Etiology of Soybean Severe Stunt and Some Properties of the Causal Virus

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ABSTRACT

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Soybean severe stunt, caused by soybean severe stunt virus (SSSV), is a new virus disease affecting Delaware soybeans (*Glycine max*). Symptoms occur on the first true leaves, and infected plants have shortened internodes resulting in severe stunting, thickened dark green leaves, superficial stem lesions, and reduced number of flowers and pods. Soybean severe stunt occurred in 30 of 50 soybean fields surveyed in Delaware, all in Sussex County, and has not been reported elsewhere. Butanol clarification and differential centrifugation were used to purify SSSV from systemically infected leaves of soybean cv. Essex plants inoculated in the greenhouse, and Essex plants inoculated in the greenhouse with sap or purified preparations of the virus showed typical field symptoms. The isometric, multicomponent virus is approximately 29 nm in diameter and has a longevity in vitro of 12–24 hr, dilution end point of 10^{-3}, and thermal inactivation of 55 C. The disease is transmitted via soil, and the dagger nematode (*Xiphinema americanum*) is strongly associated with the occurrence of the disease in the field. Antiserum to 20 isometric plant viruses has failed to react with SSSV.

Soybean (*Glycine max* (L.) Merr.) is an important commercial crop in Delaware, with 104,000 ha harvested in 1990. Soybean severe stunt (SSS) is a new virus disease affecting Delaware soybeans. Severely stunted plants with thickened dark green leaves and producing little or no yield have been observed for many years in soybean plantings in Sussex County, Delaware. Evidence indicates that SSS has been present in Delaware since 1975 and perhaps as early as 1966, when typical SSS symptoms on soybeans were attributed, on the basis of symptomatology alone, to infection by the "bud blight" strain of tobacco ringspot virus (TbRSV) (2,8). In 1983, the disease was first observed in commercial soybean fields in the Millsboro area of Sussex County.

In the field, the disease occurs generally in localized, circular areas ranging from 10 m to several hundred meters in diameter and has been observed in many of the same fields year after year, particularly those that have been continuously cropped with the soybean cultivar Essex. Initial symptoms in the field occur at the V1 stage of development (4), and affected plants typically show thickened dark green leaves and shortened internodes resulting in bunching of leaves and severe stunting (Fig. 1), accompanied by pith discoloration, superficial stem lesions, fewer flowers, and fewer mature pods. A reduction in soybean seedling emergence and stand may also be attributable to infection with SSS, and yields within affected areas of a field may be reduced by as much as 90%. This report presents evidence of the probable viral nature of the disease and some properties of the causal agent. Preliminary accounts of this work have been published (3,10,11).

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MATERIALS AND METHODS

Virus source and maintenance. A virus was isolated from leaves of severely stunted Essex soybean plants collected from affected fields near Millsboro, Delaware, during the summer of 1986. After three successive single-lesion transfers to cowpea (*Vigna unguiculata* (L.) Walp. 'Queen Anne'), the isolate was maintained in Essex soybean. Greenhouse-grown Essex soybean plants sap-inoculated with the isolate at the unifoliate stage produced typical field symptoms of SSS. The virus has been tentatively named soybean severe stunt virus (SSSV).

Host range. Carborundum-dusted leaves of test plants were mechanically inoculated with sterile sponges dipped in extracts prepared by triturating systemically infected soybean leaf tissue in 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol (1:5, w/v, tissue/buffer). At least five plants of each of 15 species or cultivars (Table 1) were inoculated. All plants were

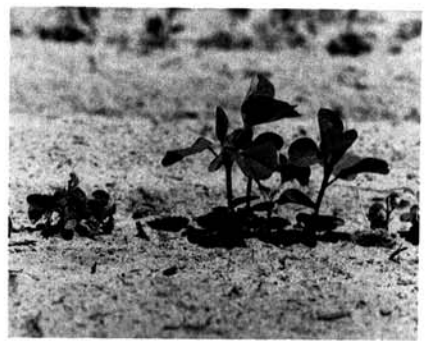

Fig. 1. (Left) Seedling of soybean cultivar Essex with soybean severe stunt compared with (right) healthy seedling.

