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# Lectin Binding to *Radopholus citrophilus* and *R. similis* Proteins[1]

DAVID T. KAPLAN AND TIM R. GOTTWALD[2]

*Abstract:* Lectin-binding glycoproteins in seven populations of two burrowing nematode sibling species were probed with five different biotinylated lectins on Western blots, and differences were correlated with nematode ability to parasitize citrus and to overcome citrus rootstock resistance. Banding patterns of molecular weight standards were fit best by an exponential decay function, and a predictive equation was used to estimate molecular weights ($r^2 = 0.999$). A band (131 kDa) that labeled with the lectin Concanavalin A (Con A) occurred in extracts from cuticles and egg shells of populations of *Radopholus citrophilus* that parasitize citrus. Wheat germ agglutin labeled a band (58 kDa) in aqueous homogenates of populations that reproduce in roots of citrus rootstock normally resistant to burrowing nematodes. The two sibling species *R. citrophilus* and *R. similis* were distinguished by a high molecular weight Con A-labeled band (608 kDa) from cuticle and egg shells. Probing blots with the lectin *Limulus polyphemus* agglutinin indicated that each population contained a band (12–16 kDa) specifically inhibited by the addition of 25 mM neuraminic acid, suggesting that glycoproteins with sialic acid moieties are present in burrowing nematodes.

*Key words:* carbohydrate, citrus, lectin, nematode, *Radopholus*, recognition, sialic acid, systematics, taxonomy.

The burrowing, nematodes *Radopholus similis* (Cobb) Thorne and *R. citrophilus* (Huettel, Dickson, & Kaplan) (14) damage a wide variety of crops throughout tropical and subtropical regions of the world (28,31). The extent of damage associated with burrowing nematode infestations of banana plantations and citrus orchards is variable (21,27), being influenced by crop cultivar, edaphic factors, and the relative aggressiveness of nematode populations.

Two sibling species of *Radopholus* spp. have been distinguished on the basis of differences in isozymes, karyotype, total protein patterns, and pheromone-mediated behavior (10–16). The presence of biotypes in *Radopholus* spp. has been verified in laboratory and greenhouse studies (21), but biochemical analyses have failed to distinguish biotypes (12,13). The occurrence of physiological races of phytoparasitic nematodes suggests that specific interactions occur between nematodes and plant cultivars (19,21). Glycoconjugates may impart specificity in recognitional aspects of host–parasite interactions (2,19,35). Antigenic determinants of animal-parasitic nematodes appear to be surface or secretory glycoproteins (1,26). For plant-parasitic nematodes, glycoconjugates have been characterized on surfaces, in stylet exudates, and in body walls (4,6,7,17,24, 32,33).

Lectin binding to aqueous-soluble and body wall proteins in homogenates from infective juveniles of root-knot nematodes (*Meloidogyne* spp.) was recently investigated with biotinylated lectin probes of Western blots (4). With this procedure, *Meloidogyne incognita* races 1 and 3, *M. javanica,* and *M. arenaria* were distinguished by heavily stained bands (4). Probing of Western blots with biotinylated lectins (25) bound to the avidin–biotin complex (9) provides a sensitive method to characterize glycoproteins separated electrophoretically. The purpose of this research was to determine if glycoproteins were correlated with species or biotypes of burrowing nematodes.

## MATERIALS AND METHODS

*Nematode cultures:* Four *Radopholus citrophilus* and three *R. similis* populations were

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[2] Supervisory Research Plant Pathologist and Research Plant Pathologist, USDA ARS, 2120 Camden Road, Orlando, FL 32803.

