GEORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

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Project No. G-32-607 GTRI/GIT DATE 9 / 13 / 84

Project Director: Dr. D. Dusenbery School/Lab Applied Biology

Sponsor: Agrigenetics Research Assoc. Limited

3375 Mitchell Lane Boulder, Co. 80301

Type Agreement: Contract signed by GTRC on 12/29/83 - unnumbered

Award Period: From 1/1/84 To 12/31/86 (Performance) _____ (Reports)

Sponsor Amount:	This Change	Total to Date
Estimated: $		$ 199,888
Funded: $		$ 199,888

Cost Sharing Amount: $ -0- Cost Sharing No: -0-

Title: Search for Chemical Stimuli That Act on Plant Parasitic Nematodes

ADMINISTRATIVE DATA OCA Contact Ralph Grede ext. 4820

1) Sponsor Technical Contact:

Agrigenetics ~~Research Assoc. Limited~~ CORP

c/o Agrigenetics Corp.

3375 Mitchell Lane

Boulder, CO. 80301

Attn: ~~Dale J. Hansen, President~~ DIANE McCLEARN

2) Sponsor Admin/Contractual Matters:

SAME

Defense Priority Rating: n/a Military Security Classification: n/a

(or) Company/Industrial Proprietary: none

RESTRICTIONS

See Attached Contract Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval – Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with GIT

COMMENTS:

Paid in full by check #3762 dated 12/20/83 for $199,888.

GTRC will give advance notice of intent to make any disclosure of research findings to sponsor.

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GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 05/03/90

Project No. G-32-607 Center No. R5715-0A0

Project Director DUSENBERY D B School/Lab AP BIO

Sponsor AGRIGENETICS RES CORP/

Contract/Grant No. CONT DTD 12/30/83 Contract Entity GTRC

Prime Contract No. CONT DTD 12/30/83

Title SEARCH FOR CHEMICAL STIMULI THAT ACT ON PLANT PARASITIC NEMATODES

Effective Completion Date 870630 (Performance) 870630 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	N	
Final Report of Inventions and/or Subcontracts	N	
Government Property Inventory & Related Certificate	N	
Classified Material Certificate	N	
Release and Assignment	N	
Other	N	

Comments

Subproject Under Main Project No.

Continues Project No.

Distribution Required:

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Semiannual Research Summary

April 1985

Agrigenetics Research Corporation

Search for Chemical Stimuli that Act on Plant-Parasitic Nematodes

David B. Dusenbery
Georgia Institute of Technology

G32-607

AGAR GRADIENTS

A major part of our efforts has been devoted to developing a technique using chemical gradients in agar as an assay for nematode responses to non-volatile chemicals. The gradients are formed in a layer of agar in the bottom of small plastic boxes. A test sample is placed at one end of the agar and several hours are allowed for chemical gradients to form. A few hundred nematodes are then added to the center of the agar and allowed to move around for several hours. The distribution of nematodes along the length of the box is then observed and scored according to the extent to which they have moved toward or away from the end where the sample was applied. Volatile stimuli have also been tested by placing the test sample on a vertical coverslip at one end of the box so that there is no direct contact between the sample and the layer of agar on which the worms are moving.

A serious problem that was initially encountered was that controls in which no sample had been added often exhibited significant responses. As described in the last report, we determined that a major contribution to this problem was the fact that these nematodes respond to very slight temperature gradients. We have developed procedures for reducing temperature gradients and controlling other factors that influence the distribution of nematodes. Now, we routinely get good controls in which the nematodes are evenly distributed between the two ends of the box when no sample has been added.

The agar gradient technique has been used to begin looking for effective stimuli in exudates of tomato roots. The predominant responses observed have been negative, i.e. the nematodes moved away from the sample. However, the responses seem to vary in a complex fashion with the way in which the sample is handled. Our working hypothesis is that the root exudates contain a complex of stimuli, including both soluble and volatile attractants and repellents. Indeed, we have discovered that bacteria in root exudate preparations can provide these kinds of stimuli.

Efforts to isolate pure cultures of the bacteria that act as stimuli for the nematodes have yielded two different cultures. In one of these, the major stimulus is attractive, while in the other it is repellent. In contrast, most other bacteria isolated do not influence the distribution of the nematodes. Initial studies to characterize the nature of the chemical stimuli produced by the bacteria indicate that both volatile and nonvolatile stimuli are produced. Some results are illustrated in Figure 1. A new student in the lab will try to characterize these bacteria, determine where they come from, and identify the chemical stimuli.

Efforts to characterize the chemical stimuli in exudates of tomato root with bacteria excluded have begun. The repellent activity fractionates on Sephadex G-15 (see Figure 2). This indicates that the

most potent material is larger than inorganic ions and is smaller than about 1500 daltons. Thus, the major stimulus is neither salt, which has previously been shown to be repellent, nor protein. This non-volatile repellent appears to be heat stable.

In the immediate future, we plan to continue to characterize this activity with the objective of identifying it chemically. In addition, we will look for attractants. Root exudates will be fractionated and concentrated in different ways in the hope of separating attractive chemicals from the repellent ones that are masking their presence. Root exudates from a variety of other plants including soy beans will also be tested.

COMPUTER TRACKING

The other major effort has been the use of computer tracking of nematodes viewed in a video camera to detect responses to volatile chemicals carried in a stream of hydrated air blowing over them. Rewriting the software for the new hardware has been completed in the last few months. Testing demonstrates that it is substantially faster than the previous system. Three hundred nematodes can now be tracked simultaneously. An initial problem that was encountered was that the nematodes often either aggregated in the center of the visual field or dispersed from it. We determined that this was probably due to thermal gradients caused by the light source. Changing to a sodium vapor light, which contains much less infra-red, has eliminated the problem.

Initial experiments to test computer tracking have employed carbon dioxide or ethylene as stimuli. These demonstrate that changes in the amount of movement of the nematodes can be detected in a single exposure to chemical stimuli in the air stream over the nematodes within several seconds. The threshold concentration for the increase in movements caused by carbon dioxide is below the concentration normally found in the atmosphere (0.03%). Thus, the nematodes are quite sensitive to it (see Figure 3). We have also discovered that they respond to ethylene with a decrease in movements (Figure 4). Initial testing with several other sources has demonstrated responses to vapors from garlic cloves, the bacterial cultures we have isolated, and roots of tomato plants. The responses could be due to interesting chemical stimuli, or simply to carbon dioxide.

To resolve this kind of problem, we have connected the computer tracking system to the outlet of a gas chromatograph. This should permit the rapid determination of responses to the individual components of a complex mixture. Initial testing of responses to pulses of CO_2 indicated that 30 seconds was sufficiently long to produce a clear response. This is longer than the usual duration of a peak in gas chromatography. However, by using a packed column with a low flow rate and temperature programming, peaks of the desired width can be obtained for a very wide

range of volatility. Test chemicals ranging in volatility from CO_2 to octanol have been isolated with well-defined peaks in a single run.

We have just started testing the combination of gas chromatograph and computer tracking. It looks very promising. If a sample containing 5 ul of carbon dioxide is injected into the gas chromatograph, it elutes in a few minutes as a peak about 15 sec wide. About 30 sec later the movement of the nematodes increases. This amount, eluting in this time, should produce a concentration at the nematodes close to the ambient level in the atmosphere. Responses have also been obtained with ethylene. We do not yet know of any other pure chemicals to test, but vapors from the bacteria cultures and soil will be tested soon.

