Journal of Nematology 24(4):522–527. 1992.

The Role of Microbes Associated with Chicken Litter in the Suppression of *Meloidogyne arenaria*[1]

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Abstract: The role of microbes associated with chicken litter in the suppression of *Meloidogyne arenaria* in amended soil was investigated. Amended soil treatments were prepared, including combinations of sterile and nonsterile chicken litter and soil. Microbial biomass in different treatments was compared by measuring carbon dioxide evolution. There was less CO_2 evolved in sterile litter than in nonsterile litter treatments. Tomato seedlings cv. Rutgers were transplanted into soil mixtures and inoculated with 2,000 *M. arenaria* eggs. After 10 days, fewer second-stage juveniles (J2) had penetrated the roots in soils amended with nonsterile litter than sterile litter. The effects of sterile and nonsterile litter-amended soil solutions on *M. arenaria* eggs and J2 were observed over a period of 6 days. A lower percentage of eggs remained apparently healthy in nonsterile than in sterile-amended soil solutions over 6 days. Microbial degradation of the egg shells was apparent. Fewer J2 survived in sterile- and nonsterile-amended-soil solutions as compared to water controls.

Key words: biological control, chicken litter, chitinase, *Lycopersicon esculentum, Meloidogyne arenaria,* microbe, nematode, organic amendment, tomato.

The addition of organic matter to soil to improve soil fertility and increase crop yield is an ancient concept (13). Research now suggests that the addition of many of these materials, particularly those high in nitrogen, may be effective alternatives to nematicides for control of *Meloidogyne arenaria* (Neal) Chitwood and other plant-parasitic nematodes (9,13). The addition of chicken litter to soil suppresses *Meloidogyne* spp., limits root galling caused by the nematode, and stimulates plant growth (11). The crop-management benefits and widespread availability of poultry litter make it of great potential use in low-input, sustainable agriculture programs.

Two hypotheses have been suggested to explain the mode of action of soil amendments in terms of nematode control (9,10, 15). These are as follows: i) the amendment or its decomposition products are directly toxic; or ii) the amendment alters the soil environment so as to favor competing microbial populations, mycoflora capable of parasitizing nematode eggs, or other soilborne antagonists that destroy or weaken these plant parasites.

Although previous studies have shown that ammoniacal nitrogen from inorganic fertilizers and organic sources, such as chicken litter, is an effective nematode suppressant (7,13), the role of microbes associated with the litter in causing this suppression is not known. The purpose of this study was to investigate the role of microbes in the suppression of *M. arenaria* in chicken-litter-amended soils.

Materials and Methods

Glass assemblies were designed to maintain a controlled environment for sterile and nonsterile soil treatments (Fig. 1). Glass cylinders (52 × 200 mm) were covered by an inverted, 250-ml glass beaker that was raised slightly off the cylinder top to allow gas exchange. Thirty grams of sand were placed in the bottom of each assembly for drainage. Four treatments were used: sterile litter/sterile soil, sterile litter/nonsterile soil, nonsterile litter/sterile soil, nonsterile litter/nonsterile soil. A greenhouse soil mix (82% sand, 10% silt, 8% clay) and chicken litter were sterilized by methyl bromide fumigation (1.7 kg a.i./m^3). The soil used in this study had been previously fumigated and stored outside in a soil bin. This fumigated soil and litter were serially diluted in a phosphate buffer

Received for publication 8 January 1992.
[1] This research was supported by state and Hatch funds allocated to the Georgia Agricultural Experiment Stations.
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The authors thank W. T. Holladay and J. Billingsley for their assistance.

