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**Control of Plant Parasitic Nematodes
by Intervention in the Host-Finding Process**

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ABSTRACT

Several phytophagous nematodes were screened with fluorescein, rhodamine or ferritin-conjugated lectins. Following blocking experiments with appropriate complementary haptens, several carbohydrate residues were found on the head, amphids or amphidial exudates of the nematodes. Differences in labeling between Anguina tritici and Meloidogyne incognita race 3 as well as between their life stages, were related to the difference in their host preference and parasitic habit. The reduction of carbohydrate presence within A. tritici life-stages, points for an age-related attrition in the specific binding sites on the cuticle, during the development of larvae into adult. The lack of this phenomenon in M. incognita is inexplicable. Increasing the intensity of the lectin fluorescence with A. tritici, but not with M. incognita, after lipase and periodate pretreatments, suggesting that glycolipids are part of the surface-coat (glycocalyx) of A. tritici while in M. incognita this layer is a glycoprotein or polysaccharide or there is just an absence of digestible glycolipids.

Invasion of potato roots by juveniles of Globodera rostochiensis was decreased by about 97% when the nematodes were treated with 1% sodium hypochlorite. This was attributed to the degradation of the amphidial exudate and subsequent damage to the exposed sensory apparatus. Other proteins which usually bind to amphidial exudate, had no effect on the ability of the juveniles to detect and migrate to disks of agar containing potato root diffusate. Various detergents reduced the ability of juveniles to perceive stimuli diffusing from

discs on which potato roots had grown, without destroying the amphidial exudate.

Significant control of tomato root-knot nematode was achieved by applications of the lectin Concanavalia A (Con A) in greenhouse, growth chamber, and microplot trials. Four consecutive weekly applications at lower concentrations of Con A yielded better control than single application at a higher total concentration. A reduction in galling caused by M. incognita and Nacobbus aberrans was observed after the leguminous plant Concanavalia ensiformis and tomato were cocultivated. The leguminous plant Mucuna deeringiana gave a lesser degree of control of the root-knot nematode under the same conditions.

The lectin Con A was demonstrated in water in which C. ensiformis had been grown. The passage of this lectin from C. ensiformis into the soil is proposed as an explanation for the observed reduction in root-knot symptoms on tomato.

Objectives of the original research proposal were:

- a) Learning the biochemistry of nematode chemotaxis by identifying the ligands that might be involved in chemotaxis (attraction or repulsion) and identification and localization of chemoreceptors on the outer cuticle of some plant parasitic nematodes, and host plant surfaces.
- b) Studing the molecular basis of host recognition by plant parasitic nematodes.
- c) Developing methods for interferring in the host-finding processes (chemotaxis, attraction, repulsion, host-recognition).
- d) Applying the acquired knowledge in control methods of phytophagous nematodes.

INTRODUCTION

Overall, it is widely accepted that plant parasitic nematodes are attracted toward plant roots. The most preferred feeding sites of phytonematodes in roots are the zone of elongation, young lateral apices, and injured tissues (28); only few nematode species feed along mature parts of the root (28). The major site of diffusive exudates is the zone of elongation (12) although the zones of lateral and adventitious root development have also been associated with high levels of exudation (9). About 10 years ago, surface carbohydrates began to attract attention as possibly playing a pivotal role in determining the specificity of many biological recognition phenomena (4,16). Since then, considerable progress has been made in evaluating the role of surface carbohydrates in plant-microorganism interactions (5). The working hypothesis states that heterosaccharides on the cell periphery 'recognize' a complimentary 'receptor' on the other cell; a lot of attention was directed to the non-immune and enzymatically inactive carbohydrate-binding proteins, the lectins (13). Plant physiologists lent support with information on the presence of carbohydrates and /or their binding sites (lectins) in root exudates and on the root surface. Different oligosaccharides, galactose, glucose, fructose, xylose, rhamnose and desoxribose were reported in barley, cucumber, red pepper, tomato, turnip cabbage and wheat root exudates (26); qualitative and quantitative differences in those carbohydrates were recorded between these crops.

