Immunofluorescent Localization of Tobacco Ringspot Nepovirus in the Vector Nematode *Xiphinema americanum*

Shouhua Wang and Rose C. Gergerich

Department of Plant Pathology, 217 Plant Science Building, University of Arkansas, Fayetteville 72701.
Accepted for publication 26 May 1998.

ABSTRACT

Wang, S., and Gergerich, R. C. 1998. Immunofluorescent localization of tobacco ringspot nepovirus in the vector nematode *Xiphinema americanum*. Phytopathology 88:885-889.

An indirect immunofluorescent technique was developed to localize tobacco ringspot nepovirus (TRSV) in the vector nematode *Xiphinema americanum* sensu *stricto*. A population of this nematode that efficiently transmitted TRSV was given an acquisition access period of 10 days on TRSV-infected cucumber. Treatment of fragments of viruliferous nematodes with a polyclonal antiserum against TRSV followed by fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G resulted in virus-specific bright fluorescence only in the lumen of the stylet extension and esophagus. Virus-specific fluorescent signals were observed in the virus-retention region of 44% of the nematode fragments examined. The percentage of nematodes labeled with virus-specific fluorescence increased as the acquisition access period increased from 0 to 22 days; the increase paralleled the increase in the transmission efficiency of the nematode population. Visualization of the entire virus-retention region of individual nematodes within a population of vector or nonvector nematodes provides a rapid and simple means of monitoring specific attachment of plant viruses.

Additional keyword: detection.

Transmission of tobacco ringspot virus (TRSV) by *Xiphinema americanum* sensu *stricto* was first reported by Fulton (7). McGuire (13) later demonstrated that *X. americanum* is a very efficient vector of TRSV. This nematode has since been reported as a vector of other important viruses, such as cherry rasp leaf (4,18) and tomato ringspot nepoviruses (2,4). The characteristics of the TRSV-*X. americanum* vector relationship were investigated further in a series of studies (8,14–17).

There is a specific association between various nepoviruses and the longidorid nematodes that transmit them (6). However, the relationship between indigenous North American nepoviruses and the *X. americanum* group of nematodes appears to be less specific than the very narrow specificity that exists between indigenous European nepoviruses and their longidorid vectors (5). Previous studies on virus transmission by nematodes have suggested that the coat protein of virus particles (10,11,19) and carbohydrate moieties on food canal walls of nematodes (22) may play an important role in determining the specificity of virus attachment in nematodes.

Infectivity tests in which viruliferous nematodes were homogenized and inoculated to indicator plants was the earliest method employed to detect plant viruses in nematode vectors (23). Later, polymerase chain reaction was used to identify tobacco rattle virus from viruliferous trichodorid nematodes (30). However, these methods only distinguished virus-carrying and -free nematodes, without indicating the sites of virus retention and their relationship to the transmission process. The detection of virus particles in vector nematodes by thin-section electron microscopy has provided important information that has led to an understanding of the basis of the specificity of virus transmission by nematodes. Thin-section electron microscopy primarily has been used to locate virus particles in vector nematodes and determine the sites of virus retention for viruses transmissible by nematodes (16,20,25–27). Roberts and Brown (21) developed an immunosorbent electron microscopy technique to detect virus particles from homogenized nematodes. This technique is more sensitive than conventional electron microscopy and more rapid, reliable, and sensitive than infectivity tests.

Indirect immunofluorescent labeling has been used to detect plant viruses in their host plants and insect and mite vectors (12,29,31) and to localize various antigens in nematodes (1,32). In this study, we developed an immunofluorescent-labeling method for rapid detection and visualization of TRSV in the retention region of the vector nematode *X. americanum*.