A major limitation of our experiments to date is that we do not have a clear method for determining the direction of a response to a volatile stimulus. The change in movement demonstrates that the nematodes can detect the stimulus, and different stimuli can elicit different reversal responses, but it is still not clear whether these responses indicate an attractive or repellent stimulus. A near-term objective is to develop a flow system that will establish a concentration gradient of volatile stimuli. The direction of movement of the nematodes across the flow and along the gradient would provide the information. An important question is how long it will take to detect such movement. Will it be several hours required for the bulk of the nematodes to move several cm or can it be detected in seconds by computer tracking? We will then determine if the measurement of changes in the rate of change of direction, which the tracking system currently provides, can be used to accurately determine the sign of the response. If not, we will attempt to develop another tracking procedure that can provide this information.

Meanwhile, other sources of stimuli will be tested by this technique. Special emphasis will be placed on the soil atmosphere around roots. Initial tests will involve simple sampling with a syringe and injection into the gas chromatograph. Other tests will involve concentrating the sample by trapping on a solid matrix such as Tenax GC.

FIG. 1 BACTERIAL CULTURES #5 & #34

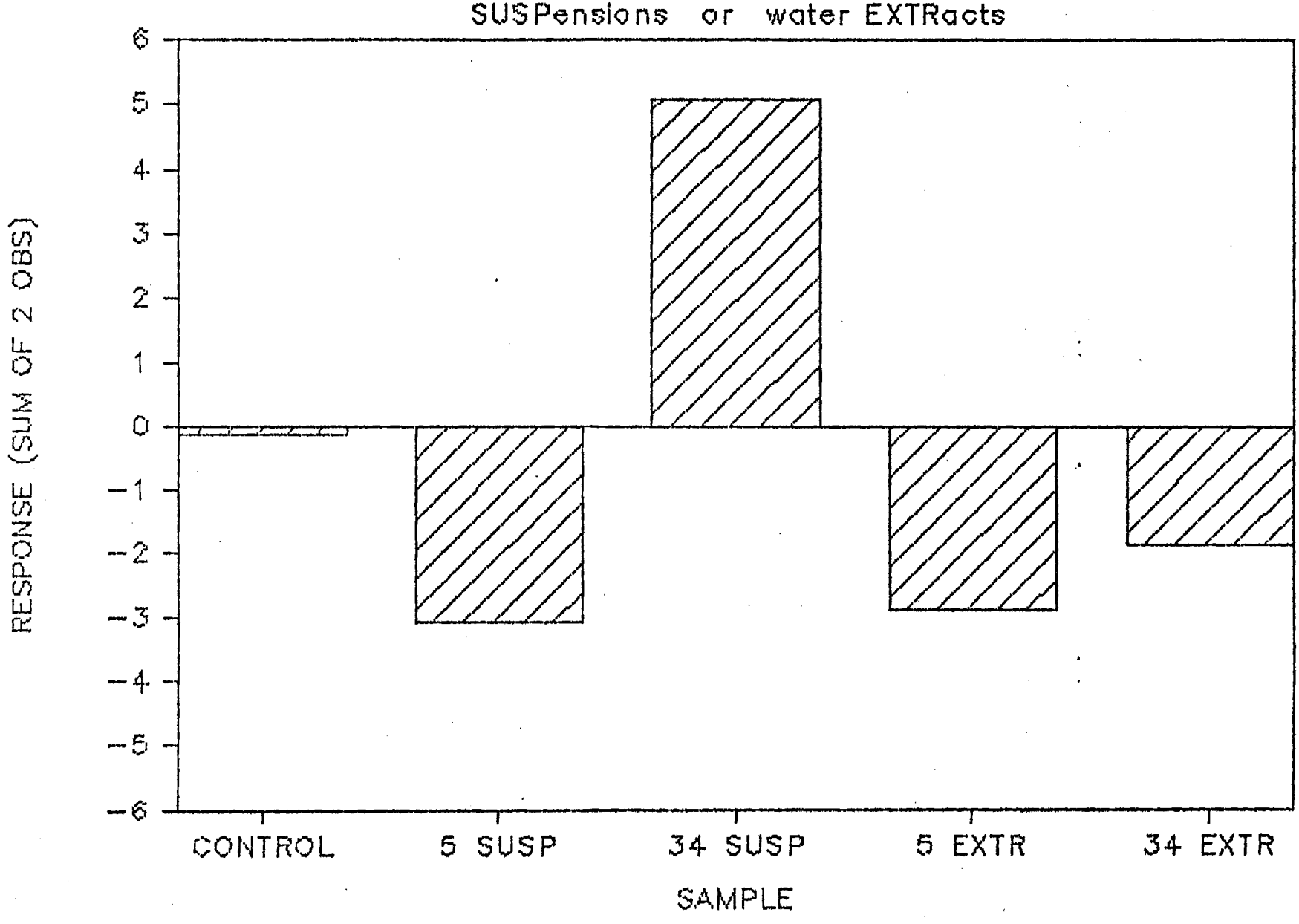


FIG. 2 TOMATO ROOT EXUDATE

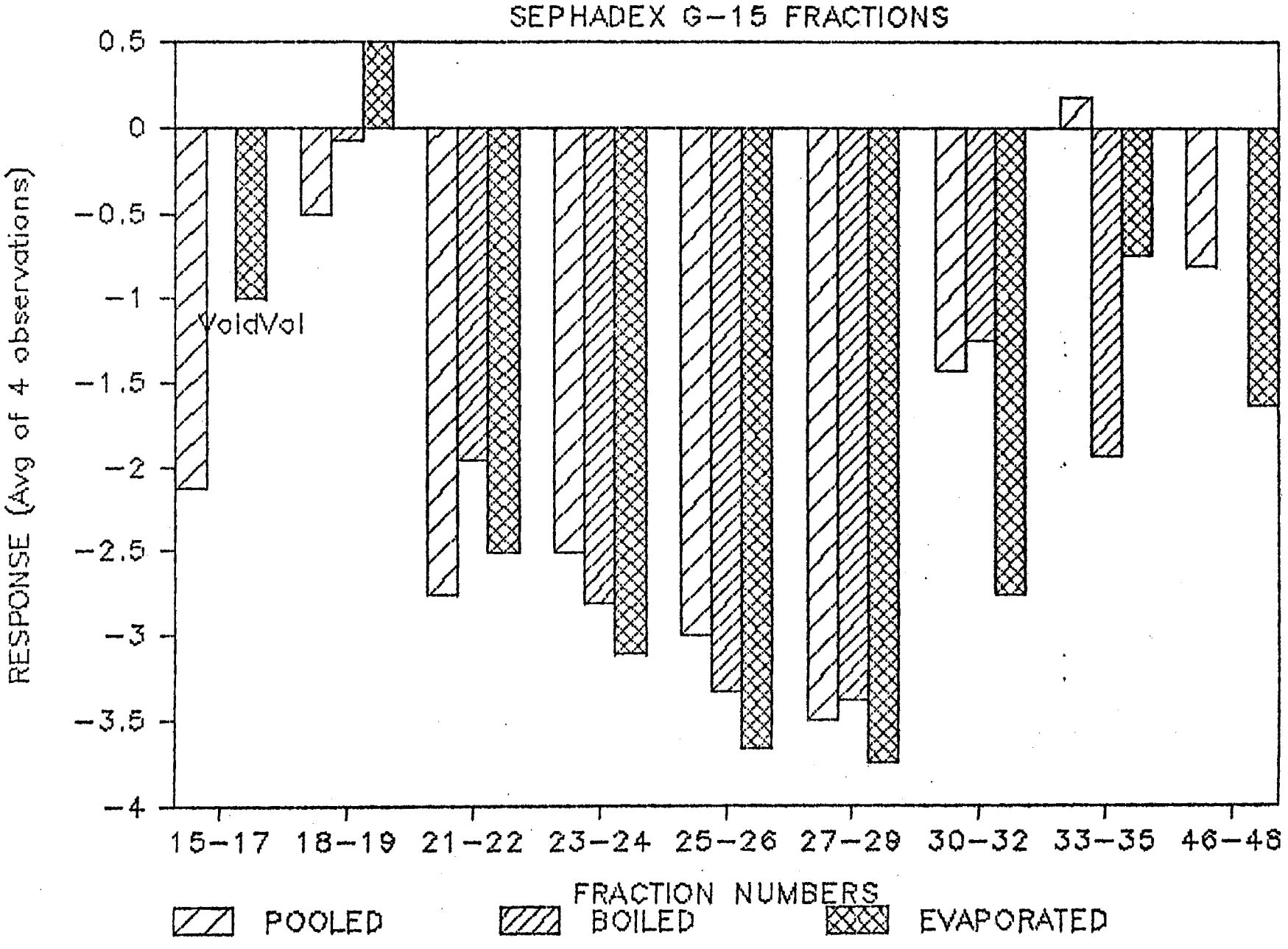


Fig. 3 TRACKING: 0.01% CO2

60 sec in 0.5 ml/s hydrated air carrier

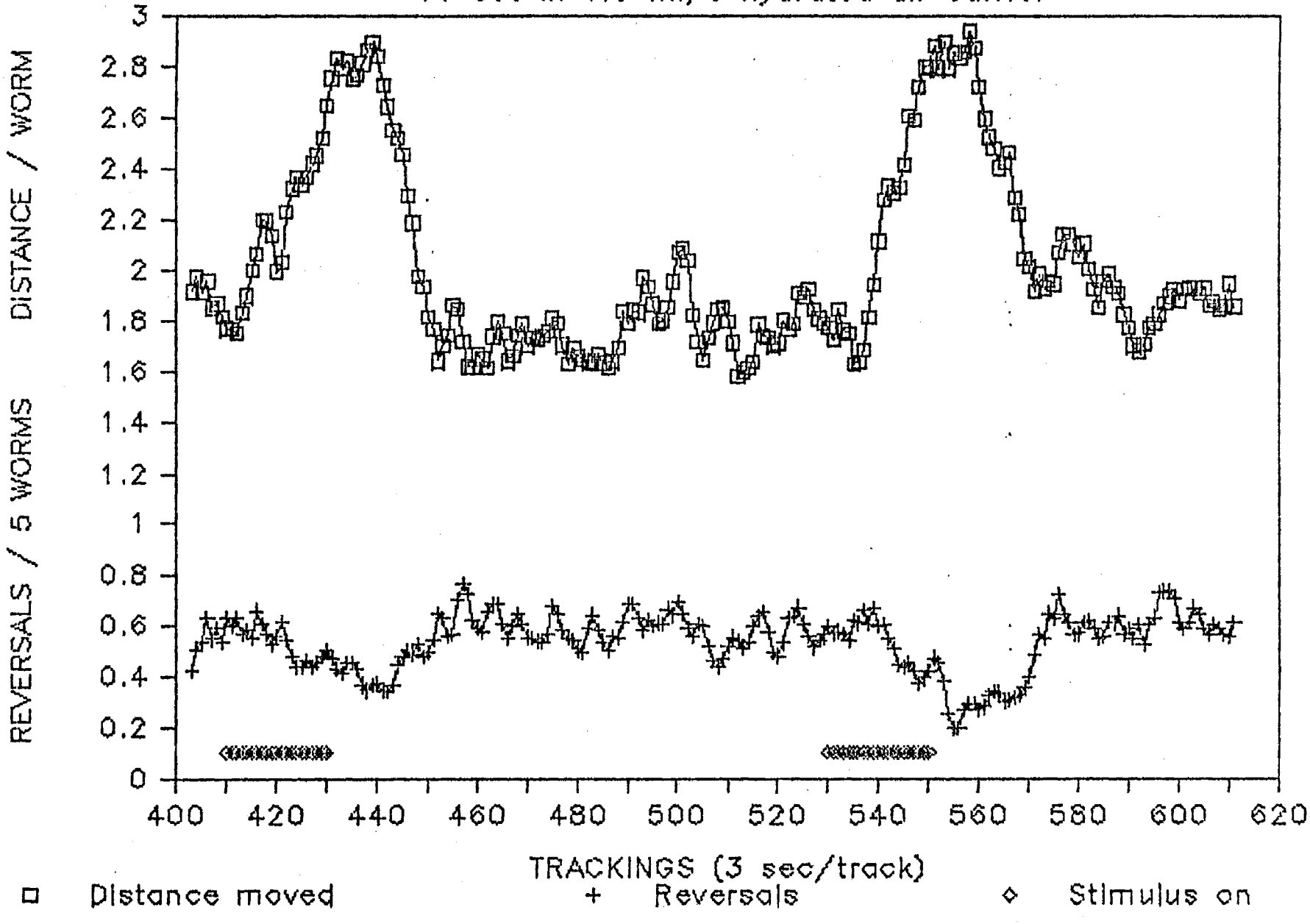
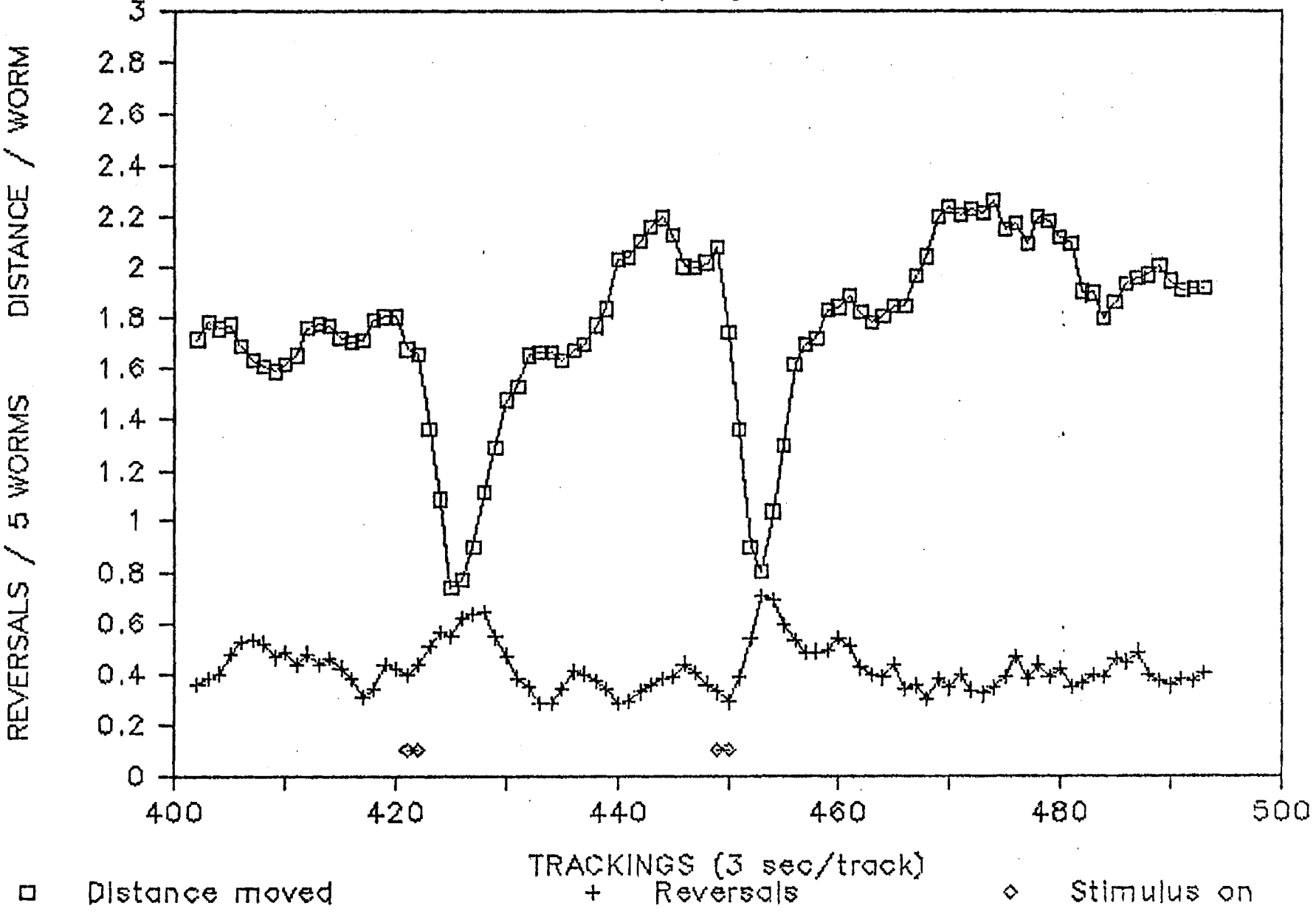


