Journal of Nematology 16(1):57–61. 1984.

Phytotoxin Production in *Bursaphelenchus xylophilus*-Infected *Pinus sylvestris*[1]

F. SHAHEEN, R. E. K. WINTER, AND R. I. BOLLA[2]

Abstract: Our findings suggest that i) phytotoxic materials can be isolated from *Bursaphelenchus xylophilus*-infected Scots pine, but not from noninfected pines; ii) the phytotoxins cause wilting of 45-day-old and 2-year-old pine seedlings in a dose and species dependent manner; iii) the phytotoxins are produced early in the infection, accumulate or increase with time, and may function to suppress water transport in the tree; and iv) the phytotoxins are lipid materials of low molecular weight which are not acidic.

Key words: pinewilt, Scots pine.

Pinewilt caused by *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970 is epidemic in Japan (4,8) and may soon eliminate *Pinus densiflora* and *P. thunbergii* (8). Since the report of this infection in *P. sylvestris* in Columbia, Missouri, in 1979 (2), the disease has been reported in the United States from 33 states in 23 pine and 7 nonpine species (3,6,13,14). Several states, including Missouri and Illinois, have reported serious, widespread infestation of *B. xylophilus* pinewilt, particularly in *P. sylvestris* and *P. nigra* (1,7).

The nematode, which resides in the lateral and radial resin canals of the infected pine, is transported from stressed or dead trees to healthy trees by an insect transport host. The main insect vectors in both the United States and Japan appear to be cerambycid beetles (5,6,9). Infective third-stage larvae overwinter as dauer larvae in association with the insect pupae in pupal chambers in the infected tree. Upon eclosion of the pupae, the larvae enter the tracheae and spiracles of the insect. As emerging young adult beetles undergo maturation feeding on growing shoots of an uninfected tree, the nematodes leave the insect and enter the tree through the site of wounding, migrate to the resin canals, and develop to adults. The adult nematodes feed on the epithelial cells and reproduce in the resin canals (8,10).

In *P. sylvestris* this infection is characterized by rapid total wilting (8); in *P. nigra* rapid wilting occurs in areas of the tree followed by progressive total wilting within 9 months (7). The initial symptoms of the

Received for publication 19 April 1983.

[1] This research was supported in part by a grant from the Weldon Springs Endowment of the University of Missouri and by grant #CRGO-82-CRCR-1-1138 from U.S. Department of Agriculture. The authors thank Drs. V. Dropkin, M. Linit, and A. Foudin, University of Missouri–Columbia, for supplying infected and noninfected Scots pine and for the seedlings used in this study.

[2] Departments of Biology and Chemistry, University of Missouri–St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121. Address correspondence to third author.

disease are loss in resin production within 3–5 days of infection followed by a loss of transpiration and water transport (11,15). The onset of these initial symptoms occurs far in advance of the time that the nematode population is large enough to cause extensive conspicuous damage to the resin canals (8–10). This relationship might suggest involvement of a phytotoxin in the mechanism of wilting. Oku et al. (12) were able to isolate a water soluble extract from infected *P. thunbergii,* which, when applied to healthy young pine seedlings, caused rapid wilt symptoms similar to those observed in a natural infection. A similar material, as determined by bioassay, could not be recovered from noninfected pines.

This paper presents our studies on the isolation of a phytotoxin from pinewilt nematode-infected *P. sylvestris.*

Materials and Methods

Extraction of pine wood: P. sylvestris infected with *B. xylophilus* and uninfected *P. sylvestris* were obtained from Columbia and Ashland, Missouri. All trees were 10–20 years old at the time of cutting. The wood was ground to a coarse sawdust and extracted in one of two ways. Initially the charcoal adsorbent method of Oku et al. (12) was used with slight modification. The wood was boiled for 16 hours in distilled water (4,800 g/10 liters), the water extract was concentrated to 0.5–1 liter by rotary evaporation and then dialyzed 48 hours against four changes of distilled water (5 liters/dialysis). The water fractions outside the dialysis bag were combined, reduced to 0.5–1 liter, mixed 50% (w/v) with activated charcoal and the slurry suction filtered through Whatman No. 2 filter paper. The filtrate was discarded and the charcoal was washed with 5 liters acetone by repeated resuspension and suction filtration. The acetone fraction was rotary evaporated to dryness and the total weight of the extract determined. This is referred to as the charcoal extract.