The external cuticular surface of nematodes is composed mainly of protein with traces of carbohydrates and lipids. Various methods, mainly with lectins, were used for the identification and localization

of specific carbohydrates (24,25,27). Experiments with free-living nematodes revealed the presence and the possible role of glycoconjugates in the interaction with bacteria (10,29) or nematophagous fungi (10).

Zuckerman and Jansson (29) have proposed that carbohydrates exuded by chemosensory sensillae bind to the cuticle glycocalyx, and that these moieties play a role in chemoreception. They support their hypothesis both by reports revealing certain carbohydrates on the cuticle proximate to the sensillae and by blockage experiments which modified the chemotactic response (10). The existence of glycoproteins in amphid secretions reported by Forrest & Robertson (7), McClure and Stynes (19), and Premachandran et al. (22), and the presence of glycoconjugates in nematode head area (24,25) give more support to Zuckerman & Jansson (29) idea. It also appears that amphid secretions play a role in the host-finding process: specific blockage of Heterodera rostochiensis second stage larvae amphid apertures by detergents, changed their structure, altered the amount of secretions normally occurred after hatching, and also interfered with their host recognition (8).

Contact interaction between carbohydrates presence on nematode body-wall (24,25) and their binding sites (lectins) on root surface (21), may lead to host-nematode recognition. Carbohydrate moieties are located mainly on the head (or tail), perhaps because these are the first parts of the nematode to make contact with the root surface. However, lectins were never reported to occur on the nematode surface

Banning of many efficient nematicides in the last decade altered completely research priorities in nematology. Consequently, biological control research or the possibility of intervention in the host-finding process, were taken advantageous lately.

Underlining the stage where specificity in the host-finding process takes place, will enable the targeting of specific rhizospher microorganisms or chemical means which will interfere with the host-recognition, thus will lead to an alternative nematode control strategy.

**SURFACE CARBOHYDRATES OF PLANT PARASITIC NEMATODES. GENERAL LABELLING
AND STAGE SPECIFIC DIFFERENCES IN LECTIN BINDING TO CUTICLE SURFACE.**

Experimentation

Nematodes. Infective, second-stage Anguina tritici juveniles (J2) were extracted from infected, seedlike wheat galls. The J2 were transferred through a 10 μ sieve in order to remove any plant tissue and then washed thoroughly with distilled water and PBS before any further treatment. Eggs, J2 and adults were obtained from inoculated wheat plants. Wheat kernels (Triticum aestivum cv. Toniichi) were sown in pasteurized sandy loam soil inoculated with seed galls containing infective J2 (17). When green galls developed on the inflorescence, they were harvested and the nematodes which they contained were dissected in phosphate-buffered saline (PBS), pH 7.2; Bursaphelenchus lignicolus originated from monoxenic culture; Longidorus elongatus and L. macrosoma were extracted directly from soil, washed three times in PBS and examined within 4 h of extraction. The root-knot nematode, Meloidogyne incognita race 3, were propagated on eggplant, Solanum melongena cv. Beauty, in the greenhouse. Eggs were collected from infected plants (18) and hatched to obtain infective, second-stage juveniles (2). Infective juveniles (InfJ2), females and males were dissected from infected plants, 2 and 4 weeks after inoculation. Pratylenchus penetrans, Tylenchorhynchus claytoni and Radopholus similis, were collected from monoxenic cultures kept in the laboratories.