Fig. 4 TRACKING: 1% ETHYLENE

5 sec in 5 ml/s hydrated air carrier



Semi-Annual Report due Oct. 15 to Agrigenetics Res. Corp.
G32-607

Search for Chemical Stimuli that Act on Plant-Parasitic Nematodes

David B. Dusenbery
Georgia Tech

August 1985

INTRODUCTION

The principle objective of this research program is to identify chemicals released by plant roots that attract or repel nematodes. Previous research has suggested that nematodes are probably attracted over significant distances, but the chemicals involved have not been identified. We have developed several techniques for assaying behavioral responses of the infective juveniles of root-knot nematodes to various stimuli. These techniques are presently being used to analyze root exudates for chemicals that either attract or repel the nematodes.

VOLATILE SUBSTANCES

The response of nematodes to volatile substances is assayed by a computer tracking system. The volatile stimuli are carried in a stream of air flowing over nematodes crawling on an agar surface. The nematodes, in darkfield illumination, are visualized by a video camera. A microcomputer interfaced to the camera is programmed to track up to 300 animals and record the rate of locomotion and the frequency of change of direction (reversals).

In order to determine more directly whether nematodes are attracted or repelled by a given stimulus, we have recently developed a variation on the standard technique described above. In the new arrangement a steady concentration gradient is established by two parallel flows carrying different concentrations of the stimulus. The computer is then programmed to measure the net locomotion of the nematodes across the gradient. With this technique, it is possible to determine in a few minutes whether the nematodes on the average are moving toward the higher or lower concentration of stimulus.

Carbon dioxide was used as a stimulus to test out the system. It causes an increase in locomotion and a decrease in reversals. In the gradient experiments, the nematodes move toward higher concentrations. These results are consistent with previous reports that CO_2 is attractive to other plant-parasitic nematodes. The threshold for a

response is below the ambient concentration in the atmosphere. The minimum gradient that causes a detectable response is about 10% change/cm. We have not yet determined what the concentration distribution around roots is likely to be.

We identified ethylene as a possible repellent by the tethered-worm technique. Since it is also released by roots, we characterized its stimulus characteristics more carefully using the computer tracking system. It causes a decrease in locomotion and an increase in reversals - just the opposite of the response to carbon dioxide. Its threshold was found to be about 0.1%. This seems relatively high and it is doubtful that concentrations around roots would be so high, although we have not yet made any determinations of this.

One of our major objectives has been to interface the computer tracking system to a gas chromatograph (GC) so that all the fractions eluting from it would be automatically tested. We have assembled a system in which the effluent from the GC is passed through a thermal conductivity detector and then added to the flow of air passing over the nematodes. Testing with carbon dioxide demonstrated that pulses of stimuli lasting about 30 s were sufficient to produce good responses. In order to produce peaks of this duration, the usual goal of gas chromatography to produce peaks of minimum duration has been abandoned. We use a packed column (of Tenax GC) and a low flow rate to produce peaks of the desired duration. In order to sample a broad range of volatilities, a 2-step temperature program is used to maintain the desired duration for temperatures from 35 to 300 ^{o}C. Testing with straight-chain alkanes has demonstrated that this set-up can separate compounds having a retention index between about 100 and 2000. Injecting carbon dioxide or ethylene into the GC does indeed lead to a response when these compounds elute.

We have begun to test this system on root vapors. In initial tests, approximately 1 ml of air that has been in contact with plant roots for several hours has been injected into the GC. Carbon dioxide is detected by the thermal conductivity detector and produces an increase in locomotion when it elutes. No response is detected at the expected time for ethylene elution. In several cases, locomotion decreased at a time corresponding to a retention index of about 800. Nothing was detected on the thermal conductivity detector at this time. This retention index would correspond to something like a 6-carbon alcohol. In order to pursue this more thoroughly, we are presently working on techniques to trap the material on Tenax and desorb with heat. This sampling technique should permit a several hundred fold increase in the amount of material that can be applied to the GC column.

NON-VOLATILE STIMULI FROM ROOT EXUDATES

Our methods for testing stable, soluble, non-volatile stimuli are now well-established and reliable. The nematode-response assay is performed in plastic trays containing 2% agar with 25 µl of a sample placed at one end and 25 µl of water at the other end of the tray; several hundred worms are placed in the center of the agar and their distribution is recorded at various times thereafter (2 hr to 3 days). We have paid particular attention to the elimination of spurious results due to very small gradients in temperature and moisture. After bacteria were found to affect nematode migration (see next section), only filter-sterilized samples have been tested.

Our initial aim was to identify as many relevant stimuli (attractant and repellent) as possible, so that the most useful could be chosen later for additional work. Preparations of root exudate from tomato plants have consistently repelled the nematodes. This has been true for exudates from young and older plants (weeks to months), collected for various amounts of time (2 to 34 hrs), in the light or dark, at 23 °C or on ice, with roots soaking in water or only misted, and from plants which had or had not been previously infected with Meloidogyne. The failure to recover an attractant stimulus suggests that it is either unstable or volatile or overwhelmed in the mixture with repellent. Since the ratio of stimuli could be species specific, we also tested raw root exudates from some other Meloidogyne hosts (soybean, watermelon, squash, eggplant, and two kinds of pepper). All of these root exudates repelled the nematodes, though they did not appear to be as strong as tomato exudates. We then decided to devote our efforts to characterizing the repellent stimuli; separations of repellents may uncover an attractant as well.

We have concentrated tomato root exudates by rotory-evaporation and lyophilization and fractionated the concentrates by column chromatography, primarily with Sephadex G-15. Nematode-repelling activity has consistently been found in void-volume fractions (molecular weight greater than 1500) and in two separated regions of intermediate size (i.e., larger than NaCl). The repellent activity in the fractions with intermediate-size molecules is greater than activity in the void-volume fractions, and there may be several unresolved peaks with molecular weights less than 1500. The repellent activity in all active fractions was recovered after evaporation under a stream of air, but the activity in the large molecular weight fractions (greater than 1500) was lost by heating at 100 °C for 15 min. None of the repellent activity could be extracted in chloroform, methylene chloride, or chloroform/methanol. Fractionation of concentrated soybean

root exudates has produced a similar pattern of elution of repellent activity; no attractants have been uncovered.

