A NEW INOCULUM PRODUCING METHOD FOR *MELOIDOGYNE HAPLA* AND OBSERVATIONS ON LARVAL MIGRATION *IN VITRO*

By

ARLIN LEON BOSTIAN

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

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Thesis Approved:

Mark C. Russell
Thesis Adviser

D. E. Wadsworth

L. F. Singleton

Norman N. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

In the field of nematology, having adequate quantities of inoculum on hand for research is frequently a limiting factor. Although many mycophagous and bacteriophagous nematodes can be easily reared, producing large quantities of plant parasitic nematodes (obligate parasites) is more difficult. Techniques frequently used for the quantitative extraction of other plant parasitic nematodes from soil are generally of marginal efficiency or of no use at all when used for root-knot nematode collection. Those techniques specifically designed for efficient extraction of root-knot nematodes (usually eggs or larvae) are time consuming, require the use of considerable laboratory equipment and space, and are destructive to the host plant. The purpose of this study was to design an experimental unit from which root-knot larvae could be harvested quickly and easily without tying up laboratory space or equipment. Also, in order to provide a viable alternative to present root-knot inoculum producing methods, the experimental units would have to produce comparable quantities of inoculum. The main purpose of this study was to design a technique where the colony could be harvested for root-knot larvae without the destruction of the colony. Because the colony remains intact, the undisturbed root-knot females continue to produce eggs resulting in a constant source of inoculum.

The second part of this study concerned the use of the root-knot larvae obtained from the experimental units in the observation of their orientation responses to an attractant in a square petri plate containing moist silica sand. Current tests used in the screening of candidate chemicals for their nematicidal properties fail to give a complete reading of the chemical's potential as a nematicide. The procedures presently used determine only if the chemical kills the nematodes. Because of the way in which these tests are constructed, it cannot be determined if the chemical has specific detrimental effects on the nematode's sensory systems. Chemicals affecting the nematode's sensory mechanisms could prevent the nematodes from locating the plant's root system, and therefore successfully prevent nematode damage. Any chemical thus affecting the nematodes could be equally as effective in protecting the plant as any chemical that kills nematodes. By determining the migration patterns of the larvae (via sampling) in a petri plate of silica sand mixed with the candidate chemical, it could be determined whether the predicted larval orientation response took place, was disrupted, or if the nematodes were killed. Such a test would allow a much more sensitive reading of a chemical's potential as an effective nematicide.

CHAPTER II

REVIEW OF LITERATURE

The difficulty in maintaining relatively pure stocks of nematodes with ready accessibility for use as inoculum has long been a problem for those working in nematological research. While many of the free-living mycophagous or bacteriophagous forms (such as the genera Rhabditis and Panagrellus) have been easily colonized, animal, insect, and plant parasites, being strict obligate parasites as a rule, present a very different problem. Glaser (12) maintained viable, reproducing stocks of the animal parasite, Haemonchus contortus through the utilization of a special medium. Stoll (27) was able to maintain the insect parasite Neoaplectana glaseri in axenic culture for seven years without any loss of pathogencity. Plant parasitic forms have generally been very difficult to colonize in vitro. Limited success has been enjoyed with rearing Ditylenchus destructor on plant storage tissues (3, 29) and Radopholus similis and Pratylenchus brachyurus on carrot discs (23). Krusberg (17) found that Ditylenchus dipsaci and Aphelenchoides ritzemabosi reproduced rapidly on alfalfa callus tissues grown on a nutrient agar medium. Callus tissues have also been used with less success in maintaining cultures of the genera Tylenchorhynchus, Hoplolaimus, Aphelenchus, Tylenchus, and Dolichodorus (26). Pratylenchus penetrans has been found to grow and reproduce on excised corn roots.

The nematodes reared in this manner were then stored in a "cold trap" and could be used for up to four months with little loss in viability (32).

In vitro colonization of the genus Meloidogyne for use as a source of inoculum has met with limited success. The root-knot nematode, when reared on callus tissue, failed to mature and complete its life cycle, thereby eliminating this method as an inoculum production technique (24). However, gall formation and limited reproduction has been demonstrated on carrot discs (24). Shepperson and Jordan (25) developed a technique involving the inoculation of aerial organs (stems and leaves) of selected plants with the eggs and larvae of Meloidogyne incognita. Both galling and reproduction was found to occur. Vrain (33) and Hussey and Barker (14) have compared several of the more traditional methods of procuring root-knot inoculum, including picking egg masses, placing galled roots in NaOCl solutions to dissolve egg masses freeing the eggs, root incubations, misting of infected roots, blender (tissue homogenization) techniques, soil extraction techniques, and some modifications of these. Others, such as the McClure, Kruk, and Misaghi (21) egg extraction technique, are time consuming, tedious, and require the destruction of the stock colonies, even though large quantities of eggs can be recovered.

The attraction of root-knot nematodes to plants has been documented many times over the years. The work of Lindford (18) indicated that Meloidogyne spp. were attracted to the root in the region of cell elongation. He also observed that plant tissues previously unattractive to root-knot larvae become attractive as they decomposed. Wieser (34) found that the apical two mm of excised tomato roots were repellent to

Meloidogyne hapla, but the next six mm were attractive. The remaining portion of the root, up to 16 mm, was either neutral or slightly repellent. Wieser (35) repeated these experiments with the root-knot nematode hosts bean, egg plant, and soybean. He found the excised roots of the bean to be repellent, while soybean and egg plant varied from repellent, to neutral, to attractive. Bird (5, 6) found Meloidogyne javanica to be attracted by carbon dioxide, but a living root tip would attract the nematodes away from the carbon dioxide source (many species are attracted by carbon dioxide because of their preference for lower oxidation-reduction potentials-important for many species like M. javanica in locating the root). Griffin and Waite (13) noted that M. hapla was equally attracted to both susceptible and resistant varieties of alfalfa, specifically to the root apical meristem. To a lesser extent, the nematodes were attracted to the upper hypocotyl and stem apical meristem of these seedlings.

Several different methods have been described (4, 10, 11, 15, 16, 19, 20, 28, 30, 31) for determining a substance's nematicidal activity. In general, however, each of these methods involves two procedures (prior to field testing). The first is the "water screen" method (20). This technique involves exposing selected nematodes (usually free-living nematodes such as Rhabditis spp. or Panagrellus spp., although any plant parasite can be used) to a solution or emulsion of the candidate chemical for various periods of time. Following the exposure the nematodes are examined and the chemical rated on the basis of nematode movement (or lack of it). The second phase of testing consists of the addition of the chemical to soil containing the nematodes. The soil is then stored for a period of time in either a closed or

open container. After the storage period, a host plant is grown in the treated soil. Evaluation is by counting the nematodes recovered from the soil, or by counting the number of galls formed on the root system, depending on the type of nematode used in the test. A variation of this procedure includes placing the host plant, chemical, and nematodes into the soil at the same time. Taylor, Feldmesser, and Feder (28) used a "water screen" method involving the placing of one or two ml of a nematode suspension in a five ml vial, adding two-thirds of a vial of washed quartz sand, then adding the chemical (never more than 1000 ppm in 0.5 ml of water). The vials were shaken, mixing the nematodes, sand and chemical thoroughly. The vials were then topped up with additional washed quartz sand and stored for 48 hours, at 20°C. The vials were then processed, and the nematodes counted. Evaluation of the chemical was based on the living nematodes recovered. Tiner (30) used an in vitro technique, where the chemicals were applied to dried bacterial cells, bacteriophagous nematodes were added, and the nematodes were observed for the next several days.

CHAPTER III

MATERIALS AND METHODS

Construction of the Experimental Unit

Sections of 157.5 cm long sewer tile (manufactured by Can-tex Solid Ceramic Tile, Mineral Wells, Texas), with an inner diameter of 20.3 cm and an outer diameter of 24.1 cm, were cut in half crosswise with a circular saw equipped with a masonry blade. The flanged tiles were halved in such a manner as to give equal barrel volumes to both of the resulting flanged and unflanged sections. Vent holes of 9.5 mm were then drilled in the sections with an electric drill using a concrete bit. The vent holes were drilled 10.2 cm from the tile bottom on the flanged sections and 5.1 cm from the bottom on the unflanged sections. The tile sections were next plugged with a layer of waterproof concrete. The recipe used for the waterproof concrete was one part masonry cement, two parts builders sand, one part 9.5 mm chat, and one part water. The plugging was done by standing the tile upright on a plastic tarp and pouring enough batter into the bottom to form a plug (5.1 cm thick for the male section and 10.2 cm thick for the female section in order to equalize the barrel volumes). Following at least a 48 hour curing period, a second layer of batter was added with the tiles tilted at approximately a 45° angle, so that a 45° concrete plane sloping downward to the vent hole was formed (Figure 1). Again the tiles