MATERIALS AND METHODS

Virus isolate and nematodes. The isolate of TRSV used in this study was obtained from naturally infected 'Jersey' blueberry in southwestern Michigan and maintained on cucumber (*Cucumis sativus* L. 'Boston Pickling'). *X. americanum* sensu *stricto* nematodes, identified previously by Brown et al. (5), were collected periodically from a perennial oregano field near Booneville, AR, and extracted from soil by a modified sieving and decanting method combined with the Baermann funnel method (3).

Acquisition feeding and transmission of TRSV. To confirm the TRSV-*X. americanum* vector relationship, transmission tests were conducted following the criteria of Trudgill et al. (28). To acquire the virus, ≈200 active nematodes were placed near the roots of TRSV-infected cucumber (cv. Model) seedlings growing in autoclaved fine river sand in 250-ml plastic beakers for 10 to 12 days at 25 to 28°C. Nematodes used as nonviruliferous controls were placed on the roots of virus-free cucumber plants under the same conditions. After the virus acquisition access period, the nematodes were extracted as described above, and hand-picked nematodes were placed near the roots of a single healthy cucumber plant (1 or 10 nematodes per plant) growing in sand in a 25-ml plastic cup. Plants were incubated in a growth chamber under 12 h of illumination at 25 to 28°C for 3 to 4 weeks. The root systems of

Corresponding author: R. C. Gergerich; E-mail address: gergeric@comp.uark.edu

Publication no. P-1998-0622-01R

the test plants were washed, and an extract of the cucumber roots in 0.05 M phosphate buffer (pH 7.5) was assayed for TRSV by mechanical inoculation to 'Model' cucumber and cowpea (*Vigna unguiculata* L. 'Monarch') that were observed and rated for virus symptoms for 3 weeks.

Immunofluorescent-labeling procedure. An immunofluorescent-labeling procedure was developed to detect the presence of TRSV particles in viruliferous *X. americanum* after a 10-day acquisition access period on TRSV-infected cucumber plants. Nematodes that had access to virus-free cucumber plants were used as nonviruliferous controls. For each treatment (discussed in Results), ≈200 active nematodes were selected individually, placed in tap water in a 1.5-ml microcentrifuge tube, and pelleted by centrifugation at 14,000 × *g* for 4 min. The water was removed, and the nematodes were incubated for fixation in 1 ml of 2% formaldehyde for 1 h at 4°C. The fixative solution was removed by centrifugation, and the nematodes were placed on a clean glass slide in a small amount of 2% formaldehyde and cut into pieces with a razor blade. Nematode fragments were suspended in 2 to 3 drops of blocking buffer (0.14 M NaCl, 0.01 M phosphate buffer, 3% bovine serum albumin, and 0.2% Triton X-100, pH 7.2) and gently pipetted into a clean microcentrifuge tube. After blocking in 500 µl of blocking buffer for 15 min at 4°C, fragments were pelleted by centrifugation, and the blocking solution was removed. A rabbit polyclonal antiserum against the TRSV isolate used in this study was purified by cross-absorption with sap from healthy cucumber plants followed by ammonium sulfate precipitation (9). Purified primary antibody against TRSV was diluted 1:50 in blocking buffer, and 200 µl was added to the microcentrifuge tube containing the nematode fragments. After incubation on an orbital shaker at 28°C for 18 h, the fragments were washed four times for 10 min each with blocking buffer at room temperature and subsequently incubated in 200 µl of a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis) in blocking buffer on an orbital shaker for 20 h at 28°C. After incubation, the nematode fragments were washed four times for 10 min each in blocking buffer and dried in a vacuum dryer for 15 min. The dried pellet was mounted on a glass slide in 10 µl of 50% glycerol in phosphate-buffered saline, and the nematodes were gently teased to disperse them in the buffer. The specimens were examined with an epifluorescent microscope (model BHT, Olympus Corporation, NY).