Lectin labellings. Lectins were obtained from E-Y Laboratories (San Mateo, CA USA) or Sigma (Mo, USA), supplied as conjugates of fluorescein or rhodamine isothiocyanate (FITC or TRITC). Nematode

suspension containing 1000-2000 eggs or J2, or 10-15 InfJ2, adults, females or males, was incubated with 100 µg/ml of the appropriate lectin, in PBS, for 45 minutes at room temperature. Con A (*Canavalia ensiformis* agglutinin) was diluted to a concentration of 100 µg/ml with 0.015M Tris, pH 7.0, containing 0.015M NaCl, 0.015M CaCl₂ and 0.015M MnCl₂. The lectins used were: DBA (*Dolichos biflorus* agglutinin), GS II (*Griffonia simplicifolia* agglutinin), MPA (*Maclura pomifera* agglutinin), PNA (*Arachis hypogaea* agglutinin), SBA (*Glycine max* agglutinin), UEA (*Ulex europaeus* agglutinin) and WGA (*Triticum vulgaris* agglutinin). Controls consisted of untreated nematodes and parallel blocking treatments similar to those described above except that the buffer used for diluting the lectin contained 0.2M lectin-specific hapten. Live nematodes treated with FITC- or TRITC-lectins were mounted under coverslips on glass microscope slides and examined by incident fluorescence microscopy with an excitation wavelength of 450-490 nm and barrier filter of 520 nm.

A. tritici and M. incognita were also exposed to ferritin conjugated to WGA and to UEA, respectively; nematode treatments and preparations for transmission electron microscopy were done as described by Forrest and Robertson (7).

Enzyme digestion. Proteolytic digestion of PBS-washed A. tritici nematodes and lipase pretreatment of A. tritici and M. incognita, were accomplished as described previously (24).

Periodate treatment. A. tritici and M. incognita J2 suspension was incubated with freshly-made 10 mM sodium meta periodate (NaIO₄) in sodium acetate buffer (pH 4.5), in dark, at 0 C (ice-water bath), for 60 min. The juveniles were then carefully washed with cold PBS and

both nematode species were incubated with FITC-conjugated Con A, GS II, PNA, SBA, UEA and WGA as described before. Parallel controls were run on the same conditions.

Results and Discussion

Sugar residues were identified on the head, amphids or amphidial exudates of most species examined. N-acetyl glucosamine residues were found on A. tritici, T. claytoni, and R. similis; D-glucose/D-mannose residues on M. incognita, P. penetrans and B. lignicolus; galactose and fucose residues on M. incognita; (Table 1). The presence of fucose was confirmed by positive labelling of the head of M. incognita larvae by the UEA-ferritin conjugate. On both Longidorus species and on R. similis, N-acetyl glucosamine was localised at pore openings. These two species also labelled for galactose residues at the side of the head. The identification of N-acetylglucosamine, galactose and glucose/mannose residues in association with cephalic chemosensory organs suggests, as proposed by Zuckerman and Jansson (29), that these carbohydrates may have a specific role in recognition of chemotactic factors. The enzyme experiments of Jansson et al. (11) and the lectin experiments by Bone & Bottjer (3) and Jeyaprakash et al. (12) support this view.

The regular occurrence of N-acetylglucosamine at the body pore openings of the Longidorus species suggests it was excreted or secreted at this site. The two Longidorus species were the only dorylaimid nematodes examined in this study, and the only ones in which body pores were consistently labelled, with the limited exception of R. similis in which the excretory pore, but not the body pores, were strongly labelled. We speculate that this observaton

indicates functional differences between the dorylaimid and tylenchid cuticles.

