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A Dissertation Presented

by

Vincent W. Saka

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To my parents

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ABSTRACT

Waste Mycelium, Sewage Sludge and Crab Chitin as Soil Amendments to Control the Plant-Parasitic Nematodes Meloidogyne incognita and Pratylenchus penetrans

May, 1978

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The effects of waste mycelium, sewage sludge and crab chitin on populations of Meloidogyne incognita and Pratylenchus penetrans infecting tomato plants were investigated in the greenhouse. When raw and digested sludge were added to the soil at the rate of 20g per 4" pot and replicated seven times, infection by Meloidogyne incognita was significantly reduced. However, the sludge treatments reduced the dry weights and the heights of tomato plants when compared to plants grown in untreated soil.

Soil amended with waste mycelium, crab chitin or sewage sludge was left to decompose for two weeks and then analyzed for nitrogenous compounds and actinomycetes before transplanting and inoculation with nematodes. Each treatment was replicated seven times. Significantly larger numbers of actinomycete colonies were obtained from chitin-amended soil than from any other treatment. Similarly,

relatively larger concentrations of NO_3-N and NH_4-N nitrogen were detected in chitin-amended soil than from the other treatments.

After two weeks, the plants were examined for galls and for numbers of lesion nematodes. Fewest galls and lowest number of lesion nematodes were found in plants grown in chitin-amended soil suggesting a correlation between the number of actinomycetes and reduction of nematodes in the plants.

Soil from each treatment was plated on chitin medium in order to isolate actinomycetes and, by serial dilution, 60 colonies were obtained and transferred onto Czapecks agar slants. Each colony was then grown in liquid medium and metabolites extracted from this medium were tested on root-knot nematode larvae and lesion nematodes. One colony yielded extracts that were nematicidal even at 1:10 dilution in water. The nematodes in crude extracts for one hour did not recover when they were transferred to distilled water. A similar reaction was observed when nematodes were transferred to distilled water after being in 1:10 dilution extracts for 24 hours.

In order to observe repulsion or attraction, the extracts were added to a well in one end of a rectangular polystyrene chamber filled with 1% agar, a well on the opposite end contained distilled water. After 24 hours a center well was made and 60 nematodes were added to it and sealed with precooled agar. Observations were made every

4 hours on numbers of nematodes that had migrated toward each end section. No nematodes were observed migrating toward the well with distilled water suggesting the absence of repulsion by the extracts. Likewise, nematodes were not attracted toward the well with extracts.

The results suggest that actinomycetes in organic amended soil contribute to the reduction of the plant-parasitic nematodes Meloidogyne incognita and Pratylenchus penetrans by producing a toxin which probably works in conjunction with nitrogenous compounds such as ammonia and nitrates.

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INTRODUCTION

Animal manures, green manures, compost and other forms of organic matter have always been an integral part of agriculture and are a subject of renewed interest. Duddington speculates that the increased importance of plant-parasitic nematode injury in the first half of this century is correlated with decreased use of animal manures (9).

The use of organic amendments to suppress soil populations of plant-parasitic nematodes has been of interest among nematologists soon after Linford, et al. (26) reported a reduction of populations of root-knot nematode after adding pineapple and sugarcane residues to the soil. They hypothesized that the incorporation of these chopped plant materials brought an increase in the total population of nematodes (plant-parasitic and free-living) in soil but also greatly increased the nematode predators such as nematode-trapping fungi, predacious nematodes and predacious mites. These collectively then decrease the number of nematodes during the early weeks of decomposition.

Since that time other workers have described various combinations of organic matter additives to the soil. Duddington, et al. (8) treated microplots of beet, heavily infested with beet cyst nematode (Heterodera schachtii), with fungus mycelium of Dactylaria thoumasia, a predac-

ious fungus having an adhesive network, and bran. Both the fungus mycelium and the bran increased the yield of the beet. The application of organic matter at very high rates significantly affected the final cyst populations of the plots but not the final egg population and the fungus mycelium had no significant effect. It is suggested that the fungus mycelium may have broken down and eventually acted as organic matter. However, Duddington, et al. (8) in another experiment decreased the number of cereal cyst nematode Heterodera avenae attacking oat seedlings by green manure treatments of chopped cabbage leaves and showed that this was due to the stimulation of predacious fungi.

The activity of nematode-trapping fungi was followed in nonsterile soil during the decomposition of organic matter by Cooke (5). Using a buried slide agar-disc method, he was able to observe the population increase of nematode-trapping fungi. The decomposition of cabbage leaf tissue induced extremely large populations of free-living nematodes; however, the period of nematode trapping activity was relatively short (4-8 weeks) but did not seem to correspond to the nematode population. With increased amounts of amendments the nematode population went up, but the fungal activity was reduced.

The situation was even more complex when he used sucrose as an amendment. A period of predacious fungus activity was produced but without increasing the nematode

population much above the one found in the unamended soil. Increasing sugar levels from 4 to 7 grams per 15 cm petri dish resulted in an increase in nematode population and an apparent reduction in nematode-trapping activity. Experiments in sterile soil strongly suggest that nematode-trapping fungi function effectively in the presence of nematodes, therefore, this lack of direct relationship between nematode population and predacious activity could be caused by some other factors not yet understood (6,7).

In order to select the most effective organic amendment for predacious fungi, Mankau (27) introduced three trapping fungi into the soil: Arthobotrys arthobotryoides, Arthobotrys dactyloides and Dactylella ellipsospora. Conidial suspensions of these fungi were placed against water agar discs which were then buried in the soil amended with various organic materials such as oat hay, chicken manure, steer manure and rotted wood shavings. He obtained better conidial germination in chicken and steer manures than with the plant residues. Using organic amendments over a 4-year period in outdoor microplots, Mankau (27) was able to reduce the infectivity and survival of Meloidogyne incognita larvae. He observed that two of the mor efficient nematode-trapping fungus species were associated with the amended soil.

Lear (25) reported significant reduction in populations of three plant-parasitic nematodes, Meloidogyne javanica, Heterodera rostochiensis and Heterodera schachtii

where large quantities of castor bean pomace were added to the soil. His observations were later confirmed by Mankau and Minteer (28) who suppressed the population of Tylenchus semipenetrans by adding steer manure, chicken manure, cotton waste, sugarbeet pulp, alfalfa pellets, alfalfa hay, liquified fish and castor pomace. Out of eight organic materials added to the soil, only steer manure failed to cause substantial reductions in numbers of larvae in 84 days. Castor pomace eliminated all citrus nematode larvae.

Johnson (16) and Johnson et al. (17) found a reduction of root-knot on tomato where 11 crop residues were incorporated into the soil. In these experiments, mature dried crop residues, oat straw, alfalfa hay, lespedeza hay and flax hay amendments each reduced root-knot infection at the rate of 10 tons rather than 5 tons. Similar observations were reported by Morgan and Collins (39) when they tested various organic materials to control Pratylenchus penetrans. Timothy hay gave the greatest suppression of populations of root lesion nematodes. In all these studies the mechanisms involved in suppression of nematodes were unknown or not investigated.