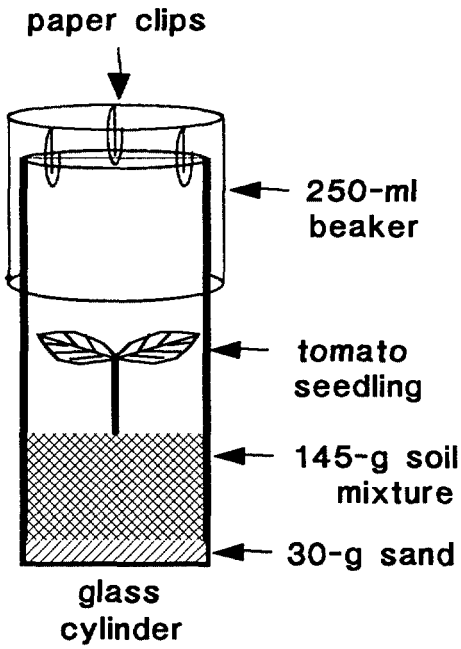

FIG. 1. Diagram of glass assembly used to maintain sterile growing conditions for soil and chicken litter amendments.

and placed on nutrient agar plus nystatin, rose bengal agar, and starch casein agar at 30 C for 5 days to confirm the absence of bacteria, actinomycetes, and fungi, respectively. The soil was amended with three rates of litter ranging from 0.25 to 1.5% (w/w), and nonamended soil was the control. The soil in the assemblies was brought to 16% moisture with sterile deionized water 2 days before planting and inoculation. Eight replicates of each treatment were prepared.

To measure microbial activity before transplanting and inoculation, four replicate glass assemblies of each treatment were sealed to restrict gas exchange, and carbon dioxide evolution was measured. A CO_2 trapping method devised by Cheng and Coleman (3) was modified by substituting barium hydroxide for sodium hydroxide. The amount of CO_2 evolved was determined by titrating the amount of $Ba(OH)_2$ that remained in the trap after 24 hours with HCl using a phenolphthalein pH color indicator.

Lycopersicon esculentum Mill. cv. Rutgers seeds were rinsed for 1 minute in 95% ethanol and surface-disinfested for 5 minutes in 1.5% NaOCl. Residual NaOCl was rinsed from the seeds with sterile deionized water. Surface-disinfested seeds were germinated on water agar for 3 days, and aseptic seedlings were transplanted into sterile vermiculite covered by a slightly raised piece of plate glass. After 7 days in vermiculite, one seedling was transplanted into each glass assembly.

Meloidogyne arenaria eggs were collected from infected tomato roots with 0.5% NaOCl (6). After collection, NaOCl was rinsed from the eggs with sterile deionized water. Each plant was inoculated with 2,000 *M. arenaria* eggs by pipetting a 1-ml egg suspension onto the soil surface. Glass assemblies were arranged in a randomized complete block design on a light room bench maintained at day and night temperatures of 30 and 26 C, respectively. Ten days after inoculation (185 degree days, base 10 C), tomato stems were cut at the soil line, roots were washed free of soil, and the fresh root weights were recorded. Roots were cut into 1- to 2-cm lengths, and the nematodes were stained with acid fuschin (1) and counted. The experiments were repeated. Root penetration was expressed as the percentage of nematodes that penetrated the root, per gram root fresh weight, relative to those observed in the roots of plants grown in nonamended soil.

To investigate the nematode suppressive action of chicken litter and the role of associated microbes, the effects of sterile and nonsterile litter-amended-soil solutions on *M. arenaria* eggs and second-stage juveniles (J2) were observed. The previously described greenhouse soil mix was amended with three rates of chicken litter (0.5, 1.0 and 1.5% [w/w]). Combinations of sterile and nonsterile soil and litter were used as previously described. Nonamended control soils and sterile deionized water were included as controls. Soil mixtures were brought to a 14% moisture level with sterile deionized water and allowed to stand on laboratory benches for

48 hours. Soil solutions were extracted from the amended and nonamended soil mixtures using a centrifugation method (5,12). Half of each extractant was filter-sterilized using a 0.22-μm Millex-GS filter (Millipore Corp., Bedford, MA). Equal volumes of sterile and nonsterile soil solutions were pipetted into six-welled flat-bottom tissue culture plates (surface area per well = 9.6 cm^2; Beckton Dickson Labware, Lincoln Park, NJ) in completely randomized designs for both the egg and juvenile studies.

