

# A Bioassay to Estimate Root Penetration by Nematodes<sup>1</sup>

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**Abstract:** An in vitro bioassay with a 96-well microtiter plate was used to study the effect of lectins on burrowing nematode penetration of citrus roots. In each well, one 4-mm root segment, excised from the zone of elongation of rough lemon roots, was buried in 0.88 g dry sand. Addition of a *Radopholus citrophilus* suspension containing ca. 300 nematodes in 50  $\mu$ l test solution completely moistened the sand in each well. The technique assured uniform treatment concentration throughout the medium. Within 16–24 hours, burrowing nematodes penetrated citrus root pieces, primarily through the cut ends. The lectins (100  $\mu$ g/ml) Concanavalin A (Con A), soybean agglutinin (SBA), wheat germ agglutinin (WGA), and *Lotus tetragonolobus* agglutinin (LOT) stimulated an increase in penetration of citrus root segments by *Radopholus citrophilus*. Concentrations as low as 12.5  $\mu$ g/ml Con A, LOT, and WGA stimulated burrowing nematode penetration of citrus roots. Heat denaturation of the lectins reversed their effect on penetration; however, incubation of nematodes in lectin (25  $\mu$ g/ml) with 25 mM competitive sugars did not. The reason for enhanced penetration associated with lectins is unclear.

**Key words:** behavior, burrowing nematode, carbohydrate, citrus, lectin, nematode, *Radopholus citrophilus*, recognition.

Attraction, penetration, and feeding behavior of plant-parasitic nematodes appear to involve molecular communication between the nematode and host within the soil environment (20,21,25). Carbohydrates associated with nematode chemosensory structures may be involved in reception of molecular stimuli and subsequent modification of nematode behavior (1,5,6,9,10,16,17,24). Lectins, proteins that bind to specific carbohydrate molecules (7,15), conjugated with fluorophores (fluorescein isothiocyanate [FITC] and tetramethylrhodamine isothiocyanate [TRITC]), peroxidase, ferritin, biotin, or colloidal gold have been used as probes to indirectly identify carbohydrates on or in nematodes in some of these studies (3,4,8,10,18,19,23).

It is appealing to consider that binding of lectins to nematode chemosensory carbohydrates may affect nematode behavior. Investigations concerning the influence of exogenous lectins on plant–nematode in-

teractions have generated conflicting results (5,6,16,17). This conflict may have arisen because exposure of nematodes to a uniform concentration of lectin in soil was probably not accomplished when nematodes were added to soil in lectin solutions (5,6), when lectins were presumably exuded into soil by plant roots (16), or when minute amounts of lectins were applied directly to soils infested with nematodes (17). The accessibility and stability of lectins in soil are unknown.

The purpose of this research was to develop a bioassay that would facilitate evaluation of the influence of lectins on root penetration by nematodes. The assay was designed so that test solutions (lectins) would not be diluted by pre-existing soil moisture and only limited amounts of lectins or other test materials would be required to conduct a well-replicated assay within a 24-hour period.

## MATERIALS AND METHODS

**Bioassay:** Each of 96 flat-bottom wells of a microtiter plate (Dynatech Immunolon, Chantilly, VA) was filled with 0.6 g heat-dried (70 C) *Astatula* fine sand (hyperthermic, uncoated typic quartzipsamments), pH 5.8. Twenty-five microliters of test solution were dispensed into each well. Root segments (4.0 mm long) excised from the zone of elongation of actively growing roots

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of rough lemon (*Citrus limon* (L.) Raf.) seedlings were placed horizontally on the surface of the sand in each well and covered with an additional 0.28 g dry *Astatula* sand. Burrowing nematodes (*Radopholus citrophilus*, Huettel, Dickson & Kaplan) extracted from excised carrot disk cultures (14) were quantified, concentrated, and resuspended in test solutions (12,000 nematodes/ml), and 25  $\mu$ l of the nematode suspensions (ca. 300 nematodes) were added to each well. Microtiter plates were then covered and placed in a moist chamber (100% humidity at 25 C) for 16–24 hours. Root pieces were collected with fine forceps and placed in stainless steel biopsy holders (Lancer, St. Louis, MO). Biopsy holders containing root segments were incubated in 1% sodium hypochlorite for 10 minutes and then rinsed for 15 minutes with rapidly running tap water. Roots were stained for 30 seconds in a boiling aqueous acid fuchsin solution (2) and left in the stain solution until examined. Nematodes in each root segment were counted at 100 $\times$  or 200 $\times$  with an inverted-light microscope after root segments were squashed between two microscope slides (25  $\times$  75 mm).