Lectin binding to surface cuticle of various life stages of A. tritici and M. incognita, is summarized in Table 2. Eggs, in both species, were the principal lectin binding sites; Con A, DBA, GS II and UEA bound differently to A. tritici and M. incognita, while MPA, PNA, SBA and WGA bound to both species. More lectins were bound to A. tritici second-stage juveniles (J2) than to M. incognita J2: While in the last species only Con A bound to the outer cuticle, GS II, PNA, UEA and WGA bound to A. tritici J2 cuticle. However, amphid and/or amphid secretions of M. incognita J2 bound more lectins than A. tritici J2. The higher amount of carbohydrate content in the amphids and/or amphidial exudates of M. incognita pre-infective J2 may have a special significance regarding the functions related to them. Playing a role in chemotaxis and host recognition (10) and elicit phytoalexins in resistant hosts. Therefore, the differences found among this two genera may be related to the differences in their host preference and parasitic habitate. In contrary to J2, females and males of A. tritici did not bind any lectin to the outer cuticle, but only to the amphids, and on some occasions, to the head and tail areas, except for DBA (both sexes), Con A and SBA, which did not bind to the males. M. incognita females, however, bound several lectins non-specifically to the outer cuticle, except for Con A and UEA which bound specifically. Binding of most lectins were confined to the entire body wall, except for the head area. PNA and WGA did not bind at all, while UEA bound to the entire cuticle, includes the head. Mild oxidation with periodate did not alter the binding of any lectin to M.

incognita J2 or eggs, except for the binding of GS II and SBA to the eggs. Binding of lectins to A. tritici J2, however, was tremendously affected by periodate, and the detectable cuticular bundings occurred in this species were highly increased (Table 2). No change occurred in the binding to amphids. Lipase pretreatment had the same effect as periodate, namely, increasing the FITC intensity of the binding to A. tritici, whereas no change was recorded in the binding pattern with M. incognita (Table 2). This may suggest at least two possibilities for the nature of the glycoconjugates: 1. The sugar residues in A. tritici are masked by a lipid layer, probably glycolipids, while in M. incognita this layer is a glycoprotein or a polysaccharide. 2. The peptides of the glycolipids in M. incognita are inaccessible to the enzymes.

The reduction of GS II, PNA, UEA and WGA-specific carbohydrates within A. tritici life stages, supports the possibility that there may be an age-related attrition in the specific binding sites on the cuticle, during the development of larva into adult. The lack of this phenomenon in M. incognita is inexplicable.

A POSSIBLE ROLE FOR THE AMPHIDS IN HOST FINDING

Experimentation

Nematodes and plant material. Cysts of Globodera rostochiensis (potato cyst nematode, PCN) grown in microplots were extracted from the soil by flotation. One-day-old, second-stage juveniles were obtained by treating cysts with potato root diffusate and exposed to chemical treatments in 1 ml plastic syringers as described by Forrest et al. (8). Nematodes used as controls were treated with tap water and rinsed in distilled water.

Potato tubers were stored in a growth cabinet at 20 C and a 16 h photoperiod. Sprouts of potato cvs Pentland Crown and Maris Piper were germinated and excised from tubers, placed in sterile 1% Murashige & Skoog plus minimum organics mixture in 1.25% Davis agar in petri dishes which were sealed and placed at 20 C for 5-7 days without light.

Methods of treatment. Con A, cetyl trimethylammonium bromide (CTAB), spermidine, cationized ferritin and Tritin X-100 were applied to nematodes in syringes for periods of 2 h as described by Forrest et al. (8). Treated nematodes were further examined in invasion and host finding assays, by lectin binding studies and by transmission electron microscopy. The initial study tested the effects of sodium hypochlorite (1%) on the ability of juveniles to invade potato roots. A second experiment with sodium hypochlorite was carried out to distinguish between failure to find the host or failure to penetrate the root. The effect of other chemicals on host finding were compared in subsequent experiments [see Forrest et al. (8)].

Invasion assay. The effect of sodium hypochlorite (1%) on invasion was tested. Rooted sprouts of the susceptible, Desiree, cultivar were inoculated with 2500 treated and untreated juveniles. After 3 days the roots were thoroughly washed and numbers of invaded nematodes were estimated after staining and destaining the roots (8).

Fluorescence and electron microscopy. Nematodes treated with sodium hypochlorite, CTAB, spermidine and Triton X-100 were labeled with FITC-, TRITC- or ferritin WGA and prepared for fluorescence or electron microscopy, as described before.