The heat lability of the repellent activity in the large molecular weight fractions is consistent with the hypothesis that the stimulus is a peptide. To test this directly, we have treated samples of the various repellent fractions with an insoluble protease before testing nematode response. So far, the results are inconclusive because of problems with controls (protease-treated water is strongly repellent); however, there are indications that the repellent activity in the large molecular weight fractions was diminished by protease treatment. This is being pursued.

RESPONSES TO BACTERIA

During the course of analyzing responses to root exudates, we noticed some bacterial growth in all of the samples which attracted nematodes and some of the samples which repelled the worms. Filter-sterilized root exudate samples contained only repellent activity. We therefore investigated the effect of bacteria alone. Forty different bacterial cultures were established from samples of root exudate and from assay trays which contained bacteria from unsterilized worms. Nematode response to each of these bacterial cultures was tested; in most cases there was no response, but in several cases attractant or repellent activity was found.

Pure cultures of these interesting bacteria have been isolated and their characterization is underway. Colonies of the most potent attractant bacterial line (#34) grow as brown droplets on nutrient agar; colonies of the most potent repellent isolate (#5) are dark yellow and very sticky. When a suspension of #5 is placed at one end of the behavior-assay tray, the vast majority of the nematodes move to the other end, but a significant minority accumulate in the discrete area containing the bacteria; this suggests the presence of a weak local attractant and a stronger long-distance repellent. When bacteria are removed from the suspension by filtration or killed by UV treatment, the attractant activity disappears. Similar treatments of #34 bacteria produce qualitatively similar results: the attractant requires the presence of living bacteria, but a soluble stable repellent is also found in the agar (after UV treatment) and in the water in which they were suspended. Since the qualitative difference in the net nematode response to #5 and #34 bacteria seem to reflect quantitative differences in the ratios of attractant(s) and repellent(s), each of the stimuli from each type of bacteria must be studied separately.

Since attractant activity was not recovered in the filtrate-water of bacterial suspensions, we hypothesized that it is volatile or unstable. The GC-computer tracking method has been used in a few preliminary experiments to test for volatile stimuli in the airspace above broth cultures of #5 and #34 bacteria. Carbon dioxide in these samples elicited a response in the nematodes. Other stimuli were either absent, too labile, or too dilute; we plan to re-examine this question after the method for concentrating volatile stimuli from root vapors has been perfected.

Using the assay for non-volatile stimuli, the repellent produced by #5 bacteria has been found to be heat labile (destroyed by treatment for 15 min at 100 ^{o}C) but relatively non-volatile (recovered after evaporation of an aqueous sample under a stream of air). Preliminary fractionations of the repellent activity on Sephadex G-15 have yielded at least 2 peaks which represent intermediate molecular weights (i.e., less than 1500 but greater than NaCl); there also seems to be some repellent activity in the higher molecular weight range and some more unresolved in the intermediate range.

We have also begun to identify and characterize all of the pure cultures which have been found to cause nematode responses. With the exception of two cultures, all are gram negative rods and oxidase positive. We are presently characterizing these strains using the API system for rapid testing of non-fermentors. One of the exceptions has been tentatively identified as Serratia marcesens; it is weakly avoided by the nematodes. The other exception is a gram positive rod which is also weakly avoided. When the interesting bacteria have been characterized and/or identified, we will use this information to determine the role of these bacteria in influencing the behavior of nematodes in the rhizosphere.

PLANS FOR 1986

During the coming year we expect to continue this work in several directions. Techniques for trapping volatile compounds on Tenax and then transferring to the GC will be developed. These efforts will concentrate on thermal desorption with direct transfer, since this method allows the entire sample to be injected onto the GC column. The techniques developed will then be used to test for activity in the atmosphere (headspace) of several kinds of samples. These will include the rhizosphere of potted plants, bare roots, and bacteria cultures. Active fractions will then be characterized in terms of retention index on both a nonpolar

(e.g. Tenax) and a polar (e.g. Carbowax 20M) column. This will provide information on both the molecular weight and the polarity of the active agent. As time permits, we also plan to develop a collaboration to test active fractions on a GC-Mass Spec. in order to identify the active chemicals.

The next steps in our work with non-volatile stimuli from root exudates will be to purify, chemically characterize, and ultimately identify the repellents which have been fractionated by gel filtration. Molecular weights will be determined by gel filtration on calibrated Sephadex columns. Further purifications and characterizations will employ techniques such as anion and cation exchange columns, adsorption chromatography, solvent extractions, and possibly HPLC and electrophoresis. Treatment of samples with specific reagents (e.g., insoluble protease) will be used to test for activity attributable to particular compounds. The major foreseeable problem in doing these standard biochemical analyses will be obtaining control conditions which do not cause nematode responses to salts, pH, etc. As this work progresses, it is likely that we will also experiment with several of the variables in the root exudate preparations we use, in order to optimize the recovery of particular stimuli.

Much of our work with stimuli from bacteria will parallel that outlined above for roots. Non-volatile stimuli from bacterial exudates/leachates will be purified and characterized by the same methods used to analyze stimuli from root exudates. In addition to the bacterial cultures we already have, we plan to test nematode response to some other bacteria which may already be of (other) interest to Agrigenetics (e.g, good root colonizers). Finally, we aim to characterize and perhaps identify the bacteria which produce stimuli which affect the nematodes.

G-32-607

Search for Chemical Stimuli that Act on Plant-Parasitic Nematodes

David B. Dusenbery
Georgia Tech

April, 1986

INTRODUCTION

The principle objective of this research program is to identify chemicals from the rhizosphere which attract or repel nematodes. Previous research has suggested that nematode are probably attracted over significant distances, but the chemicals involved have not been identified. We have developed several techniques for assaying behavioral responses of the infective juveniles of root-knot nematodes (Meloidogyne incognita) to various stimuli. These techniques are presently being used to analyze root and bacterial exudates for chemicals that either attract or repel the nematodes.

VOLATILE STIMULI FROM ROOTS AND BACTERIA

The response of nematodes to volatile substances is assayed by a computer tracking system. The volatile stimuli are carried in a stream of air flowing over nematodes crawling on an agar surface within a glass chamber. The nematodes, in darkfield illumination, are visualized by a video camera. A microcomputer interfaced to the camera is programmed to simultaneously track up to 300 animals and record the rate of locomotion and the frequency of changes of direction (reversals).

Problems with thermal gradients, to which the nematodes are extremely sensitive, have been further reduced. The glass chamber containing the nematodes has been completely enclosed by a plexiglass box. Air is recirculated from the box through a heat exchanger coupled to a thermostatically controlled water bath and back to the box. The sodium vapor lamp previously used has been replaced with a 14W fluorescent lamp. The air stream carrying volatile stimuli has been standardized by relacing the air pump with high purity charcoal filtered compressed air. This has eliminated variations in carrier air composition and allowed precise manipulation of CO_2 content which has some effect on nematode sensitivity to stimuli. Extensive evaluation of the tracking program software has allowed further refinement of the program for reduction of noise and increased accuracy and sensitivity of measurements.

Carbon dioxide is a known attractant for several phytoparasitic nematodes and is a major exudate of both plant roots and rhizosphere bacteria. It has been used as a model stimulus to test and refine the tracking system and to determine basic responses of the nematodes to a known attractant. Recent experiments have revealed a sensitivity to CO_2 that is higher than previously reported for other nematodes. Sensitivity is dependent on the concentration of CO_2 to which the nematodes are

acclimated. A sudden increase of as little as 0.01% CO_2 caused an increase in the rate of locomotion and a decrease in the frequency of reversals when baseline concentration was 0.003% CO_2. Under a more ecologically relevant baseline concentration of 1.0% CO_2, the same response occurred with an increase of 0.05% CO_2. By establishing steady concentration gradients in a modified glass chamber, the threshold gradient was found to be below 0.01% CO_2/cm which corresponded to relative gradients of less than 1.0% change/cm. This high degree of sensitivity to CO_2 lends new support to the possibility that CO_2 acts as an attractant to roots, to optimal depths in the soil, or as a collimating stimulus. Calculations indicate that it is physically possible for the nematodes to be even more sensitive than we have domonstrated.

