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Effect of Ammonium Ions on Egg Hatching and Second-Stage Juveniles of *Meloidogyne incognita* in Axenic Tomato Root Culture

SUDIRMAN AND J. M. WEBSTER[1]

Abstract: Eggs, either dispersed or in masses, and second-stage juveniles (J2) of *Meloidogyne incognita* were exposed to different concentrations of ammonium ions in a nutrient agar medium upon which excised tomato roots were growing. Egg hatch and J2 penetration of the roots was slowed or inhibited at high (54 and 324 mg/liter) but not at low (1.5 and 9 mg/liter) concentrations of ammonium nitrate. The effect of ammonium on J2 appeared to be temporary and reversible. High potassium nitrate concentration (1,116 mg/liter) did not modify egg hatch or J2 penetration. There was no adverse effect from the high ammonium nitrate concentrations or an equivalent potassium nitrate concentration on root dry weight. Ammonium ions influence nematodes both directly and via plant roots and do so independently of microbial organisms.

Key words: ammonium ion, axenic culture, hatching, *Meloidogyne incognita,* root-knot nematode.

Since the writings and experiments of Lawes (16) in 1845, the use of ammonium-based fertilizers as alternatives to selected organic manures for improved crop yield has become established. Recent reports indicate that both organic and inorganic nitrogen, especially ammonia, suppresses nematode populations when applied to crops (6,11,12). The withdrawal from registered use of many effective nematicides has focused attention on the development of measures to increase crop yield and decrease nematode populations in an environmentally acceptable and economically practical way. A variety of organic amendments have been shown to decrease egg hatch of plant-parasitic nematodes. Raw sewage sludge (20) or chicken litter (12) added to *Meloidogyne*-infested soils decreased the number of infective juveniles (J2) penetrating the host plant. It is not known whether these and similar effects are due to the direct toxic effect of decomposition products on the nematode, to the indirect effects of increasing the populations of soil-borne nematode antagonists-microflora, or to effects on the host plant roots. It has been speculated (20) that the suppressing effect on *Heterodera glycines* populations of adding anhydrous ammonia to soils may be due to its selective influence on nematode antagonists. However, earlier work (24) in sterile and non-sterile soil suggested that application of ammonium salts had a nematicidal effect against *Pratylenchus penetrans.* This paper reports on a study to determine the effect of ammonium on the hatching of *Meloidogyne incognita* eggs and on the penetration by J2 into excised tomato roots under aseptic conditions.

Materials and Methods

Plant root culture: Seeds of the root-knot nematode susceptible tomato cultivar, *Lycopersicon esculentum* Mill. UC 82 (A. L. Castle, Morgan Hill, CA) were surface sterilized in a 1:1 mixture of 95% ethanol and 6% NaOCl for 8 minutes, rinsed five times with sterile, distilled water, plated in Petri dishes containing a 3–4 mm thick layer of 1% water agar and incubated in the dark at 25 C. After germination, 1-cm pieces of primary root, cut at the hypocotyl, were transferred aseptically to Petri dishes containing a modified Skoog, Tsui and White (STW) (15) nutrient medium amended with different concentrations of ammonium or potassium nitrate. The pH was adjusted to 6.0 with 0.1 N NaOH. The excised root cultures were kept in the dark at 25 C for 3 days before inoculating them with nematodes (14,15).

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[1] Centre for Pest Management, Department of Biological Sciences, Simon Fraser University, Burnaby, Vancouver, V5A 1S6, Canada.

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To obtain different concentrations of ammonium, the concentration of ammonium nitrate in the STW medium was modified for the different treatments as follows: ammonium deficient, 6.67 mg/liter (1.5 mg/liter NH_4^+); normal, 40 mg/liter (9 mg/liter NH_4^+); 240 mg/liter (54 mg/liter NH_4^+); and 1,440 mg/liter (324 mg/liter NH_4^+). To discern whether the effect of the higher ammonium nitrate concentrations was due to the ammonium or the nitrate ion, the concentration of potassium nitrate was increased to 1,897 mg KNO_3/liter (1,116 mg/liter NO_3^-) in one treatment while retaining the normal concentration of ammonium nitrate. This high concentration of nitrate was equivalent to that in the high concentration of ammonium nitrate treatment but without the associated increase in ammonium.