Approximately 100 *M. arenaria* eggs, collected with 0.5% NaOCl (6), were added to each well. The initial number of eggs and J2 added to each well were counted. Each plate was wrapped in a moist towel and kept in a sealed plastic bag to minimize the evaporative loss of the soil solutions. The culture plates were shaken on an orbital shaker at 115 rpm to aerate the solutions. At 1, 2, 4, and 6 days after adding the eggs, each well was observed for the number of eggs that appeared to be free of microorganisms and remained intact in each solution, and the number of hatched J2. Percentage of eggs hatched (% hatch) by each sampling date (D) was calculated as $\%\ \text{hatch}_D = ((J2_D - J2_0)/\text{egg}_0) \times 100$, where $J2_D$ is the number of J2 observed at a given sampling date, $J2_0$ is the number of J2 counted at day zero, and egg_0 is the number of eggs counted at day zero. Percentage of eggs remaining (% egg) in each of the solutions was calculated as $\%\ \text{egg}_D = (\text{egg}_D/\text{egg}_0) \times 100$, where egg_D is the number of intact, microbe-free eggs observed at a given sampling date (D). On day six, the pH of each treatment was recorded. The experiment was repeated.

Approximately 100 *M. arenaria* J2, collected from infected Rutgers tomato roots on a mist extractor (2), also were pipetted into treatment wells in tissue-culture plates. The number of J2 in each well was recorded. Plates with the J2 suspensions were prepared and maintained as described for the egg hatch study. At 1, 2, 4, and 6 days after adding the J2 to the soil solutions, both the number of dead (not moving even when probed) and living (moving, or moving when probed) J2 were recorded. Percentages of surviving J2 (% J2) were calculated at each sampling date (D) as $\%\ J2_D = (J2_D/J2_0) \times 100$, where $J2_D$ is the number of living J2 observed at a given sampling date. The experiment was repeated.

Data were analyzed for all experiments by ANOVA, and means were separated by Duncan's multiple-range test (14). Data from repeated experiments were combined for presentation where there were no significant interactions. All differences reported in the results were significant at the $P \leq 0.05$ level.

Results

No differences were observed between the sterile and nonsterile soil treatments, and there were no interactions between soil and litter treatments. Results are presented for sterile versus nonsterile litter, with soil treatments combined.

The number of *M. arenaria* in roots grown in soil amended with nonsterile litter (405 J2/g root) was only 55% of the number found in roots of nonamended controls (734 J2/g root), whereas amendment with sterile litter did not affect numbers of nematodes (93% of nonamended controls, 683 J2/g root). There was a response to increasing percentage of litter (w/w) only among the nonsterile litter treatments (Fig. 2A). A decreasing trend in *M. arenaria* penetration in response to increasing sterile litter percentages was observed, but the regression was not significant. The mg CO_2 evolved in 24 hours increased as a linear function of litter percentage in both the sterile and nonsterile litter treatments (Fig. 2B). There was more CO_2 evolved overall in nonsterile than in sterile litter treatments (3.7 mg nonsterile vs. 3.1 mg sterile).

There were no differences among the percentages of eggs that hatched in water, and in sterile and nonsterile litter-amended soil solutions. The percentages of apparently healthy eggs remaining in the nonsterile 1.5% amended soil solutions