Table 1. Response of herbaceous host plants to inoculation with soybean severe stunt virus

Host plants	Symptoms	
	Local	Systemic
Vigna unguiculata (L.) Walp. 'Queen Anne'	Necrotic lesions	Chlorotic lesions, mottle
Phaseolus vulgaris L. 'Bountiful'	Pinpoint lesions	No infection
Cucumis sativus L. 'National Pickling'	Chlorotic ring spots	No infection
Cucurbita maxima Duchesne	Necrotic ring spots	No infection
Chenopodium quinoa Willd.	Chlorotic lesions	Necrotic ring spots
C. murale L.	No infection	Symptomless infection
Gomphrena globosa L.	Necrotic lesions	Symptomless infection
Datura stramonium L.	No infection	No infection
Nicotiana benthamiana Domin.	No infection	No infection
N. glutinosa L.	No infection	No infection
N. tabacum L. 'Havana'	No infection	No infection
'Hibshaman'	No infection	No infection
'Pennbell'	No infection	No infection
Lycopersicon esculentum Mill. 'Heinz 1350'	No infection	No infection
Petunia × *hybrida* Hort. Vilm.-Andr.	No infection	No infection

maintained in a greenhouse with 16 hr of light at 24–28 C and evaluated for symptom expression for 6 wk. Plant species or cultivars that did not show obvious symptoms were back-inoculated to Queen Anne cowpea, cucumber (*Cucumis sativus* L. 'National Pickling'), or Essex soybean to determine whether they had symptomless SSSV infection.

Physical properties. The methods of Walkey (9) were used to determine the physical properties of SSSV, including thermal inactivation point (TIP), longevity in vitro (LIV), and dilution end point (DEP), in sap from systemically infected soybean. Temperatures between 35 and 70 C were tested at 5 C intervals for determination of TIP; sap, in 1-ml aliquots, was maintained in the dark at 21–24 C for LIV determination; and sodium phosphate buffer (0.02 M, pH 7.0) was used for determining DEP. Infectivity was determined by inoculation of sap aliquots onto the local lesion hosts cowpea and cucumber.

Transmission. To evaluate seed transmission of SSSV, 100 seeds were collected from Essex soybean plants naturally infected in the field and 100 from plants mechanically inoculated with SSSV and maintained in the greenhouse. These and an equal number of healthy, certified seeds of Essex were sown in a sterile greenhouse potting mix (2:3:4, vermiculite/peat moss/perlite, v/v) in 12.7-cm-diameter plastic pots at five seeds per pot. The greenhouse was maintained at 24–28 C with a 16-hr photoperiod, and the percentages of emergence and infection were determined. The test was repeated three times. Transmission via seed was determined by the occurrence of SSS symptoms after 3–4 wk, at which time leaf tissue from each plant was rub-inoculated onto leaves of cowpea, cucumber, and Essex soybean to verify or refute the presence of SSSV.

Transmission of SSSV via soil was evaluated in the greenhouse. Samples were collected from each of four fields by taking a minimum of 100 cores (3 cm diameter) to a depth of 10 cm adjacent to plants showing typical SSS symptoms. Samples for each field were pooled, and one-half of each pooled sample was autoclaved for 1 hr at 121 C. This was done twice, 2 days apart, and soil samples were used 1 wk later. Six seeds of certified Essex soybean were planted into each of 10 plastic pots (12.7 cm diameter) of autoclaved and nonautoclaved field soil. The pots were maintained in the greenhouse for 5 wk, and plants were observed for the development of typical field symptoms. The percentages of seedling emergence and infected plants were recorded, and leaf and root samples of symptomatic plants were back-inoculated to the indicator cowpea and cucumber plants to verify the presence or absence of SSS.

Nematode survey. Soil samples were collected from around SSS-infected and healthy soybean plants from four fields by means of the procedure described above. Nematodes were extracted from soil samples by a modified Baermann funnel method (12) and examined with a dissecting microscope to detect known virus vectors. Additionally, soil samples were taken from around roots of plants showing typical SSS symptoms chosen at random (10 plants per field) from each of 26 fields affected by the disease. Nematodes were extracted as before, and the number of *Xiphinema* spp. per 250 ml of soil was determined by means of a dissecting microscope.