In a second and more expedient method of extraction, the sawdust was boiled for 3 hours at 121 C and 15 psi in an autoclave in distilled water (4,800 g/2.5 liters). The water was recovered and the extraction repeated. The water extracts were combined, adjusted to pH 12.0 with 1 N NaOH, and held at this pH for 1 hour by further addition of NaOH. The basified extract was partitioned five times, each with ⅛ volume of $CHCl_3$; the $CHCl_3$ fractions were combined and dehydrated over anhydrous Na_2SO_4 and the $CHCl_3$ removed by rotary evaporation. Total extract dry weight was determined. This is referred to as the $CHCl_3$-base extract.

Bioassay: Forty-five-day-old *P. sylvestris, P. banksiana, P. echinata, P. radiata, P. thunbergii,* and *P. jeffreyi* seedlings and 2-year-old *P. sylvestris, P. strobus,* and *P. jeffreyi* seedlings were used for bioassay of the extracts. The roots of the 45-day seedlings were cut to 5 cm from the stem and the seedlings suspended in test tubes with the cut roots immersed in the solution to be tested. Experimental extracts were applied to 2-year seedlings by scraping the bark from one side of the stem half way up the stem. A plug of absorbent cotton was affixed to the scraped area and taped in place. The experimental solution was applied to the cotton and allowed to diffuse into the seedling.

Experimental and control extracts were solubilized in 20% acetone in water for testing. Controls consisted of similar extracts from noninfected trees and 20% aqueous acetone.

Infection of seedlings: Forty-five-day-old seedlings were infected with 5,000–10,000 *B. xylophilus* by suspending the seedlings in water containing a mixed population of nematodes recovered from fungal mat cultures (8). Older seedlings were infected using the scraped stem method with 5,000–20,000 nematodes from a mixed population applied to the cotton. Infection was verified after 3–20 days by suspending the seedlings in a modified Baermann apparatus and examining the eluate for *B. xylophilus.*

Rate of toxin production: Two-year-old *P. sylvestris* seedlings were infected with 5,000 *B. xylophilus* recovered as mixed populations from fungal mat cultures (2). The seedlings were observed daily for wilting. Beginning 3 days post infection, when no visual wilt symptoms were present, three seedlings were examined for the presence of *B. xylophilus* and then extracted by the $CHCl_3$-base extraction procedure to quantify the total $CHCl_3$-base extractable ma-

terial. Additional infected and noninfected seedlings were similarly examined at 5-day intervals from 5 to 45 days post infection. The extracted material was quantified gravimetrically.

Effect of infection on water transpiration: To obtain preliminary information on the effect of *B. xylophilus* infection of *P. sylvestris* on water transpiration, we observed the state of the leaf stomates at various times post infection. Two-year-old seedlings were infected with 5,000 nematodes (mixed population). On days 4, 10, 15, 20, and 30 after inoculation, the leaves of the seedlings were randomly selected, coated with clear nail polish, and removed from infected and control seedlings. The epithelial layer was then stripped from the leaf and mounted on a microscope slide for study. Closed versus open stomates were scored. All preparations were made at 9:00 a.m. to avoid diurnal variations. Control leaves from both infected and noninfected seedlings were treated with sucrose to verify stomate reactions.

Chemicals and supplies: All solvents used were HPLC grade from Fisher Scientific Co. Older seedlings were obtained from the Missouri State Forestry Nursery; 45-day seedlings were grown from seed.

Results

Effect of pinewood extracts on pine species: The charcoal extract from infected *P. sylvestris* pine caused wilting on 2-year-old *P. sylvestris* pine seedlings within 7–21 days. The rate of wilting was dose dependent (Table 1), and the onset of symptoms was progressive. The initial wilt symptoms appeared as a browning of the leaves at the apex of the seedlings. This browning progressed downward until all leaves had a characteristic red brown color. Resin flow at a wound site ceased within 3–7 days after toxin application. The obvious wilt symptoms in the toxin-treated seedlings were identical to those in the nematode-infected seedlings. Control seedlings treated with an identically prepared extract from noninfected Scots pine showed no wilt symptoms. Except that the response to the phytotoxin was slower, *P. strobus* responded similarly to *P. sylvestris* (Table 1). Interestingly, *P. jeffreyi* seedlings were not affected by the charcoal extract from nematode infected or noninfected *P. sylvestris* at any dose applied (Table 1).

Table 1. Days required for total wilting* of 2-year-old pine seedlings treated with various amounts of an extract prepared by the charcoal absorbent method from *Bursaphelenchus xylophilus* infected *P. sylvestris.*

	Extract applied (mg)†		
Pinus spp.	250	500	1,000
P. sylvestris	10–15	9–12	3–5
P. strobus	28–35	30–35	12
P. jeffreyi	no wilt	no wilt	no wilt

* Data presented at the range of days required for total wilting of five of five seedlings of each species at each experimental dose. Seedlings treated with comparably prepared extracts from noninfected Scots pine did not show any signs of wilt.