Nematodes: Meloidogyne incognita (Kofoid and White, 1919) Chitwood 1949 race 2, (NC 632 from North Carolina State University) was maintained on greenhouse-grown, tomato plants, cv. Bonny Best. Two-week-old seedlings, grown in 20-cm-d by 25-cm-deep plastic pots filled with autoclaved growing medium (80% sand and 20% loam), were inoculated with one egg mass each. The plants were maintained in a greenhouse at 24–28 C, watered every 2 days, and fertilized periodically with a 15:15:10 N:P:K mix. Egg masses were collected after 2–3 months.

Dispersed eggs of *M. incognita* were obtained by washing the soil off root-knot nematode infected tomato roots, cutting the roots into 1- to 2-cm-long segments, comminuting in a blender with 200 ml water at high speed for 15 seconds, and shaking for 2 minutes in a 0.525% sodium hypochlorite solution (14). The suspension was rinsed through a sieve series, and the eggs were collected on a 26-μm-pore sieve. The eggs were washed off the sieve, centrifuged at low speed to remove clumps of eggs and soil particles, decanted into sterile water, centrifuged, surface-sterilized with 0.525% sodium hypochlorite for 2 minutes, and rinsed four times with sterile, distilled water. To provide sterile nematode cultures, about 200 eggs per 5 μl of water were inoculated onto the surface of excised roots.

To provide inoculum for the experiments, egg masses of *M. incognita* were picked from washed tomato roots, surface sterilized with 0.525% sodium hypochlorite for about 3 minutes (14), blotted dry on sterile filter paper, and aseptically inoculated onto 3-day-old, excised roots in Petri plates with STW medium. The plates were sealed with Parafilm and incubated in the dark at 25 C. After 55 to 65 days, egg masses produced in these cultures were sorted for uniformity in size and color and used as inocula.

To obtain second-stage juveniles (J2) egg masses, taken from sterile cultures, were placed in a layer of sterile water (about 1–2 mm deep) in Petri dishes in the dark at 25 C. Inocula were prepared by diluting a suspension of one-day-old J2 to give about 150 juveniles per 5 μl aliquot.

Inoculation and experiments: Dispersed eggs, egg masses, or J2 were placed about 1 cm from the tips of 3-day-old excised tomato roots in Petri plates. At sampling, excised roots were removed from culture, cleaned of adhering medium, and weighed immediately. To obtain dry weight, clean roots were placed in paper towels, dried at 70 C for 48 hours, and weighed. Roots freshly removed from culture were stained with acid fuchsin (1) for stereomicroscope examination.

To investigate treatment effects on hatching, two types of inocula were used: dispersed eggs, to obtain fully differentiated individuals at a similar stage of development, and egg masses. The experiments were conducted both with and without excised roots on the medium.

To determine ammonium effects on dispersed-egg hatching, Petri dishes were divided into five groups with five dishes in each; each of four groups contained STW medium with one of the ammonium concentrations, and a fifth group had the high potassium nitrate concentration. Five-ml aliquots containing about 200 dispersed eggs were pipetted onto the medium in

each Petri dish containing 3-day-old excised roots. The open Petri dishes were placed in a laminar air flow for about 40 seconds to allow excess water covering the eggs to evaporate. The dishes were sealed with Parafilm, randomized, and kept in the dark at 25 C. Hatched juveniles were counted and removed from the dish daily for up to 15 days using a fine, mounted hair. The percentage of hatched J2 was determined after examining and counting the remaining eggs in the dishes.

Similar experiments were done with egg masses as inocula. One egg sac of *M. incognita* was inoculated onto an STW medium in the treatment series. The juveniles that hatched from each egg mass were counted and removed from the dish daily for 15 days. To determine the percentage of hatched juveniles from an egg mass, each egg mass was put in a 1-ml plastic vial half-filled with 5% NaOCl, shaken vigorously for 15 seconds, and incubated for about 60 minutes at 25 C (13); the liberated eggs were then examined and counted under a stereomicroscope.

To investigate ammonium effects on nematode infectivity, egg masses and J2 were used. A total of 225 Petri dishes were divided into five groups of 45. Each of four groups contained one of the four ammonium levels, and one group the high potassium nitrate level. One egg mass was inoculated onto excised roots in each dish, which was then sealed and placed in the dark at 25 C. Five replicate roots of each treatment were harvested at 2, 3, 4, 5, 6, 7, 9, 11, and 14 days following inoculation. Each root was washed in a dish to remove nematodes from the surface, which then were counted. The roots were stained and the juveniles counted.

A similar experiment was done using 5-µl aliquots containing about 150 J2 as inoculum. After the experiment, roots were sampled and examined as described for the egg mass experiment. Estimates of the number of juveniles penetrating the roots were based on the number of juveniles inside the roots as a proportion of available juveniles from the inoculum.

