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Effect of Phytotoxin from Nematode-induced Pinewilt on *Bursaphelenchus xylophilus* and *Ceratocystis ips*[1]

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Abstract: A phytotoxic extract from *Pinus sylvestris* infected with *Bursaphelenchus xylophilus* inhibited growth of the blue-strain fungus *Ceratocystis ips* and caused temporary paralysis in vitro of *B. xylophilus*. Although the nematodes recovered from paralysis, final population size of *B. xylophilus* was suppressed by the toxin. Extracts from noninfected *P. sylvestris* affected neither the fungus nor the nematode.

Key words: phytoalexin, nematode, terpenoids.

Pinewilt caused by *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) is epidemic in Japan (10,11,15,18) and is becoming an important disease in the United States (20,21). The mode of transmission of the nematode and symptomology of this disease were described in Japan (16,17) with similar observations made in the United States (5,12–14). In addition to the nematode in the infected trees, the blue-stain fungus *Ceratocystis ips* (Davidson, 1953) and several species of bacteria have been reported (8,11,12). Whether *C. ips* is present in the tree before *B. xylophilus* infection or subsequently invades the infected tree is still debatable (25).

The initial symptoms of *B. xylophilus* pinewilt disease occur before the nematode population is large enough to cause conspicuous damage to the resin canals of the tree (16,17). A phytotoxin has been demonstrated (19,22) in 2-year-old *Pinus sylvestris* seedlings infected with 5,000 *B. xylophilus*. Resin production at the site of a wound induced on the seedling and stomate closing indicative of decreased transpiration occurred 3 days after infection (22), and phytotoxic resin components were extracted from those seedlings. When applied to noninfected 2-year-old *P. sylvestris* seedlings, these extracts caused wilting symptoms identical to a natural nematode infection (22).

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Initial analysis suggests that the toxins are oxygenated monoterpenes (22, and our unpublished results) possibly derived from natural resin components of the pine. Because these phytotoxic materials appear soon after infection, we have considered that they are produced by the tree in an attempt to control infection by the nematode, by *C. ips*, or by both. This paper reports our studies on the effect of extracts from *B. xylophilus*-infected *P. sylvestris* on the in vitro growth of *B. xylophilus* and of *C. ips*.

Materials and Methods

Growth and maintenance of organisms: C. ips (American Type Culture Collection, isolate No. 12860) was maintained at 26 C on slants of potato dextrose agar (PDA) (22 g instant mashed potatoes, 10 g glucose, 16 g Bacto agar/liter). *B. xylophilus* obtained from Dr. V. H. Dropkin, University of Missouri–Columbia, was maintained at 26 C on fungal mats of *Botrytis cinerea* grown on PDA. Both nematode and fungus were subcultured monthly.

Preparation of phytotoxin extracts: Partially purified phytotoxin extracts were prepared from 20–30-year-old *P. sylvestris* naturally infected with *B. xylophilus* by a $CHCl_3$-base method (22). Noninfected *P. sylvestris* wood was extracted by the same procedure and the resulting material was used in control experiments. *P. sylvestris* extracts stored in $CHCl_3$ were aseptically evaporated to dryness and then were solubilized in sterile 0.1 M KOH/0.1% Tween 80 adjusted to pH 7.0 with 1 N NaOH.

Bioassay of fungal growth: Two approaches were used to investigate the effect of pinewilt phytotoxin on the growth of *C.*

ips. First, PDA agar plates (150 × 25 mm) when cooled to 50 C were supplemented with 10, 100, or 500 μg/ml of partially purified pinewilt phytotoxin (22) or the comparably prepared extract from noninfected *P. sylvestris*. A 7.2-mm-d plug of *C. ips* from stock cultures in 60- × 15-mm petri dishes was transferred with a sterile cork borer to the experimental and control PDA plates or to PDA supplemented with 0.1 M KOH/0.1% Tween 80 (pH 7.0 with 1 N NaOH). On days 3, 6, and 9 after inoculation, the area of fungal growth was measured with sterile calipers. As an alternative approach, 10-ml aliquots of semiliquid PDA containing 0.2% agar were dispensed into 16- × 150-mm culture tubes, cooled to 50 C, and supplemented with experimental and control extracts from *P. sylvestris* at final concentrations of 10, 50, 100, or 500 μg/ml or supplemented with 1.5 mM KOH and 0.0015% Tween 80. These cultures were inoculated with 0.1 ml of a suspension of fungal spores (10,000 spores/ml) in sterile 0.9% NaCl. On days 3, 6, and 9 after inoculation, the culture tubes were heated to 45 C and the liquified medium filtered through 150-μm opening nylon screening (Tetko Inc., Elmsford, N.Y.). The fungus was rinsed from the screen with distilled water and transferred to preweighed vials. The vials were heated for 24 hours at 100 C and cooled to room temperature under vacuum. Fungus dry weight was determined gravimetrically. Ten experimental replicates were done for each experimental condition and for the controls.

