected by soil amendments of collagen and hyphae of *Cunninghamella elegans* grown on different media. Galling was rated on a scale from 0 (no galling) to 5 (100% of roots covered with galls). Values with the same letter do not differ ($P = 0.05$) according to Duncan's multiple-range test. Each value is the mean of eight replicates, and the data represent one of three trials performed. Soil amendments were as follows: Collagen plus hyphae of *C. elegans* grown in Czapek medium (column 1), collagen plus hyphae of *C. elegans* grown in chitin medium (column 2), collagen plus hyphae of *C. elegans* grown in collagen medium (column 3), collagen alone (column 4), and *C. elegans* hyphae (from collagen medium) alone (column 5). Unamended soil was used as a control (column 6).

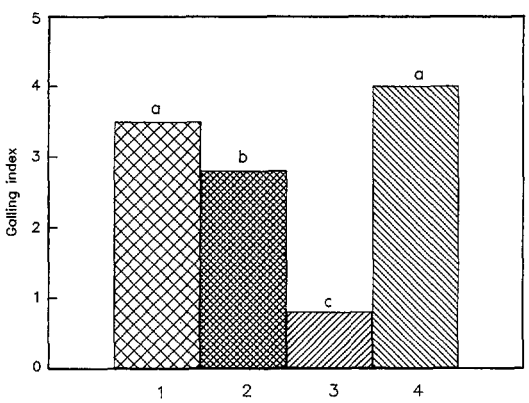

FIG. 2. Galling of tomato roots by *Meloidogyne javanica* as affected by soil amendments of *Cunninghamella elegans* homogenized in different culture media. Galling was rated on a scale from 0 (no galling) to 5 (100% of roots covered with galls). Values with the same letter do not differ ($P = 0.05$) according to Duncan's multiple-range test. Each value is the mean of eight replicates, and the data represent one of three trials performed. The following fungal culture homogenates were added to soil without collagen: Czapek (column 1), chitin (column 2), and collagen (column 3). Unamended soil was used as a control (column 4).

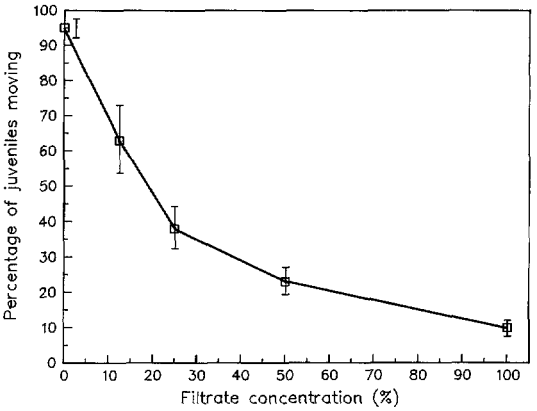

FIG. 3. Motility of *Meloidogyne javanica* as affected by culture filtrates of *Cunninghamella elegans* grown in collagen medium. Values are means ± SE of eight replicates, ca. 50 juveniles per replicate. The data represent one of three trials performed.

2.5; this difference was significant ($P = 0.05$). Treatment with mycelial suspension did not affect the motility in vitro of *M. javanica* juveniles or of the other genera tested (data not shown).

Undiluted collagen culture filtrate reduced hatch of *M. javanica* by 75%, whereas autoclaved filtrate reduced hatch by 35% (Fig. 4). Diluted filtrates did not affect egg hatch (data not shown). Hatch was not affected by treatment with hyphae alone (Fig.

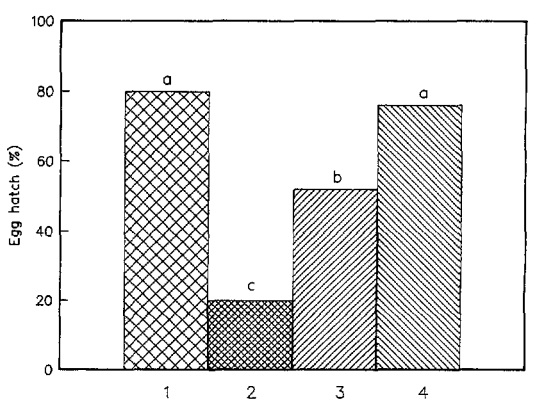

FIG. 4. Egg hatch of *Meloidogyne javanica* as affected by culture filtrate of *Cunninghamella elegans* grown in collagen medium. Values are means of eight replicates, with ca. 50 eggs per replicate. Values with the same letter do not differ ($P = 0.05$) according to Duncan's multiple-range test. The data represent one of three trials performed in water (column 1), culture filtrate (column 2), autoclaved culture filtrate (column 3), and mycelial suspension (column 4).

4). When eggs that did not hatch after exposure to collagen culture filtrate were washed and placed in water, 80 ± 10% hatched within 48 hours.

Collagen culture filtrates of *Cunninghamella elegans* exhibited proteolytic, collagenolytic, keratinolytic, and elastolytic activities. Chitin culture filtrate showed chitinase activity only. None of these enzymes was detected in Czapek culture filtrate.

Discussion

In a previous report (4), collagen was the most effective of several proteinaceous soil amendments against root-knot nematodes. In the present work, the effect of collagen was enhanced when a collagenolytic fungus was added to the amendment, even though half the concentration of collagen was added to soil this time. The fungus did not appear to be parasitic on the nematode, and the inhibition was evidently indirect. The fungal hyphae did not affect juveniles in soil or in vitro; the antagonistic effect was obtained only when the fungus was added with collagen to the soil or when a collagen culture filtrate was applied directly to *M. javanica* juveniles or eggs.

The effect of the homogenized fungal cultures on galling of roots varied with the media; the collagen culture homogenate substantially suppressed galling, whereas the Czapek culture homogenate did not affect galling. The presence or absence of protease and collagenase in the culture filtrates might explain these results. Collagenase and protease decrease *M. javanica* egg hatch, juvenile motility, and infectivity (4). Collagenase-treated *M. javanica* juveniles are unable to cause galling in tomato roots (4). Keratinase and elastase in the filtrate also might affect the nematode cuticle, because the external cortical layer in some respects resembles keratin (1) and it is also sensitive to digestion by elastase (3). Chitinase was reported also to be lethal to plant-parasitic nematodes (9). The results obtained with the homogenized chitin medium suggest that chitinase, which we detected in the chitin culture, might affect *M. javanica*, although chitin was never found in the nematode cuticle. In contrast to the chitin and Czapek cultures, the collagen culture exhibited collagenolytic and general proteolytic activities. We suggest that the inhibition of *M. javanica* juveniles by the collagen culture in soil or in vitro resulted from the interaction of these enzymes with collagen-like proteins of the cuticle (1,3,5). *Meloidogyne javanica* eggs were less affected than the juveniles by the fungal filtrates. This confirms our previous report (4) that *M. javanica* juveniles apparently are more sensitive than eggs to collagenase and protease. The adverse effect on hatching by the autoclaved culture filtrate indicates that other nonproteinaceous factors in the filtrate may affect egg hatch.

The filtrates immobilized most but not all nematodes examined. We have no explanation as to why *A. tritici* and *D. dipsaci* were less responsive than other nematodes to the filtrates. However, these two species differ from the others in that they are parasites of above-ground plant organs and are adapted to exposure to drier conditions. Cayrol et al. (2) found that the activity of culture filtrate of the nematophagous fungus *Paecilomyces lilacinus* was very specific. The authors attributed this specificity to differences in the chemistry of the nematode cuticle. A better understanding of the composition of the nematode cuticle might facilitate the selection of soil microorganisms capable of damaging plant-parasitic nematodes.

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