**Lectin tests:** The bioassay was used to evaluate the influence of the following lectins on root penetration by nematodes: Concanavalin A (Con A), *Lotus tetragonolobus* agglutinin (LOT), soybean agglutinin (SBA), or wheat germ agglutinin (WGA) (E-Y Laboratories, San Mateo, CA) at 100  $\mu$ g/ml in MOPS buffer (0.1 M 3-[N-morpholino]propanesulfonic acid containing 0.01 M CaCl<sub>2</sub>) at pH 6.5 and pH 7.5. Control treatments were 0.01 M MOPS buffer at pH 6.5 and pH 7.5. Two pH levels were utilized because of the influence of pH on lectin (especially Con A) stereochemistry (11).

The influence of each lectin at 100, 50, 25, and 12.5  $\mu$ g/ml in 0.01 M MOPS buffer at pH 6.5 on nematode penetration was estimated with the bioassay. Control treatments included buffer alone.

The bioassay was also used to compare the effect of the following treatments on root penetration by nematodes: LOT or

TABLE 1. Effect of four lectins at two pH levels on mean number of *Radopholus citrophilus* penetrating root segments of rough lemon (*Citrus limon*) in vitro.

Treatment†	Nematodes/root	
	pH 6.5	pH 7.5
Con A	18.3 a	16.9 ab
LOT	18.5 a	11.6 cd
SBA	18.3 a	15.9 abc
WGA	17.0 a	18.9 a
Buffer alone	11.0 cd	9.8 d

Means followed by the same letter are not significantly different according to Duncan's multiple-range test ( $P = 0.05$ ).

† 100  $\mu$ g/ml Concanavalin A (Con A), *Lotus tetragonolobus* agglutinin (LOT), soybean agglutinin (SBA), or wheat germ agglutinin (WGA), in 0.1 M MOPS (3-[N-morpholino]propanesulfonic acid) buffer with 0.01 M CaCl<sub>2</sub>.

Con A (25  $\mu$ g/ml), LOT or Con A (25  $\mu$ g/ml; denatured at 97 C), LOT (25  $\mu$ g/ml plus 25 mM L-fucose) or Con A (25  $\mu$ g/ml plus 25 mM  $\alpha$ -methyl mannopyranoside), or 25 mM L-fucose or 25 mM  $\alpha$ -methyl mannopyranoside. Control treatments were the 0.01 M MOPS buffer at pH 6.5 or distilled water. Reduced agglutination activity of lectins that were heat treated or incubated with 25 mM competitive carbohydrate (i.e., L-fucose or  $\alpha$ -methyl mannopyranoside) was confirmed by a hemagglutination assay (4,22) with trypsinized and glutaraldehyde-treated human type O red blood cells (Sigma, St. Louis, MO) and 0.01 M phosphate-buffered saline, pH 7.2.

Each of the penetration bioassays was repeated at least once, and all treatments were replicated eight times.

## RESULTS

*Radopholus citrophilus* penetrated citrus root segments in microtiter plates within 16 hours. Nematodes entered the root segments through both cut ends. In the first experiment, (four lectins, 100  $\mu$ g/ml, two pH levels), nematode penetration of excised roots was enhanced by treatment with all four lectins at pH 6.5; however, at pH 7.5 only Con A, SBA, and WGA were associated with increased penetration of root segments (Table 1).

Nematode penetration was enhanced by Con A, WGA, and LOT at all concentrations tested at pH 6.5. In contrast, SBA

TABLE 2. Effect of four decreasing concentrations of four lectins on mean number of *Radopholus citrophilus* penetrating root segments of rough lemon (*Citrus limon* (L.) Raf.) in vitro.

Treatment†	Nematodes/root			
	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml
Con A	23.3 a	17.7 a-d	19.0 a-c	16.7 b-d
LOT	16.7 b-d	18.9 a-c	19.0 a-c	19.6 a-c
SBA	13.4 c-e	13.9 c-e	11.4 d-f	7.0 f
WGA	17.0 a-d	21.3 ab	17.4 a-d	14.3 c-e
Buffer alone—6.7 f				

Means followed by the same letter are not significantly different according to Duncan's multiple-range test ( $P = 0.05$ ).  
 † Concanavalin A (Con A), *Lotus tetragonolobus* agglutinin (LOT), soybean agglutinin (SBA), or wheat germ agglutinin (WGA), in 0.1 M MOPS (3-[N-morpholino]propanesulfonic acid) buffer with 0.01 M CaCl<sub>2</sub>, pH 6.5.

treatments below 50 µg/ml did not enhance penetration (Table 2). Heat treatment of the Con A and LOT, but not incubation with L-fucose or  $\alpha$ -methyl mannopyranoside, eliminated lectin-enhanced penetration (Table 3). Heat treatment suppressed lectin-induced hemagglutination to a greater extent than did coincubation of lectins with their competitive carbohydrate.