Time-course studies. To study the correlation between virus transmission efficiency and immunofluorescent labeling, nematodes were allowed access to TRSV-infected cucumber seedlings for 0, 1, 3, 5, 7, 10, 15, or 22 days. Four infected cucumber plants were grown in each of eight 250-ml plastic beakers for each acquisition access period, and ≈200 nematodes were added to each beaker. After the virus acquisition access period, nematodes from the eight beakers for each acquisition period were extracted and pooled. Half of the pooled population for each acquisition time period was processed for immunofluorescent labeling as described above, and the other half was used in transmission tests.

TABLE 1. Immunofluorescent labeling of tobacco ringspot virus (TRSV) in *Xiphinema americanum* sensu *stricto*

Experiment	Treatments[a]			Nematode immunofluorescence	
	A	B	C	Frequency[b]	Percentage
Test	+	+	+	34/77	44
Control-1	+	–	+	0/100	0
Control-2	–	+	+	0/115	0
Control-3	+/–	+	–	0/120	0
Control-4	+/–	CMV	+	0/100	0

[a] A: viruliferous (+) or nonviruliferous (–) nematodes; B: incubated with a polyclonal antibody against TRSV (+), only with buffer (–), or with antibody against cucumber mosaic virus (CMV); C: incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (+) or only with buffer (–).

[b] Combined data from two experiments. Ratio of nematode fragments labeled with virus-specific fluorescence to total number of fragments examined. Only fragments that contained virus-retention sites were evaluated for fluorescence.

RESULTS

Virus transmission. On average, 26% of the cucumber bait plants with one nematode were infected with TRSV, whereas infection increased to 65% when 10 nematodes were added (Table 1). Nematodes that had fed on virus-free cucumber seedlings during the acquisition access period did not transmit TRSV, indicating that the field-collected population of nematodes used in this study was not carrying detectable levels of TRSV.

Immunofluorescent labeling of viruliferous nematodes. Microscopic examination of nematodes prepared for immunofluorescent microscopy revealed that the majority of the nematodes were fragmented and the internal structures of the nematodes appeared to be intact (Fig. 1A and C). Bright, virus-specific fluorescence was observed in individual nematodes in the esophageal region between the junction of the stylet and stylet extension and the junction of the esophagus and intestine (Fig. 1D through F) but not in the nematode stylet. Sometimes distribution of the fluorescent signal was patchy in the stylet extension and esophagus (Fig. 1E), but the majority of the specimens exhibited a uniform distribution of fluorescence. The triradiate nature of the esophagus lumen was apparent in some specimens that exhibited a strong fluorescent signal in this area (Fig. 1F).

No virus-specific FITC-fluorescent signals were observed in nematodes that had fed on virus-free cucumber plants (Fig. 1A and B), although some nonspecific fluorescence was apparent at the cut edges of the nematode fragments (Fig. 1B). Weak autofluorescent signals were apparent at the amphids, the oral orifice, and the guiding ring, but these were present in all treatments, even in the controls where FITC conjugate was not used. No fluorescence was observed in the various controls designed to detect nonspecific fluorescent labeling (Table 2). In the region between the esophagus and intestine junction and the tail end, no fluorescence was observed.

Immunolocalization and transmission of TRSV in populations of *X. americanum* with increasing virus acquisition access periods. Microscopic examination of fragments from nematodes that had acquired virus for 10 days showed that 44% of fragments containing the anterior portion of the nematode exhibited bright green, virus-specific fluorescent signals (Table 2). In time-course experiments with virus acquisition access periods ranging from 0 to 22 days, the percentage of virus-specific fluorescent labeling increased dramatically from 0 to 5 days but showed little increase from 5 to 22 days (Table 3). Transmission tests with nematodes from the same populations used in the immunofluorescent labeling assays showed a similar trend, with increasing transmission efficiency from 0 to 5 days and approximately the same efficiency from 5 to 22 days (Table 3).