To show the effect of ammonium on nematode activity after root penetration, 175 Petri dishes were divided into five groups of 35. Each of four groups contained one of the four ammonium levels, and one group the high potassium nitrate level. About 150 J2 in 5 µl of water were pipetted onto the excised roots in each dish. Five replicate excised roots of each treatment were removed at 2, 3, 4, 5, 6, 7, and 14 days after inoculation, washed to remove nematodes from the surface of the roots, placed in a Petri dish filled with a 3–4 mm layer of sterile, distilled water, and incubated in the dark at 25 C. After 24 hours, all of the juveniles that had emerged from the roots were counted, and nematodes inside the roots were counted after staining. The juveniles that had migrated from the roots grown in high ammonium were reinoculated onto excised roots grown in STW medium with normal ammonium (control) to determine their infectivity.

Each experiment was designed as a completely randomized block with five treatments, each replicated five times. The data were subjected to analysis of variance, and each treatment mean was compared with the control using a protected LSD ($P = 0.05$). Arcsin transformation of percentages was used where necessary. All experiments were repeated at least twice.

Results

Increased ammonium concentrations inhibited the hatching of dispersed eggs and of eggs within masses (Figs. 1,2). The relative decrease in cumulative percentage of hatched J2 with increasing ammonium concentrations started from the third or fourth day of the experiment, with dispersed eggs or egg masses in both the presence and absence of roots (Fig. 1). From the third day until the end of the experiments, the cumulative percentage of hatched juveniles from treatments with 54 mg/liter and 324 mg/liter of ammonium was lower than that in the control and in the two lower ammonium concentrations.

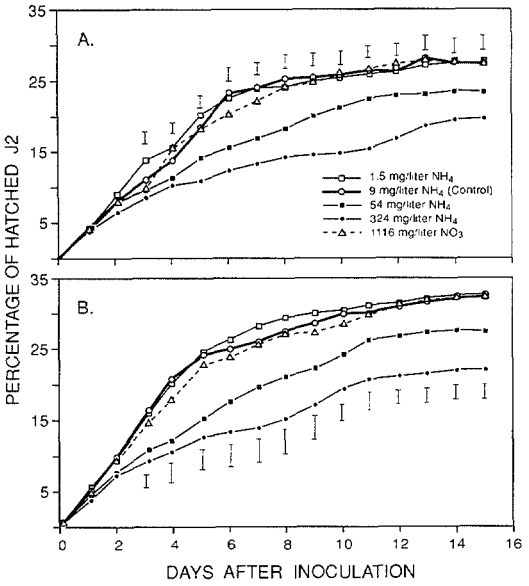

FIG. 1. Cumulative percentage of hatched *Meloidogyne incognita* juveniles from dispersed eggs exposed to different ammonium or nitrate concentrations on STW medium, A) without and B) with excised tomato roots. Vertical bars represent LSD (P = 0.05).

There were no significant differences among the deficient ammonium, high nitrate, and control treatments.

The cumulative percentage of hatched juveniles from egg masses was higher than that from dispersed eggs (Figs. 1,2). Most of the dispersed eggs hatched in the first 7 days and from egg masses in the first 5 days in the control, deficient ammonium, and high potassium nitrate treatments, whereas the rate of hatching was slower in the 54 and 324 mg/liter ammonium treatments. The eggs hatched faster in the presence of roots with both types of inocula, but this effect was less apparent with the 54 and 324 mg/liter treatments, using dispersed egg inoculum.

There was a decrease in the cumulative percentage of J2 penetrating roots with increased ammonium concentrations in both experiments at all time intervals (Figs. 3,4). There were lower percentages of juveniles in the roots with the 54 mg/liter and 324 mg/liter treatments than in the control, whereas there were no differences between 1.5 mg/liter ammonium or high nitrate and the control. At normal and deficient ammonium and high nitrate treatments, most juveniles penetrated the roots during the first 4 days, and continued root