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compared. *Radopholus citrophilus* RC1 and RC2 were previously described as biotypes one and two, respectively (21). A third *R. citrophilus* (RCF) population called Ford was similar to biotype two but was found in a greenhouse where rootstock screening had been conducted. The fourth population was previously reported from *Anthurium andreanum* (RCA) and does not reproduce on citrus (16). *Radopholus similis* populations included in the study were isolated from banana in Hawaii (RSH), Orlando (RSO), and Guatemala (RSG). Mixed developmental stages of each population were extracted separately from carrot disk culture (20). Nematodes and eggs (4,000,000/ml) were transferred to Tris-HCl Extraction Buffer (0.05 M tris-[hydroxymethyl]aminomethane, 1 mM PMSF [phenylmethylsulfonyl fluoride], pH 7.0) and frozen at −80 C prior to use.

*Protein preparation:* Protein extraction from frozen nematodes was similar to that described previously (4). Approximately 4 million nematodes (mixed developmental stages) per ml of extraction buffer were thawed to 25 C, and the suspension was pipetted dropwise into liquid nitrogen in a cold ceramic mortar. Once the liquid nitrogen had evaporated, the resultant frozen beads were collected, ground manually with a Potter homogenizer, and allowed to thaw. This procedure was repeated until 99% of the nematodes and eggs were free of internal contents as determined with a light microscope (400×). The suspension was then sonicated with a Micro Ultrasonic Cell Disruptor (Kontes, Vineland, NJ) for 1 minute at 0 C and then centrifuged at 2,000 *g* for 10 minutes, and the aqueous-soluble supernatant was collected, divided, and frozen at −80 C prior to use.

Pellets of isolated cuticles and egg shells were rinsed twice in Extraction Buffer and resuspended in the same buffer containing 0.25% (w/v) cetyltrimethylammonium bromide (CTAB) (4,29). Body walls in CTAB-buffer were heated in a water bath at 37 C for 4 hours and centrifuged at 2000 *g* for 10 minutes, and the CTAB-soluble extract was collected. Extracts of cuticles and eggshells were stored frozen at −80 C before use. Protease activity in the homogenates and extract was determined with a colorimetric Azocoll assay (Sigma Chemical Co., St. Louis, MO).

*SDS-PAGE and Western blots:* Electrophoresis and electroblotting were performed essentially as described (25). All protein concentrations were determined according to the Lowry method (23). Samples from each population were run in discontinuous SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described by Laemmli (22). Individual samples of each of the seven nematode populations from the same extraction method were run in adjacent lanes to form a "set" for population comparisons, and two identical "sets" were included in each electrophoretic run and Western blot. Proteins were separated at a constant 20 mA per gel for 50 minutes in a 7 cm × 8 cm × 0.75 mm vertical slab gel (4.0% stacking, 12.0% separating polyacrylamide gel) in a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). Biotinylated SDS-PAGE molecular-weight standards (Vector Laboratories, Burlingame, CA) were included in each gel to be electroblotted and probed with biotinylated lectin. Proteins in the cuticle-eggshell extracts were separated as above but SDS-PAGE molecular-weight standards (Bio-Rad) were used.

Immediately after electrophoresis, gels were equilibrated for 30 minutes in transfer buffer (14 mM Tris, 11 mM glycine, 0.0375% SDS [w/v], 20% methanol [v/v], pH 8.0). The PVDF (polyvinylidenedifluoride) transfer membrane (Immobilon-P, Millipore, Bedford, MA) was soaked in methanol for 20 seconds, rinsed 3× in distilled water, and equilibrated in the transfer buffer for 15 minutes before electroblotting. Proteins were transferred from gels to PVDF membranes at a constant 250 mA for 1.5 hours with a Mini-Trans Blot apparatus (Bio-Rad) filled with cold transfer buffer and continuously cooled with ice. Western blots to be probed with biotinylated lectins were rinsed 3× in HEPES

(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffered saline (HBS: 10 mM HEPES, 150 nM NaCl, 0.1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $MnCl_2 \cdot 4H_2O$, pH 7.5) and incubated in HBS plus 1.0% BSA (w/v) at 4 C overnight to block nonspecific membrane binding sites. Blots for general protein stain were immersed for 30 minutes in aqueous 0.1% (w/v) naphthol blue-black containing 40% methanol and 10% glacial acetic acid. General protein patterns were visualized by destaining blots in staining solution minus naphthol blue-black.