DISCUSSION

Immunofluorescent labeling provides a simple and rapid method for visualization of TRSV in viruliferous nematodes and offers three advantages over previous methods used to detect viruses in vector nematodes: (i) the entire region of virus attachment can be viewed in individual nematodes, (ii) populations of nematodes can be examined to determine the proportion of nematodes carrying the virus under different experimental conditions, and (iii) detection of the virus is strongly correlated with actual virus transmission.

Transmission tests confirmed the findings of Brown et al. (5) that the nematodes in the population of *X. americanum* used in this study are efficient vectors of TRSV, although in the experiments in which they used *Petunia hybrida* for transmission test

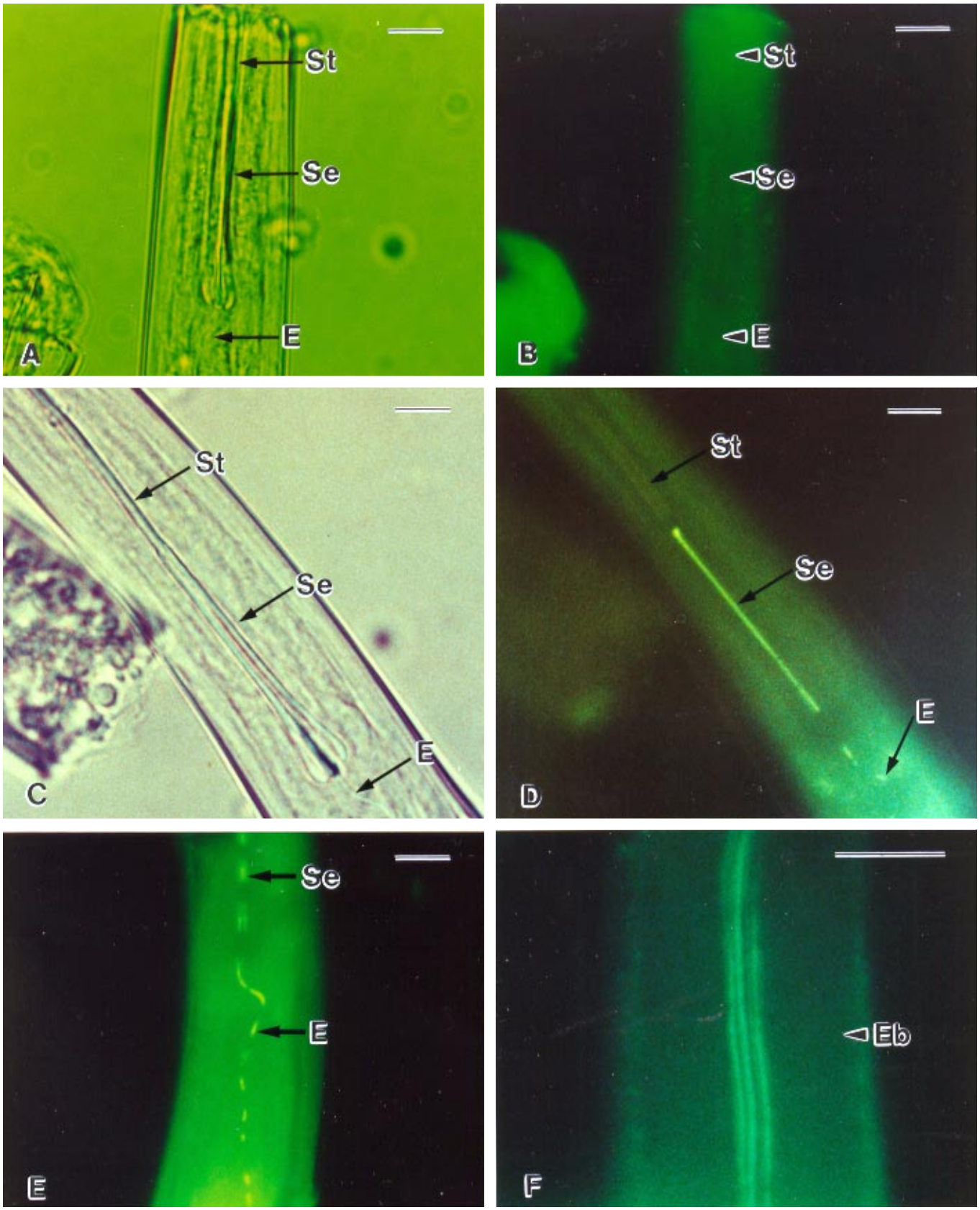

Fig. 1. Immunofluorescent labeling of *Xiphinema americanum* sensu *stricto* revealing tobacco ringspot virus (TRSV)-retention sites. **A and B,** Nonviruliferous nematode head region. **A,** Micrograph under bright field. **B,** Micrograph under UV light. **C through F,** Viruliferous nematode head regions. **C,** Micrograph under bright field. **D,** Micrograph under UV light showing specific sites of virus retention in the stylet extension. **E**, Fluorescent micrograph showing patchy distribution of TRSV in the lumen of the esophagus. **F,** Fluorescent micrograph showing virus-specific fluorescence in the triradiate lumen of the esophageal bulb. St = stylet; Se = stylet extension; E = anterior esophagus; and Eb = esophageal bulb. Bars = 10 µm.

plants, the transmission efficiency of single nematodes was lower (5%) compared to the average transmission efficiency of 26% obtained with single nematodes in our experimental system, in which we used cucumber as a test plant. Immunofluorescent labeling of populations of nematodes that had access to virus-infected plants for more than 3 weeks resulted in only approximately one-half of the nematodes exhibiting virus-specific fluorescence. The nematodes that did not acquire virus may not have fed on bait plants during the acquisition access period, or they may have acquired the virus but lost it during subsequent feeding or molting.