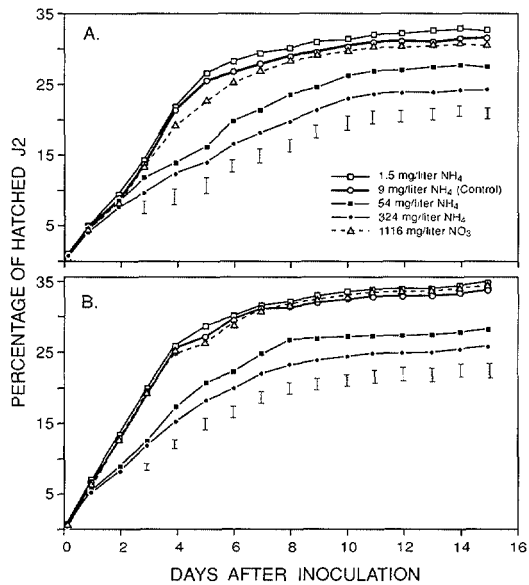

FIG. 2. Cumulative percentage of hatched *Meloidogyne incognita* juveniles from a single egg mass exposed to different ammonium or nitrate concentrations on STW medium, A) without and B) with excised tomato roots. Vertical bars represent LSD (P = 0.05).

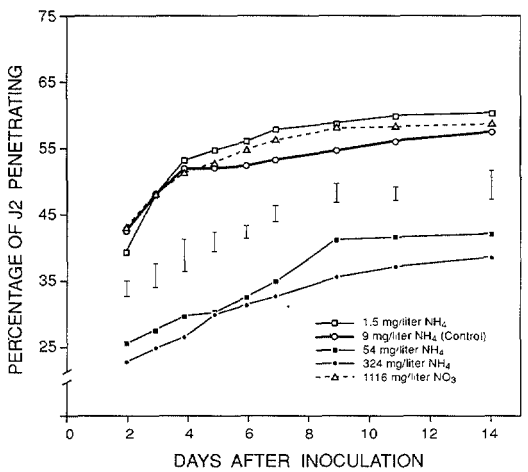

FIG. 3. Cumulative percentage of *Meloidogyne incognita* juveniles, emerging from a single egg mass that penetrated excised tomato roots grown on STW medium containing different ammonium or nitrate concentrations. Vertical bars represent (LSD) (P = 0.05).

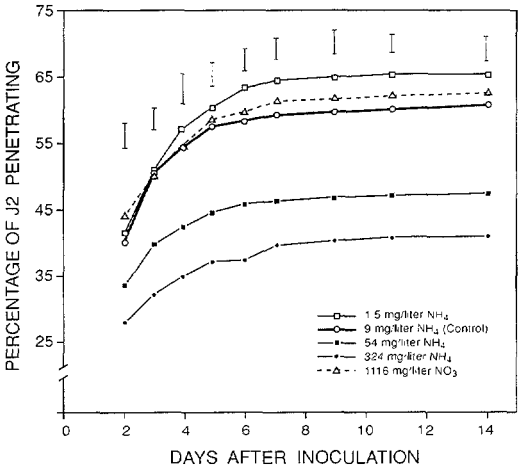

FIG. 4. Cumulative percentage of inoculated *Meloidogyne incognita* juveniles penetrating excised tomato roots on STW medium containing different ammonium or nitrate concentrations. Vertical bars represent LSD (P = 0.05).

invasion in progressively smaller numbers until day 14. Root invasion by nematodes treated with 54 mg/liter and 324 mg/liter was at a significantly slower rate than for the other treatments throughout both 14-day experiments. There was no difference between the cumulative percentages of juveniles penetrating tomato roots of the deficient ammonium and control treatments up to day 5. Thereafter, more juveniles penetrated the roots in the deficient ammonium treatment. The cumulative percentages of J2 that penetrated the roots inoculated with egg masses were lower than for those inoculated with second-stage juveniles.

More juveniles migrated from roots in treatments with 54 mg/liter and 324 mg/liter than from the control or the other treatments (Fig. 5). The cumulative percentage of juveniles that left the roots of the high nitrate treatment was similar to that of the control. By day 4 virtually no more nematodes exited the roots of any treatment, and by that time twice as many nematodes had migrated from each of the high ammonium treatments than from the control. Juveniles that migrated from the roots grown in high ammonium induced roots galls when subsequently inoculated onto excised roots grown at normal levels of ammonium.

DISCUSSION

Higher than normal ammonium concentrations in the nutrient medium, in the presence and absence of excised roots, decreased the rate and total number of J2 that hatched from dispersed eggs and egg masses. As well, the number of J2 that infected excised roots increased. The two higher concentrations of ammonium may have been sufficient to modify malate dehydrogenase activity (22), and thus decrease available energy for hatching and plant invasion processes. High salt concentrations also modify osmotic pressure sufficiently to inhibit hatching and movement of *Meloidogyne* spp. (5). However, it is notable that the high level of potassium nitrate, which would also increase osmotic pressure, did not diminish nematode hatch or root penetration in these experiments.