Singh and Sitaramiah (47) and Singh et al. (48) reduced galling on tomato and okra plants when they applied finely divided oil cakes and sawdust in combination with urea, they suggested that ammonia released during the decomposition of the organic amendments was the active

mechanism. Their theory was confirmed by Walker (5) who investigated the effects of decomposing nitrogenous compounds in the form of nitrite, nitrate and ammonium compounds. In his studies, nitrite and ammonia were more effective against lesion nematodes. He believed that the nematode reduction occurs during ammonification when the nitrogenous and organic amendments decompose. These results are in agreement with those of Miller *et al*. (33) who used cellulosic amendments such as sawdust, chopped filter paper, chopped tobacco stems, cotton seed meal and castor pomace to control invasion of eggplants by larvae and to inhibit larval emergence from *Heterodera tabacum* cysts. Sawdust was more effective than other amendments. They speculated that this was because the ammonia produced at high concentrations was toxic to *Heterodera tabacum*. However, the source of high ammonia was not known because, for sawdust to decompose, some nitrogen source usually must be added to it. Patrick *et al*. (42) observed reduction of plant-parasitic nematode populations by naturally occurring plant-derived decomposition products which were not identified.

Mankau and Das (29) amended soil with chitin and found it effective against the root-knot nematode. More recently Miller *et al*. (34) used industrial mycelium residues, wood fibers and chitin to control *Tylenchorhynchus dubius* and *Pratylenchus penetrans*, Rhizoctonia damping-off

of cotton and Fusarium rot of cucumber. They found that the nematode populations were reduced more quickly than the fungal populations. They suggested that ammonia, which is produced when mycelial residues degrade, could be responsible for the reduction of nematode populations. Habicht (13) using lime treated and composted raw sewage was able to reduce the level of galling by Meloidogyne incognita acrita on tomato roots. His results are in agreement with those of other workers who have reported that chemicals such as ammonia, high salt concentration or organic acids play a role in suppressing the nematode populations (34,54).

Sayre, et al. (44) found that during decomposition of rye and timothy hay, nematicidal factors were released which are ten times more toxic to plant-parasitic nematodes Meloidogyne incognita and Pratylenchus penetrans than to the free-living nematode Panagrellus redivivus. Butyric acid was isolated and identified as one of the nematicidal components released from the residues. Their results confirmed Johnston's (15) who reported that reduction in populations of Tylenchorhynchus martini were due to volatile fatty acids in water-saturated soil. He isolated a soil bacterium, Clostridium butryicum that produced a mixture of formic, acetic, propionic and butyric acid in its culture medium. Banage, et al. (2) confirmed Johnson's results when they tested fatty acids on the nematode

Dorylaimus. They too found butyric acid to be toxic to the nematode. More recently, Johnson (18) demonstrated that a toxic substance extracted from manure, dried oat straw and leaves and stem segments of flax was effective against eggs and larvae of root knot. A toxin was also extracted, but in lower concentrations from soil amended and incubated 10 weeks with similar amounts of these organic materials. He found that oat straw-amended soil contained more toxin than did flax-amended soil. The toxic substances remained unidentified.

The mechanisms suggested for the control of bean root rot with chitin by Mitchell and Alexander (35) and Mitchell (37) was however, quite different. They found that amendment of soil with chitin led to an increase in actinomycetes, chitinase-producing microorganisms and chitinase activity. They suggested that mycolytic activity and toxin production might have been responsible for the suppression of the disease. Their hypothesis was confirmed when they demonstrated lysis of fungal mycelium. A number of bacteria were isolated which were capable of lysing Fusarium oxysporium. Among the bacteria isolated was a strain of Bacillus cereus which was studied in detail. Living and dead fusarium mycelia, as well as cell-wall preparations, were digested by the bacterium. Chitin and hyphae of a number of other fungi also served as carbon sources for the active organisms but there was no lysis of the species Pythium, Streptomyces, Agrobacterium or Pseudomonas presumably because these organisms have no chitin in their

cell walls. Lysis appeared to be associated with chitinase and laminarinase production activity of Bacillus cereus.

Adams, Lewis and Papavizas (1) added spent coffee grounds to soil and found that there was an initial increase in chlamydospore germination of Fusarium solani. However, the germination was soon followed by lysis due to increased fungistatic activity in the amended soil which might have brought about by toxic substances such as phenolic compounds which increased up to 792 ppm soon after addition of the spent coffee grounds. Skujins, Potgieter and Alexander (46) digested fungal walls of Fusarium solani with B (1-3) glucanase and chitinase isolated from streptomycete cultures. The products of the digestions with both enzymes were N-acetylhexosamine and glucosamine and glucose. No significant lysis was produced when B (1-3) glucanase and chitinase were used separately.

Mitchell (37) was able to demonstrate the idea that the soil environment can be changed by organic amendment and thereby increase artificially the population of a species already present in order to favor antagonistic microflora and hence control pathogens. He treated the soil with either of two fungal cell wall constituents, chitin or laminarin, and was able to observe a decline in severity of diseases caused by several soil-borne fungal pathogens, especially Fusarium solani f. phaseoli

and Fusarium oxysporium f. conglutinans. Both of these pathogens contain chitin in their cell walls. Pythium debaryanum does not contain chitin in its cell walls and was not suppressed by chitin amendment.

Successful biological control has been obtained by making use of the selective effect of the plant rhizosphere. Numbers of microorganisms are much greater in this zone than in soil devoid of roots. It is therefore to be expected that antagonistic activities would be greatest in this area (21). Using this principle, Koths and Gunner (23) obtained partial control over Fusarium roseum, the pathogen of fusarium stem rot of carnations. Mitchell and Hurwitz (38) isolated two strains of Arthrobacter from the tomato rhizosphere which lysed mycelium of Pythium debaryarum. Tomato disease caused by this pathogen was, in aseptic culture, reduced by inoculation with the appropriate lytic bacterium. In nonsterile soil, seed inoculation with lytic bacteria protected young seedlings against damping off caused by Pythium debaryanum. Plate counts indicated that the lytic bacteria predominated in the root zone for approximately 14-21 days.

Dugan (11), using lobster shells, was able to control fusarium root rot on beans. He observed an increase in actinomycetes and chitinase producers which lysed the fungal cell walls of the pathogen.

Lytic action against nematodes was demonstrated by Katznelson, et al. (19) when they observed three species

of bacteria-feeding nematodes, Caenorhabditis briggsae, Rhabditis oxycera and Panagrellus sp., being lysed by two out of three soil isolates of myxobacters in liquid agar medium. Aphelenchus avenae, a fungus-feeding nematode, and Heterodera trifolii, a cyst-forming plant parasite, were unaffected by the myxobacters. Lysis of the nematodes was also shown spectrophotometrically by the decrease in optical density of a nematode homogenate following addition of an enzyme from cell-free fluid culture of one of the myxobacters and by the increase in trichloracetic acid soluble tyrosine residues in the mixtures after 15-20 minute incubation. The enzyme concentrate could be separated into a "lytic" fraction that dissolved the nematodes and proteolytic fraction that did not.

More recently Mankau and Prasad (30) have described the endoparasitic action of Bacillus penetrans that readily infected larvae of Meloidogyne spp. after exposure to an aqueous suspension of spores from infected root knot nematode females. Infection severely reduced motility of second-stage larvae through soil. Bacillus penetrans exhibited a distinct host specificity in that only 5 of 16 nematode species tested became infected with the population used. Meloidogyne javanica, Meloidogyne arenaria and M. incognita became more heavily infected than Meloidogyne hapla or Pratylenchus scribneri under similar conditions. Sayre and Wergin (45) also observed the same

parasite, however, they believe that the endoparasite is a procaryotic organism having structural features that are common to members of actinomycetales and is therefore an actinomycete.