Disease survey. Plants were sampled from symptomatic and asymptomatic soybean fields during the 1987 growing season. Each of 29 fields selected at random in Sussex County were surveyed once when plants were at the V3–V4 growth stage (4). Each field was evaluated by making general observations for SSS and by determining the percentage of plants infected with SSSV by observing and sampling 10 arbitrary plots consisting of 50 consecutive plants within a row. Ten to 15 symptomatic plants per field were collected and stored at 4 C until tested for SSSV by rub-inoculation to indicator soybean, cowpea, and cucumber plants. During 1988 and 1989, an additional 21 fields were surveyed for SSS. Leaf samples were taken from a minimum of 20 randomly selected symptomatic plants and rub-inoculated onto cowpea, cucumber, and soybean plants to test for SSSV.

Purification techniques. Purified suspensions of SSSV were prepared from systemically infected soybeans according to a method adapted from Hollings (6). Fresh leaf tissue (100 g) was triturated in a Waring blender for 3–5 min in 120 ml of 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol. The homogenate was squeezed through cheesecloth and clarified with the addition of 8.5% *n*-butanol (v/v) with rapid stirring for 15 min. The precipitate was pelleted by low-speed centrifugation of 10,000 *g* for 15 min. The virus was purified and concentrated from the resulting supernatant by two cycles of differential centrifugation. Ultracentrifugation was done in a Sorval OTD-75B ultracentrifuge with an AH 625 rotor at 25,000 rpm for 2 hr at 4 C. The final pellet was resuspended overnight in 200 µl of the extraction buffer. Leaf tissue (100 g) from healthy, greenhouse-grown soybeans was purified by the same method and served as the control.

Density gradient centrifugation. Virions were further purified by rate zonal centrifugation in 0–30% (w/v) linear-log sucrose density gradients (SDG) in 0.02 M sodium phosphate buffer (pH 7.0) in a SW-41 rotor for 2 hr at 38,000 rpm in a Beckman L8-70M ultracentrifuge. For comparison of relative sedimentation profiles, SSSV and tomato ringspot virus (TmRSV) (provided by E. V. Podleckis) were purified by the above procedure, and sister gradients were loaded with fresh virion preparations and centrifuged simultaneously (1,7). Gradients were fractionated and scanned at 254 nm with an ISCO density gradient and UV analyzer. Fractions of SSSV gradients were diluted 1:3 (v/v) with 0.02 M sodium phosphate buffer (pH 7.0), pelleted by ultracentrifugation at 38,000 rpm for 3 hr and rub-inoculated onto unifoliate leaves of Essex soybean to determine infectivity. Virus concentrations were calculated on the basis of an extinction coefficient of 10.0 as given for members of the nepovirus group (5).

Ultraviolet spectrophotometry. Healthy and diseased soybean leaves (100 g) were purified and fractionated after SDG centrifugation, and equivalent fractions from the middle and bottom virus zone and from healthy preparations were diluted and pelleted as described above. Pellets were resuspended in 1 ml of extraction buffer without 2-mercaptoethanol and scanned on a spectrophotometer, and $A_{260/280}$ was calculated.

Electron microscopy. The morphology of SSS virions was determined by placing partially purified preparations on Formvar-backed, carbon-coated grids, staining them with 2% aqueous uranyl acetate or 2% ammonium molybdate, and examining them with a Philips 201 electron microscope at 80 kV. To determine virion size, germanium shadowed carbon replicas made from defraction gratings with 54,864 lines per inch were photographed at the same relative magnification as the virus particles. Then, 100 particles of the virus were measured from micrographs magnified to 100,000×.