*Lectin probes of Westen blots:* Blots incubated in BSA blocking solution were rinsed 3× in HBS. The two sets of nematode proteins on Western blots were probed as follows. One blot was incubated for 1 hour at room temperature in 5.0 ml HBS containing either 2.0 μg/ml biotinylated Con A (Concanavalin A), SBA (soybean agglutinin), WGA (wheat germ agglutinin), LOT (*Lotus tetragonolobus* agglutinin), or 5.0 μg/ml LPA (*Limulus polyphemus* agglutinin) (E–Y Laboratories, San Mateo, CA). The other blot was incubated in lectin–sugar solutions: Con A and α-methyl mannopyranoside, SBA and N-acetylgalactosamine, WGA and N-acetylglucosamine, LOT and d-L-fucose, and LPA and N-acetylneuraminic acid, which had been previously incubated together for 1 hour.

One hundred microliters of each reagent, A and B, from the Vectastain ABC alkaline phosphatase kit (Vector Laboratories) were mixed in 20 ml HBS for 30 minutes prior to use to form the avidin–biotin complex (ABC). Blots were rinsed 3× in HBS to remove unbound lectin. Each blot was then incubated in 10.0 ml ABC solution for 1.5 hours at room temperature. Blots were then rinsed 3× in HBS to remove unbound ABC and equilibrated for 15 minutes in substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2 \cdot 6H_2O$, pH 9.5). Substrate solution containing 1.65 mg NBT (nitroblue tetrazolium chloride) and 0.85 mg BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) (Bethesda Research Laboratories, Gaithersburg, MD) per 10 ml substrate buffer was used to visualize alkaline phosphatase-labeled proteins. Control blots were used to indicate nonspecific binding of ABC to protein bands: blots were incubated in HBS or alkaline phosphatase without the biotinylated lectin and then probed with ABC and developed with BCIP and NBT as described above.

All blots were incubated in 5.0 ml substrate solution in the dark until bands developed or background darkened. The substrate reaction was stopped by immersing blots in solution containing 20 mM Tris-HCl and 5.0 mM EDTA at pH 7.5. Blots were photographed immediately after developing with substrate. The experiments and results were repeated at least once.

The distance traveled by each band in the protein standard was regressed against its molecular weight (minimum of six bands per standard) by nonlinear regression. These values were fitted to an exponential decay equation with a minimum acceptable coefficient of determination of 0.98. The fitted equation was then used to estimate the range of possible molecular weight values of bands by increments of 0.25 cm to generate a migration distance (cm) vs. molecular weight table. Estimated molecular weights of individual protein bands were then determined by measuring the distance from the origin to the band.

*Relatedness of nematode populations:* Individual protein bands within each lectin vs. nematode SDS-PAGE test were assigned an intensity index of 1 to 3 where 1 = a faint band, 2 = a band of moderate intensity, and 3 = a heavily stained band. An assumption was made that the intensity of staining was related, directly or indirectly, to the number of available lectin-binding sites on proteins or the protein amount in a particular band. Bands common to all seven nematode populations were not considered in further analyses because they could not serve to discriminate nematode populations. Data sets for individual lectins and across nematode isolates were

converted to dissimilarity coefficient matrices by the average taxonomic distance method of the NTSYS-PC program (Numerical Taxonomy and Multivariant Analysis System, Exter Publishing, Ltd., Setauket, NY). Cluster analysis was performed on each lectin dissimilarity matrix by the unweighted average pair group method. The results of cluster analysis were utilized to prepare phenograms to delineate the similarity (1 − dissimilarity) coefficients and, thus, the relatedness among nematode isolates determined by each lectin profile.