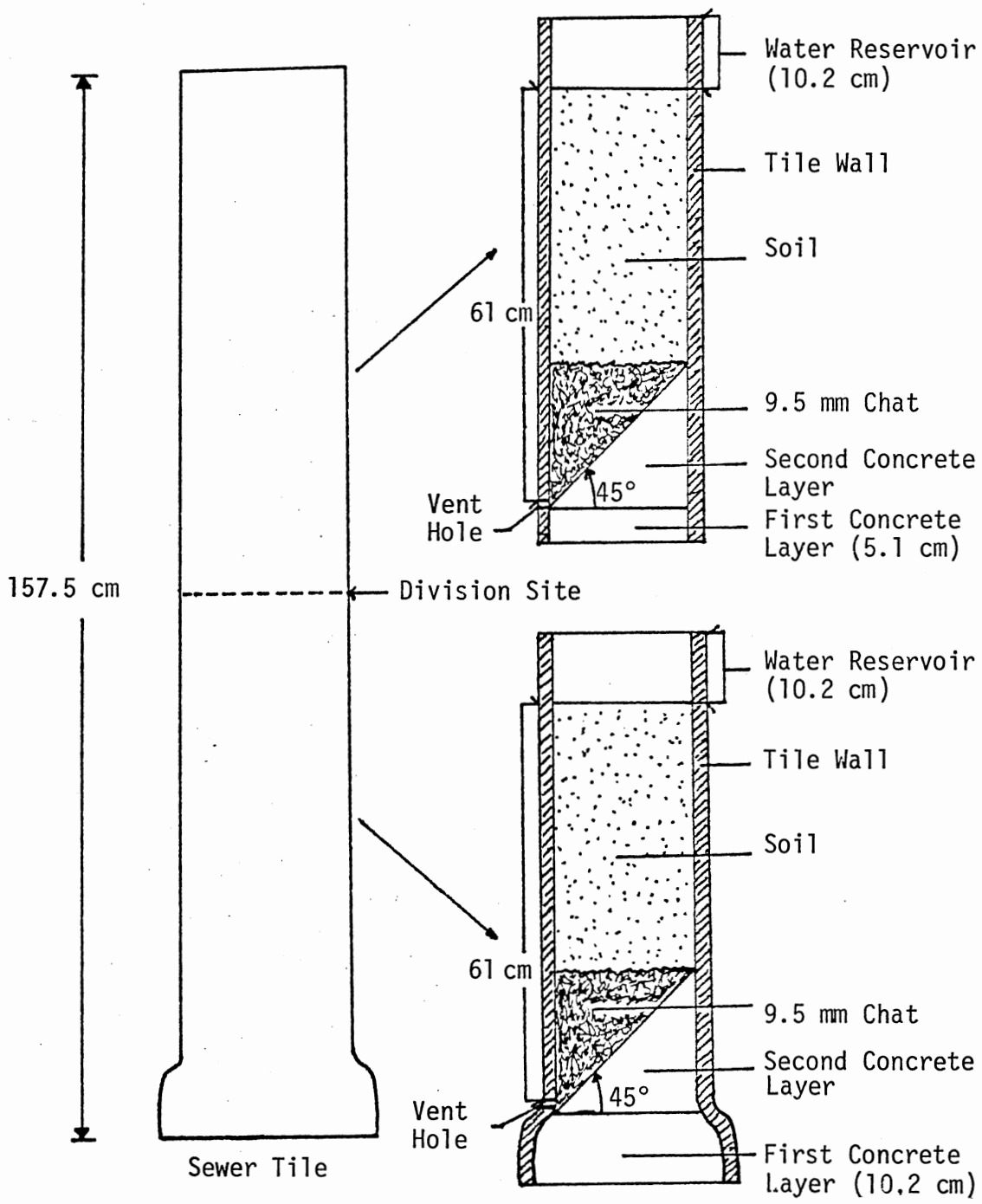


Figure 1. Conversion of the Sewer Tile to the Experimental Units. The two finished units contain equal volumes.

were allowed to cure for at least 48 hours. The tiles were then fitted with hose connections to facilitate sample collection. A male hose connection was inserted into the vent hole and cemented in place using Dow Corning silicone rubber marine sealant. A rubber hose with a female coupling was attached during each harvest in order to direct the leachate flow into the collection container (Figure 2).

Four treatments of two soil types were used: Lincoln fine sand (LFS), builders sand (BS), a half and half mixture of the two, and a layering of the two (a top half of LFS on a bottom half of BS). The LFS was sterilized with methyl bromide at a rate of two 1.5 pound (681 g) cans per cubic yard of sand. The BS was not sterilized, but careful sampling revealed no nematodes present. In all cases the soil was added to a layer of 9.5 mm chat in the bottom of each tile. Enough chat was used to level the 45° angle of the concrete and served to keep the sand from washing out the vent hole during watering and, more importantly, during harvest. Soil was added to each tile so as to leave a 10.2 cm "water reservoir" at the top of each (Figure 1). Additional temperature control (TC) treatments were added, one corresponding to each of the previously described soil treatments. These TC treatments were added to offset the anticipated deleterious effect of temperature fluctuations within the units during the production of the root-knot inoculum. The temperature selected was 25°C, which has been reported to be optimum for the reproduction and development of M. hapla (1, 7). The TC was done by circulating temperature controlled water through specially bent 7.9 mm copper tubing heat sinks inserted into each of these tiles (Figure 3). The heat sinks were