Evidence indicates that when organic amendments are added to the soil, a stimulation of different microflora such as bacteria, fungi and actinomycetes takes place and the relationship between plant-parasitic nematodes and these microorganisms is still not yet well understood. The objective of this study was to try to investigate the role that actinomycetes play in the reduction of plant-parasitic nematodes such as Pratylenchus penetrans and Meloidogyne incognita. The soil from the University of Massachusetts Farm in South Deerfield was selected because previous studies had shown that Pratylenchus penetrans and Meloidogyne incognita were absent in the area but could be found several hundred yards away.

MATERIALS AND METHODS

Soil Selection

In order to observe the residual effect of organic amendments, soil from two different sites (A and B) at the University farm in South Deerfield, Mass., was collected and analyzed for the presence of Pratylenchus penetrans. Site A had been previously (3 years before the soil for this experiment was collected) analyzed for lesion nematode and then treated with fresh mycelial waste

produced in the manufacture of organic acids and antibiotics by Charles Pfizer Company, Groton, Connecticut. The mycelial waste was spread on the surface at the rate of 200 tons/acre and disked in with a rotary tiller and left to decompose. Soil from site A was selected for isolation of actinomycetes because Pratylenchus penetrans populations were unable to survive in this soil (43). Site B was a nearby untreated area which supported large populations of Pratylenchus penetrans.

Soil Analysis for NO_3-N and NH_4-N

Before the additions of organic amendments, soils were analyzed for nitrogenous compounds and for actinomycetes. In order to provide a stock standard of KNO_3, 0.722 grams of analytical reagent grade of KNO_3 was dissolved in 500 ml of distilled water to give 200 ppm NO_3-N (200 ug NO_3-N) and diluted to provide 1 to 10,50,100 and 200 ppm NO_3-N. Similarly 0.476 grams $(NH_4)_2SO_4$ was dissolved in a liter of distilled water to make a standard stock solution of 100 ppm and was diluted to 5,10 and 50 ppm. In order to make the solutions basic, 1 ml of 1N NaOH was added to each dilution.

Twenty-five grams of air-dried soil from each pot was dissolved in 50 ml of distilled water and shaken on a wrist-action laboratory shaker for 30 minutes and then filtered through glass wool into 100 ml beaker collecting 30 to 40 ml of the filtrate. The filtrate was stirred mechanically at a constant, slow to moderate speed and

then a nitrate specific ion electrode was inserted into the beaker and readings were recorded. This apparatus consists of a nitrate ion electrode, a calomel reference electrode and a pH meter with an expended millivolt scale (3).

Isolation of Actinomycetes

A. Preparation of colloidal chitin. Sixty grams of crab chitin (Sigma Chemical Company) were placed in a Waring blender at high speed for several minutes and then dissolved in 400 ml of concentrated HCl by stirring for 30 to 50 minutes. The chitin was precipitated as a colloidal suspension by adding it slowly to 2 liters of water at 5 to 10 C. The suspension was collected by filtration with suction on a coarse filter paper, washed by suspending it in about 5 liters of tap water, and then refiltered. The washing was repeated several times until most of the acid was removed. The suspension was pH 4. The aqueous suspension was stored at room temperature (14).

B. Medium for isolation from the soil. Sufficient volume of colloidal chitin suspension to give 4 g of chitin was mixed with the following mineral salts: K_2HPO_4 (0.7g), KH_2PO_4 (0.3g), $MgSO_4$ 5 H_2O (0.51g); $FeSO_4$ $7H_2O$ (0.01g); $ZnSO_4$ (0.001g); $MnCl_2$ (0.001g) and 20 g of agar in 1 liter of distilled water. After autoclaving the melted agar was adjusted to pH 8 with 1N NaOH.

One gram of soil before treatment was dissolved in

99 ml of sterile buffer (0.5 M K_2PO_4 adjusted with 1N NaOH to pH 8) and then shaken for 15 min on a wrist-action laboratory shaker. Ten ml of the suspension was transferred to 90 ml of sterile buffer. This procedure was repeated several times to make serial dilutions up to 10^{-5}. One milliliter aliquots of dilute soil suspension from all dilutions were placed in petri dishes (replicated 5 times) and melted and cooled agar (40 to 45 C) was mixed with the suspension by swirling. Plates were incubated for 7 to 10 days at 25 C when counts of actinomycetes were made with the aid of a colony counter. This procedure was used any time actinomycetes were isolated from amended soil (14).

Organic Amendments

After the soil was analyzed for nitrogenous compounds and actinomycetes, the soil was treated with dried waste mycelium waste (Chas. Pfizer Company, Groton, Connecticut), dehydrated sewage sludge (Holyoke and Amherst Sewage Plants) and crab chitin (Sigma Chemical Company) at the rate of 20 g/4" pot for all treatments. Each treatment was replicated 14 times. After two weeks the soil was again analyzed for NO_3-N, NH_4-N and actinomycetes using the procedures described agove, and at this time a 2-week old tomato (Lycopersicon esculentum) cv. Rutgers was planted in each pot. At the same time, seven pots from each treatment were inoculated with 800 Pratylenchus penetrans and

another seven were inoculated with equal volumes of Meloidogyne incognita eggs. After two weeks, 3 g of roots from each plant infected with Pratylenchus penetrans were washed clean of soil, cut in 1 cm pieces and placed in 100 ml jars with 5 ml of tap water and tightly capped. The nematodes emerging from roots were counted after 24 hours. Similarly plants inoculated with Meloidogyne incognita were harvested, examined for galls and indexed as described by Daulton and Nusbaum (10).

Nematode Cultures

A. Pratylenchus penetrans. Pratylenchus penetrans was cultured on alfalfa callus tissues using modified Krusberg's medium consisting of 1 g yeast extract, 20 grams sucrose (cane sugar), 2 mg 2,4-D, 10 g agar per liter of distilled water (22). Another population of this nematode was raised in the greenhouse on sunflower, Helianthus annus cv. Mammoth. Soil with a substantial population of Pratylenchus penetrans from the University farm in South Deerfield was brought to the greenhouse, potted and transplanted with one week old sunflower seedlings. Nematodes for both greenhouse and laboratory experiments were extracted from sunflower roots using Baerman funnels.

B. Meloidogyne incognita. Meloidogyne incognita was cultured in the greenhouse on tomato Lycopersicon esculentum cv. Rutgers. Two-week-old tomato plants were transplanted into 12" pots with soil containing root knot eggs. The culture was maintained for as long as the nematodes were

needed for both the greenhouse and laboratory experiments. Root knot extraction was done by using a modified method described by McClure et al. (32). About 15 g of tomato roots infected with Meloidogyne incognita were cut into small pieces and placed in an electric blender with 100 ml of tap water. The blender was operated at maximum speed for 40 seconds. The resulting suspension of eggs and debris was quickly poured into nested 100 and 325 mesh sieves and the eggs and some fine debris retained on 325 mesh were collected in tap water in a 250 ml beaker. Equal portions were then placed in 50 ml polycarbonate centrifuge tubes and then centrifuged at 1000 g for five minutes. The pellet, containing eggs plus minute debris, was resuspended in sucrose solution (500 g/liter) and centrifuged for 40 seconds. The supernatants were quickly poured through a 325 mesh sieve and eggs were washed several times with tap water and then added to tomato plants. Larvae for laboratory experiments were obtained from eggs and kept overnight at room temperature.