## Results

Biotinylated lections labeled numerous bands in each population. The numbers of bands detected on Western blots of nematode-egg aqueous-soluble homogenates and cationic detergent-treated body wall–egg shell extracts varied for the seven nematode populations probed with five different biotinylated lectins (Tables 1,2). The presence of 25 mM sugar specific to each lectin inhibited lectin labeling for most bands detected on the blots. Proteases were not detected, and banding patterns were consistent throughout the study. Alkaline phosphatases were identified (42–44 kDa) in all populations and were not included in further analyses.

Several bands were correlated with ability to parasitize citrus or relative aggressiveness of burrowing nematode populations. Con A labeled a band (131 kDa) from CTAB-treated body wall–egg shell extracts from three *Radopholus* populations that parasitize citrus: RC1, RC2, and RCF (Fig. 1). Another band (58 kDa) that labeled with WGA occurred in aqueous homogenates of only the RC2 and RCF populations (Fig. 2); both reproduce on citrus rootstocks that are normally burrowing nematode-resistant. No unique bands were associated with inability of the RCA, RSO, RSH, and RSG populations to reproduce on citrus.

The two sibling species of *Radopholus* were distinguished by a high molecular weight Con A-specific band (extrapolated mass of 608 kDa) in CTAB-treated body wall–egg shell extracts of *R. similis* (Fig. 1) and by a smaller band (41 kDa) in CTAB-treated body wall–egg shell extracts of *R. citrophilus* stained for total protein. The molecular weight of the 608 kDa band was extrapolated from the fitted equation, as the band was resolved outside of the range of bands in the molecular weight standards. In addition, four biotinylated Con A-labeled bands (217, 70, 36, and 26 kDA) were detected in two or more of the *R. citrophilus* populations but were not detected in any of the *R. similis* populations. Several bands in CTAB-treated burrowing nematode cuticles and egg shells were labeled with Con A, WGA, and LPA (Table 2); labeling was inhibited when appropriate carbohydrates were added to the biotinylated lectin solution. Three WGA-

Table 1. Numbers of glycoprotein bands† detected in aqueous extracts of homogenates of seven *Radopholus* populations,‡ probed on Western blots from SDS-PAGE gels with biotinylated lectins.

| | Nematode population | | | | | | |
|---|---|---|---|---|---|---|---|
| Lectin§ | RC1 | RC2 | RCF | RCA | RSO | RSG | RSH |
| Con A | 19 | 4 | 6 | 17 | 12 | 12 | 13 |
| SBA | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| WGA | 10 | 11 | 15 | 7 | 9 | 8 | 5 |
| LPA | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| LOT | 3 | 4 | 6 | 0 | 3 | 0 | 0 |

† Bands on Western blots not labeled with lectin in the presence of 25 mM competitive sugar.

‡ RC1 = *Radopholus citrophilus* biotype 1; RC2 = *R. citrophilus* biotype 2; RCF = *R. citrophilus* "FORD" population; RCA = *R. citrophilus* from *Anthurium;* RSO = *R. similis* from Orlando greenhouse banana; RSG = *R. similis* from Guatemala; RSH = *R. similis* from banana in Hawaii.

§ Con A = Concanavalin A; SBA = soybean agglutinin; WGA = wheat germ agglutinin; LPA = *Limulus polyphemus* agglutinin; LOT = *Lotus tetraglobulus* agglutinin.