Host plant nutrition alters the degree of nematode attraction, the number of available lateral root-sites, and the nematode's surface feeding behaviour (19). Scott and Martin (21) showed that treatment with different ions significantly affected the

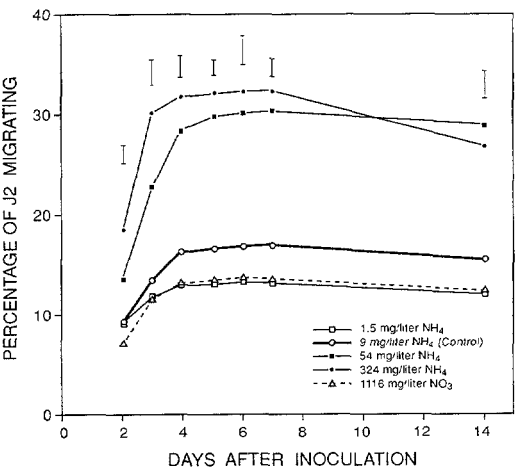

FIG. 5. Percentage of *Meloidogyne incognita* juveniles migrating, after 24 hours incubation in water, from excised tomato roots grown initially on STW medium containing different ammonium or nitrate concentrations. Vertical bars represent LSD (P = 0.05).

electrical potential around the root tip area where J2 penetrated. Consequently, the relative concentration of ammonium ions may influence nematode penetration of the root through diminished attractiveness of the root tips.

Since higher ammonium concentrations significantly decreased hatching, estimates of the number of juveniles penetrating roots were based on the number of juveniles inside the roots as a proportion of the total number of hatched juveniles. Despite this correction for hatch rate, the results showed that high ammonium concentrations strongly inhibited root penetration by juveniles.

Our observations agree with a previous report (23) that the presence of excised roots in nutrient medium stimulated hatching of *Meloidogyne* eggs. Nevertheless, the effect of the roots did not overcome the detrimental affect of the ammonium on the nematodes. Approximately 50% more juveniles emerged from egg masses than from dispersed eggs. This result may have been due to an inhibitory effect from sodium hypochlorite used during egg dispersal, and may also account for the occurrence of deformed J2 hatching from the dispersed eggs. Previous investigations (7,9) have shown that 0.53–1.05% sodium hypochlorite treatment of *Meloidogyne* eggs decreased hatching.

Castro et al. (2) showed that *M. incognita* juveniles were strongly repelled by salts containing K^+ or NH_4^+ and especially by KNO_3. However, in our experiments, high nitrate as potassium nitrate did not significantly modify egg hatching or juvenile penetration of the host and did not significantly affect root growth. This is in accordance with the results of other studies (10,18).

Loewenberg et al. (17) reported that in vitro survival of *M. incognita* depends on mineral balance in the medium. They suggested that the mineral balance may influence infection of the host by *Meloidogyne* juveniles. When a salt gradient barrier was inserted in the soil between plant roots and *M. incognita,* the juveniles were repelled (3). In that experiment, ammonium nitrate was especially effective. Our experiment was designed so that an imbalance of nutrients (except for ammonium or potassium nitrate) would not be a factor influencing hatch or juvenile penetration.

The effect of ammonium on nematode activity became more apparent when, after 24 hours of incubating the nematode-infected roots in water, there were more juveniles leaving roots from the high than from the low ammonium treatments. The ammonium probably changed the physiology of the roots, resulting in failure of many of the J2 to induce feeding sites in these treated roots, even after removal from exogenous ammonium. These emerging juveniles were apparently unharmed by the ammonium because they were capable of infecting untreated excised roots and producing galls.

Egg hatch and root penetration by juveniles was diminished by high ammonium concentrations independently of microbial organisms, although the mechanisms of suppression cannot be fully determined from these experiments. Ammonium affected the nematodes directly via the plant roots, as observed also by Glazer and Orion (8) using urea and its derivatives under aseptic conditions. However, they examined only the parasitic development of the nematode in its host plant. Juveniles that penetrate excised tomato roots after treatment with high levels of ammonium retain their ability to induce giant cells (8) but migrate out of the root without doing so. It has been hypothesized (20) that rates of ammonia-based fertilizer high enough to also have a nematicidal effect would probably result in phytotoxicity. However, root growth was not greatly affected in our experiments with ammonia, but the experiments lasted only 14 days, and conditions were not representative of field conditions.

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