A primary objective in testing for active volatile substances has been to interface the computer tracking system to a gas chromatograph (GC) so that all fractions eluting from it can be tested for possible nematode response. To sample a broad range of volatilities, low column flow rates and a 2-step temperature program are used to maintain desired peak widths of about 30 seconds for temperatures from 35° to 300°C. To avoid possible degradation of fractions as they pass through the high temperature thermal conductivity detector, the packed column (of Tenax GC) has recently been split to direct about 25% of the effluent through the detector and the remaining 75% directly into the flow of air passing over the nematodes.

Several approaches have been used in the preparation of volatile samples to be injected into the GC. The most direct approach, taking 1 ml samples of headspace air around enclosed plant roots, elicited substantial nematode response only to the CO_2 fraction. Liquid samples (1 ul) of the condensate from rotory-evaporated solutions containing water soluble root exudate have elicited some inconclusive responses to various fractions, but this approach is limited because of the very small sample size permitted in GC separation of liquids. The approach currently being pursued is the concentration of volatiles on small Tenax traps and desorption with heat. In one method, the roots of whole living plants are bathed in distilled water for 24 hours, and the exudate solution is filter-sterilized through 0.2 um membrane filters. Filtered compressed air is then bubbled through the solution and through small stainless steel tubes containing Tenax. A second more direct method is to blow compressed air into the water bathing the roots then directly into the Tenax traps. Several liters of air can be passed through each trap in a 24 hour period. Carbon dioxide and water are not adsorbed onto Tenax and so pass through leaving a heavy concentration of potentially active volatile substances. The trap is installed at the head of the GC column and the trapped volatiles are desorbed in the process of running the 2-step temperature program.

Using this method of concentration with tomato plants, five large peaks and a dozen or so smaller peaks of compounds are detected by the GC in a relatively narrow range of elution temperatures between 155° and 225°C, corresponding to retention indices of between 550 and 1100. Increased nematode activity occurs in conjunction with these elutions but has been inconsistent. Efforts are underway to further resolve these compounds and hopefully elicit more distinct nematode responses.

Some preliminary trials using this method of concentration with the volatile exudates of a strongly repellent bacteria strain (designated GT-10) isolated from plant roots have been successful in detecting 5 distinct compounds with retention indices between about 500 and 1100. Work with this strain and two strains of attractant bacteria will continue as methodology with root exudate improves.

NON-VOLATILE STIMULI FROM TOMATO ROOT EXUDATES

Methods

Nematode responses to non-volatile aqueous stimuli are tested by observing the net movement of worms on agar which contains a gradient of the stimulus.

At the time of our last report, we believed that "our methods for testing stable, soluble, non-volatile stimuli are now well-established and reliable." Since then, we have gained additional appreciation for the extreme sensitivity of these worms for some common environmental stimuli (e.g., temperature, humidity, carbon dioxide) and have refined the assay methods to solve some other problems. For example, there have been occasional problems in maintaining zero-response scores in the absence of test-stimuli. This indicated the need for even more extreme attention for controlling variations in temperature and humidity which normally occur in the lab, and change with the seasons. We now conduct the assays in styrofoam boxes which contain aluminum or copper plates to help disperse thermal gradients. These boxes are placed inside other styrofoam boxes in a draft-free room. We have also refined our methods for dealing with bacterial contamination. We discovered that when worms are treated with hibitane, their response to chemical stimuli is severely blunted. However, treatment of the eggs prior to hatching has no deleterious effect. We hatch the eggs in a mixture of kanamycin + gentamicin; these antibiotics inhibit most bacteria in the worm hatching water, but do not affect the worms' response to stimuli. In general, we spend about 20% of our time on quality-control, verifying the validity of our results and/or solving control problems as they arise. At times, this seems frustrating and excessive, but it is necessary, given the extreme sensitivity of these nematodes to various environmental stimuli.

Characterizing the stimuli

When we began this work, we expected that we might find both nematode- attracting and repelling stimuli in root exudate. At the time of our last report, we noted that we had found repellent but not attractant stimuli in root exudate from several host plants. We decided to focus our work on characterizing the repellent activity in tomato root exudate because this could lead to useful approaches for controlling nematode infection and because separations of repellents may also reveal attractant stimuli which had been "hidden" in crude mixtures. This strategy has been fairly successful.

We have determined the apparent molecular weights of two peaks of repellent activity fractionated on Sephadex G-15. Peak #1 (P1), about 900 daltons, produces a "moderate" worm response; Peak #2 (P2), in the range of 400 daltons, is "strongly" repellent. The separation of P1 and P2 has been repeated many times with different tomato root exudate preparations (e.g., young and old plants) and is surprisingly reproducible. When the UV absorbance of the column effluent is monitored, several major and minor peaks are found. There is no absorbance peak in the region of P1 repellent activity, suggesting that it is a relatively minor (but very potent) component of the exudate. The P2 peak is wider and aligns with two fused UV absorbance peaks, indicating that this remains a relatively crude mixture of components. We have sometimes arbitrarily split the fractions of this peak into P2A (~500 daltons) and P2B (~300 daltons). P1 and P2 repellents are stable to heating at 100 ^{o}C for 20 min.

In proposing to characterize the repellent activity by ion exchange chromatography, we recognized that the nematodes' response to various ions could pose problems. For example, we had earlier found that NaCl was a good repellent. Since we also wanted to find buffer systems which could be used with Sephadex chromatography, we tested responses to a number of salts and buffers. The worms showed no significant responses to the following buffers (25 mM): sodium acetate (pH 3.7 to 5.6), sodium phosphate (pH 5.8 to 7.5), and glycine-NaOH (pH 8.6 to 10). Since neither H^+ nor Na^+ affect the worms, both of these could be used as the exchangable ion in ion exchange chromatography. Since Cl^- is repellent, the anion exchange resin was converted to the OH^- form before applying root exudate samples.

In our first experiments with ion exchange columns, we applied the P1 and P2 repellent to anion (OH^- form) and cation (H^+ and Na^+ forms) exchange columns. Repellent activity was found in the effluent from the anion exchange columns. Most of the repellent was removed by passage through cation (Na^+) columns, but surprisingly, the effluent from the cation (H^+) columns attracted the worms. These results led to the following hypotheses, most of which have been tested further: a) P1 and P2 contain a mixture of attractant and repellent stimuli; b) the repellent activity acts like a cation with a selectivity intermediate between H^+ and Na^+; c) the attractant acts like an anion. This was exciting, since it was one of the few cases of attractant activity isolated from a root exudate preparation.

Whole root exudate preparations (raw or concentrated) were used in other ion exchange experiments to simplify the sample preparation and optimize the chance for a good recovery of attractant. As expected, when ion exchange columns were loaded with root exudate, repellent activity could be eluted with ammonium hydroxide from cation (H^+) columns, and attractant activity could be eluted with formic acid from anion columns. All fractions were dried and reconstituted with water before testing the nematode response. However, all of the fractions which showed attraction (i.e., cation effluent and anion eluant) were strongly acidic. When these samples were neutralized, attracting activity disappeared. It is not the case that the worms were simply attracted to acid: some equally acidic solutions or fractions caused no response or were repellent. It appears that a low pH ($<\sim 3$) is a necessary but not

sufficient condition for the attraction. This requires further study, but has been temporarily set aside as we pursue some easier and hopefully more productive approaches. The results with repellent activity were not so complicated; it is clear that most of the repellent in whole root exudate acts like a cation.