TABLE 2. Numbers of glycoprotein bands† detected in CTAB‡ extracts of burrowing nematode cuticles and egg shells from seven *Radopholus* populations,§ probed on Western blots from SDS-PAGE with biotinylated lectins.

| Lectin‖ | Nematode population | | | | | | |
|---|---|---|---|---|---|---|---|
| | RC1 | RC2 | RCF | RCA | RSO | RSG | RSH |
| Con A | 4 | 10 | 7 | 3 | 8 | 11 | 12 |
| SBA | 3 | 1 | 1 | 0 | 1 | 3 | 0 |
| WGA | 2 | 2 | 2 | 2 | 2 | 2 | 1 |
| LPA | 1 | 2 | 3 | 1 | 1 | 1 | 1 |
| LOT | na¶ | na | na | na | na | na | na |

† Bands on Western blots not labeled with lectin in the presence of 25 mM competitive sugar.
‡ Cetyltrimethylammonium bromide.
§ RC1 = *Radopholus citrophilus* biotype 1; RC2 = *R. citrophilus* biotype 2; RCF = *R. citrophilus* "Ford" population; RCA = *R. citrophilus* from *Anthurium;* RSO = *R. similis* from Orlando greenhouse banana; RSG = *R. similis* from Guatemala; RSH = *R. similis* from banana in Hawaii.
‖ Con A = Concanavalin A; SBA = soybean agglutinin; WGA = wheat germ agglutinin; LPA = *Limulus polyphemus* agglutinin; LOT = *Lotus tetraglobulus* agglutinin.
¶ na = bands too faint to be measured accurately.

labeled glycoproteins (97, 55, and 52 kDa) were detected in only three of the burrowing nematode populations. Eleven glycoproteins (90, 70, 63, 53, 50, 37, 36, 35, 31, 26, and 24 kDa) were labeled with biotinylated Con A in three or more populations of the burrowing nematode sibling species. Of these, the 50 and 26 kDa glycoproteins occurred only in *R. similis*. For LOT, a high molecular weight band was identified only in RC1, RC2, and RCF, the three populations studied that parasitize citrus. Another LOT-binding band (47 kDa) was identified in two *R. citrophilus* populations, but not in any of the *R. similis* populations. Total protein staining of aqueous homoge-

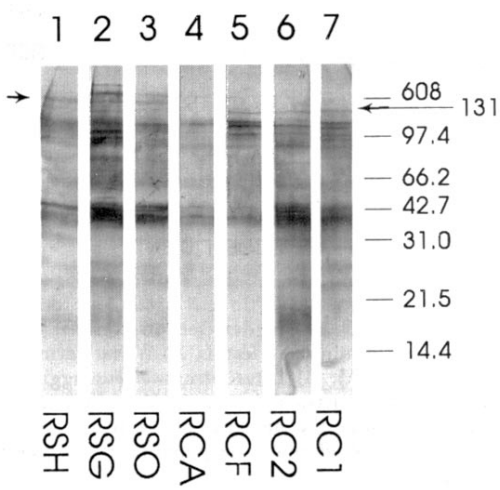


FIG. 1. Western blot of SDS-PAGE separation of cetyltrimethylammonium bromide (CTAB) extracts of body wall and egg shell from seven burrowing nematode populations probed with biotinylated Concanavalin A (Con A). RC = *Radopholus citrophilus;* RS = *R. similis;* RC1 = biotype that does not reproduce on BN-resistant citrus rootstocks; RC2 and RCF reproduce on BN-resistant citrus rootstock; RCA, RSO, RSG, and RSH do not reproduce on citrus. Band values are in kilodaltons (kDa). Arrows indicate protein bands unique to RC1, RC2, and RCF.