Nematicidal Activity

A. Media for growth. Actinomycete isolates were cultured on either sucrose-nitrate or glucose-asparagine agar. The sucrose-nitrate medium contained: sucrose (30g), $NaNO_3$ (2.0), K_2HPO_4 (1.0 g), $MgSO_4 7H_2O$ (0.5 g), $FeSO_4$ (0.01 g) in a liter of distilled water and the pH was adjusted to 7.5. The glucose-asparagine agar contained glucose (10 g), asparagine (0.5 g), K_2HPO_4 (0.5 g),

agar (15 g) and the pH was adjusted to 7.5. The slants were incubated at 23 C and transferred every 4 weeks.

B. Extraction Method. Actinomycetes isolates were each grown in the following medium on a reciprocal shaking machine: glucose 2.5%, 1% peptone, 1% brewer's yeast, 0.1% K_2HPO_4, 0.4% $CaCO_3$, 0.4% Kcl with the pH adjusted to 7.0 and incubated for 5 to 7 days at 30 C. Five hundred milliliters of each culture broth were filtered through Miracloth (R) with suction and were extracted with 500 ml of hot acetone and concentrated in vacuo to 150 ml (40). One hundred milliliters of ethyl acetate were added to the filtrate forming a layer which was then washed with 0.02N HCl, 0.06M solution $NaHCO_3$ and distilled water successively. Using a separatory funnel, the top layer was collected and taken to dryness in a rotary evaporator under reduced pressure at 50 C. The residues were dissolved in 20 ml of sterilized water and tested for activity.

Sometimes a modified method was used that was quicker although less efficient in yield of the extracts (41). The isolates were inoculated into 250 ml flasks each containing 100 ml of the following substances: soluble starch 2%, soytone 1.5%, brewer's yeast 0.25%, glycerin 0.5% and NaCl 0.25%. The flasks were incubated for 5 days at room temperature on a reciprocal shaker. The broths were filtered through the Miracloth, the filtrate was extracted with ethyl acetate and the mycelium was extracted with hot acetone. The acetone fraction was taken to dryness and then

dissolved in ethyl acetate. The two ethyl acetate fractions were combined and evaporated to dryness. The residues were dissolved in sterilized distilled water and this solution was tested for toxicity.

C. Toxicity to nematodes. In order to test for nematicidal activity, the extracts were diluted with water 1:1, 1:5 and 1:10 and sterilized distilled water served as the control. The dilutions plus the undiluted extract were placed in separate watch glasses. One hundred active Pratylenchus penetrans larvae and adults were added to each watch glass and observed at hourly intervals. The nematode was considered dead if it did not move when touched with a needle and if it did not show any mobility when placed in sterilized distilled water. This procedure was repeated for Meloidogyne incognita larvae which were collected from the eggs extracted using the method above.

Repellent Activity

Metabolites extracted from actinomycete cultures were then tested for repellent action against Pratylenchus penetrans. The method used was a slight modification from the one used by Lavallee and Rohde (24). Polystyrene chambers (46x22x5 mm), marked on the undersurface in 14 mm end sections and an 18 mm center, were filled with 1% water agar to the depth of about 5 mm. Using a number 3 cork borer, a well was made in each end section of the chamber and sealed with a drop of melted 1% water agar.

After gelation one well on each chamber was filled

with actinomycete metabolite and the other well on the opposite end was filled with distilled water. The treatments were replicated 5 times. The chambers were incubated for 24 hrs and then another well was made in the center section and similarly sealed at the bottom. Approximately 60 Pratylenchus penetrans from alfalfa callus tissues were added to the center well with a Pasteur capillary pipette.

After addition of nematodes to the center well, excess water was removed with a pipette and the well was filled with cool, but ungelled, 1% water agar. Observations were made every 4 hours after inoculation to see how many nematodes migrated to each end section. Nematodes were considered repelled if they moved toward the water end of the chamber and attracted if they moved toward the extract end of the chamber (24).

RESULTS

Residual effects of waste mycelium were apparent in that Pratylenchus penetrans was unable to reproduce in Site A soil (Table 1). This site had been treated with waste mycelium three years previously. Adjacent untreated plots supported normal reproduction of the nematode.

When raw and dehydrated sewage sludges were compared as soil amendments to control Meloidogyne incognita (Table 2), there was no significant difference between the two of them. However, there were fewer galls on tomato plants

in either treated soil than in untreated soil. Similarly there was no significant difference in heights between plants in raw and dehydrated sewage-sludge treated soil. Plants in untreated soil were taller (Table 3). Both raw and dehydrated sludge severely reduced the dry weight of tomato plants (Table 4). In some cases the plants were half the size of those grown in the untreated soil.

When the soil was treated with chitin, waste mycelium, and sewage sludge, chitin and sewage sludge significantly reduced galling on tomato. Chitin was significantly more effective than sludge (Table 5). The content of both NO_3-N and NH_4-N were higher in chitin treated soil than in all other treatments both times that the readings were taken (Tables 6 and 7). The increase in NO_3-N and NH_4-N was still evident four weeks after the addition of organic amendments. After 4 weeks NH_4-N could no longer be detected in sewage-treated soil (Table 7).

The effect of the organic amendments on other micro-organisms was observed by culturing the soil on chitin medium before and two weeks after the addition of amendments. All treatments significantly stimulated the growth of actinomycetes. Chitin was more effective than other treatments (Table 8). The actinomycete population was correlated with the suppression of both Meloidogyne incognita and Pratylenchus penetrans. Fewer Pratylenchus penetrans were recovered from chitin and waste mycelium treated soil than sewage sludge treated and untreated soil (Table 9). However,

irradiated digested sludge from Boston's Deer Island wastewater treatment plant had a different effect on Pratylenchus penetrans. While the number of actinomycetes colonies increased in sludge treated plants compared to ones treated with mineral fertilizer, fewer numbers of Pratylenchus penetrans were recovered in mineral fertilizer treated plots than sewage-sludge treated plots (Table 10).

Since the reduction of both lesion and root-knot nematodes was accompanied by the increase of actinomycete populations, sixty different actinomycetes colonies were isolated from all the treatments plus the control. The majority of the isolates came from chitin treated soil and the next largest number from waste mycelium treated soil. Metabolites were extracted from liquid medium cultures of each isolate and tested for nematicidal activity. One isolate from chitin treated soil yielded a metabolite which was able to immobilize both lesion and root-knot nematodes. Nematodes were considered immobile if they did not move when touched with a needle. The undiluted metabolite immobilized lesion nematodes within 3 hours, whereas a 1:10 dilution in distilled water took 7 hours to inactivate the nematodes (Fig. 1). A similar toxic effect was observed against root-knot nematode larvae. However, no significant difference was observed between root-knot larvae and lesion nematode in 1:10 dilution (Fig. 2). After 100 of each lesion and root-knot nematodes were separately placed in a 1:10 dilution of the metabolite for

6 hrs and then transferred to aerated distilled water only a few recovered. On the other hand, if they were kept for 24 hr in the metabolite, they did not recover (Fig. 3).