Host-finding assays. We used a modification of Balan (1) method to determine the effect of the chemicals on the host-finding ability of PCN juveniles. Two agar discs 0.8 cm diam. were cut with a cork-borer either from positions adjacent to potato root tips or from plates containing nutrient agar only. Discs from both plates were placed in pairs on another plate containing 1% water agar and a small drop of water containing treated or untreated nematodes (200-500) were placed between the discs. Excess liquid was removed by blotting and subsequent movement of the nematodes observed. After 40 min. the numbers which had migrated to each disc and those which remained in the central area between the discs were counted.

Results and Discussion

The studies on host-finding and invasion partially confirmed the results of Fenwick(6) and showed that treatment with 1% hypochlorite reduced the numbers of juveniles able to infect potato roots. Many of the treated nematodes were active, but remained in the centre of the plates and failed to detect the attractant discs. The most obvious changes observed in the treated nematodes were the degradation of

amphidial exudate and the inability to respond to attractants which were possibly low molecular weight substances diffusing from the agar discs. We assume that a damage to the sensory apparatus of the amphids probably caused this inability to recognize the host. CTAB, spermidine and lower concentration of hypochlorite reduced chemotaxis but did not remove the exudate; in TEM observations it appears that CTAB changed the characteristic appearance of the exudate. However, neither Triton X-100 nor Con A had any effect on the ability of PCN to detect the discs.

EVALUATION OF CONTROL OF MELOIDOGYNE INCOGNITA AND NACOBUS ABERRANS
IN TOMATO BY THE LEGUMINOUS PLANT LECTIN, CON A.

Experimentation

The direct treatments with Con A consisted of a range of concentration levels of the lectins Con A (Sigma, Mo USA) and Limax flavus agglutinin (LFA) (E-Y Laboratories, CA USA). The lectins were tested in greenhouse, growth chamber and micro plot trials. Each experiment included a lectin treatment, an aldicarb (Temik 15 G) application, and untreated control plants inoculated with M. incognita. The procedure was to apply lectins to 2-3-week-old tomato seedlings grown in steam-sterilized soil. Lectins were dissolved in 10mM Tris buffer (pH 7.2) and applied at the desired concentration to the soil around the plant roots. The soil was irrigated to incorporate the lectins as specified in (15). Aldicarb treatments were concurrent with those of the lectins. Hatched second-stage juveniles in tap water were added to the soil 24 h after application of lectins or aldicarb. The trials were terminated 42-60 days after nematode inoculation. Data were taken on the total number of root galls, fresh root weights, and plant tops.

In a direct application tests, two leguminous plants were assayed for their effects on control of root-knot nematode: Canavalia ensiformis, a plant which is the source from which the lectin Con a is commercially extracted, and Mucuna deeringiana. The control by this two leguminus plants was evaluated in two ways. First was the application in the N. aberrans experiments of ground M. deeringiana or C. ensiformis seed at 2 or 4 g/pot containing 2-3 week old tomato seedlings grown in 250 cm³ steam sterilized soil. The ground seed was

applied as a soil drench in 20 ml water. In the second approach, a legume plant and tomato plant were grown in the same pot. Controls were tomatoes with no treatment, tomatoes infected with nematodes alone, and legumes cocultured with tomato plants but with no nematodes to evaluate for fertilizer effects. The procedure of the experiments is further detailed in (14). For assay of root exudates, legumes at the one true leaf stage were transferred to 75 ml sterile distilled water held in a flask wrapped in aluminum foil. After e days, 400 ul of the water was tested by enzyme linked immunoabsorbant" assaya (ELISA) for the presence of Con A.

Results and Discussion

Significant control of tomato root-knot was achieved by applications of Con A and LFA lectins in greenhouse, growth chamber, and microplot trials. The lowest consistently effective single dosage application of Con A which reduced root galling was 30 µg; LFA was much less effective than Con A. Combinations of Con A and LFA did not enhance root-knot control, suggesting that no synergistic action occurs between the two lectins. The experimental observations of Con A binding specifically to the head of M. incognita (20) and to 4 populations of G. rostochiensis and G. pallida (7), as well as specific localization of sialic acid residues on the head region of Xiphinema index (25), suggest a logical line of speculation as to the mode of action of Con A on root-knot of tomatoes. Binding of the ligand presumably could result in blocking or altering the conformation of the nematode chemoreceptors.