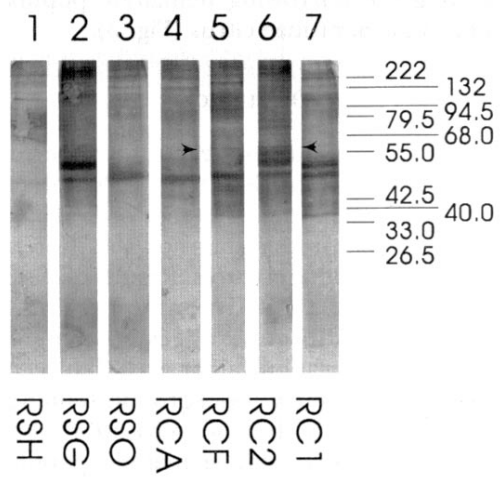


FIG. 2. Western blot of SDS-PAGE separation of aqueous-soluble homogenate extracts from seven burrowing nematode (BN) populations probed with biotinylated wheat germ agglutinin (WGA). RC = *Radopholus citrophilus;* RS = *R. similis;* RC1 = biotype that does not reproduce on BN-resistant citrus rootstocks; RC2 and RCF reproduce on BN-resistant citrus rootstock; RCA, RSO, RSG, and RSH do not reproduce on citrus. Band values are in kilodaltons (kDa). Arrows indicate protein bands unique to RC2 and RCF.

nates indicated that only three of the 29 discernable bands (97, 79, and 33 kDa) occurred in three or more populations. Two of these (79 and 33 kDa) were present only in *R. similis*.

A broad LPA-binding band (12–16 kDa) in aqueous homogenates and CTAB-extracts was present in all burrowing nematode populations. Labeling of the band was inhibited when 25 mM neuraminic acid was included in the biotinylated lectin solution. However, the limited number of bands that labeled with LPA precluded the generation of a phenogram for the lectin. A similar situation occurred for SBA-labeled bands when N-acetylgalactosamine was included in the biotinylated lectin solution.

Comparison of relative similarity of the burrowing nematode isolates based on qualitative and quantitative (stain intensity) differences within individual Western blots of nematode homogenates for each lectin resulted in three distinct phenograms. The phenogram for WGA-specific glycoproteins demonstrated 92% similarity among the burrowing nematode populations that parasitize citrus (Fig. 3).

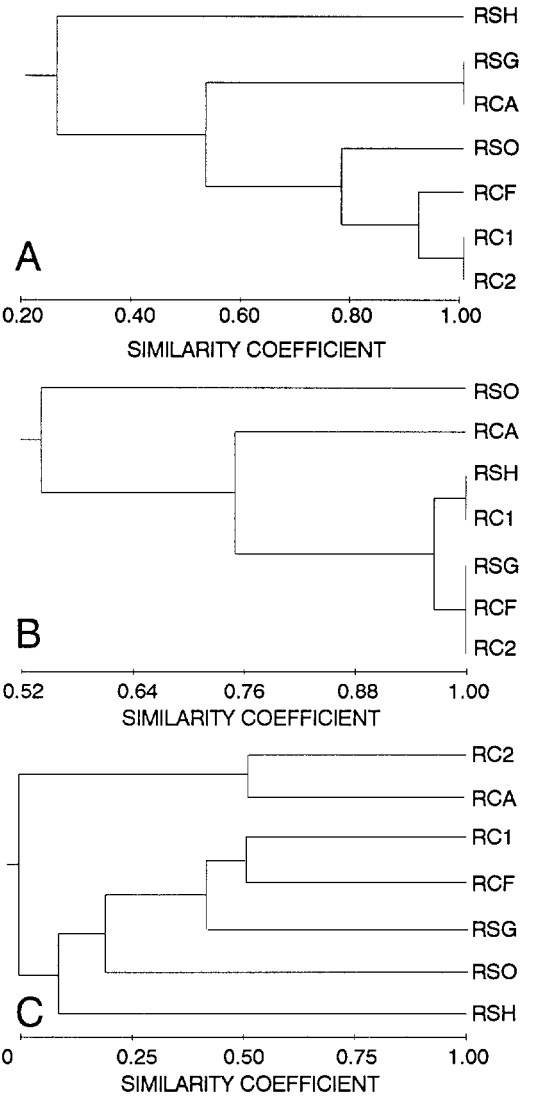


FIG. 3. Phenogram of the relative similarity of aqueous homogenates of three *R. similis* and four *R. citrophilus* populations as detected on Western blots probed with biotinylated lectins. A) Wheat germ agglutinin (WGA). B) *Lotus tetragonolobus* agglutin (LOT). C) Concanavalin A (Con A). Vertical connecting lines indicate the similarity of coefficient values between individual nematode populations or among groups.