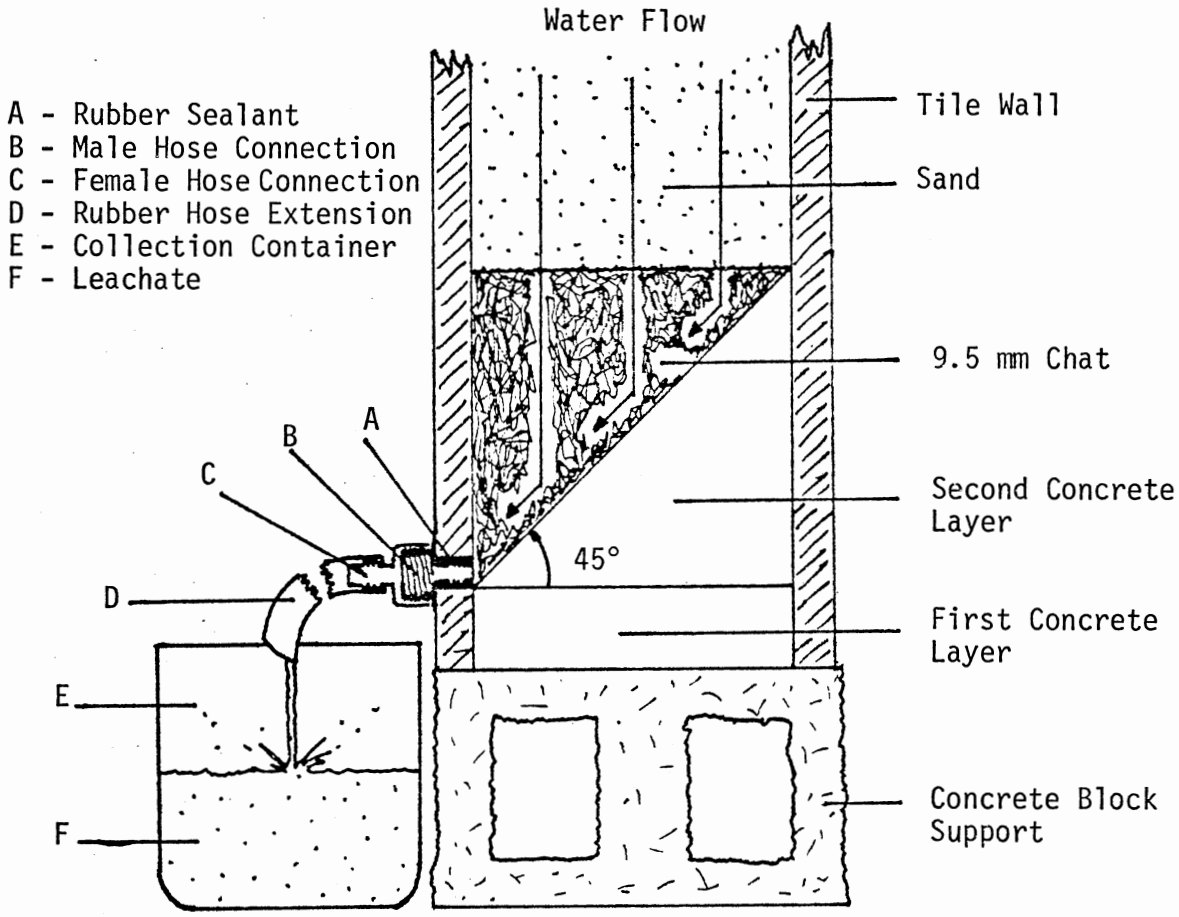


Figure 2. Detail of the Vent Hole Assembly

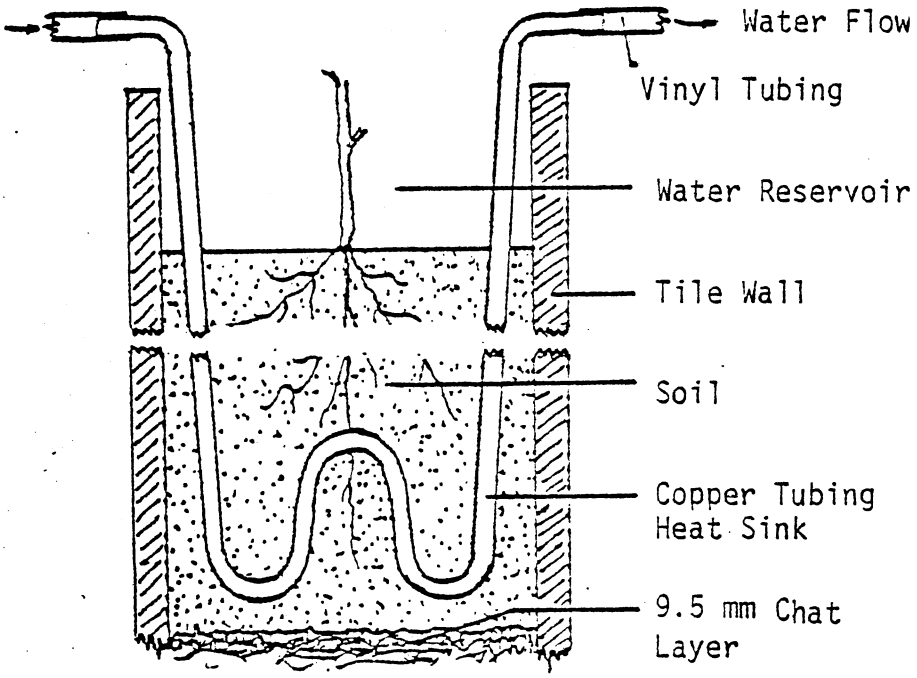
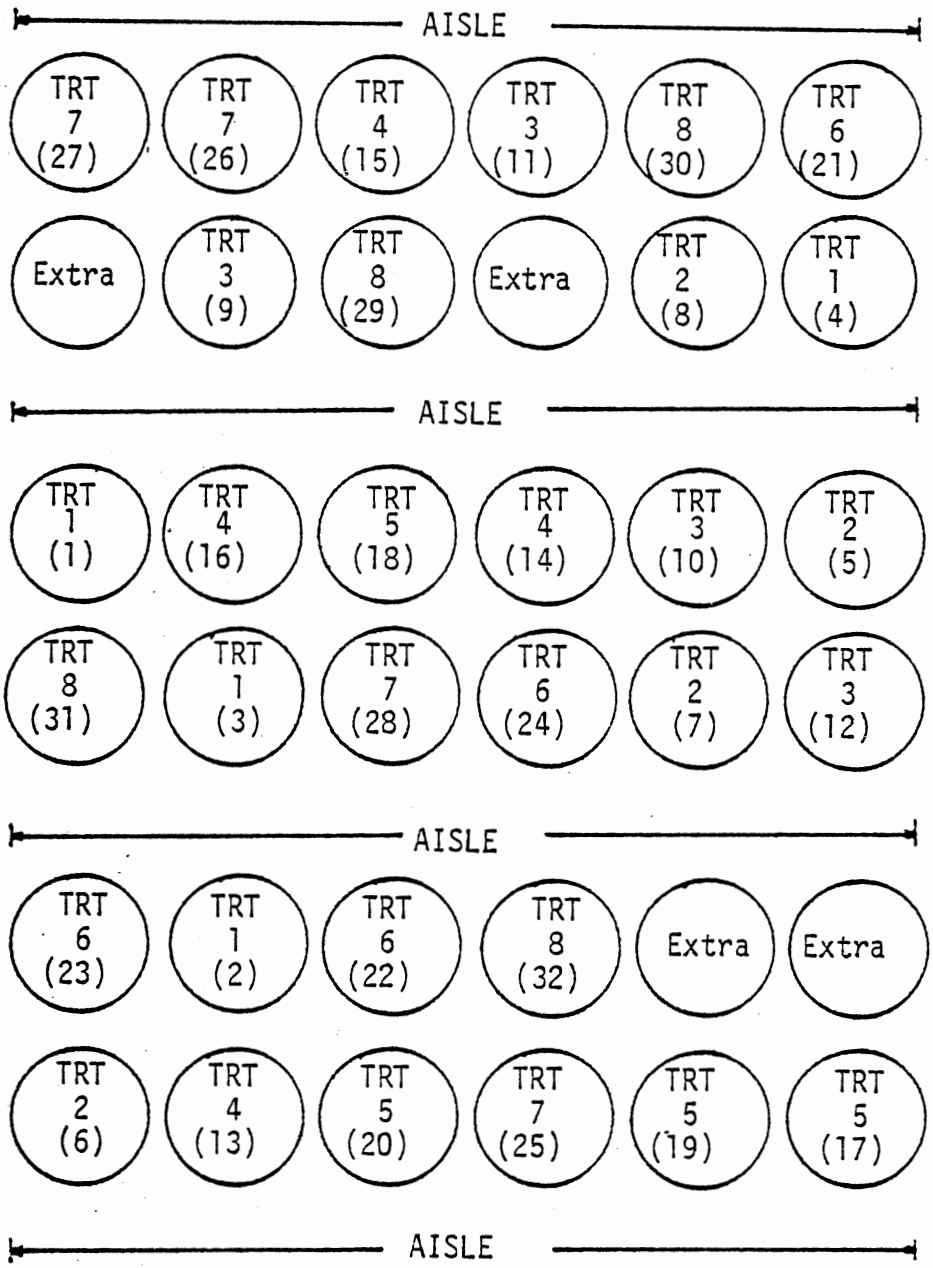


Figure 3. Detail of the Temperature Control Assembly Showing the Design of the Copper Tubing Heat Sink

coated with marine epoxy to eliminate any adverse effects that might result from the plants coming into contact with the copper (copper toxicity). Vinyl tubing was used to connect the heat sinks, forming a circulation loop (approximately ten heat sinks per loop). Three circulation loops were necessary to prevent reduction of flow due to friction loss and provide efficient temperature control in these units. In order to deliver 25°C water to the copper tubing, the thermostat on the temperature controlled water vat was set at approximately 23°C. Thermometers were inserted to a depth of eight cm in several of the TC tiles, as well as in the tiles receiving no TC, in order to monitor the effectiveness of this method of temperature control. Tiles selected for monitoring included both the first and last tiles in a circulation loop and one or two in the center. The eight treatments of four tiles each were set up in a completely randomized design as dictated by space limitations and other greenhouse factors (Figure 4). Each tile was elevated by placement on a concrete block to aid in harvesting. Prior to planting, the tiles were thoroughly flushed with water until the drain water was relatively clear.

Inoculation of the Experimental Unit

Four week old Rutgers tomato plants growing in LFS in 10.2 cm clay pots were inoculated with approximately 500 M. hapla larvae. The plants were inoculated by washing a small hole around the base of the plant with a wash bottle and pipetting a larval suspension into the resulting hole. This procedure was followed to avoid root injury and thereby eliminate the possible entry of the nematodes into a severed root.



Treatments (TRT):

1 - LFS (1-4)
2 - LFS with TC (5-8)
3 - LFS on BS (9-12)
4 - LFS on BS with TC (13-16)
5 - BS (17-20)
6 - BS with TC (21-24)
7 - LFS-BS mixture (25-28)
8 - LFS-BS mixture with TC (29-32)

Lincoln Fine Sand - LFS; Builders Sand - BS; Temperature Control - TC

Figure 4. Completely Randomized Design Showing Location of Individual Treatments and Tiles. The aisles were used during harvesting as space for the leachate collection containers.

The hole was then filled with sterilized LFS. The initial inoculum was obtained from greenhouse stocks and the species was verified by the examination of the perineal patterns of several adult females. The northern root-knot nematode, M. hapla, was selected for use primarily because of the external location of its eggs masses on Rutgers tomato, which should aid in the ready collection of the larvae. After an additional four weeks, the tomatoes were transplanted into the tiles (one per tile). Established young colonies were used instead of planting a tomato seedling in the tile and then inoculating it for two reasons: (1) the upper layer of the tiles containing BS dried out rapidly, making it difficult to prevent a small seedling from becoming water stressed, and (2) inoculation of the seedling in the tile could be inefficient due to the leaching of the inoculum away from the host plant during routine watering. The tiles were watered and fertilized (Peter's soil test soluble fertilizer 20-20-20) as needed. The plants were allowed to grow for ten weeks before sampling was initiated. The tiles were then harvested bi-weekly for a period of six months.

Harvesting

Because of the differences in the moisture retention characteristics of the soils studied, the tiles were "flushed" until six liters of leachate had been collected, rather than flushed with a certain quantity of water. The water was added to the "water reservoir" and allowed to drain through the tile (leaching out M. hapla larvae). During the harvesting, the water reservoir was kept full at all times to ensure uniform pressure and water flow through the tile. When four

liters of leachate had been collected, the water reservoir was filled for the last time. This resulted in approximately six liters being collected in all. The leachate was collected in plastic containers at the base of the tile from the rubber hose extensions previously described (Figure 2). The leachate was then poured through a 400 mesh screen and the screen contents concentrated and processed using the Oklahoma State University (OSU) tub technique (a modification of the Christie-Perry technique (9)) described by Alby (2). After 24 hours, the samples were decanted and poured into 50 ml beakers. Following a minimum of two hours resettling time, the samples in the 50 ml beakers were decanted to a volume of 40 ml. After thoroughly agitating the 40 ml, two two ml aliquants were withdrawn and counted. The total number of larvae in the original sample was determined by taking the combined aliquant counts times ten. Counting by aliquant was necessitated by the high numbers of nematodes recovered. During later harvests, a contaminating predacious nematode began showing up in several samples. When present, the predators were counted and their numbers recorded.

Controls

In order to ascertain the efficiency of the tile technique, the method was compared to the standard technique used at OSU: incubation of washed galled root masses of Rutgers tomatoes in aerated 250 ml flasks of water. The controls consisted of sixteen 10.2 cm pots of Rutgers tomatoes inoculated in the same manner as previously described with the same number of infective M. hapla larvae. Eight of these pots received the 25°C TC via the heat sinks previously described. These heat

sinks were also coated with marine epoxy and consisted of a single loop inserted into each pot (Figure 5). These plants were also watered and fertilized as needed. After ten weeks, the plant tops were discarded, the soil processed for root-knot larvae (again using the OSU tub technique (2)), and the roots incubated. The roots were then processed at weekly intervals by pouring the flask contents through nested 30 mesh and 400 mesh screens. The roots, which remain on the 30 mesh screen, were returned to the flask, the flask re-filled with water, and the roots incubated for another week. The contents of the 400 mesh screen were processed and the nematodes counted in the same manner as previously described for the tiles. The roots were processed weekly until larvae were no longer being recovered.

Migration Studies

Silica sand (Penn-Sand number one grade silica sand, 100 pound (45.36 kilos) bag, Pennsylvania Glass Sand Corporation) was selected for this study because of its uniformity, lack of organic matter, and commercial availability. Both sterilized (autoclaved for one hour) and unsterilized silica sand were used in preliminary tests with no differences in larval migration or percent survival. Analysis of the unsterilized silica sand prior to inoculum introduction failed to reveal the presence of any nematodes or living material. Therefore, the unsterilized sand was used for all further testing. Field capacity (FC) of the silica sand was determined by saturating a known quantity (weight in grams) of the sand in vented styrofoam cups, covered to retard evaporation. After eight hours the sand was reweighed and 100% FC was determined by subtracting the dry sand's weight from the final

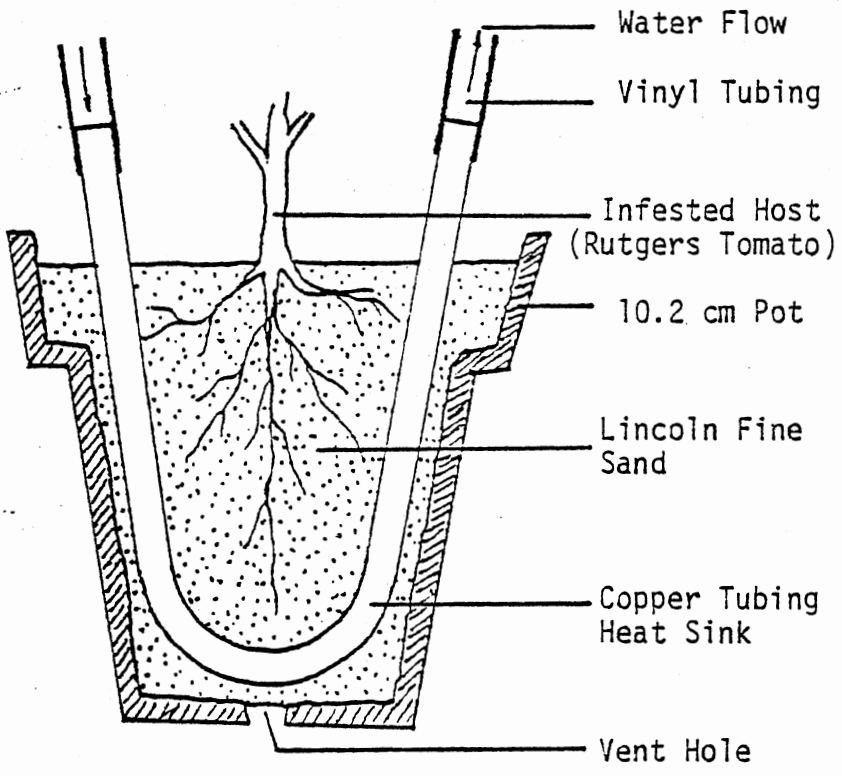


Figure 5. Detail of the Temperature Control Apparatus Used in the 10.2 cm Pot Controls

weight. Various increments of FC were tested, including 95%, 90%, 85%, 80%, and 75%. These were determined by multiplying the percentage desired times the number of ml of water necessary to give 100% FC.