When the leguminous plant C. ensiformis and tomato were cocultivated in greenhouse pot tests, a reduction in galling caused by

M. incognita and N. aberrans was observed. The leguminous plant M. deeringiana generally gave a lesser degree of control of root-knot under the same conditions, as did treatments with powdered, ground beans of the two legumes. The lectin Con A was demonstrated in water in which C. ensiformis had been grown. The passage of this lectin from C. ensiformis into the soil is proposed as an explanation for the observed reduction in root-knot symptoms on tomato.

Evaluation of the research achievements resulting from the research project

The identification of several carbohydrates on the outer cuticle of various phytonematode species, especially on the head, amphids and/or amphidial exudates, and the intervention in the detection of chemotactic signals between nematode and their host-roots using carbohydrate-specific proteins, form a potential base to plan a new strategy designed to confuse the host - root finding by the nematode. Not being able to detect the root, the nematode would embark on an aimless meandering through the soil, with only a random chance of encountering a suitable food source.

Encouraged by the fact that the intervention studies with the lectin Con A, reported in this project, were later supported by other research groups (W. Robertson, private communication) led us to believe that the concept originally stated in our proposal may lead to develop a novel and non-environmentally hazardous methods for the control of plant parasitic nematodes. These methods would involve no risk to human health or to non-target soil organisms.

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TABLE 1

Labelling of plant parasitic nematodes
by TRITC-lectin conjugates^{1,2}

Species	PNA	Con A	Lectin LFA	UEA	WGA
<u>Anguina tritici</u>	-	-	-	-	b ⁺⁺⁺
<u>Bursaphelenchus lignicolus</u>	-	a ⁺	-	-	-
<u>Longidorus elongatus</u>	f ⁺	-	NT	-	f ⁺
<u>L. macrosoma</u>	f ⁺⁺	-	NT	-	f ⁺⁺
<u>Meloidogyne incognita</u>	a ⁺⁺	a ⁺⁺	-	c ⁺⁺⁺	-
<u>Pratylenchus penetrans</u>	-	a ⁺	-	-	-
<u>Tylenchorhynchus claytoni</u>	-	-	-	-	a ⁺⁺⁺
<u>Radopholus similis</u>	-	-	-	-	c ⁺⁺ d ⁺⁺⁺ e ⁺⁺⁺

1. + = light label
 ++ = intermediate label
 +++ = heavily labelled
 - = negative or intermediate results

2. Letters in body of table.

a = head labelled
 b = label all over body
 c = amphids labelled
 d = excretory pore labelled
 e = amphid exudate labelled
 f = body pores labelled

Table 2. Effect of lipase and sodium periodate (IO_4^-) pretreatments on labelling of Anguina tritici and Meloidogyne incognita race 3 juveniles with several plant lectins

Lectin*	<u>A. tritici</u>			<u>M. incognita</u>		
	Non-treated	IO_4^-	Lipase	Non-treated	IO_4^-	Lipase
Con A	—	—	—	++a	++a	++a
DBA	—	—	—	—	—	—
GSII	+++	+++	++++	—	—	—
MPA	—a	—a	—a	—a	—a	—a
PNA	++++a	++++a	+++++a	—	—	—
SBA	—a	—a	—a	—a	—a	—a
UEA	++++	+++++	+++++	—a	—a	—a
WGA	++++	+++++	+++++	—a	—a	—a

* Lectins were conjugated to fluorescein isothiocyanate (FITC)

— = no labelling; +, ...,+++++ = increased intensities of labelling all over body.

a = amphids labelled

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List of publications derived from this BARD grant

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