It strikes us as curious that the only attracting activity from tomato roots that we have been able to find thus far is carbon dioxide. If there is a water soluble, relatively non-volatile attractant that could be identified with the agar-gradient method, then it must be: a) unstable under the conditions we have tried, b) hidden by the presence of repellent in the samples tested, or c) not recovered from the Sephadex column. Instability is unlikely, given the benign conditions we have employed, but the other two possibilties have some potential. 1) As noted above, when the P2 repellent fractions were run through a cation exchange column, attractant activity was recovered. 2) The separation of the P1 and P2 repellents is coincident with a peak in UV absorbance. Thus, there could be a broad region of several unresolved repellents (~300 to 900 daltons) which is "broken" by elution of an attractant at ~750 daltons. This is consistent with the observations that P1 and P2 contain repellents which partition differently in chloroform-methanol (see below). 3) The void volume fractions from the G-15 column have typically shown variable results. There is often some weak attracting activity, but it has not been clear or reproducible enough to study. We are therefore beginning to fractionate the root exudate on Sephadex G-50, in order to separate the larger molecular weight components of the mixture. 4) We have recently observed 3 additional peaks of UV absorbance eluting from the G-15 column with elution volumes of 1.5 to about 3 times bed volume of the column, with the last peak eluting during the NaOH wash of the column. This suggests that these compounds are strongly adsorbed to the Sephadex. Adsorption will be minimized with the G-50 Sephadex and with BioGel P4 which has a similar fractionation range as G-15 but less adsorbing properties. Biogel might therefore yield a different elution profile which could separate an attractant.

As previously reported, we had tried to extract root stimuli into several organic solvents, but all of the repelling activity stayed in the aqueous phase. This is consistent with our observation that the repellent acts like a cation during ion exchange chromatography. Nonetheless, with better Sephadex fractions to work with, we tried some organic solvent extractions again. These results look interesting. It is still true that repellent activity from P1, P2A, and P2B is not extracted by 5 volumes of chloroform or 2.2 volumes of chloroform-methanol (1:1). However, with 5 volumes of chloroform-methanol, significant amounts of repellent are extracted from P2A and P2B, but not from P1. This indicates a difference in the polarity of the P1 and P2 repellents. A difference in the chemical nature of P2A and P2B has been suggested in a recent experiment where repellent was extracted from samples made acidic or basic before extraction with 3 volumes of chloroform-methanol. With this solvent:sample ratio, there was no extraction from neutral or acidic samples. When the aqueous samples were alkaline, repellent was extracted from P2B but not P2A or P1. This is consistent with what would happen if some of the active substance in P2B were an amine; the unextracted repellent may be chemically different although it has a similar molecular

weight. These experiments need to be repeated and extended before any firm conclusions can be drawn.

NON-VOLATILE STIMULI FROM BACTERIA

In our previous report, we presented some data on the nematode attracting and repelling effects of two lines of bacteria which we called #34 and #5, respectively. In the process of trying to find optimal growth and storage conditions for these bacteria and others isolated from tomato roots, we have developed several sublines of the original and new cultures. These have been designated with a new numbering system ("GT-" series) and have been tested for their ability to attract or repel nematodes. From these we have chosen 3 lines for further study: the repellent bacteria GT-10 and attractant bacteria GT-16 and GT-21. The stimuli produced by these bacteria seem similar to those we had found earlier. That is, attraction by GT-16 and GT-21 requires the presence of the bacteria; we have been unable to prepare a sterile aqueous extract (exudate or lysate) which contains the attractant. This suggests that the attractant is volatile or unstable. The repellent from GT-10 can be recovered in sterile aqueous exudate or lysate. Some activity was lost by heating at 100 ^{o}C for 15 min, but otherwise it is quite stable. We have tried to find appropriate methods for growing large amounts of these bacteria so that we can characterize the repellent(s). Since nutrient broth alone repels the nematodes, the bacteria must be washed before extracting the repellent. This causes some problems, but formulation of minimal media which do not affect the nematodes should do much to resolve them. Repellent has been concentrated from large volume broth cultures; attempts to fractionate this on Sephadex G-15 have not yet been successful.

We have tested 40 cultures of bacteria (including replicates) supplied by Agrigenetics for attractant or repellent activity. None elicited significant nematode responses. This was disappointing, since at least some of these bacteria are known to be good root colonizers. However, it is not surprising since most of the bacteria we have examined have had no effect on the nematodes. The attracting and repelling bacteria which we have isolated are therefore quite special and interesting.

G-32-609

Search for Chemical Stimuli that Act on Plant-Parasitic Nematodes

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Sunil Lal
Mark McCallum
Technician: Marc Pline

September, 1986

INTRODUCTION

The principle objective of this research program is to identify chemicals released by plant roots that attract or repel nematodes. Previous research has suggested that nematodes are probably attracted over significant distances, but the chemicals involved have not been identified. We have developed several techniques for assaying behavioral responses of the infective juveniles of root-knot nematodes to various stimuli. These techniques are presently being used to analyze root exudates for chemicals that either attract or repel the nematodes.

NON-VOLATILE STIMULI FROM ROOT EXUDATES

As proposed in our report last year, our major aim has been to characterize and purify the repellent stimuli which we have found in tomato root exudate. We had also hypothesized that "separations of [these] repellents may uncover an attractant as well."

Molecular weights of the repellents have been estimated by fractionating concentrated tomato root exudate preparations with gel filtration chromatography. In our early fractionations, we had found two separated repellents of "intermediate" size (MW in the range of 100 to <1500 daltons) and one repellent in the void volume (MW > 1500). However, the activity of the void volume fractions became variable, ranging from repellent to neutral to attractant in various experiments. Since we also found significant adsorption problems with our samples on Sephadex G-15, we tried some other gels (Sephadex G-50; BioGel P2 and P4) and eluant buffers in order to fractionate larger molecular weights with less adsorption. The intermediate-sized repellents were found in all cases, but were actually separated best on long columns of Sephadex G-15 eluted with water. The second of the two peaks originally seen has now been partially resolved into 2 peaks. We have not found any consistent activity for molecules greater than ~1000 daltons. The fractions with

repellent activity have been designated R1, R2, and R3, with apparent molecular weights of ~1000, ~500, and ~400. The uncertainties in estimating size are partly due to possible adsorption to the Sephadex. If there is an error in these estimates, it is an underestimate. Thus, these repellents are certainly larger than ions or single amino acids or simple sugars. Fractions containing the repellents show some overlap with some of the major peaks in UV absorbance but do not coincide with them exactly. This indicates that the repellents are probably relatively minor components of the total root exudate, and that they must still be separated better from other extraneous material.

The R1, R2, and R3 repellents are stable to heating at 100 ^{o}C for 20 min. They can be recovered after evaporation under an air stream, indicating that they are not volatile and not easily oxidized.

The repellents appear to be fairly polar molecules since they are not extractable into non-polar solvents such as chloroform, methylene chloride or ether. Extractions with chloroform-methanol mixtures suggest that R2 and R3 are less polar than R1. Dried residue of R3 can be extracted into methanol and N-propanol, but is only slightly soluble in acetone, which is less polar. The repellent activity of R3 is retained on silica gel and amine solid phase extraction columns and can be eluted with polar solvents (propanol, methanol, water). Reverse-phase chromatography columns were used to extract non-polar materials from R3; the repellent activity was not retained by the column.

The liquid and solid phase extractions are also providing efficient methods for purifying the R1, R2, and R3 fractions. This is, of course, a necessary step before chemical identification can be made.