† One gram of wood yields approximately 13.75 mg of a crude phytotoxic extract prepared by the charcoal absorbent procedure.

Similar results were obtained when $CHCl_3$-base extracts from infected or noninfected *P. sylvestris* were bioassayed on either 2-year or 45-day seedlings. When several species of 45-day-old pine seedlings were used in bioassay of the $CHCl_3$-base extracts, a tendency for a species dependent rate of wilting was observed (Table 2). As with the 2-year seedlings, wilting rate was dose dependent over the range of doses tested (Table 2). The 45-day-old *P. jeffreyi* seedlings were not wilted. *P. sylvestris* 45-day seedlings were most sensitive to the extract. Seedlings of the various pine species were not wilted either by extracts from noninfected *P. sylvestris* or by the 20% acetone in water solvent (Table 2).

Rate of toxin production: As seen in Table 3, production of phytotoxin was observed as early as 3 days post infection and increased in concentration on a per gram wood basis until 30 days post infection after which no change occurred. Visual wilt symptoms were present in the infected *P. sylvestris* seedlings at 10 days post infection and progressed until 30 days post infection when the seedlings were completely wilted. The toxicity of the extracts from the various seedlings was bioassayed on 45-day *P. sylvestris* seedlings. All extracts from infected seedlings (3–45 days post infection) caused total wilting of the seedlings within

TABLE 2. Days required for total wilting* of 45-day-old pine seedlings treated with various amounts of an extract prepared by the $CHCl_3$-base procedure from *Bursaphelenchus xylophilus* infected *P. sylvestris.*

Pinus spp.	Extract applied (mg)†			
	1.0	2.5	5.0	10.0
P. sylvestris	6.3 ± 2.9	2.9 ± 1.3	1.6 ± 0.7	1.4 ± 0.5
P. radiata	10.3 ± 5.7	8.6 ± 4.3	5.8 ± 1.8	7.8 ± 0.5
P. banksiana	19.0 ± 5.2	6.0 ± 2.4	4.0 ± 1.2	4.4 ± 0.9
P. echinata	12.8 ± 3.1	12.8 ± 6.3	4.2 ± 0.4	5.4 ± 1.9
P. thunbergii	9.5 ± 2.1	5.4 ± 1.9	3.4 ± 0.5	3.8 ± 1.3
P. jeffreyi	no wilt	no wilt	no wilt	no wilt

* Data presented as mean number of days required for total wilting ± standard error of the mean, N = 10 seedlings. Seedlings treated with comparably prepared extracts from noninfected *P. sylvestris* did not show any signs of wilt.

† One gram of wood yields approximately 1.1 mg of a partially purified phytotoxin when extract is prepared by the $CHCl_3$-base procedure.

5–7 days in a dose-dependent manner. No wilting was caused by extracts from the noninfected seedlings.

Effect of infection on water transpiration: Stomate closure was used as a means of estimating water transpiration in *B. xylophilus*-infected *P. sylvestris* seedlings. Throughout the study only 0–5% of the stomates were observed to be closed in leaves from control seedlings whereas, by 3 days post infection, 50% of the stomates on the leaves of infected seedlings were closed. By 7 days post infection, 100% of the stomates were closed; this complete closure of the stomates was observed through 30 days post infection. The seedlings were completely wilted 30–45 days after infection.

DISCUSSION

Oku et al. (12) observed production of phytotoxic materials in *B. xylophilus*-infected *P. thunbergii.* Results of the present study indicate that phytotoxins are similarly produced in *B. xylophilus*-infected *P. sylvestris* and thus support Oku's initial observation. The timing of the production of these materials following infection of 2-year-old *P. sylvestris* seedlings corresponds to the onset of initial wilt symptoms and to the reaction of the leaf stomates to the infection. That is, resin production at the site of a wound ceased 3 days post infection, 50% of the leaf stomates were closed at this time, and phytotoxic material could be extracted. An increase in the concentration of extractable phytotoxin accompanied further changes in stomate closing and wilting of the seedlings. This suggests an involvement of the phytotoxin in restricting water flow in the infected seedlings.

TABLE 3. Rate of phytotoxin synthesis in *Bursaphelenchus xylophilus* infected 2-year-old *P. sylvestris.*

Days postinfection	Mg phytotoxin/g wood[a]
3	0.611 ± 0.06
7	0.652 ± 0.10
10	0.837 ± 0.13
15	1.35 ± 0.18
30	2.77 ± 0.29
45	2.56 ± 0.21
0	0.54 ± 0.08

[a] Milligrams phytotoxin was calculated based on the dry weight of the base resistant material extractable by $CHCl_3$ from 2-yr-old *P. sylvestris* seedlings infected with 5,000–20,000 nematodes (mixed population).