Freshly extracted meatbolite was tested for ability to repel and attract nematodes. Either sixty lesion nematodes or 60 root-knot larvae were placed in the center well of each chamber. One end of the chamber had been treated with the crude metabolite 24 hours before the nematodes were added. No repellent activity was observed and nematodes did not move away from the treated end. Similarly no nematodes were seen moving toward the metabolite suggesting no apparent attraction.

DISCUSSION

The decrease in plant parasitic nematode populations which often occurs after addition of various organic matter has been well documented (25,26,28). Habicht (13), using raw and composted sewage sludge, reduced galling on tomato plants. He found raw sludge to be more effective than composted and attributed the nematicidal activity to be related to chemicals like ammonia salts or organic acids released during sludge decomposition. His results support those of Miller et al. (34) who reported a reduction of Tylenchorhynchus dubius and Pratylenchus penetrans and other soil-borne pathogens such as Fusarium and Rhizoctonia when they added to soil waste mycelium from the commercial

Table 1. Residual effects of waste mycelium on the population densities of Pratylenchus penetrans. Site A had been treated 3 years previously and site B adjacent to A remained untreated.

	Sites	
	A (treated)	B (untreated)
Replicates	Numbers of nematodes/50g soil	
1	0	38
2	0	26
3	0	24
4	0	54
5	0	40
6	0	89
7	0	30
Total	0	300
Mean	0	42.86**

**Indicates significantly effect at P = 0.01 level.

Table 2. The effects of sewage sludge on *Meloidogyne incognita* on tomato plants.

Replicates	Root Knot Index(a) Treatments 20g/pot Raw	Dehydrated	Untreated
1	3	1	6
2	2	1	4
3	3	2	5
4	4	3	6
5	5	2	5
6	4	2	5
7	4	3	4
Total	25	14	35
Mean	3.6*	2*	5

*Indicated significant control at P = 0.05 level than the untreated.

(a) Infection class	Index value	Description of index value
0	0	Free from galls
1	1	Trace, less than 5 galls/ plant
2	5	Very slight, trace to 25 galls
3	10	Slight, 26 to 100 galls
4	25	Moderate, galls numerous, mostly discrete
5	50	Moderately heavy, galls numerous, many coalesced
6	75	Heavy galls very numerous, mostly coalesced, root growth retarded

Table 3. The effects of sewage sludge (20 g/4" pot) on the height of tomato plants inoculated with *Meloidogyne incognita*.

Replicates	Treatments (heights, cm) Raw	Dehydrated	Untreated
1	15	17	25
2	17	17	26
3	20	17	26
4	18	16	31
5	17	20	45
6	16	18	30
7	17	17	35
Total	120	122	218
Mean	17.14	17.43	21.14*

*Significantly different at P = 0.05 level than the treated.

Table 4. Dry weight of tomato plants (g) grown in sewage sludge amended soil and inoculated with *Meloidogyne incognita*.

Replicate	Treatments (20g/pot) Raw	Dehydrated	Untreated
1	0.25	0.09	2.50
2	0.25	0.1	1.25
3	0.5	0.2	2.0
4	0.05	0.1	1.0
5	0.1	0.2	4.9
6	0.25	0.2	1.5
7	0.25	0.5	1.5
Total	1.65	1.39	14.65
Mean	0.235	0.198	2.093*

*Significantly different at P = 0.05 level than the treated.

Table 5. Influence of organic amendments (20g/4" pot) on gall formation on tomato plants by Meloidogyne incognita after two weeks incubation in the greenhouse.

	Root Knot Index			
Replicates	Sewage sludge	Chitin	Waste mycelium	Untreated
1	1	1	1	2
2	0	0	1	3
3	2	0	1	1
4	0	0	1	1
5	0	0	2	3
6	0	0	0	2
7	0	1	1	2
Total	3	2	7	14
Mean	.429*	.286**	1*	2

*Indicates significant difference from the untreated at P = 0.05 level.

**Indicates significant difference from the untreated at P = 0.01 level.

Table 6. The effects of various organic amendments (20 g/ 4" pot) on NO_3-N and NH_4-N content in the soil two weeks after they were added.

Treatments			
Waste mycelium	Chitin	Sewage sludge	Untreated
	NO_3-N (ppm)		
64 ppm	250 ppm	100 ppm	10ppm
55 ppm	250 ppm	80 ppm	11.5 ppm
94 ppm	255 ppm	78 ppm	12 ppm
	NH_4-N ppm		
27 ppm	220 ppm	20 ppm	5 ppm
25 ppm	170 ppm	11 ppm	6 ppm
25 ppm	150 ppm	10 ppm	6 ppm

Table 7. The effects of various organic amendments (20g/ 4" pot) on NO_3-N and NH_4-N content in the soil four weeks after they were added.

Treatments			
Waste mycelium	Chitin	Sewage Sludge	Untreated
	NO_3-N (ppm)		
110 ppm	115 ppm	91 ppm	100 ppm
110 ppm	115 ppm	98 ppm	82 ppm
110 ppm	115 ppm	81 ppm	86 ppm
	NH_4-N (ppm)		
40 ppm	115 ppm	0	0
50 ppm	125 ppm	0	0
0	155 ppm	0	0

Table 8. Numbers of colonies ($X10^5$/g soil) of actinomycetes detected two weeks after the addition of organic amendments at the rates 20g/4" pot.

Replicates	Untreated		Treatments		
	Initial count	2 wks later	Sewage sludge	Chitin	Mycelium
1	3	4	32	105	21
2	3	9	37	91	19
3	2	6	31	85	20
4	2	7	36	105	27
5	2	4	35	101	15
6	4	5	37	77	18
7	3	9	40	81	22
Total	21	44	248	644	142
Mean	3	6.29	35.43*	92**	20.29*

*Significantly effective at P = 0.05.

**Significantly effective at P = 0.01.

Table 9. Numbers of lesion nematodes Pratylenchus penetrans in tomato plant roots (3 g root/replicate) grown in organic-amendment (20g/4" pot) soils.

	Treatments			
Replicates	Untreated	Sewage sludge	Chitin	Mycelium
1	89	34	11	4
2	66	42	11	10
3	67	24	6	17
4	150	24	10	16
5	163	21	10	13
6	189	21	13	12
7	101	25	11	14
Total	825	191	72	86
Mean	117.86	27.29*	10.29*	12.19**

*Significantly effective at P = 0.05.

**Significantly effective at P = 0.01.

Table 10. The effects of irradiated digested sewage sludge on actinomycetes and Pratylenchus penetrans.