One of the problems in performing the bioassay for nematode response to possible stimuli is that the worms are known to be repelled by NaCl at fairly low concentrations (e.g., 10 mM NaCl is a "moderate" repellent). We therefore had to study the response to various salts in order to determine which ions which could and could not be used in elution buffers, or as counter-ions in ion exchange chromatography, etc. At 80mM, the nematodes did not show a significant response to the following buffers: sodium acetate (pH 4.5), sodium phosphate (pH 7), and glycine-NaOH (pH 9.5).

Since the repellents were found to be polar molecules, we used ion exchange chromatography to determine whether we could achieve any separation on the basis of charge of the molecules. Whole root exudate was applied to columns of anion and cation exchange resin; effluents, water-washes, and eluants were tested to see which fractions contained active chemotactic stimuli. Repellent stimuli seem to behave like a

cations: nematodes are repelled by effluent from the anion column and eluant from the cation column. Surprisingly, effluent from the cation column and eluant from the anion column attract the nematodes. It seems that attractant acts like an anion. This is the first evidence we have found for a stable water-soluble attractant from root exudate preparations. The significance of this finding is not yet clear, however, since there are some complications. The pH of the ion exchange samples which attract the nematodes is typically very low (i.e., < 3); when the pH is raised, attracting activity decreases and then disappears. Control experiments have demonstrated that the nematodes are not attracted to a low pH per se. It seems that the attracting samples recovered from ion exchange resins require an unphysiologically low pH.

VOLATILE STIMULI

Several technical improvements have been made in the computer tracking system in order to improve the signal-to-noise ratio. These changes seem to have helped, as judged by tests with carbon dioxide as stimulus.

Various methods of sampling root vapors and trapping interesting molecules on Tenax GC have been tested. The Tenax trap has been eluted thermally onto a GC column, the effluent of which has been passed over nematodes being tracked by the video-computer system. The use of the trap to concentrate volatiles has not produced any repeatable responses by the nematodes. Thus the only clear responses of Meloidogyne incognita to root vapors is the response in the vicinity of carbon dioxide elution. In order to determine if CO_2 is the only highly volatile stimulus present, we plan to use cryogenic cooling of the column to improve separation of chemicals in this range of volatility.

BACTERIA

We had previously isolated two lines of bacteria which attract and repel Meloidogyne juveniles. Because of the circumstances of this serendipitous discovery, it was not totally clear whether these bacteria were originally associated with roots, nematodes, or Sephadex columns. Whether the bacteria are associated with the rhizosphere or not is relevant in determining their role(s) in enabling nematodes to find host plants or in using the bacteria to design a strategy for control of nematode populations.

Tomato root/soil washes were used as the source of a new series of bacterial isolates. The response of the nematodes to the bacteria was determined for morphologically pure cultures and some mixed cultures. The majority of the forty

bacterial preparations did not effect the net migration of the nematodes. One of the pure cultures and two of the mixed cultures repelled the worms. It is interesting that the morphology of these bacteria resembled that of the original repelling bacteria. Six of the pure cultures and one of the mixed cultures attracted the worms. The mixed culture showed the strongest response, suggesting that the individual attractants are additive.

We also tested nematode response to forty cultures of root-colonizing bacteria which were supplied to us by Agrigenetics. The worms showed no significant response to any of these bacteria; a few cultures produced very weak (i.e., dubious) attracting and repelling responses. This set of bacteria is clearly different from our sample of general rhizosphere bacteria, which had several lines producing chemotactic stimuli. With all these results taken together, the bacteria which attract or repel Meloidogyne larvae appear to be fairly special.

Our efforts to chemically characterize the bacterial stimuli are not as advanced as our work with the stimuli from root exudate. The attractant(s) seem to be volatile and/or unstable. Carbon dioxide is the only attracting stimulus we have identified. The differences between attracting and non-attracting bacteria could simply be due to the amount of CO_2 produced or to the ratio of CO_2 to repellent(s), but we suspect that other attractant(s) are involved. The bacterial repellent activity is water soluble and fairly stable. Most of our work with this has centered around devising protocols for obtaining sufficient amounts of the material to use in biochemical studies. On nutrient agar, our best recovery of repellent is from 3-5 day old cultures. We can get more bacteria with broth cultures, but since broth alone repels the nematodes, the bacteria must be washed before harvesting repellent(s). Washing must remove the broth repellents (probably salts) without entirely removing the unique repellents produced by the bacteria. Using bacteria grown on agar and in broth, we have studied the localization of the repellent (exudate, intracellular, membrane-bound), the effects of washing the bacteria, and the time course and conditions for recovering repellent from water-soaks of washed bacteria.

16 Sept. 86

AGRIGENETICS SPONSORED RESEARCH REPORT
QUARTER ENDED SEPTEMBER 30, 1986

Principal Investigator: DAVID B. DUSENBERY
Institution: GEORGIA INSTITUTE OF TECHNOLOGY
Project: Nematode Biochemistry

I. PERSONNEL INVOLVED WITH SPONSORED RESEARCH PROGRAM

Please review the following list, making appropriate corrections and inserting missing information which is marked by blue highlighting. Add new personnel and list terminations in the appropriate section.

Current Records:

Name	Job Title	Date of Assignment	Percent Time on Project	Signed Confidentiality Agreement?
Champion, Becky	Grad. Student	07/01/85	10	In Process
Diez, Jim	Res. Sci. I	01/03/84	100	Yes
Dusenbery, David	PI	12/31/83	50	Yes
Lal, Sunil	Grad. Student	01/10/85	33	In Process
McCallum, Mark	Grad. Student	06/23/86	50	In Process

Additions:

Name	Job Title	Date of Assignment	Percent Time on Project	Signed Confidentiality Agreement?

Terminations:

Name	Job Title	Date of Assignment	Percent Time on Project	Status/Location of Notebook
	Grad. Student	01/10/85	33	Georgia Tech

A. Do any persons listed as NOT having signed a confidentiality agreement come within the classification of "potential inventor" (e.g., researchers, postdoctoral fellows, etc.)?

_____ Yes _____ No

If yes, please list person and job position:

Name	Job Title

B. Do you have any job vacancies on your Sponsored Research Project at present?

_____ Yes _____ No

If yes, please describe:

If yes, would you like the position announced when the next quarterly report request is sent?

_____ Yes _____ No

C. Do you need additional laboratory notebooks?

_____ Yes _____ No

If yes, please list how many and for whom notebooks are needed.

D. Your additional comments, if any:

II. PROJECT RELATED TRAVEL

Date	Place	Purpose

III. ANTICIPATED PUBLICATIONS AND OTHER FORMS OF PUBLIC DISCLOSURE (PROJECT RELATED ONLY)

A. Please provide information on public disclosures which are anticipated and/or in preparation. As soon as a draft is available, please submit a copy to us for approval prior to release.

Title or Subject	Expected Completion Date

B. Please list completed publications, oral presentations, abstracts, etc., which have occurred during this quarter. (Please provide reprint.)

Title	Publication or Place of Presentation	Date	Received Approval?
Root Exudate Fractions repellent to root-knot nematodes	SON meeting	Aug-86	Yes
Bacteria that attract and repell root-knot nematodes	SON meeting	Aug-86	Yes

C. Please list and briefly describe ANY item that you feel is patentable. This could include DNA sequences, novel ideas, new techniques, new plant varieties, etc.

IV. GERMPLASM/BACTERIAL STRAIN TRANSFERS (PROJECT RELATED ONLY)

Date	From	To	Description of Germplasm/Strain (Treatments, Generation, Plasmids, Resistance, etc.)