Preliminary cytological observation of the infected seedlings suggests that 3–5 days post infection the resin canals are intact (unpublished data). The onset of phytotoxin production by 3 days post infection suggests that the phytotoxin is produced in advance of the time that the nematode population is large enough to cause extensive damage to the resin canals. This would agree with earlier observations of Mamiya and co-workers (8–11) that in older trees wilt symptoms begin and progress in advance of large destructive nematode populations and might suggest that the production of phytotoxic materials is one of the main factors in the early disease symptoms.

The species-dependent sensitivity to the phytotoxin corresponds to the species-de-

pendent susceptibility to *B. xylophilus* (6). That is, 2-year-old *P. sylvestris,* which is highly susceptible to infection, is highly sensitive to the toxin, whereas *P. strobus,* which is less susceptible, is less sensitive and the infection-resistent *P. jeffreyi* is not affected by the phytotoxin. This correspondence is also reflected in 45-day-old seedlings of other species. The observation that phytotoxic extracts could not be obtained from noninfected *P. sylvestris* indicates that these materials are specific to stressed or wilted pines and may be unique to *B. xylophilus*-infected pines.

Several conclusions about the chemical nature of the toxin can be drawn, based on the methods of its extraction. The phytotoxic materials are small, dialyzable, heat-resistant lipid materials as defined by several criteria (unpublished observations, 15). These materials are resistant to base treatment and therefore are either neutral or basic and contain no base-sensitive functional groups. Finally, the methods of extraction preclude the possibility that these materials are phenols or carboxylic acids.

Literature Cited

1. Dropkin, V. H. 1983. Pine wilt disease: Overview. Proc. 1982 Natl. Pine Wilt Dis. Workshop, pp. 7–10.

2. Dropkin, V. H., and A. S. Foudin. 1979. Report of the occurrence of *Bursaphelenchus lignicolus* induced pine wilt disease in Missouri. Plant Dis. Rept. 63:904–905.

3. Dropkin, V. H., B. Kondo, M. J. Linit, M. T. Smith, K. Robbins, and A. S. Foundin. 1981. Pinewood nematode. Is it a threat to U.S. forests? Plant Dis. 65:1023–1027.

4. Kiyohara, T., and K. Suzuki. 1978. Nematode population growth and disease development in pine wilting disease. Eur. J. For. Path. 8:285–292.

5. Kobayashi, F. 1978. Pine bark beetle problem in Japan, referring to the discovery of the pine wood nematode *Bursaphelenchus lignicolus.* Anz. Schadlingskale. Planzenschutz. Umweltschutz. 51:76–79.

6. Kondo, E., A. Foudin, M. Linit, M. Smith, R. Bolla, R. E. K. Winter, and V. H. Dropkin. 1982. Pine wilt disease: Nematological, entomological, and biochemical investigations. Missouri Agric. Expt. Sta. Bull. SR 282.

7. Malek, R. B. 1983. Symptomatology. Proc. 1982 Natl. Pine Wilt Dis. Workshop, pp. 14–16.

8. Mamiya, Y. 1976. Pine wilting disease caused by pine wood nematode *Bursaphelenchus lignicolus* in Japan. Jap. J. Agric. Res. Quart. 10:206–211.

9. Mamiya, Y., and N. Enda. 1972. Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). Nematologica 18:159–162.

10. Mamiya, Y., and T. Kiyohara. 1972. Description of *Bursaphelenchus lignicolus* (n. sp. Nematoda: Aphelenchoididae) from pine wood and histopathology of nematodes in infested trees. Nematologica 18:120–124.

11. Mamiya, Y., and H. Tamura. 1977. Transpiration reduction of pine seedlings inoculated with the pinewood nematode *Bursaphelenchus lignicolus.* J. Jap. For. Soc. 59:59–63.

12. Oku, H., T. Shiraishe, and S. Kurozumi. 1979. Participation of toxin in wilting of Japanese pines caused by a nematode. Naturwissenschaften 66:210.

13. Robbins, K. 1979. Pine wood nematode. Pest alert. Forest Service USDA NA-FB/U7.

14. Robbins, K. 1983. Distribution of the pinewood nematode in the United States. Proc. 1982 Natl. Pine Wilt. Dis. Workshop, pp. 3–7.

15. Suziki, K., and T. Kiyohara. 1977. Influence of water stress on development of pine wilting disease caused by *Bursaphelenchus lignicolus.* Eur. J. For. Path. 8:97–107.