The attractants used were living Rutgers tomato seedlings and excised Rutgers tomato roots. The tomato seedlings were pregerminated for five days prior to use in an aerated flask of water, and only those with approximately three mm of primary root were used. The roots used were from healthy Rutgers tomatoes grown in the greenhouse and were cut into three-six mm sections prior to use. In both cases, dialysis tubing (0.4" (10.2 mm) flat width, Union Carbide, cat. no. D1615-4) was used to provide a barrier between the attractant and the nematodes (8). This was necessary in order to prevent root penetration by the nematodes so that the larvae that were attracted to the site could easily be recovered. Controls having only the dialysis tubing were also tested as well as plates having only nematodes. The former was to test the effects of only the dialysis tubing on the nematodes, while the latter served to monitor the extent of any random nematode movements.

Inoculum

The nematodes used for this phase of testing were obtained from the inoculum production samples. Several of these samples were concentrated to the point where approximately 1000 larvae could be dispensed in 0.5 ml of water. In all plate experiments, the nematodes were introduced in 0.5 ml of water. One thousand larvae per plate were used whenever possible; however, sometimes fewer were used due to low numbers available at the time the test was initiated.

The Petri Plate Test Unit

The basic unit in these experiments was a square petri plate (Lab-Tek, 100 mm x 100 mm x 15 mm, polystyrene, cat. no. D1936). Because of reported lethal effects of newly unwrapped plastic containers (22), care was taken to use either all new or all used petri plates during each test. Each plate was filled with 160 grams of silica sand and the sand in the plate leveled by gently shaking the plate from side to side. A water volume of 0.5 ml less than that required to provide the desired FC was added to the plate. A chart of the FC percentages and the water necessary to reach them for 160 grams of silica sand can be found in Table I. The remaining 0.5 ml of water was introduced, with the nematode inoculum suspension giving the percent FC desired. The water was carefully applied to all of the sand's surface, except for the area immediately around the inoculum introduction site. This dry area covered approximately one square centimeter and was wet by the addition of the inoculum. The inoculum introduction site was left dry to minimize nematode movement due to the water flow when the plate's moisture equilibrated. The attractant was then inserted into one side of a 24 mm section of dialysis tubing which had been folded in half (Figure 6). The fold served to seal the bottom and prevent nematode entry into the attractant. A slit was made down into the wet sand at one of the two attractant sites (Figure 7), and the folded dialysis tubing was inserted into the sand. The beaker containing the nematode suspension was agitated and a 0.5 ml volume was withdrawn using a pipette. The 0.5 ml volume was then placed on one of the two inoculum introduction sites (Figure 7). In the controls, only folded dialysis

TABLE I

AMOUNT OF WATER NECESSARY TO GIVE DESIRED FIELD CAPACITY IN 160 GRAMS OF SILICA SAND

% Field Capacity	ml Water
100	30.0
95	28.5
90	27.0
85	25.5
80	24.0
75	22.5
70	21.0

