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Evaluation of Dry Ice as a Potential Cryonematicide for *Meloidogyne incognita* in Soil

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Abstract: Solid CO_2 (dry ice) was added to pots containing soil that was infested either with eggs of the root-knot nematode, *Meloidogyne incognita,* or with tomato (*Lycopersicon esculentum* 'Rutgers') root fragments that were infected with various stages of the nematode. Two hours after dry ice was added, thermocouples in the soil recorded temperatures ranging from –15 °C to –59 °C. One day after treatment with the dry ice, the temperature of the soil was allowed to equilibrate with that of the greenhouse, and susceptible tomato seedlings were planted in pots containing infested soil treated or untreated (controls) with dry ice. After 5 weeks, roots were removed from the pots and nematode eggs were extracted and counted. Plants grown in soil infested with eggs and receiving dry ice treatment had less than 1% of the eggs found in the controls; plants from soil infested with root fragments and receiving dry ice treatment had less than 4% of the eggs found in controls. Dry ice used to lower soil temperature may have potential as a cryonematicide.

Key words: carbon dioxide, control, cryogen, cryonematicide, dry ice, *Lycopersicon esculentum, Meloidogyne incognita,* nematicide, nematode, population dynamics, root-knot nematode, temperature, winter survival.

Low temperatures associated with winter significantly affect the survival and population dynamics of plant and animal-parasitic nematodes (Wharton, 1995). Daulton and Nusbaum (1961) compared the cold tolerance of three populations of *Meloidogyne javanica* from Southern Rhodesia, North Carolina, and Georgia with *Meloidogyne hapla.* When these populations were exposed to winter conditions in North Carolina, only *M. hapla* survived. Nusbaum (1962) showed that *M. hapla* survived North Carolina winters better than either *M. incognita* or *M. javanica.* Similarly, Sayre (1963) found that *M. hapla* survived winter in Ontario, but *M. incognita* and *M. javanica* did not. More subtle effects of winter on nematode population dynamics have been studied by McSorley and Phillips (1993) and Trudgill (1995).

Studies such as these suggest that low temperatures associated with winter influence the survival and population densities of many plant-parasitic nematodes. The objective of our research was to determine if a cryogen applied to soil would lower soil temperatures sufficiently to reduce survival and subsequent reproduction of the root-knot nematode *M. incognita.* The cryogen, solid CO_2 (dry ice), was selected for this experiment because: (i) its temperature, –78 °C, is lethal to active juvenile forms of the nematode; (ii) dry ice will ''expand'' in volume about 500-fold during sublimation and warming to create highly localized concentrations of CO_2 gas in the soil that could adversely affect nematodes; and (iii) dry ice is innocuous, as upon warming it becomes carbon dioxide gas.

Materials and Methods

Infestation with eggs: For each of two experiments, eggs of the southern root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood, were obtained from two infected tomato plants (*Lycopersicon esculentum* 'Rutgers') that had been maintained under greenhouse conditions. Roots of the infected plants were removed from the soil, washed several times in water, and then immersed in a 0.525% sodium hypochlorite solution for 3 minutes (Hussey and Barker,

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1973) to extract the eggs. Approximately 500,000 eggs were collected and thoroughly mixed with sterile soil (16 parts coarse sand and 9 parts compost) in a cement mixer for 20 minutes, after which the soil was divided among eighteen 20.3-cm-diam polyethylene pots. Each pot contained 20,000 eggs in 5 kg of soil.

Infestation with infected roots: Heavily infected roots from five tomato plants maintained under greenhouse conditions were washed several times in water and chopped into 2.5 to 5.0-cm fragments. The infected root fragments were thoroughly mixed with another portion of the soil mixture and placed into eighteen 20.3-cm-diam. polyethylene pots. Each pot contained 50,000 eggs and juveniles in 5 kg of soil.

Cryogen treatment: Dry ice pellets (Dry Ice, Baltimore, MD) were precooled in liquid nitrogen at −196 °C and then mixed (454 g/pot) with: (i) soil in each of nine pots that had been infested with eggs, or (ii) soil in nine pots containing the infected root segments (eggs and juveniles). Next, the 18 pots (9 from each inoculum) were placed in a box constructed from 5-cm-thick sheets of styrofoam. The styrofoam box was wrapped in plastic and remained on the greenhouse bench for 20 hours. After 20 hours, the plastic was removed and the soil was allowed to reach room temperature.

Temperature recording: Temperature monitoring was performed on a separate test pot, consisting of the same soil and placed in a separate small styrofoam box. To record soil temperatures, thermocouples were placed in the pot at three levels: 2.5 cm below the soil surface, midway, and 2.5 cm from the bottom of the pot. Readings were taken every 5 minutes for the first 105 minutes (Fig. 1). Air temperature in the large styrofoam box was recorded at the beginning of the experiment. Final readings were taken 22 hours after the experiment began.