#### DISCUSSION

The bioassay developed to evaluate the influence of lectins on infection was prac-

TABLE 3. Effect of heat denaturation of lectins and carbohydrate-lectin binding on mean number of *Radopholus citrophilus* penetrating root segments of rough lemon (*Citrus limon* (L.) Raf.) in vitro.

Treatment	Nematodes/root
Con A (25 µg/ml)	9.0 ab
Heat denatured	6.0 bc
With sugar†	8.8 ab
Sugar alone‡	5.7 bc
LOT (25 µg/ml)	10.2 a
Heat denatured	2.8 c
With sugar‡	9.0 ab
Sugar alone‡	5.8 bc
Buffer alone§	4.5 c
Distilled water	2.7 c

Means followed by the same letter are not significantly different according to Duncan's multiple-range test ( $P = 0.05$ ).

† 25 mM  $\alpha$ -methyl mannopyranoside

‡ 25 mM L-fucose.

§ Concanavalin A (Con A), *Lotus tetragonolobus* agglutinin (LOT), LOT 1 and Con A 1 = *Lotus tetragonolobus* agglutinin and Concanavalin A (25 µg/ml), respectively; LOT 2 and Con A 2 = heat denatured LOT and Con A (25 µg/ml), respectively; LOT 3 and Con A 3 = 25 µg/ml LOT with 25 mM L-fucose or 25 µg/ml Con A with 25 mM  $\alpha$ -methyl mannopyranoside, respectively; LOT 4 and Con A 4 = 25 mM L-fucose and 25 mM  $\alpha$ -methyl mannopyranoside, respectively; Buffer = 0.01 M MOPS buffer, pH 6.5, containing 0.01 M CaCl<sub>2</sub>.

tical and reliable. The assay could be used to evaluate the influence of a wide variety of other compounds or biological entities on nematode penetration of roots. The microtiter plate facilitated the use of many replicates in a relatively small amount of space. The assay required a small volume of test solution (50 µl), few nematodes (300 per well), and little plant tissue. In addition, test solutions were not subject to dilution upon introduction into the assay medium as in similar experiments with soil in pots, trays, or fields (5,6,16,17).

The assay is limited because it monitors only the numbers of nematodes in roots, and it cannot distinguish whether treatments that affect penetration result from changes in nematode attraction, motility, or ability to penetrate roots. To evaluate the condition of nematodes that did not penetrate roots, nematodes could be recovered from the sand in individual wells. Nematode penetration of root pieces in the assay reflected an oriented response of the nematode to the cut ends of the root.

The present study reports on the penetration of citrus roots by *R. citrophilus*; however, we have used other types of roots (tomato and soybean) and other species of nematodes (*Meloidogyne incognita* and *M. javanica*) with success (Kaplan, unpubl. data).

Use of root tissue from plant species other than citrus would make the assay more practical. For citrus, we determined that good penetration depended upon use of young, nonsuberized fibrous root segments of medium girth from 3-month-old, greenhouse-grown citrus seedlings. Com-

parable data were attained with root segments from 4-day-old soybean plants germinated as previously described (5).

Results of the three experiments indicated that Con A, LOT, and WGA enhanced *R. citrophilus* penetration of citrus root segments at very low concentrations. The minimal concentration of these lectins required for enhanced penetration was not determined, but the inability of competitive carbohydrate treatment of the LOT and Con A to reduce lectin stimulation of penetration suggests that relatively few molecules may be required for this effect.

Previous studies have indicated that lectins influence host-finding or food-finding in nematodes. Con A, mannosidase, and sialidase interfered with food-finding mechanisms in *Caenorhabditis elegans* (12, 13). Applications of minute amounts of Con A to soil in pots and field plots were also associated with reduced galling of tomato roots by *Meloidogyne incognita* (16). It has been hypothesized that suppressed root galling of tomato associated with interplanting of Jack bean, *Canavalia ensiformis*, results from release of Con A from roots (17). In contrast, Davis et al. (5) did not observe any influence on root-knot nematode infection of intact soybean roots growing in trays by Con A, LOT, WGA, and SBA; two other lectins and the carbohydrate sialic acid did reduce the number of nematodes that penetrated roots. This was confirmed in greenhouse pot studies where Con A (200  $\mu\text{g}/\text{ml}$ ) was not consistently associated with reductions in egg mass ratings (6).

In the present study, Con A and LOT were correlated with enhanced nematode penetration of root segments *in vitro*. The mechanism responsible for this enhanced penetration is unclear. Lectin binding to exposed receptors and (or) secretions may enhance overall nematode activity, chemosensory capability, or feeding behavior. Fluorescent conjugates (TRITC) of LOT (Kaplan, unpubl. data) and WGA (23) strongly labeled amphids of the burrowing nematode, and this labeling was blocked in the presence of complimentary competi-

tive sugars. It is also possible that lectins may influence penetration by binding to substances released from the cut surface of the root.

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