Serology. Attempts to produce SSSV-specific antiserum failed, and all serological tests were done without homologous antisera. Sap from plants systemically infected with SSSV was diluted 1:5 (w/v) with 0.02 M sodium phosphate buffer (pH 7.0) and used in Ouchterlony gel double-diffusion tests. Antisera to TbRSV and TmRSV were obtained from the American Type Culture Collection, Rockville, Maryland, numbers PVAS 157 and PVAS 174, respectively; antisera to asparagus virus II (AV-II) and tobacco streak virus (TSV) were obtained from G. I. Mink of Washington State University, Prosser. These antisera were tested against SSSV at dilutions of 1:2, 1:4, and 1:16. The gels consisted of 1% agarose in a 0.85% NaCl solution containing 0.05% NaN^3. On the basis of particle size and the multicomponent nature of the virus, antisera to the following viruses were tested in indirect ELISA against SSSV: TbRSV, TmRSV, AV-II, TSV, arabis mosaic, grapevine fanleaf, peach rosette mosaic, cherry raspleaf, strawberry latent ringspot, raspberry ringspot, cherry

leafroll, Prunus necrotic ringspot, bean western yellows, cowpea chlorotic mottle, peanut stunt, cucumber mosaic, spinach latent, turnip yellow mosaic, Andean potato latent, and hop mosaic. All ELISA testing was courtesy of G. I. Mink.

RESULTS

Host range. Results of a limited study indicated that SSSV had a moderately wide host range, with most species or cultivars being only locally infected (Table 1). SSSV infected the members of the Leguminosae, Cucurbitaceae, Amaranthaceae, and Chenopodiaceae, but not the Solanaceae and Gramineae, that were tested. Symptomless systemic infection of *Chenopodium murale* L. and *Gomphrena globosa* L. was revealed by back-inoculation to cowpea. No suitable propagative host was found among the species and cultivars tested. The original host, Essex soybean, remains the best propagative host.

Physical properties. The results of TIP, LIV, and DEP determinations showed that SSSV was infective after 10 min at 50 C, but not 55 C, after standing at room temperature for 12 hr, but not 24 hr, and after dilution of sap to 10^{-3}, but not to 10^{-4}.

Transmission. Seedlings grown from seeds harvested from systemically infected field or greenhouse-grown soybean plants developed no symptoms, and back-inoculation studies did not detect SSSV in these plants. The percentage of seedling emergence was slightly lower in seeds collected from naturally infected field plants than in certified seed.

Transmission of SSSV via soil and a concomitant reduction in emergence were confirmed. Certified seed planted in three of four nonautoclaved soils resulted in transmission of SSSV, with 6, 13, and 19% of the seedlings becoming infected. Seedling emergence was reduced 10–30% for the same soils. SSSV was not transmitted in autoclaved soils.

Nematode survey. During the spring of 1988, the dagger nematode, *Xiphinema americanum* Cobb (speciation courtesy of A. M. Golden; voucher specimen in USDA Nematode Collection, Beltsville, Maryland), was present at 30–60 per 250 ml of soil sampled from four fields affected by SSS in Sussex County. The dagger nematode was detected in 23 fields affected with SSS and was not detected in soil samples taken around healthy soybean plants.

Disease survey. In 1987 and 1988, SSS was detected in 19 of 29 fields surveyed, with a disease incidence of 0.4–5.9% in 11 of those fields and greater than 21% in only one field. During 1989 and 1990, SSS was detected in 11 of 21 fields surveyed. Thus, SSS was detected in 30 of the 50 soybean fields surveyed. All fields were in Sussex County, and most were restricted to a 100-km^2 area of its southeastern portion.

Purification. Purification of SSSV from infected Essex soybean yielded approximately 50–80 mg of virus per kilogram of fresh leaf tissue. Two and sometimes three components were observed after rate zonal centrifugation (Fig. 2). The top component was noninfectious, but the middle and bottom components were infectious, as evidenced by the development of typical SSS symptoms on inoculated soybeans. Purified preparations of the middle and bottom components after SDG centrifugation showed a typical ultraviolet absorption spectrum for nucleoproteins, with a maximum at 260 nm and an $A_{260/280nm}$ of 1.50. Sedimentation coefficients of the top, middle, and bottom components were estimated at 53, 115, and 134 S, respectively. Preparations from diseased tissue yielded a typical ultraviolet spectrum, and no ultraviolet absorbing zone was obtained with preparations from healthy tissue (Fig. 3).

Electron microscopy. In negatively stained preparations, SSSV appeared isometric (Fig. 4) and ranged in diameter from 28 to 30 nm, with a modal diameter of 29 nm. Particles were often deformed and without sharp detail in electron micrographs. No particles were observed in preparations from healthy soybean leaf tissue.