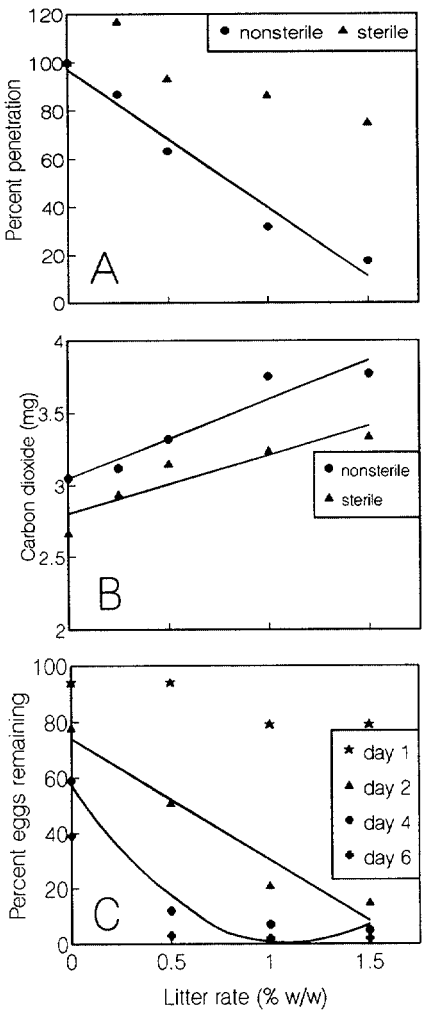

FIG. 2. Responses to increasing percentages of chicken litter soil amendment. A) *Meloidogyne arenaria* penetration per gram root fresh weight in nonsterile and sterile litter-amended treatments as a percentage of the number of nematodes that penetrated the roots of tomatoes in nonamended soil. Linear regression indicated a response in nonsterile litter treatments of $y = 97 - 59x$, $R^2 = 0.38$, $P = 0.0001$. Although a decreasing trend was observed in sterile litter treatments, regression was not significant ($P = 0.05$). Data are means of 16 replicates. B) Total mg CO_2 evolved within 24 hours in soil amended with increasing rates of sterile and nonsterile chicken litter. Linear regression for nonsterile litter treatments indicated a response of $y = 3.05 + 0.57x$, $R^2 = 0.57$, $P = 0.0001$. Linear regression for sterile litter treatments indicated a response of $y = 2.8 + 0.39x$, $R^2 = 0.35$, $P = 0.0001$. Data are means of 16 replicates. C) Percentage of *M. arenaria* eggs remaining in nonsterile litter-amended soil solutions over a period of 6 days. Linear regression at day two indicated a response of $y = 74 - 44x$, $R^2 = 0.61$, $P = 0.0001$. A quadratic regression model, $y = 57 - 103x + 46x^2$, $R^2 = 0.77$, $P = 0.0001$, described the rate response at day four. Data are means of eight replicates.

were lower than in the sterile 1.5% amended soil solutions and in sterile deionized water controls after 1, 2, 4, and 6 days (Fig. 3A). No difference was observed between the percentage of eggs remaining in the sterile 1.5% litter-amended soil solutions and in the water controls until day four. Similar trends were observed for other amendment percentages (data not shown). A response to increasing rates of amendment occurred only among the nonsterile litter-amended soil solutions (Fig. 2C). Eggs were observed in close association with unidentified fungal and bacterial microorganisms within nonsterile litter-amended soil solutions.

No differences in percentages of surviving J2 were observed among water controls and among sterile and nonsterile 1.5% litter-amended soil solutions until day two (Fig. 3B). At days two, four, and