	Treatments		
	0X	1X	2X[a]
Nematodes/150g soil	16	3	1
	2	26	1
	0	5	2
Mean	6	11.3	1.3
Actinomycetes ($X10^5$/g soil)	7	8	12
	8	9	17
	8	9	38
	7.6	8.6	22.3

(a) 0X = Fertilizer treatment or normal culture
1X = 340 gal/plot (plot = 600 sq. ft.)
2X = 690 gal/plot
organic matter content = 4%
From Boston's Deer Island Wastewater Treatment Plant

FIG.1. Survival of Pratylenchus penetrans in different dilutions of Actinomycetes Extract

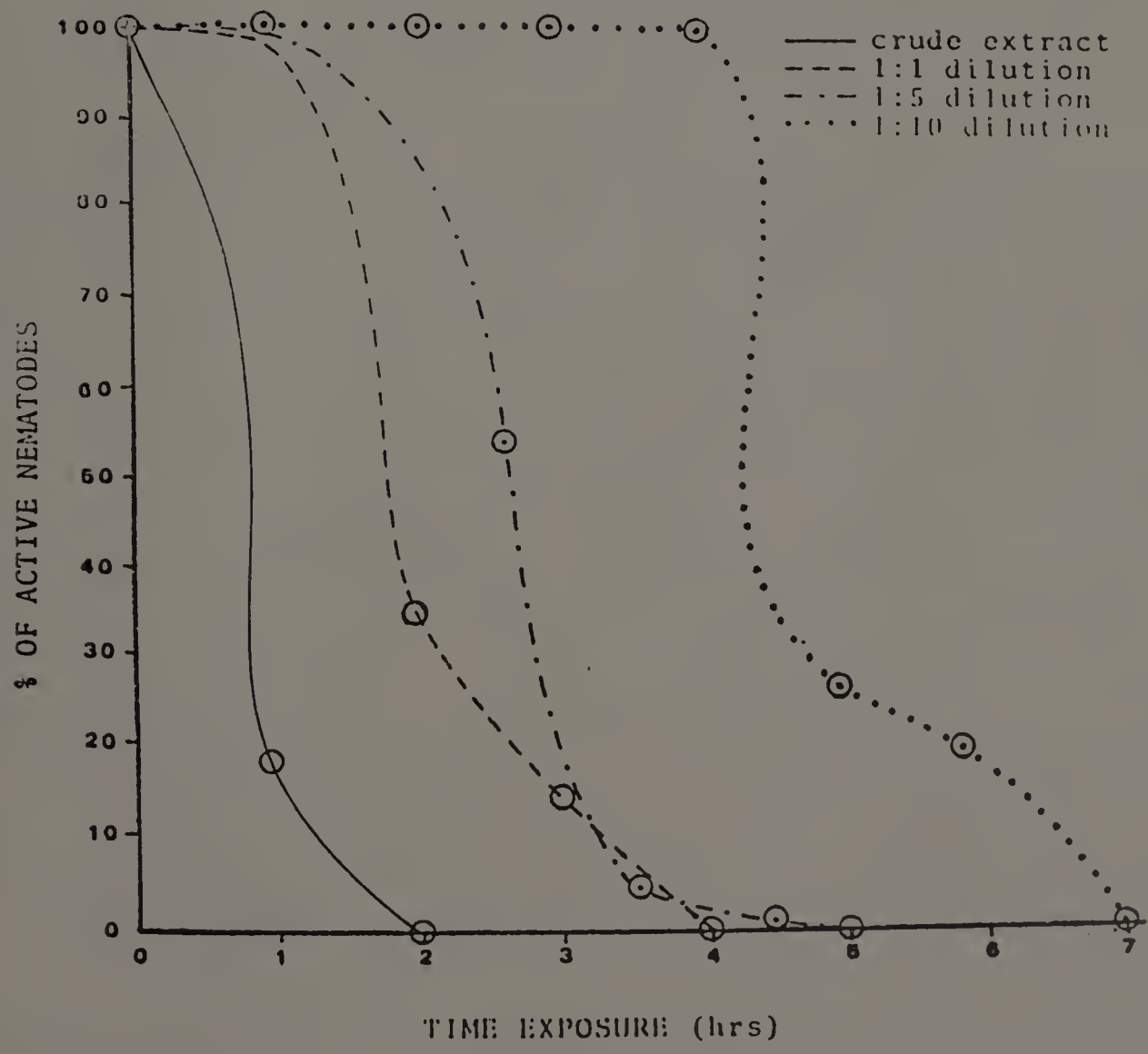


FIG.2. Survival of Meloidogyne incognita in different dilutions of Actinomycetes Extract