## DISCUSSION

In previous studies (12,13), intraspecific variation was not detected in *Radopholus citrophilus* (then the citrus race of *R. similis*) with general protein stains or 16 different enzyme reaction mixtures. Results of silver staining of two-dimensional SDS-polyacrylamide gels suggested that intraspecific variation might exist, but technical problems (e.g., background staining) interfered with their identification (13). All of the burrowing nematode populations (except RCF) used in the present study (RCA, RC1, RC2, RSH, RSO, RSG) were included in these studies (11–13). Neither the 16 enzymes nor the general protein bands detected in these populations (12,13) have been demonstrated to be involved in nematode–citrus root recognition, as they did not correlate with the host range of the four *R. citrophilus* populations.

Although bands that correlated with the inability of burrowing nematodes to reproduce on or damage citrus were not identified, their detection may have been obscured by the presence of comigrating glycoproteins. The involvement of carbohydrates in recognition involving plant–

nematode interactions remains to be determined. The present findings support previous reports of the occurrence of glycoproteins on or in plant-parasitic nematodes (4,17,30,34) or in their secretions (17). Therefore, bands that correlate with sibling species or host range require further characterization to determine if they contain more than one glycoprotein. Purification of unique proteins could facilitate immunological characterization of glycoproteins in situ and thereby improve our understanding of nematode–plant communication. Physiologically important carbohydrates may also be present as glycolipids or other nonproteinaceous compounds not detected by Western blot methodology.

The observed LPA-specific binding to bands in nematode homogenates and cuticles suggests that sialic acids are conjugated to proteins in plant parasitic nematodes. The presence of sialic acids in nematodes is controversial (3); they may play a role in recognition or chemosensory modulation in nematode–plant systems in a manner similar to that in plant–bacteria interactions (5,18). Bacic et al. (3) analyzed nematodes for sialic acids and determined that sialic acid detection in nematodes was dependent on the presence of sialic acid in the growth medium. In the present study, nematodes were cultured on excised carrot disks. Sialic acids have not been reported from carrots or any other plant. All *Radopholus* homogenates and extracts contained glycoprotein bands that were labeled with LPA. In root-knot nematodes, glycoprotein bands that labeled positively with LPA were detected in CTAB extracts of cuticles (4). Furthermore, the banding pattern on Western blots of root-knot nematode aqueous homogenates probed with LOT were similar in appearance to CTAB extracts of nematode cuticles probed with LPA (4). In contrast, glycoprotein band patterns of nematode homogenates and CTAB extracts of body walls and egg shells on Western blots probed with LOT and LPA were dissimilar. However, all burrowing and root-knot nematode species or populations contained glycoprotein bands that were specifically labeled with biotinylated LPA. That is, labeling was competitively inhibited by the addition of neuraminic acid to the incubation medium.

Although the emphasis of this research was on intraspecific variation, two distinct interspecific glycoprotein bands were detected in CTAB-extracts: one high molecular weight band (608 kDa) that labeled with Con A from *R. similis* populations and another that labeled with naphthol blue-black (41 kDa) from *R. citrophilus*. These proteins occurred in body wall–egg shell extracts. If they are present on the outer surface of eggs or cuticles, they might be used to serologically distinguish *Radopholus* species. Glycoprotein analysis has been used to distinguish plant species (25) and may be useful for separation of nematode species (4). Qualitative and quantitative aspects of glycoprotein banding patterns may provide a useful tool for nematode systematists when used in conjunction with numerical taxonomy methods.

Our findings indicate that the avidin–biotin complex affinity technique is useful for detection of nematode glycoproteins. This procedure, coupled with a predictive equation (exponential decay) to estimate molecular weight equivalents, provides a useful system to estimate molecular weights and to identify bands on Western blots.

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