Serology. Attempts to produce antiserum against SSSV failed, and one-way tests were used for serological determinations. SSSV failed to react with 20 different isometric viruses in Ouchterlony gel double-diffusion tests and/or indirect ELISA.

DISCUSSION

The isometric particles purified from leaves of naturally infected soybean plants have been shown to be the cause

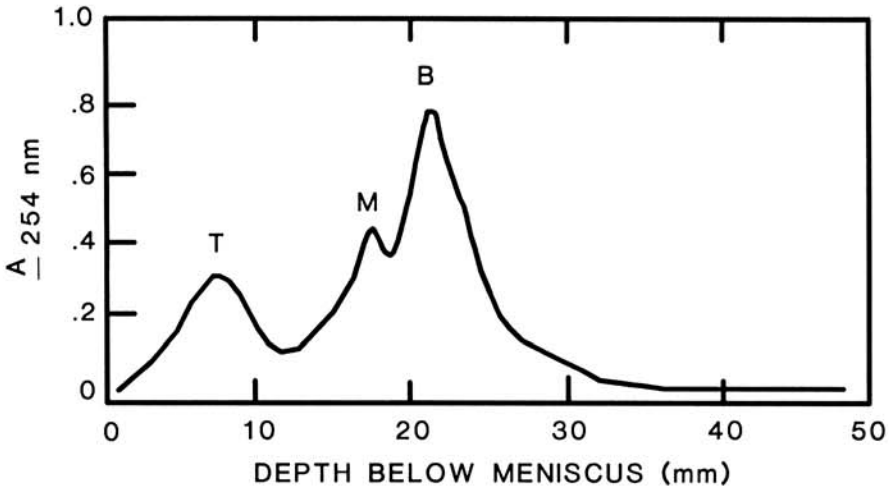

Fig. 2. Ultraviolet absorption profile of soybean severe stunt virus in a linear-log 0–30% sucrose density gradient. Direction of sedimentation is from left to right. T = top, M = middle, and B = bottom components.

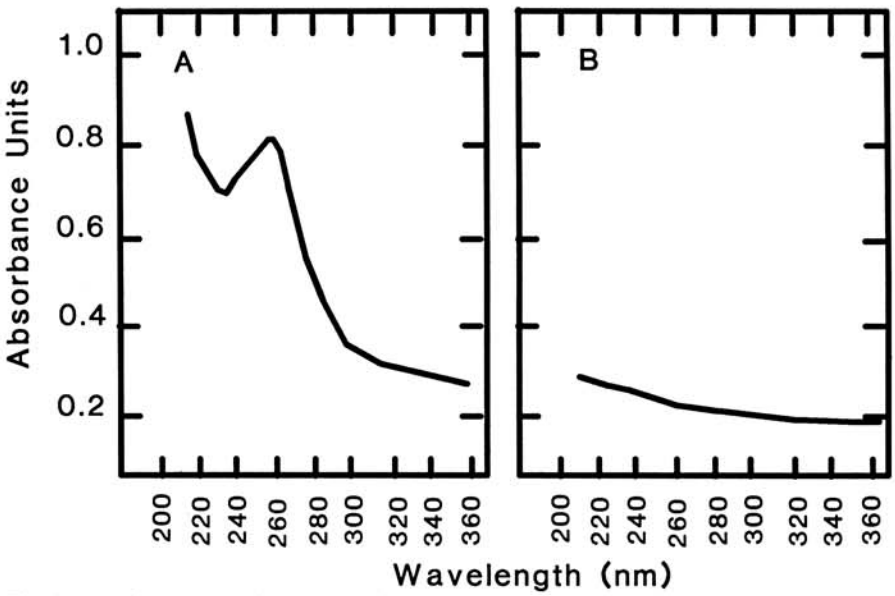

Fig. 3. Ultraviolet absorption spectra of **(A)** soybean severe stunt virus purified from leaves of diseased soybean (cv. Essex) and **(B)** purified sap from leaves of healthy soybean (cv. Essex). The spectra of both preparations were measured after separation from a sucrose density gradient.