The patchy distribution of the fluorescent signal seen in some nematodes may reflect alternate periods of nematode feeding and quiescence or, possibly, selective loss or degradation of the virus acquired during earlier feeding. Another possibility is that TRSV is attached specifically to a mucus layer lining the anterior portion of the food canal, as suggested by Taylor and Robertson (26) for raspberry ringspot nepovirus in *X. diversicaudatum*. Robertson and Henry (22) described the mucus layer in *X. diversicaudatum* as discontinuous, and the patchy distribution of TRSV in *X. americanum* as detected by immunofluorescent labeling may reflect binding of the virus to a discontinuous mucus layer.

Dissociation of virus from virus-retention sites in vector nematodes is necessary for successful transmission. In addition to monitoring nematode populations for efficiency of virus acquisition from virus-infected plants, it is anticipated that the immunofluorescent labeling technique will be useful for monitoring the release of virus over time from retention sites in nematodes held under different experimental conditions, such as in the absence of a host plant, under fallow conditions in the field, during the winter season, or when the nematodes feed on different types of host plants after virus acquisition.

It has been suggested that nematode vectors fail to transmit some variants of viruses normally transmitted because the viruses are not dissociated from the sites of retention in the nematode during feeding (24). Although much effort has been expended to detect viruses in vector nematodes with electron microscopy, the possible role of attachment without subsequent dissociation as an explanation for lack of virus transmission by nematodes has not been fully investigated. The immunofluorescent labeling technique provides a valuable tool for monitoring both virus attachment and release in populations of nematodes and should help establish the role of attachment and detachment in the transmission process for various virus-vector combinations.

TABLE 2. Transmission of tobacco ringspot virus by *Xiphinema americanum* sensu *stricto*

No. of nematodes per test plant	Experiment	Transmission frequency[a]	Percent bait plants infected
1	1	7/23	30
	2	5/23	22
10	1	75/100	75
	2	43/78	55
10[b]	1–2[c]	0/40	0

[a] Number of virus-infected bait plants/total number of plants tested.
[b] Control nematodes that were fed on healthy cucumber.
[c] Combined data from two tests (0/20 for each experiment).