Transplants: After pots reached room temperature, 7.5 to 10-cm-high tomato seedlings (cv. Rutgers) were transplanted into the 18 pots that had been infested with either eggs or infected roots. Eighteen pots containing soil that had been infested but had not received dry ice served as the controls. The plants were grown for 5 weeks under greenhouse conditions. The first experiment was begun on 6 March 1997; the entire experiment was repeated beginning on 6 June 1997 under summer conditions in the greenhouse.

Evaluation of infection: After 5 weeks, the plants were harvested. Length of plant tops was measured, and nematode reproduction on root systems was measured as follows. The root system from each plant was gently washed in water to remove soil. Each clean root system was placed in a 0.525% sodium hypochlorite solution for 3 minutes, and the roots were gently rubbed. The roots and the hypochlorite solution were poured into nested 300-µm and 25-µm-pore sieves and rinsed with water. Eggs collected on the 25-µm-pore sieve were rinsed into plastic centrifuge tubes, which were spun at 160g for 3 minutes. The volume of supernatant was brought to 30 ml, an aliquot of 1 ml was withdrawn and diluted to 10 ml, and 1.0 ml was placed on a Hawksley slide for counting.

Statistical methods: The data, which were combined over both experiments for shoot length, shoot dry weight, and shoot wet weight, were analyzed with linear mixed model procedures (SAS Institute, Cary, NC). The model contained the fixed effects of CO_2 (with or without), nematodes (eggs or infected roots), and the interaction of CO_2 × nematodes. The random portion of the model contained the effects of experiment and residual error. Nematode count data were not normally distributed and therefore were analyzed with the Generalized Linear Mixed Model macro of SAS. The error distribution was defined as Poisson and a log link was used. The fixed portion of the model contained the effects of CO_2, nematodes, and their interaction. To correct for variance heterogeneity, a separate residual variance was fitted for each combination of CO_2 and nematodes. The least significance difference test (LSD) was used to identify differences between treatment means at the 5% level of significance.

Results

Temperature of soil: Temperatures recorded in the test pot varied significantly, presumably due to the relative position of the thermocouple to the nearest pellet of dry ice. The lowest temperatures were recorded 50 to 65 minutes after the experiment began (Fig. 1). Temperatures 2.5 cm below the surface and at midpoint in the pot were −59.2 °C and −48.5 °C, respectively. The temperature 2.5 cm from the bottom of the pot dropped continuously during the recording period to −15.4 °C after 105 minutes. A similar pattern was apparent in the second experiment (Fig. 1). After 105 minutes, the air temperature in the styrofoam box was −8.5 °C 5 cm from the top and −19.3 °C 5 cm from the bottom. After 22 hours, just before the styrofoam box was disassembled, temperatures at the three locations in the pots were 9.9, 8.3, and 8.5 °C, respectively, while the air temperature in the sealed box was 14.2 °C.

Appearance of roots: Plants from the controls of both inoculations had thickened roots with significant galling, characteristic of heavy root-knot nematode infections. Roots from plants grown in the two infested soils treated with dry ice had fine fibrous root systems that did not exhibit any obvious galls.

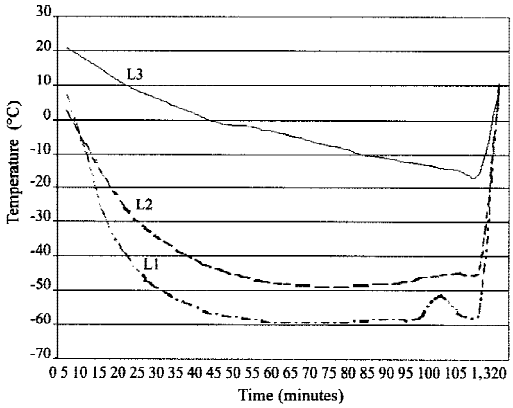

Fig. 1. Effect of dry ice treatment on soil temperature at three points in experimental apparatus. Temperatures were recorded with thermocouples 2.5 cm below soil surface (L1), midway (L2), and 2.5 cm from bottom (L3) in test pots.

Nematode reproduction: Use of either eggs or infected roots did not significantly affect the final counts of eggs from control plants (Table 1). However, for plants grown in the dry ice-treated soil, egg counts were lower in roots from soil infested with eggs than in roots from soil infested with eggs and juveniles. The dry ice-treated soil had plants with egg counts less than 1% and 4% of the controls for the egg and infected root infestations, respectively.

For the egg-infested soil, treatment with dry ice had no effects on shoot lengths or on wet and dry weights (Table 1). For soil infested with infected roots, these parameters were significantly increased by treatment with dry ice. The dry ice treatment reduced nematode infection by more than 99% (Table 2). Interactions between the experiments × nematode infestations or between experiments × CO_2 × nematode infestations were not significant for other variables.