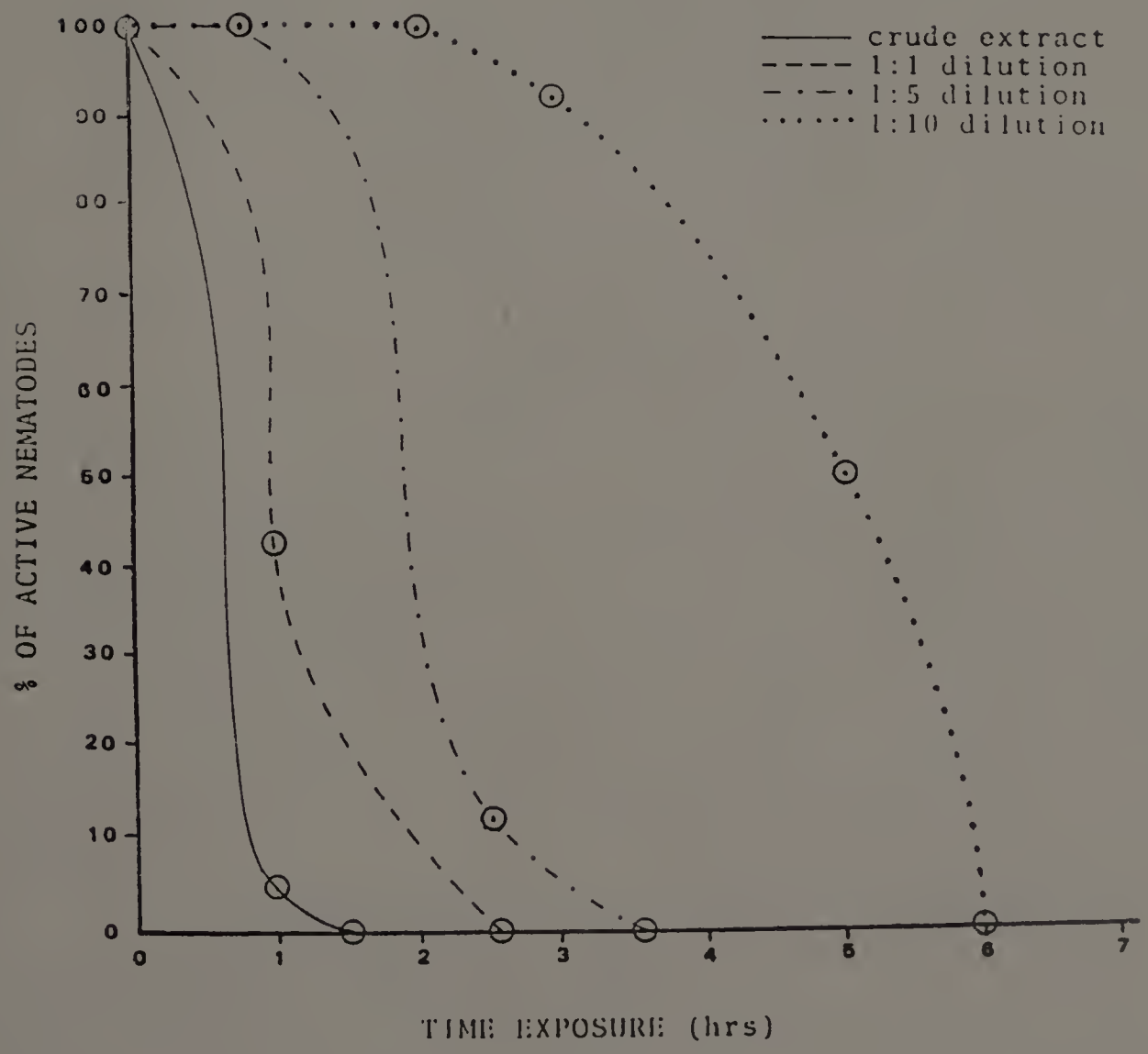
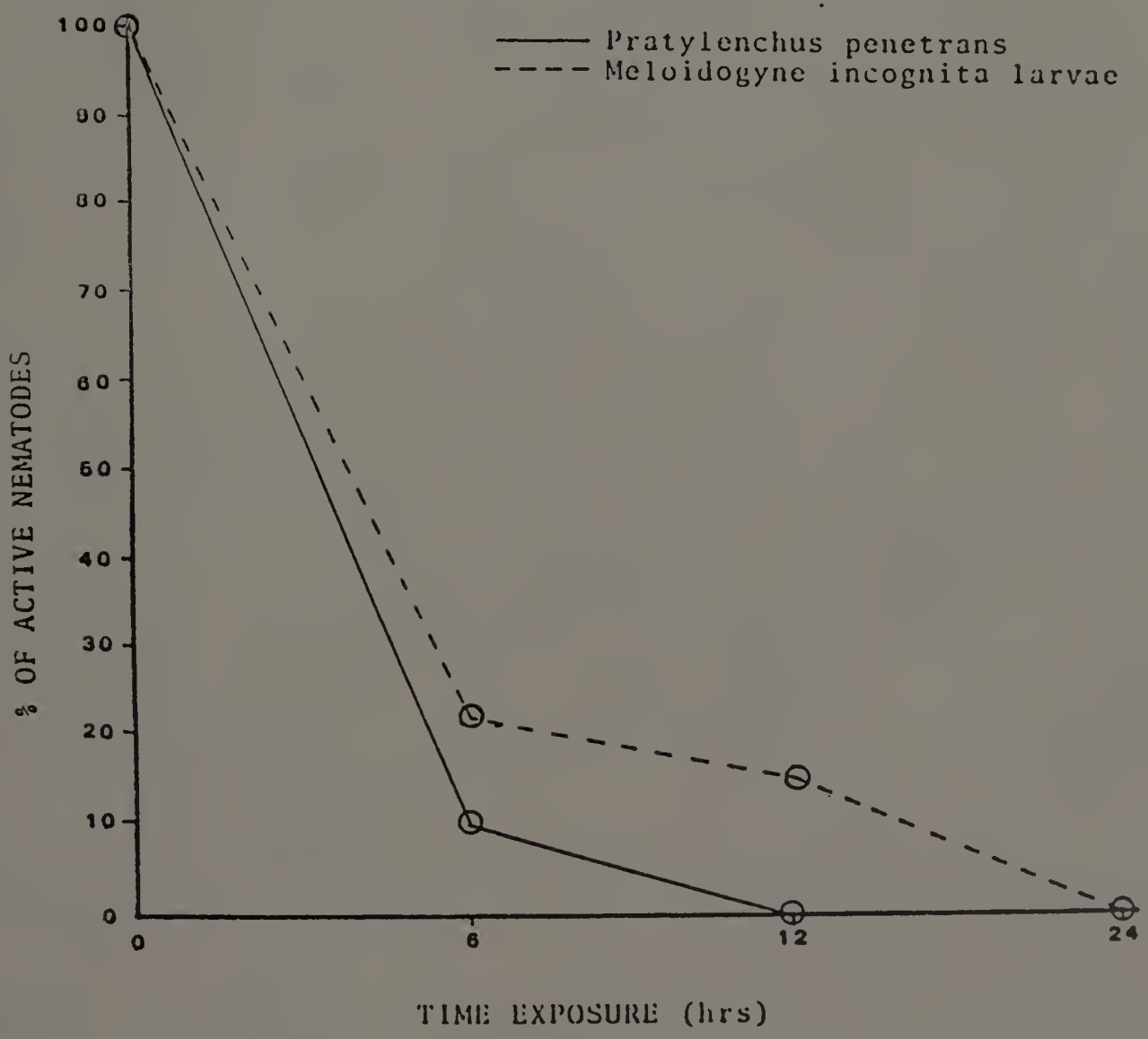


FIG.3. Recovery of Pratylenchus penetrans and Meloidogyne incognita larvae from 1:10 dilution of Actinomycetes Extract after 6, 12, and 24 hours



production of antibiotics and citric acid, cellulosic wastes from paper factory, chitin and mixtures of the cellulosic wastes and chitin. Although they did not measure the amount of ammonia in the soil, they speculated that ammonia,released during breakdown of the organic amendments, may have been toxic to soil nematodes and fungi.

As shown in Table 2, galling is markedly reduced by sewage sludge with no significant difference between raw and digested sludge. These results, therefore, support Habicht's findings. Usually the main effect of the organic amendments is the addition of nutrients to the soil. Although sludge reduced galling on tomato plants, it severely reduced the dry weight and height of the plants (Tables 3 and 4). Similar effects were reported by Habicht (13) who used sewage sludge from the Washington, D.C. area and thought that the adverse effects might have been caused by water retention, breakdown products and heavy metals. The sludge used in this experiment came from Amherst and Holyoke areas which are relatively free of heavy industries, and the only substances added during sewage sludge treatment in these areas are 1-½% ferric chloride and 3% lime. These are added to the sludge after it has undergone a slow anaerobic decomposition in which methane bacteria are most active. When the sludge gets into the vacuum filter, lime is added to adjust the pH to 7 in order to promote aerobic decomposition. Ferric chloride is then added to flocculate the sludge into a cake.

The adverse effects on the dry weight and height of the plants may have been caused by poor drainage, which was evident in the 4" pots which were used (4) and by excessively high chloride concentrations.

The microbial breakdown of nitrogen-containing substances in the soil through the process of ammonification (mineralization) and nitrification releases ammonia, ammonium ions, nitrites and nitrates (49). During ammonification, the nitrogenous compounds synthesized by plants serve as the nitrogen source for most animals. During the assimilation of these compounds, complex nitrogenous compounds are hydrolyzed but the nitrogen remains largely in reduced forms in compounds such as amino acids and proteins. Whenever a plant or animal dies its body constituents are immediately attacked by microorganisms with the liberation of ammonia and some of the nitrogen is assimilated by the microorganisms themselves. The ammonia is then converted to nitrate during nitrification which is brought about by two highly specialized groups of obligately aerobic chemoautrophic bacteria, Nitromonas and Nitrobacter. In the first step ammonia is oxidized to nitrite which is then converted to nitrate (49).