TABLE 3. Transmission of tobacco ringspot virus and fluorescent antibody labeling of sites of virus retention in *Xiphinema americanum* sensu *stricto* after varying virus acquisition access periods (AAP) on virus-infected cv. Model cucumber

Virus AAP (days)	Virus-specific labeling		Virus transmission	
	Frequency[a]	Percentage	Frequency[b]	Percentage
0	0/52	0	0/20	0
1	1/56	2	1/20	5
3	4/51	8	2/20	10
5	18/50	36	11/20	55
7	20/50	40	11/20	55
10	26/50	52	12/20	60
15	33/61	54	10/20	50
22	28/51	55	10/20	50

[a] Ratio of nematode fragments with virus-retention regions specifically labeled with fluorescence to total number of fragments examined.
[b] Number of virus-infected bait plants/total number of plants tested. Ten nematodes were added to each bait plant.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Arkansas Agricultural Experiment Station, Research Initiative Program. We thank S. Wickizer for technical assistance.

LITERATURE CITED

1. Atkinson, H. J., Harris, P. D., Halk, E. J., Novitski, C., Leighton-Sands, J., Nolan, P., and Fox., P. C. 1988. Monoclonal antibodies to the soya bean cyst nematode, *Heterodera glycines*. Ann. Appl. Biol. 112:459-469.
2. Breece, J. R., and Hart, W. H. 1959. A possible association of nematodes with the spread of peach yellow bud mosaic virus. Plant Dis. Rep. 43: 989-990.
3. Brown, D. J. F., and Boag, B. 1988. An examination of methods used to extract virus-vector nematodes (Nematoda: Longidoridae and Trichodoridae) from soil samples. Nematol. Mediterr. 16:93-99.
4. Brown, D. J. F., and Halbrendt, J. M. 1992. The virus vector potential of *Xiphinema americanum* and related species. (Abstr.) J. Nematol. 24:584.
5. Brown, D. J. F., Halbrendt, J. M., Jones, A. T., Vrain, T. C., and Robbins, R. T. 1994. Transmission of three North American nepoviruses by populations of four distinct species of the *Xiphinema americanum* group. Phytopathology 84:646-649.
6. Brown, D. J. F., Robertson, W. M., and Trudgill, D. L. 1995. Transmission of viruses by plant nematodes. Annu. Rev. Phytopathol. 33:223-249.
7. Fulton, J. P. 1962. Transmission of tobacco ringspot virus by *Xiphinema americanum*. (Abstr.) Phytopathology 52:375.
8. Fulton, J. P. 1967. Dual transmission of tobacco ringspot virus and tomato ringspot virus by *Xiphinema americanum*. Phytopathology 57:535-536.
9. Harlow, E., and Lane, D. 1988. Storing and purifying antibodies. Pages 283-318 in: Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Harrison, B. D., and Murant, A. F. 1978. Nematode transmissibility of pseudorecombinant isolates of tomato black ring virus. Ann. Appl. Biol. 86:209-212.
11. Harrison, B. D., Murant, A. F., Mayo, M. A., and Roberts, I. M. 1974. Distribution and determinants for symptom production, host range and nematode transmissibility between the two RNA components of raspberry ringspot virus. J. Gen. Virol. 22:233-247.
12. Mahmood, T., Hein, G. L., and French, R. C. 1997. Development of serological procedures for rapid and reliable detection of wheat streak mosaic virus in a single wheat curl mite. Plant Dis. 81:250-253.
13. McGuire, J. M. 1964. Efficiency of *Xiphinema americanum* as a vector of tobacco ringspot virus. Phytopathology 54:799-801.
14. McGuire, J. M. 1973. Retention of tobacco ringspot virus by *Xiphinema americanum*. Phytopathology 63:324-326.
15. McGuire, J. M., and Douthit, L. B. 1978. Host effect on acquisition and transmission of tobacco ringspot virus by *Xiphinema americanum*. Phytopathology 68:457-459.
16. McGuire, J. M., Kim, K. S., and Douthit, L. B. 1970. Tobacco ringspot virus in the nematode *Xiphinema americanum*. Virology 42:212-216.
17. McGuire, J. M., and Schneider, I. R. 1973. Transmission of satellite of tobacco ringspot virus by *Xiphinema americanum*. Phytopathology 63: 1429-1430.
18. Nyland, G., Lownsbery, B. F., Lowe, B. K., and Mitchell, J. F. 1969. The transmission of cherry rasp leaf virus by *Xiphinema americanum*. Phytopathology 59:1111-1112.
19. Ploeg, A. T., Brown, D. J. F., and Robinson, D. J. 1993. RNA-2 of tobacco rattle virus encodes the determinants of transmissibility by trichodorid vector nematodes. J. Gen. Virol. 74:1463-1466.
20. Raski, D. J., Maggenti, A. R., and Jones, N. O. 1973. Location of grapevine fanleaf and yellow mosaic virus particles in *Xiphinema index*. J. Nematol. 5:208-211.
21. Roberts, I. M., and Brown, D. J. F. 1980. Detection of six nepoviruses in their nematode vectors by immunosorbent electron microscopy. Ann. Appl. Biol. 96:187-192.
22. Robertson, W. M., and Henry, C. E. 1986. An association of carbohydrates with particles of arabis mosaic virus retained within *Xiphinema diversicaudatum*. Ann. Appl. Biol. 109:299-305.