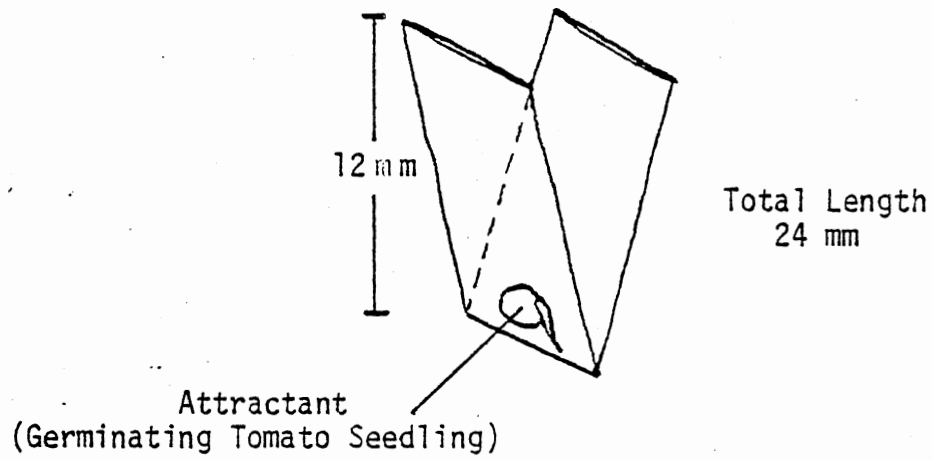


Figure 6. Detail of the Dialysis Tubing

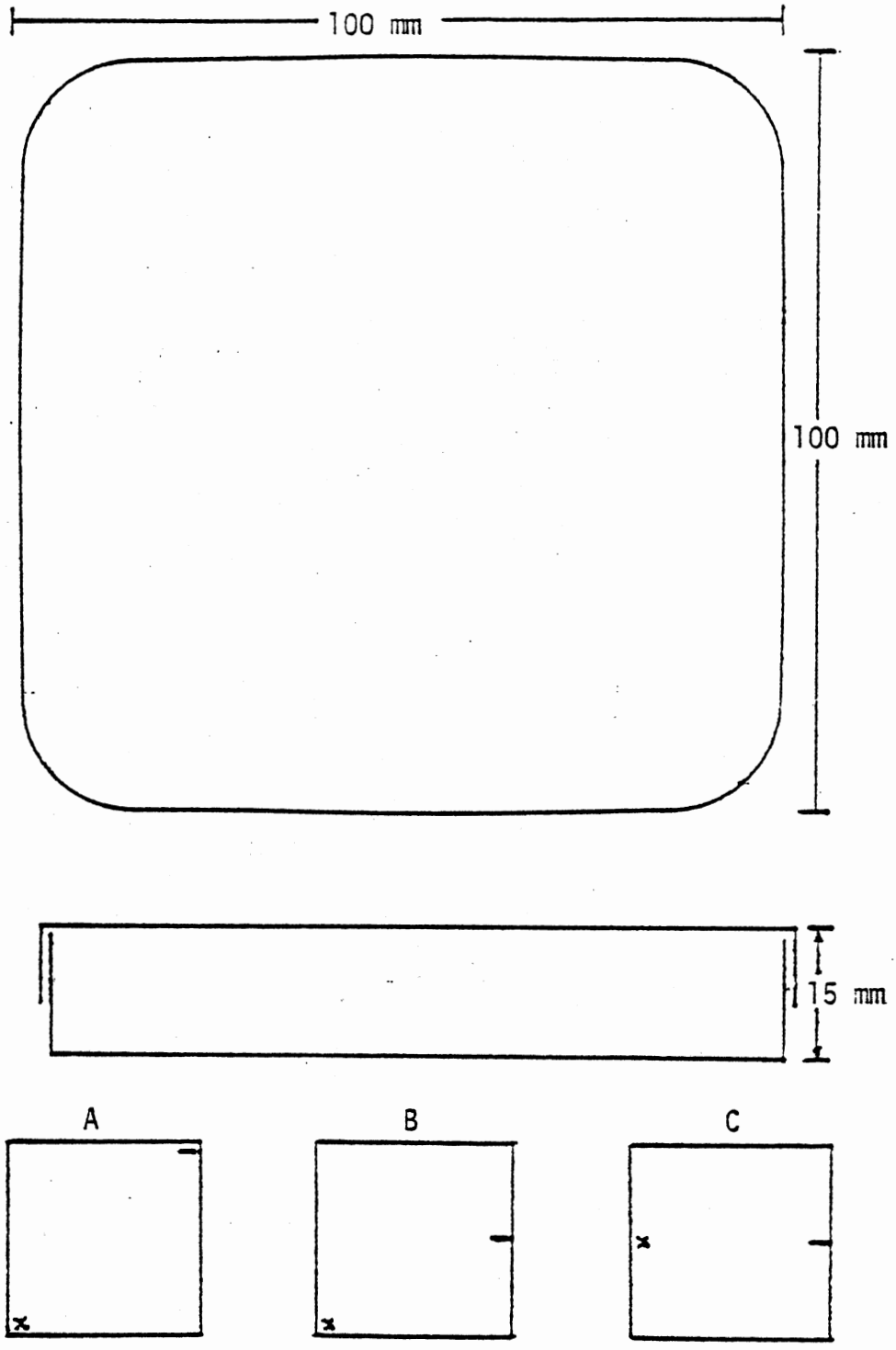


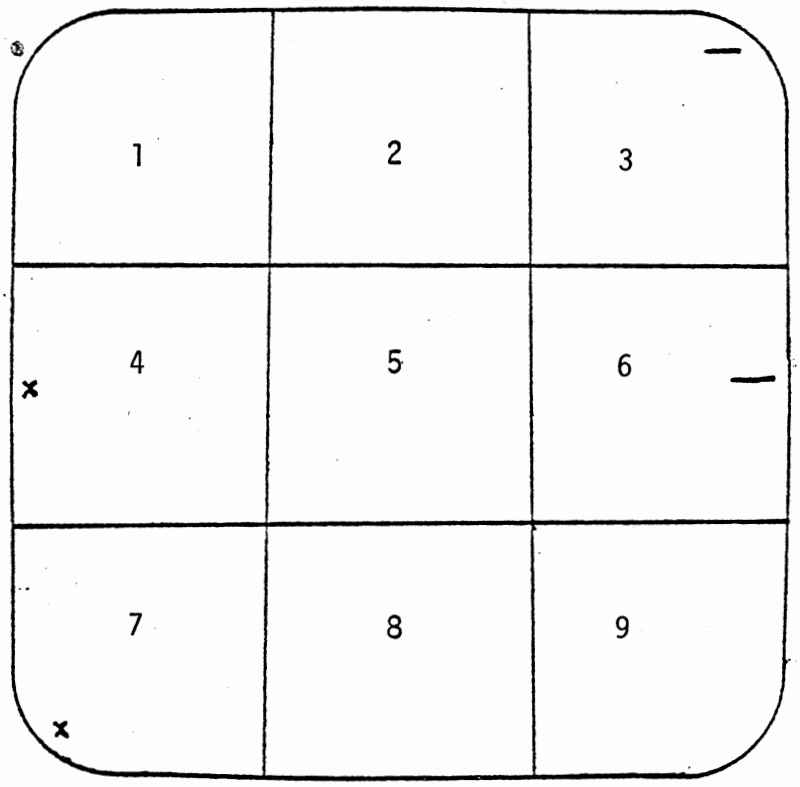
Figure 7. Plate Dimensions and Detail of the Three Combinations of Inoculum Introduction and Attractant Sites Tested

tubing was inserted into the attraction sites and random movement plates received only nematodes. Following treatment, the covers were placed on the plates, and the plates were sealed using four strips of adhesive freezer tape. The tape seal served to retard much of the evaporation.

Three plate positions were included in the tests: flat, upright, and tilted (approximately 45°). The plates were covered to exclude light and incubated at room temperature (26°C). The units were sampled at five days, although some tests also included samplings at one, two, three, and four days.

Data Collection

Following the removal of the petri plate cover, the bottom of the plate (containing the sand) was inverted and dropped lightly on a smooth, clean surface. The plate bottom was removed, leaving the square section of moist sand. The sand was then sectioned into nine equal blocks with a spatula (Figure 8). Each block was then removed and placed directly on the tissue surface of the Oklahoma tub (2). The block of sand was then gently spread into a thin layer on the surface of the tissue by the use of a wash bottle. Any excess water was carefully withdrawn at the edge of the tub(from outside the tissue) with the aid of a bulb syringe. The samples were decanted, placed in counting dishes, and counted 24 hours later.



× - Inoculum Introduction Sites

— - Attractant Sites

Figure 8. Plate Sectioning and the Block Numbering System Used

CHAPTER IV

RESULTS AND DISCUSSION

Harvest Times

The tile harvesting process required differing amounts of time for the different soil types. There were also variations in the harvesting time necessary among individual tiles of the same soil types. This variation was probably the result of the soil's differing moisture retention characteristics and degree of compaction. Generally, the time required for flushing enough water through the tiles to collect six liters of leachate ranged from ten to 15 minutes for the builders sand (BS) to a maximum of 45 minutes for the lincoln fine sand (LFS). The time needed to harvest any one given tile remained fairly constant during the study. In addition to a rapid collection time, the screened leachate was processed and ready for use within 24 hours. With continuous, regularly spaced (bi-weekly) harvesting, it was possible to obtain such clean, soil-free leachate that the larvae could be screened and placed directly into a beaker for counting or use. Utilizing this system, the time necessary to obtain clean inoculum could be reduced to one hour or less. The root incubation, by contrast, required one week's aeration and another 24 hours processing time. Obviously, the tiles could provide larvae very rapidly, and this could be a decided advantage when compared to other relatively slow techniques.

The greatest advantage of the tile inoculum production technique was the fact that the host plant was not damaged in any manner during harvest. No roots were removed or damaged; the flushing harmlessly leached out quantities of the root-knot larvae. This nondestructive harvest allowed the host to maintain growth and yield a continuing supply of larvae because those harvested were constantly being replenished by the production of eggs from older and maturing (from reinfecting larvae) females. The root incubation method (and other techniques) required the removal of the root system for larval extraction, thereby destroying the host. This method was needlessly wasteful, arresting further egg mass and larval production and preventing the larval production potential of the host from being realized. The numbers of eggs and larvae that were present at harvest in the root incubation method were the maximum numbers of nematodes that could be recovered. The tiles, however, continued to produce larvae after harvest and, under greenhouse conditions, could be a viable inoculum producing colony for two years or more. Utilizing this factor in a comparison of the numbers of larvae produced per unit time, including the initial growth period, the best tile treatment produced an average of 3,350 larvae per week. By comparison, the best root incubation produced only 489 larvae per week. If a comparison on larvae produced per unit area is calculated, the numbers are a bit closer: 3,350 larvae per week (per unit area, approximately 929 square cm) for the root incubation technique. The latter comparison was made on the basis that four 10.2 cm pots require approximately the same area as one tile. In either comparison, the tile technique remains superior to the root incubation. In addition,

the longer the tile unit is used continuously without having to re-establish the colony, the more efficient it becomes when compared to the destructive sampling of the root incubation technique.

Yields

The average numbers of M. hapla larvae harvested for each tile treatment are shown in Table II, as well as the average yields of the root incubation technique. The two methods were compared on the basis of the numbers of larvae produced during two week periods and by the length of time larvae could be generated. Although the initial numbers of larvae recovered were higher for the root incubation method, a rapid decline occurred and high numbers could not be recovered beyond the third or fourth week. The tiles, by contrast, offered a continuing source of inoculum, the numbers of which steadily increased during the study. A comparison of the two techniques is shown in Figure 9.

The average yields for each tile treatment were as follows: treatment number one (LFS) - 21,016; treatment number two (LFC with TC) - 20,257; treatment number three (a layer of LFS on a layer of BS) - 21,042; treatment number four (a layer of LFS on a layer of BS with TC) - 12,719; treatment number five (BS) - 64,187; treatment number six (BS with TC) - 1,951; treatment number seven (LFS - BS mixture) - 46,898; and treatment number eight (LFS - BS mixture with TC) - 37,282. The average yields of the root incubations were 14,843 and 14,760 for the TC. Statistical comparison of the yields indicates six of the eight tile treatments yielded significantly higher numbers of larvae than the root incubation (control) method ($P = 0.10$). These treatments were one, two, three, five, seven, and eight. Three of these treatments, five,

TABLE II

AVERAGE NUMBERS OF *MELOIDOGYNE HAPLA* LARVAE RECOVERED DURING A TWO WEEK PERIOD FOR THE EIGHT TILE TREATMENTS AND TWO ROOT INCUBATION TREATMENTS

Treatments	Harvests							
	1	2	3	4	5	6	7	Totals
1 : LFS	4620	3674	1772	2969	1436	6420	125	21016
2 : LFS with TC	1071	443	275	568	899	11800	5201	20257
3 : LFS on BS	608	3302	1088	2751	1880	10080	1333	21042
4 : LFS on BS with TC	8	150	282	2431	1662	7460	726	12719
5 : BS	161	6593	2700	17045	3819	25804	8065	64187
6 : BS with TC	31	42	65	125	187	1300	201	1951
7 : LFS/BS Mixture	5807	8258	4373	19119	2460	5480	1401	46898
8 : LFS/BS Mixture with TC	900	901	880	4595	1860	19003	9143	37282
Root Incubation	12710	1860	238	22	10	3	0	14843
Root Incubation with TC	8215	5001	1413	109	17	4	1	14760

LFS = Lincoln Fine Sand BS = Builders Sand TC = Temperature Control

Because of the tile's bi-weekly harvesting, the corresponding weekly root incubation averages have been added to give a two week total for an easier comparison of larval production. Least Significant Difference (LSD) 0.10 = 758.75, LSD 0.01 = 1209.74. CV = 2.33%.