Discussion

Artificially lowering soil temperature by use of a cryogen may adversely affect nematode survival and could serve as a means of control. In our experiments, use of dry ice to treat *M. incognita*-infested soil decreased the numbers of nematode eggs produced on susceptible tomato plants by more than 94%. These experiments were not designed to estimate the LT_{50} (temperature resulting in a 50% reduction in extractable eggs). Consequently, temperatures recorded within the pots were as low as −59 °C, which

Table 1. Effect of dry ice and composition of *Meloidogyne incognita* inoculum on numbers of eggs and infected roots on tomato shoot length and wet and dry weights, 5 weeks after inoculation.

Treatment	Number of eggs	Length (cm)	Wet weight (g)	Dry weight (g)
Eggs	298,000 a	67 ab	153 a	18.3 a
Eggs + CO_2	100 c	73 a	142 a	18.5 a
Eggs + J	221,000 a	51 c	71 c	9.1 c
Eggs + J + CO_2	7,900 b	63 b	113 b	14.1 b

Means in a column with identical letters are not different according to an LSD test ($P > 0.05$).

TABLE 2. Main effect means for combined nematode inoculum, consisting of purified eggs or eggs and juveniles of *Meloidogyne incognita* in root fragments of *Lycopersicon esculentum,* on mean number of eggs of *Meloidogyne incognita,* and on tomato shoot length and wet and dry weights 5 weeks after inoculation.

Treatment	Number of eggs	Length (cm)	Wet weight (g)	Dry weight (g)
Inocula	257,000 a	59 b	112 b	13.7 b
Inocula + CO_2	890 b	68 a	127 a	16.3 a

Means in a column with any identical letters are not different at the 0.05 level by LSD.

was considered well below that normally occurring in agricultural soils.

Dry ice was more effective in pots that had been infested with eggs (99% reduction) than those infested with root fragments from infected plants (96% reduction). This variation may result from differences in the concentration of the inocula that were used, i.e., 20,000 eggs/pot vs. 50,000 eggs + juveniles in root fragments. Alternatively, some stage associated with the root fragment inoculum could be more cold-tolerant than eggs and could be responsible for the slightly greater infection rates. However, this latter possibility is not consistent with previous observations (Vrain, 1978) that mature eggs of *M. incognita* and *M. hapla* were more cold-tolerant than embryonic eggs. On the other hand, perhaps extraction of the eggs in sodium hypochlorite affected their ability to survive freezing.

In our experiments, no attempts were made to acclimate the eggs or the root fragments that were used to infest the soil. Forge and MacGuidwin (1990, 1992a, 1992b) showed that the survival of *M. hapla* second-stage juveniles (J2) exposed to frozen conditions was affected by thermal history. Even a 12-hour exposure to 4 °C increased the survival of J2 from about 30% to nearly 80% (Forge and MacGuidwin, 1992b). However, even when the J2 were preconditioned at 4 °C, survival in frozen conditions dropped sharply when subsequent temperatures were lowered from −4 to −6 °C. These results suggest that although acclimation may increase the tolerance of J2 to frozen conditions, the lethal temperature might not be depressed sufficiently to result in survival at the temperature resulting from the dry ice treatment.

Sayre (1964) described three categories of nematodes based on their response to low temperature: (i) those that were susceptible to chilling injury and died at temperatures above freezing, (ii) those that were capable of supercooling to a certain point but were killed by freezing when it occurred (referred to as freezing susceptible), and (iii) those that were not injured by freezing (freeze-tolerant). He also observed that eggs and J2 of *M. incognita* survived for short times in supercooled water but that the formation of internal ice was lethal to the nematode. We did not determine whether the *M. incognita* J2 and eggs in these experiments were: (i) supercooled but killed by low temperature; (ii) killed by internal freezing; or (iii) killed by other stresses associated with frozen conditions, such as desiccation (Forge and MacGuidwin, 1992a). Our observations suggest that the cryogen used in our experiments lowered the soil temperature, causing the formation of ice that was lethal to the nematode.

In our study, the low temperatures induced by dry ice treatment are assumed to cause the reduction of infective eggs. However, perhaps high concentrations of CO_2 gas could have reduced the number of infective eggs. Robinson and Heald (1991) noted anesthetic effects of CO_2 at concentrations greater than 5%. Given the density of CO_2 as a solid at −20 °C of 1.0310 and the density of the gas at 0 °C of 0.00198, we calculate that the volume of CO_2 expands more than 500 times during the phase change or sublimation from the solid to the gas. Therefore, in our experiments sublimation would generate nearly 250 liters of CO_2 gas/pot. Assuming that the gas could freely diffuse throughout the pore spaces of the soil, we estimate that the resulting concentration of CO_2 gas approached 100% for several hours. This concentration could be lethal to the nematode.

These observations suggest that use of an environmentally safe cryogen, such as dry

ice, may have potential to control or reduce populations of species that have little or no tolerance to subzero temperatures. The cryogens probably would be applied during the winter season, when lowering the soil temperatures only a few degrees might have a significant impact on nematode survival. Future work will be directed at determining the LT_{50} for *M. incognita* and exploring the effects of acclimation.

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