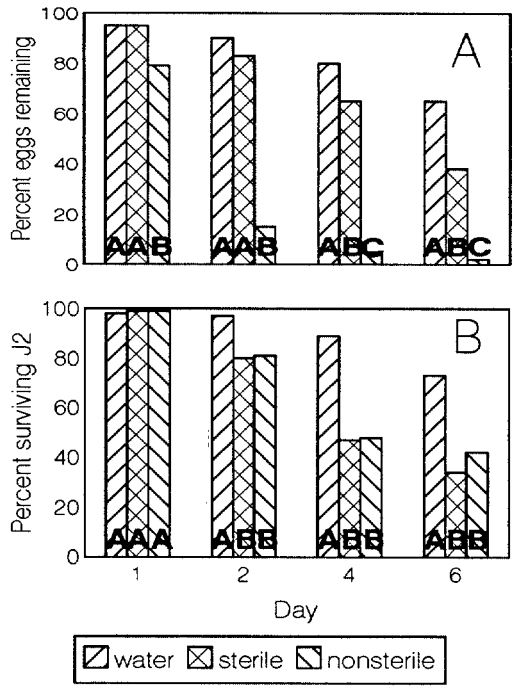

FIG. 3. A) Percentage of apparently healthy, intact *Meloidogyne arenaria* eggs remaining in water, and in sterile and nonsterile 1.5% (w/w) litter-amended soil solutions over 6 days. B) Percentage of surviving second-stage *M. arenaria* juveniles (J2) in water, and in sterile and nonsterile 1.5% (w/w) litter-amended soil solutions over 6 days. Comparisons apply within the same sampling date. Bars with the same letter are not different ($P = 0.05$). Data are means of eight replicates.

six, litter-amended soil solutions had lower percentages of surviving J2 than the water control. No difference was observed between the percentage of surviving J2 in sterile and nonsterile litter-amended soil solutions. Similar trends were observed for other amendment percentages (data not shown). After 6 days, treatment pH ranged from 5.6 in the 1.5% litter-amended nonsterile soil solutions to 7.8 in the water controls.

Discussion

A comparison of the effects of sterile and nonsterile litter-amended soil showed fewer *M. arenaria* in tomato roots grown in nonsterile litter-amended soil, 10 days after inoculation. Previous investigators have suggested that the amount of "protein" N in an organic soil amendment is directly related to its effectiveness in suppressing nematode population densities (13). Our results show that the addition of increasing rates of N, in the form of increasing percentages of sterile chicken litter, was not solely responsible for suppression of *M. arenaria* penetration on tomato, as associated microbes appeared to be involved. The soil used in this study had been fumigated previously, and although the "nonsterile" soil was indeed nonsterile, it did not contain a natural community of soil microorganisms. Thus, no direct conclusions can be drawn concerning the role of soilborne microbes or the interaction between litter and soilborne microbes in the suppression of *M. arenaria*. Use of nonfumigated soil, however, could introduce other nematodes, as well as fungal and bacterial soilborne plant pathogens, into the experimental system.

Many potential biological control agents are selective not only with respect to host genus, but also with respect to the nematode life stage affected. For example, in the case of *Pasteuria penetrans* Sayre & Starr, adhesion of spores to the cuticle of the root-knot nematode J2 is vital (16). On the other hand, *Paecilomyces lilacinus* Thom has been shown to infect and destroy only the eggs of root-knot nematodes (8). The destruction of eggs in nonsterile-amended soil solutions, not observed in sterile-amended soil solutions, suggested that microbes associated with chicken litter were involved. The microbes must have been associated with the litter, because the same effects were observed whether the litter was combined with sterile or nonsterile soil, and there were no effects attributed to sterile versus nonsterile soil. The pH of the solutions, being sufficiently high, would not account for the observed effects on the nematodes (4). Because there was no evidence of microbial interaction in the effects on J2, parasitism of *M. arenaria* eggs was possibly the result of microorganisms whose mode of infection is through the production of chitinase (10). Chitin is found in nematode egg shells but not in the J2 cuticle. Chitinase also would not affect juveniles within eggs that are fully developed and ready to hatch.

In summary, both biotic and abiotic factors appear to be involved in the nematode-suppressive effects of chicken-litter soil amendment. Microbes associated with chicken litter were related to the suppression of nematode penetration observed in tomato roots 10 days after inoculation. Because eggs were used as inoculum, this restriction may have resulted from the destruction of eggs, or from effects on J2 before penetration. Further investigation is needed to identify microorganisms in chicken litter and/or soil involved in suppression of *M. arenaria*.

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