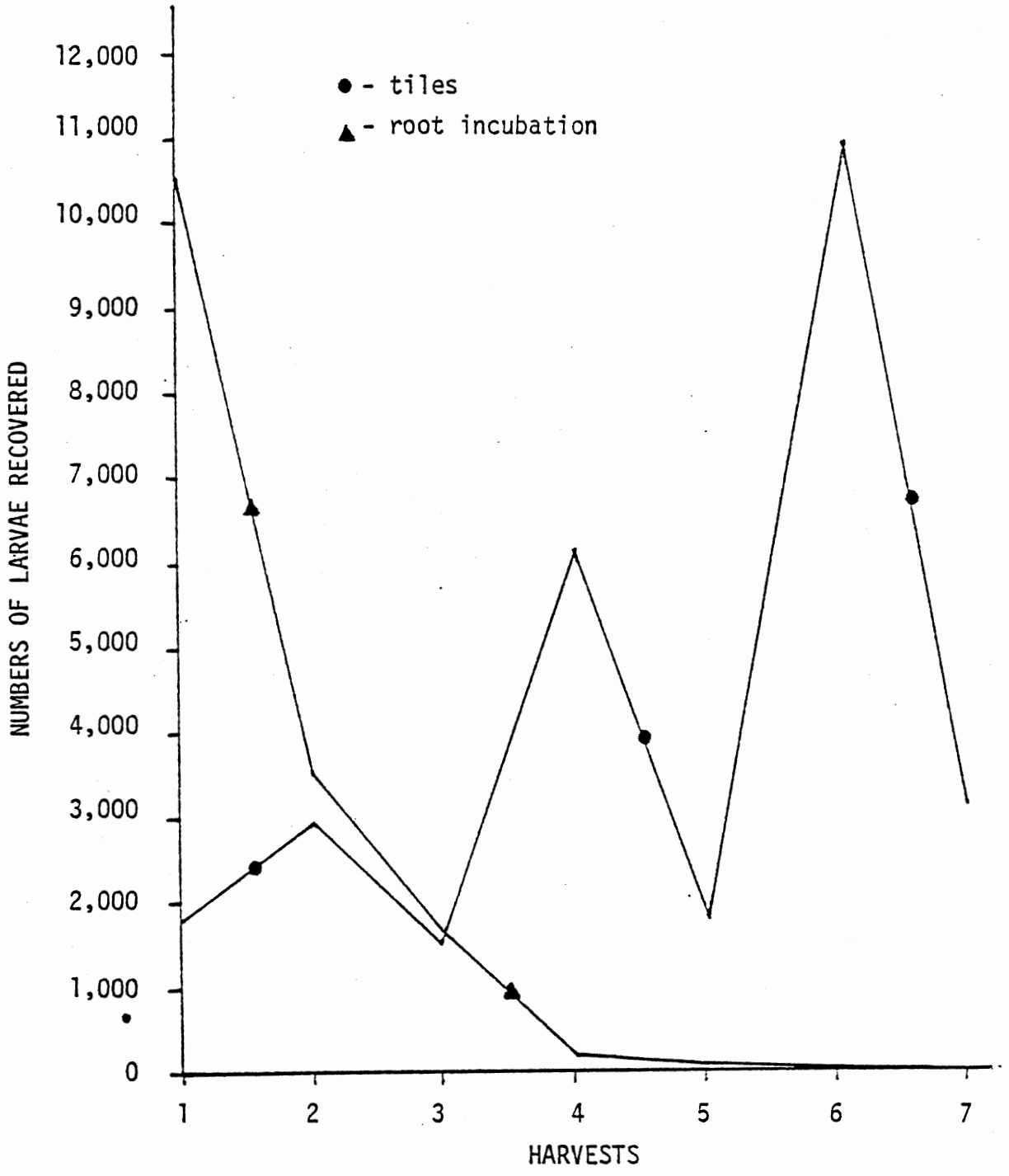


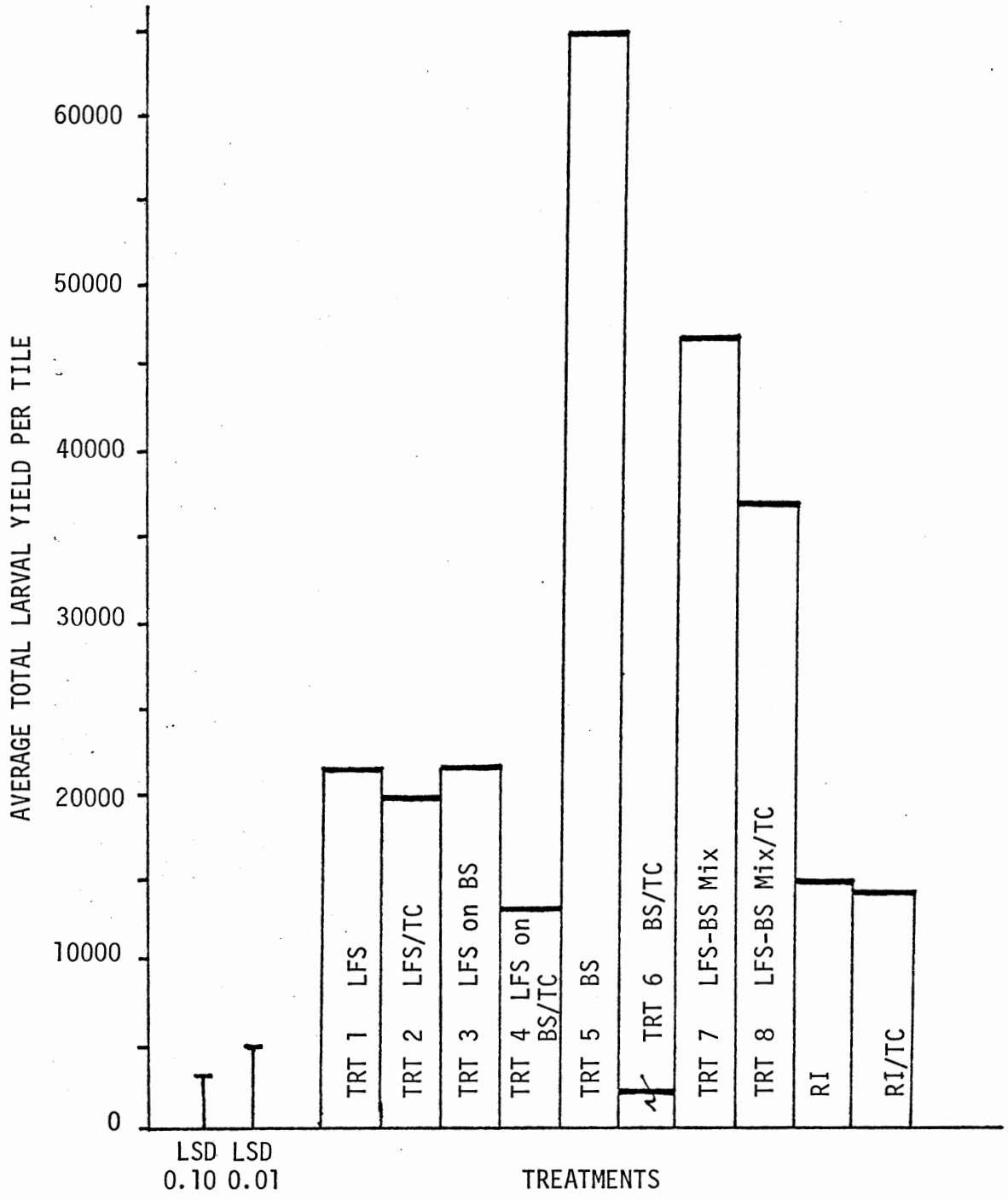
Figure 9. Average Numbers of Meloidogyne hapla Larvae Recovered for Each Harvesting of the Tiles Compared to Those Recovered from the Root Incubation Method

seven, and eight, yielded numbers significantly higher (P = 0.01). The treatment yielding the greatest significant difference from the controls was number five (BS, no TC), which was also significantly higher (P = 0.01) than any of the other treatments. This is advantageous for two reasons: (1) BS involves the least amount of time to harvest, and (2) the TC is unnecessary, thus reducing unit cost and maintenance. A presentation of the statistical comparisons can be found in Figure 10.

Temperature Control

The TC treatments maintained the tiles to within ± 2°C of the 25°C optimum temperature. The ambient units, however, varied as much as 15°C from this optimum. A comparison of the overall larval production with respect to TC is shown in Figure 11. The tiles supplied with TC yielded substantially lower numbers of larvae than did the ambient tiles. One of the TC tile treatments (BS with TC) yielded significantly less (P = 0.10) larvae than did either of the control techniques (root incubation with or without TC). Two of the remaining three TC treatments did yield significantly higher numbers than the controls, LFS with TC (P = 0.10) and LFS-BS mix with TC (P = 0.01). The best TC treatment, LFS-BS mix, was only the third best yielding treatment in the study, however, and was significantly out yielded (P = 0.01) by both BS (no TC) and LFS-BS mix (no TC).

Additional advantages of the tile technique include the simplicity of the harvest and the fact that little equipment is necessary. Furthermore, the harvest can be done entirely in the greenhouse. The root incubation method, however, requires the use of the laboratory and flask aeration in addition to the amount of greenhouse bench space used



Treatment = TRT Lincoln Fine Sand = LFS Builders Sand = BS
Temperature Control = TC Root Incubation = RI

Figure 10. Average Larval Yield Per Tile Each Treatment.
CV = 2.33%

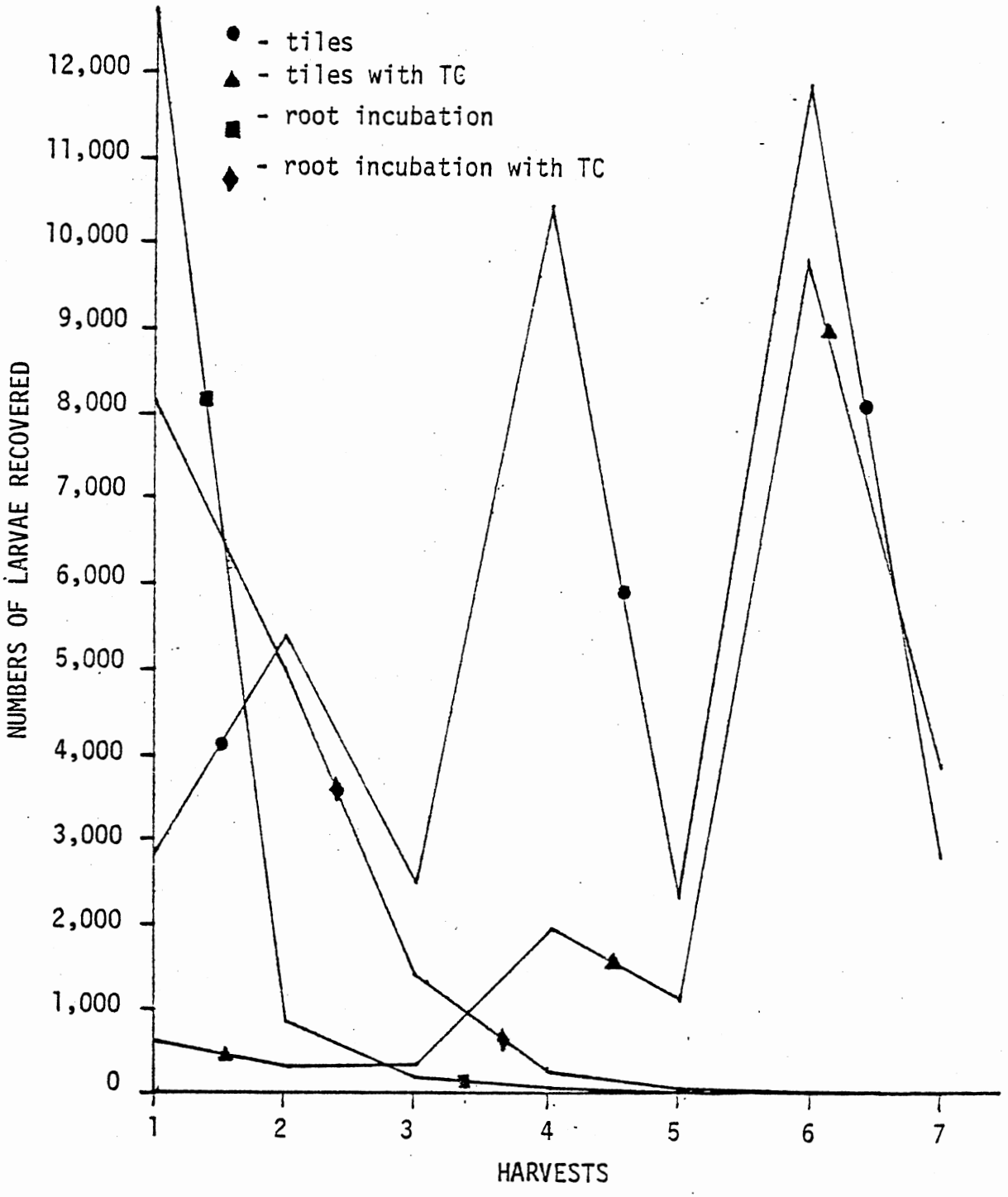


Figure 11. Comparison of the Effects of Temperature Control (TC) on That of No TC on Larval Yields in Tiles and Pots

for growing the 10.2 cm pots of infested tomatoes. The tiles, by comparison, do not require any bench support as they stand on concrete blocks on the floor. As a result, the tile units can be placed next to the walls, by the cooling pads, in the aisles, or in any space not normally used. This would help conserve valuable greenhouse space and free benches for other uses.