Several workers have found a sharp decrease of nematode populations where NH_3 levels in soil reach 400 ppm Eno et al. (12) and Walker (53). In their experiments, nitrite and ammonium nitrogen levels of about 500 ppm were detected from the soil within 14 days. As shown in

Tables 6 and 7, the concentrations in all soils of nitrate and ammonia nitrogen in the present study were relatively low compared to that reported by Eno *et al*. (12) and Walker (54) suggesting that ammonia and other nitrogenous compounds did not play a major role in the reduction of populations of *Meloidogyne incognita* and *Pratylenchus penetrans*.

Antibiosis, an important concept of biological control of disease, is based on the assumption that the pathogen can be inhibited by other organisms already present in the soil and that these microorganisms can be selectively stimulated by the addition of organic amendments (26,37). Using this concept, Marshall and Alexander demonstrated that *Agrobacterium radiobacter* could reduce drastically the growth of *Fusarium oxysporum* f. *cubense*; the competition was enhanced by glucose amendment of the soil. The bacterium limits the fungus growth by immobilizing soil nitrogen when the supply of carbon is very high (31). Using chitin, as a soil amendment, Mitchell and Alexander (35) observed a sharp increase of actinomycetes and chitinase producers accompanied by a decline of disease symptoms of *Fusarium solani* f. *phaseoli*. More recently, Dugan (11) reported similar observations when he amended soil with lobster shells to control bean root rot caused by *Fusarium solani*.

The data in Tables 9 and 10 are in agreement with those of Mitchell and Alexander (35) and Dugan (11). There

was a sharp increase of actinomycetes in all amended soils which supports the hypothesis that there is a selective stimulation of specific microflora when organic amendments are added to the soil. These results show that chitin is more effective in both controlling nematodes and in activating actinomycetes than are waste mycelium and sewage sludge. This relationship between chitin decomposition and increase in actinomycete activity is not unexpected since chitin has been reported to selectively increase this group of microorganisms. (14)

Since the suppression of plant-parasitic nematodes was accompanied by a sharp rise in numbers of actinomycetes, studies were undertaken to determine the relationship between these two groups of microorganisms. Crude extracts from one actinomycete isolate proved to be nematoxic even at 1:10 dilution. The idea of isolating nematicidal metabolites from actinomycetes has been reported by Mori (40) and by Walker *et al*. (52). Mori was able to isolate four strains of actinomycetes which produced active compounds toxic to the free-living nematodes *Rhabditis* sp. and *Panagrellus redivious* at 10 ppm (40). Walker et al (52) obtained some fluids from four isolates of *Streptomyces* sp. and two isolates of *Bacillus* and found them to be nematicidal (in vitro) to adults and larvae of *Pratylenchus penetrans*. Fluids from bacterial cultures were more nematicidal than those from actinomycetes and the degree of activity was dependent upon both temperature and duration of incubation.

The results in the present study (Figs. 1 and 2) are in agreement with those reported by both workers in that extracts from some actinomycetes are nematoxic to plant-parasitic nematodes although in all these studies the mode of action of these metabolites still remains unknown.

Katznelson and Henderson (19) found the influence of actinomycetes and fungi isolated from soil and from plants on Rhabditis oxycera de man, a bacteria-feeding nematode, was that of attraction. They observed that striking accumulation of nematodes occurred in the vicinity of colonies of certain of these organisms or agar media. This attraction was thought to be possibly due to an optimal CO_2-O_2 balance or elaboration of metabolic by-products which are attractive to nematodes as are root diffusates for certain plant-parasitic nematodes (19). The metabolite extracted in the present study did not influence movement of nematodes. No nematodes were seen moving through agar toward the well that had been filled with the metabolite 24 hrs previously or moving in the opposite direction toward the well with distilled water. They remained in the center well where they were initially placed suggesting the absence of either attraction or repulsion.

Although nematode mortality in organically amended soil has been attributed to be the result of unfavorable pH (48,54) or the release of toxic substances from the decomposition of organic residues, the results here suggest that there is antagonistic interaction between plant-

parasitic nematodes Meloidogyne incognita and Pratylenchus penetrans in which actinomycetes produce a metabolite which is toxic to these nematodes. This toxin probably has a synergistic effect with nitrogenous compounds such as ammonia and nitrites. Once the nature of such metabolites and their activities are determined, the knowledge of ecology and population dynamics of soilborne plant-parasitic nematodes, currently in its early stages, will probably be better understood.

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APPENDIX

The analysis of variance as presented by Steel and Torrie was used to test differences between treatments. F values were compared with those of Steel and Torrie. Orthogonal polynomial was used for comparison between treatments. To clarify the use of this method the following example is given. (55)

Total Sum $\sum_i \sum_y$ 1099

Total uncorrected ss $\sum_i \sum_j Y_{ij}^2 = 72205$

Total ss = $\sum_i \sum_j Y_{ij}^2 - \frac{(\sum_i \sum_j Y_{ij})^2}{ni}$

$$72205 - \frac{(1099)^2}{35} = 72205 - 34508.6 = 37699.4$$

$$\text{Treatment ss} = \frac{(21)^2 + (44)^2 + (142)^2 + (644)^2 + (248)^2}{7} - \frac{(1099)^2}{35}$$

$$= 68034.285 - 34508.6 = 33525.685$$

Within ss = Total ss - Trt ss = 37699.4 - 33525.685 = 4173.715

Analysis of variance.

Source	df	ss	ms	f
Total	35	37699.4	1077.126	8.0000
Group	4	33525.685	8381.421	62.2510
Within	31	4173.715	134.639	

Partition of ss among treatments

$$Levels_{ss} = \frac{(T_1)^2+(T_2)^2+(T_s)^2+(T_c)^2+(T_m)^2-(_1+T_2+T_s+T_c+T_m)^2}{r_1\ r_2\ r_s\ r_c\ r_m \qquad\qquad r_1+\ r_2+\ r_s+\ r_c+\ r_m}$$

$$L_{ss} = \frac{(21)^2+\ (44)^2\ +\ (248)^2\ +\ (644)^2+\ (142)^2}{7} - \frac{(1099)^2}{35}$$

$$L_{ss} = 71254.43 - 34508.6 = 36745.83$$

Comparison between control vs treatment

$$O_1 = -1(T_s+\ T_c+\ T_m)\ \ +\ 3\ (T_c\ )$$

$$D\ \ = 7(1)^2\ +\ (1)^2\ +\ (1)^2\ +\ (3)^2$$

$$O_1 = (-1034\ +63)$$

$$D_1 = 7\ (12)$$

$$O_L^2 = \frac{(-971)^2}{84} = 11224.30$$

Comparison between sewage sludge, mycelium and chitin.

$$O_1 = T_c - \frac{T_s + T_m}{2}$$

$$O_1 = (1)\ (644) - (\tfrac{1}{2})\ (248) + \tfrac{1}{2}\ (142)$$
$$644 - \ 195$$

$$D_1 = 7\ (1)^2 + (\tfrac{1}{2})^2 + (\tfrac{1}{2})^2\ \ = 7\ (1.5) = 10.5$$

$$SS_{O_j} = \frac{O_j^2}{D_j} = \frac{499}{10.5} = 42.76$$

Analysis of variance.

Source	df	ss	ms	F
Treatment	4	33525.685	8381.421	62.25
Levels	1	36745.83	36745.83	272.9
Control vs trt	1	11224.30	11224.30	84.36
Chitin vs sewage/mycelium	2	42.76	21.38	1.567
Within	31	4173.715	134.639	