23. Sanger, H. L., Allen, M. W., and Gold, A. H. 1962. Direct recovery of tobacco rattle virus from its nematode vector. (Abstr.) Phytopathology 52:750.
24. Taylor, C. E. 1980. Nematodes. Pages 375-416 in: Vectors of Plant Pathogens. K. F. Harris and K. Maramorosch, eds. Academic Press, New York.
25. Taylor, C. E., and Robertson, W. M. 1969. The location of raspberry ringspot and tomato black ring viruses in the nematode vector, *Longidorus elongatus* (De Man). Ann. Appl. Biol. 64:233-237.
26. Taylor, C. E., and Robertson, W. M. 1970. Sites of virus retention in the alimentary tract of the nematode vectors, *Xiphinema diversicaudatum* (Micol.) and *X. index* (Thorne & Allen). Ann. Appl. Biol. 66:375-380.
27. Taylor, C. E., and Robertson, W. M. 1970. Location of tobacco rattle virus in the nematode vector, *Trichodorus pachydermus* Seinhorst. J. Gen. Virol. 6:179-182.
28. Trudgill, D. L., Brown, D. J. F., and McNamara, D. G. 1983. Methods and criteria for assessing the transmission of plant viruses by longidorid nematodes. Rev. Nematol. 6:133-141.
29. Tsuda, S., Fujisawa, I., Ohnishi, J., Hosokawa, D., and Tomaru, K. 1996. Localization of tomato spotted wilt tospovirus in larvae and pupae of the insect vector *Thrips setosus*. Phytopathology 86:1199-1203.
30. Van der Wilk, F., Korsman, M., and Zoon, F. 1994. Detection of tobacco rattle virus in nematodes by reverse transcription and polymerase chain reaction. Eur. J. Plant Pathol. 100:109-122.
31. Wang, R. Y., Gergerich, R. C., and Kim, K. S. 1994. Entry of ingested plant viruses into the hemocoel of the beetle vector *Diabrotica undecimpunctata howardi*. Phytopathology 84:147-153.
32. Willats, W. G. T., Atkinson, H. J., and Perry, R. N. 1995. The immunofluorescent localization of subventral pharyngeal gland epitopes of preparasitic juveniles of *Heterodera glycines* using laser scanning confocal microscopy. J. Nematol. 27:135-142.