A slight problem caused by the flushing during harvest was the leaching of soil nutrients from the system as well as larvae. As a result, it was difficult to maintain good soil fertility in the porous soils. To compensate for this effect, the plants were fertilized the day following each harvesting thus maintaining good host growth.

Tile Contamination

The numbers of larvae recovered began to decline following contamination of the colonies by a predacious dorylaim (tentatively identified as Mesodorylaimus sp.). These nematodes were initially recovered in very small numbers during the fourth and fifth harvestings, but began to occur in very high numbers beginning in the sixth harvesting. In addition, the predacious nematodes were scattered throughout 22 of the 32 experimental units. Even in tiles where root-knot larvae recovery had been high, a rapid decline in production occurred with the increase of the population of the predator. The harvesting history of one particular contaminated tile (number 25, treatment number seven) illustrates this pattern very well (Figure 12). Harvesting of the tiles continued, but the data collected was of little use. It should be noted, however, that in the tiles that remained free of the contaminant, recovery of the root-knot larvae continued unabated. Larvae continued to be

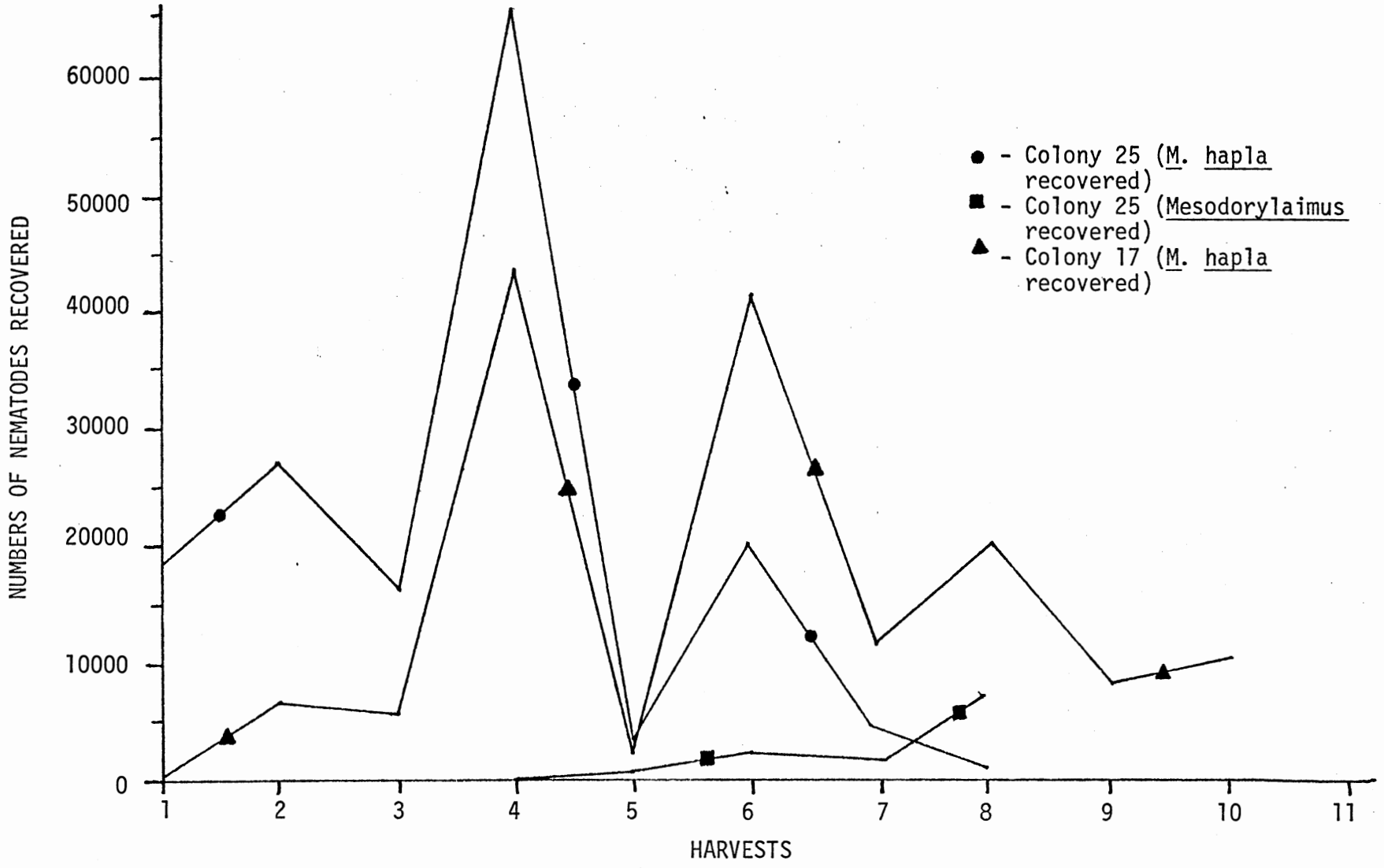


Figure 12. Comparison of the Numbers of Meloidogyne hapla Larvae Recovered from a Mesodorylaimus sp. Contaminated Tile Colony to Those Recovered from the Uncontaminated Tile Colony

recovered in large numbers in these colonies as long as harvesting was continued.

The source of the contamination is unknown at present. The soils used were free from any nematodes. In addition, the experimental units were located in an isolated section of the greenhouse, eliminating any possibility of the predator having been splashed into the tiles from other greenhouse pots during routine watering. Splashing, however, could be a possible explanation as to how the nematodes were spread from tile to tile once they were established. Other possible sources of the original infestation could include man, mice, insects, etc.

Insect pests, such as aphids and white flies, were present, but kept under control through the use of contact insecticides (such as malathion). These insecticides were applied with the use of an atomizer, which produced a very fine mist. The fine mist was necessary to prevent runoff of the chemical into the soil, and thereby prevent exposure of the root-knot larvae to any toxic compounds.

Incubation Time

The incubation time of the petri plate migrations was set at a maximum of five days. The small size of the experimental unit coupled with the ability of the root-knot larvae to traverse it readily made five days more than an adequate time to achieve results. In addition, the five day incubation period corresponded to the work week, allowing the tests to be initiated on Monday and processed on Friday. In order to determine the speed and extent of the nematode's random movements, a study was conducted whereby several plates were sampled on each of the five days. These plates were at 95% FC, incubated flat at room

temperature (26°C). The inoculum introduced was 500 M. hapla larvae per plate. The plates contained only nematodes; no attractants were present. By the third day, larvae were being recovered in every block of the plate, regardless of the inoculum introduction site. The final (fifth day) average counts of this random movement study are shown in Table III. The results of these plate migration tests show relatively high numbers of larvae were present in the block (number six) that in later tests would contain the attractants when the inoculum was introduced in block number seven. It is interesting to note that even after five days, an average of better than 60% of the initial inoculum was recovered.

Field Capacity

Petri plate samples were tested using varying percentages of FC ranging from 70% to 95% (by increments of 5%). The plates contained only root-knot larvae and silica sand - no attractants were present. These tests indicated that the larvae moved about most freely at the upper FC percentages. Therefore, 70%, 75%, and 80% field capacities were eliminated from further testing. Additional tests using the field capacities 85%, 90%, and 95% revealed the greatest amount of random nematode movement occurred at 95% FC. Although these results were not statistically significant, the differences observed served as the basis for the discarding of both the 85% and the 90% field capacities. The results of this test are shown in Table IV. The figures shown correspond to the number of larvae recovered from the plates' block number one - the one farthest from the inoculum introduction site (block number nine). The counts of the larvae recovered

TABLE III

NUMBERS OF MELOIDOGYNE HAPLA LARVAE RECOVERED FROM PLATES WITHOUT ATTRACTANTS SHOWING THE EXTENT OF RANDOM MOVEMENTS FROM TWO DIFFERING INOCULUM INTRODUCTION SITES

Site	Sample	Plate Block 1	2	3	4	5	6	7	8	9	Totals
1 (Block 4)	1	104	124	32	68	48	4	12	12	8	412
	2	80	92	24	52	36	0	12	60	4	360
	3	40	68	20	36	56	12	8	40	12	292
	4	44	24	28	8	12	28	4	0	8	156
	Average	67	77	26	41	38	11	9	28	8	305
2 (Block 7)	1	8	8	56	12	16	36	72	72	44	324
	2	4	20	92	8	36	120	52	56	52	440
	3	8	12	28	8	16	100	40	32	56	300
	4	8	12	24	4	36	56	80	8	56	284
	Average	7	13	50	8	26	78	61	42	52	337

The plates were at 95% field capacity, incubated flat for five days at room temperature. The inoculum introduced was 500 M. hapla larvae per plate.