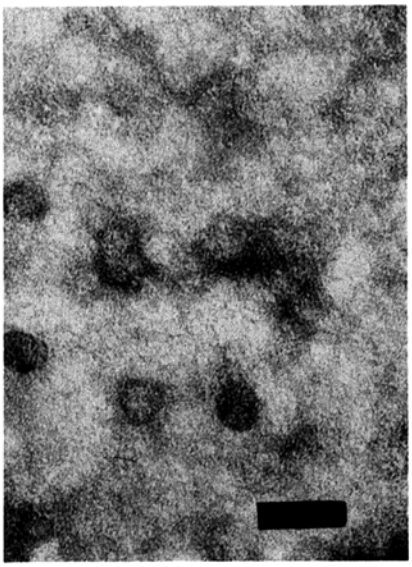

Fig. 4. Soybean severe stunt virions stained with 2% uranyl acetate after purification from leaves of diseased soybean (cv. Essex). Scale bar = 50 nm.

of soybean severe stunt. The isolation of the particles from diseased, but not from healthy, plants and the occurrence of the typical symptoms of the disease on soybean seedlings mechanically inoculated with SDG purified virus are evidence of pathogenicity.

Our study indicates that soybean severe stunt is probably soilborne. Greenhouse studies demonstrating the infection of susceptible soybean plants grown in field soil taken from around the roots of soybean plants showing typical SSS symptoms, but not in plants grown in the same soil after autoclaving, present evidence of soil transmission. Further, SSS typically occurs in circular patches within a given field year after year, and the dagger nematode is almost always present in soil surrounding soybean plants showing typical SSS symptoms but not in soil near healthy soybean plants from the same field. Additional research is required to determine the role of *X. americanum* as a vector of SSSV.

The presence of two or three major ultraviolet absorbing zones in SDG indicates that SSSV is a multicomponet virus. The multicomponent nature of SSSV, its 28–30 nm particle size and isometric shape, and the association of dagger nematodes with the disease in the field indicate that the virus is a probable member of the nepovirus group. However, serological testing of all nepoviruses known either to infect soybean in the United States or to be transmitted by *X. americanum* has yet to demonstrate any relatedness. We believe the attempts to produce SSSV-specific antisera with unfixed antigen in rabbits have failed largely because of the relatively unstable nature of SSSV.

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LITERATURE CITED

1. Brakke, M. K. 1960. Density gradient centrifugation and its application to plant viruses. Adv. Virus Res. 7:193-224.
2. Crittenden, H. W., Hastings, K. M., and Moore, D. M. 1966. Soybean losses caused by tobacco ringspot virus. Plant Dis. Rep. 50:910-913.
3. Evans, T. A., Weldekidan, T., Carroll, R. B., and Mulrooney, R. P. 1991. Etiology of soybean severe stunt disease and some properties of the causal virus. (Abstr.) Phytopathology 80:1021.
4. Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci. 11:929-931.
5. Harrison, B. D., and Murant, A. F. 1977. Nepovirus group. No. 185 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England. 4 pp.
6. Hollings, M. 1965. Anemone necrosis, a disease caused by a strain of tobacco ringspot. Ann. Appl. Biol. 55:447-457.
7. Stace-Smith, R. 1984. Tomato ringspot virus. No. 290 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England. 6 pp.
8. Stace-Smith, R. 1985. Tobacco ringspot virus. No. 309 in: Description of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England. 5 pp.
9. Walkey, D. G. A. 1985. Applied Plant Virology. John Wiley & Sons, New York. 329 pp.
10. Weldekidan, T. 1989. A new virus disease of soybeans in Delaware. M.S. thesis. University of Delaware, Newark. 100 pp.
11. Weldekidan, T., Evans, T. A., Carroll, R. B., and Mulrooney, R. P. 1988. Observations of an unknown virus-like disease affecting Delaware soybeans. (Abstr.) Phytopathology 78:864.
12. Zuckerman, B. M., Mai, W. F., and Harrision, M. B. 1985. Plant Nematology Laboratory Manual. Univ. Mass. Agric. Exp. Stn., Amherst. 212 pp.