TABLE IV

THE NUMBERS OF MELOIDOGYNE HAPLA LARVAE RECOVERED IN PLATE BLOCK NUMBER ONE DURING RANDOM MOVEMENT STUDIES, BY PERCENT FIELD CAPACITY

% Field Capacity	Replicates				
	1	2	3	4	Average
85	0	4	0	8	3.0
90	40	4	0	0	11.0
95	12	24	16	12	16.0

The plates were incubated flat for five days at 26°C. The inoculum consisted of 1000 larvae per plate introduced in plate block seven. LSD (0.10) = 17.8, CV = 43.26%.

in blocks number one only are shown, because sampling indicated this block was the last one during any of the tests to show larvae. Therefore, it was a good indicator of the extent of the nematode's movements. Additional tests conducted on the three field capacities in plates containing larvae and attractants indicated the similar, more extensive larval movements at the 95% FC. An added advantage of using the 95% FC was generally a higher recovery rate of the initial inoculum. That is, greater numbers of the larvae introduced into these plates survived the five day test period at 95% FC than at any other percentage tested. Therefore, all further testing was limited to the use of 95% FC.

Petri Plate Positioning

In tests conducted with the petri plates in a vertical or tilted (45° angle) position, the results were biased by the effects of gravity on the larval movements. In all of these tests, the majority of the larvae were recovered in the lower sections of the plates (blocks seven, eight, and nine). This effect was observed regardless of the presence or absence of an attractant. The results of one such test are shown in Table V. The numbers of larvae found plate blocks seven, eight, and nine are significantly different from the remaining blocks ($P = 0.01$). Subsequent tests were conducted with the plates in a horizontal position (flat plating). Use of the flat plating position largely eliminated the bias due to gravity.

Attractant and Inoculum Introduction Sites

Three combinations of sites were used (shown previously in Figure 7). These sites were far enough apart to allow for easy determination

TABLE V

NUMBERS OF *MELOIDOGYNE HAPLA* LARVAE RECOVERED PER PLATE BLOCK SHOWING THE EFFECTS OF GRAVITY ON LARVAL MOVEMENTS IN PLATES INCUBATED VERTICALLY

Plate Block Number	Replicates 1	2	3	4	Average
1	0	0	0	4	1
2	0	0	0	0	0
3	0	0	0	0	0
4	4	0	0	0	1
5	0	0	4	0	1
6	0	0	4	0	1
7	16	92	4	56	42
8	12	16	16	40	21
9	36	12	56	72	44

The plates were incubated vertically for five days at 26°C with a field capacity of 95%. Initial inoculum was 300 larvae per plate. Block nine was the inoculum introduction site, and block one contained the attractant. LSD (0.01) = 33.78, CV = 15.33%.

of a positive attraction response and hopefully would be low in counts due to random nematode movements. Two types of attractants were used: excised Rutgers tomato roots and germinating Rutgers tomato seeds. The results of tests conducted comparing these three site combinations using excised roots or seedlings are shown in Table VI. The average numbers of larvae recovered in the attractant or inoculum introduction blocks only are shown. Only two treatments were significantly different from the controls ($P = 0.01$) - site combination B using roots and site combination B using germinating seeds. The site combination B/seedlings was significantly different ($P = 0.01$) from the B site/roots. None of the other site/attractant combinations were significantly different from the controls. There were also no significant differences between the random movement controls and the dialysis tubing only controls. Additional tests confirmed the results of this comparison although occasionally high random movement counts biased the results. The results of these tests indicated the best site combination to be that of B (a corner inoculum introduction with the attractant located centrally at an opposing wall). Analysis also indicated the better attractant to be the germinating Rutgers tomato seed ($P = 0.01$).

Additional Factors Affecting Nematode Response

A comparison of random movement studies was conducted in both newly unwrapped petri plates and previously used petri plates. Results indicated ten to 20% fewer larvae were recovered from the "new" plates. All subsequent testings were provided "used" plates (plates that had been unwrapped for a while, then washed). This is in agreement with the findings of Miller (22).

TABLE VI

AVERAGE NUMBERS OF MELOIDOGYNE HAPLA LARVAE RECOVERED AT BOTH THE INOCULUM INTRODUCTION AND ATTRACTANT SITES USING THREE COMBINATIONS OF SITES AND TWO TYPES OF ATTRACTANTS

Site Combination	Treatment	Average Numbers of Larvae Recovered	
		Attractant Blocks	Inoculation Introduction Blocks
A	Seedling/DT	5	277
A	Roots/DT	7	238
A	DT only	11	426
A	Larvae only	4	340
B	Seedling/DT	43	169
B	Roots/DT	27	143
B	DT only	14	128
B	Larvae only	6	124
C	Seedling/DT	9	177
C	Roots/DT	16	179
C	DT only	10	248
C	Larvae only	8	226

DT = dialysis tubing. A =  B =  C = 

x = inoculum introduction site

- = attractant site

The plates were incubated flat for five days at 26°C with a field capacity of 95%. Initial inoculum was 500 larvae per plate. LSD (0.05) = 11.05, LSD (0.01) = 14.88, CV = 4.8%.

In many of the flat plating tests, larvae were recovered around the edges of the plates in numbers that seemed much higher than would be dictated by a normal plate distribution. This could be explained in two ways: (1) the physical limitation of the nematodes' movement by the plate side, which would tend to concentrate the nematodes in this area, and (2) the attractant accumulated in the area of the plate sides resulting in slightly higher concentrations of the stimulus, thereby attracting more larvae to this area.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

Eight treatments of a new method of obtaining quantities of M. hapla larvae for inoculum were compared to a 10.2 cm pot/root incubation inoculum production method. Four differing soil types were used: BS, LFS, a half and half mixture of BS and LFS, and a layer of LFS on a layer of BS. The treatments included one each of the above soil types, and one each of the above soil types with TC. The TC was supplied by inserting a copper tubing heat sink into each of these units and circulating temperature controlled water through it. This maintained the TC tiles at close to 25°C while the tiles not receiving TC were at ambient greenhouse temperatures. The 10.2 cm pots used were all LFS, half of which received TC, half of which did not. The two inoculum production methods were compared on the basis of the number of larvae produced at each two week period for the fourteen week duration of the test. The fourteen week period was used because it was the maximum length of time larvae were obtained from the 10.2 cm pot/root incubation method.

The results of the comparison showed that six of the eight tile treatments significantly ($P = 0.10$) out yielded the control root incubation method. The most productive of the tile treatments was the BS

(without TC). In addition to out yielding the control method during the fourteen week test period, the tiles promise recovery of high numbers of larvae for a considerable length of time because the host plant, nondestructively harvested, remains intact. The tile method also requires little equipment for harvest and supplies inoculum much faster than the control method (and many other inoculum methods).

A reproducible larval orientation response to an attractant in a square petri plate filled with moist silica sand was developed to be the basis for a new, more sensitive nematicide screening procedure. Several factors were tested, including plate positioning, field capacity of the silica sand, inoculum introduction sites, attractant sites, and the type of attractant to be used. The results of the study indicate the best combination of factors to be flat plate positioning, 95% field capacity, inoculum introduction/attractant site B (the inoculum introduction site in one plate corner with the attractant site located in the center of an opposing plate side), and use of a germinating tomato seed as the attractant. Using this combination of factors, the larval orientation response is reproducible, although further research is needed to alleviate variation due to larval random movements.

Conclusions

Under the conditions of this study:

1. The tile inoculum producing method yielded usable quantities of M. hapla larvae eight times as fast as the control (root incubation) method.

2. The tile inoculum producing method out yielded the control

technique seven to one on the basis of larvae produced per unit time and 1.7 to one on the basis of larvae produced per unit area.

3. The tile inoculum producing method requires little equipment, the harvesting can be done entirely in the greenhouse, and the technique should efficiently utilize waste greenhouse space. The control method requires considerable equipment, use of the laboratory, and bench space in the greenhouse.

4. The tile inoculum producing method yielded considerable quantities of inoculum for a long period of time because of non-destructive harvesting, while the control method, with its destructive harvest, generated inadequate quantities of larvae after only four weeks.

5. The most reproducible larval orientation response to an attractant was provided by these factors: flat plate positioning, 95% field capacity, inoculum introduction/attractant site B (the inoculum introduction site in one plate corner with the attractant site located in the center of an opposing plate side), and use of a germinating tomato seed as the attractant.

Recommendations for Future Research

The prevention of predator contamination is paramount to successful yields using the tile inoculum production technique. Research is needed to determine if flushing the tiles with a normal soil strength contact nematicide would eliminate the predator contamination. The chemical should kill the predators and any other nematodes free in the soil, including any root-knot larvae present. The female root-knot nematodes, inside the root galls, would be afforded protection, however, and should

not be affected. The chemical could then be flushed out of the tiles by the addition of more water. Production of inoculum could resume quickly due to the recovery of larvae and eggs protected from the chemical by the egg masses. Larval production would be depressed for a short period of time, but should again build up to a high level.

In the event contamination does occur, the predator could be removed by pouring the leachate through nested 200 mesh and 400 mesh screens. The larger predator nematodes would be caught on the 200 mesh screen, while the small M. hapla larvae should pass through and remain on the 400 mesh screen. The cleaned larvae could then be concentrated and used.

Additional research should be done to determine the effects of low light intensity and plant top size on the production of larvae. The use of lower light intensity (waste) areas of the greenhouse might be detrimental to larval production (however, any larvae produced would still constitute an efficient use of this otherwise wasted space). Extensive plant top growth (perhaps trellising the tops) could allow the development of a more extensive root system able to support more nematodes and subsequently boost larval production.

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VITA

Arlin Leon Bostian

Candidate for the Degree of

Master of Science

Thesis: A NEW INOCULUM PRODUCING METHOD FOR *MELOIDOGYNE HAPLA* AND OBSERVATIONS ON LARVAL MIGRATION *IN VITRO*

Major Field: Plant Pathology

Biographical:

Personal Data: Born in Guthrie, Oklahoma, October 29, 1953, the son of Mr. and Mrs. T. Leon Bostian.

Education: Graduate of Perkins-Tryon High School, Perkins, Oklahoma, in 1971; received the Bachelor of Science degree in Biological Science from Oklahoma State University in 1975; completed requirements for the Master of Science degree at Oklahoma State University in December, 1979.

Professional Experience: Graduate research assistant, Department of Plant Pathology, Oklahoma State University, 1976-1979.

Professional Organizations: American Phytopathological Society, Society of Nematologists.