Bioassay of B. xylophilus growth and reproduction: Short term effects of the phytotoxin on *B. xylophilus* were determined as follows. *B. xylophilus* was recovered aseptically from *B. cinerea* cultures, axenized, and put into soy peptone-yeast extract medium (3). After overnight growth at 26 C, 2,700 nematodes were inoculated into 18- × 150-mm culture tubes (cotton plugged) containing 5 ml soy peptone-yeast extract medium supplemented with various concentrations of pinewilt phytotoxin extract, extract from noninfected trees, or 0.1 M KOH/0.1% Tween 80. The final concentrations of KOH and Tween 80 in the culture medium were 1.5 m*M* and 0.0015%, respectively. The cultures were incubated at 26 C. At 1.5, 3, 6, and 24 hours the cultures were mixed on a vortex mixer and the number of active and apparently dead nematodes in ten 50-μl aliquots from each culture was determined. Ten cultures were used for each experimental condition. The results are presented as the mean percentage of actively moving nematodes. Nematodes which were not moving and showed neither body flexure nor contraction of the esophageal bulb were considered dead.

The long-term effects of phytotoxin from *B. xylophilus*-infected *P. sylvestris* were determined as follows. *B. cinerea* was grown on PDA supplemented with various concentrations of extract from *B. xylophilus*-infected and noninfected *P. sylvestris* or with KOH/Tween 80. Fungal growth covered 50–65% of the PDA surface within 6 days. At this time each culture was inoculated with 2,000 or 4,000 *B. xylophilus* recovered from stock cultures and axenized (3). On days 4, 6, 8, and 10 after inoculation the nematodes were recovered by a modified Baermann funnel procedure from 10 cultures for each experimental condition. Aliquots of the culture eluate were scored in triplicate for the number of active adult and juvenile nematodes.

Statistical analysis: Data from fungal growth studies were calculated as mean ± standard error of the mean, and differences were analyzed by the Student's *t*-test. Nematode growth and survival data were analyzed by the statistically conservative Studentized range test and by two-way ANOVA analysis.

Results

Effect of phytotoxin growth of C. ips: Pinewilt phytotoxin from *B. xylophilus*-infected *P. sylvestris* significantly retarded the growth of *C. ips* (Tables 1, 2). This effect was observed beginning 6 days after inoculation of the cultures and continued through 9 days. A 2–3-fold difference from controls in area of fungal growth was observed at 6 and 9 days, depending on phytotoxin concentration (Table 1). Nearly equal fungal growth occurred in the presence of 100 or 500 μg/ml of partially purified phytotoxin, and growth in the presence of 10 μg/ml of phytotoxin was significantly greater than in higher concentrations of the phytotoxin at 6 and 9 days. Growth on PDA supplemented with the $CHCl_3$-base

TABLE 1. Effect of phytotoxin from *Bursaphelenchus xylophilus*-infected *Pinus sylvestris* on the growth (mm^2) of *Ceratocystis ips* as a function of time in culture.†

Extract	Concentration (μg/ml)	Days after inoculation of culture with fungus		
		3	6	9
Phytotoxin‡	10	40.9 ± 9.1	85.4 ± 8.2*	304.5 ± 12.6*
	100	36.2 ± 4.5	58.3 ± 9.8*	207.7 ± 58.3*
	500	27.2 ± 2.1*	56.2 ± 7.3*	187.3 ± 72.1*
Noninfected‡	10	40.9 ± 10.9	170.9 ± 7.9	561.1 ± 83.4
	100	41.5 ± 11.7	166.8 ± 10.4	574.8 ± 54.6
	500	35.3 ± 12.4	157.3 ± 12.6	526.9 ± 77.4
Control‡ (KOH/Tween 80)		51.7 ± 16.0	169.5 ± 5.2	637.2 ± 65.3

* Significant differences of experimental from the controls.
† Values reported as mean ± standard error of 10 separate measurements. Differences are significant at $P \leq 0.05$ as determined by Students' *t*-test.
‡ Phytotoxin: extract from *B. xylophilus*-infected *P. sylvestris*. Noninfected: similarly prepared extract from noninfected *P. sylvestris*. Control: PDA supplemented with KOH/Tween 80.

extract from noninfected *P. sylvestris* was no different from the solvent controls. These same trends were reflected in dry weight of *C. ips* propagated on semiliquid PDA, where significant repression of growth was seen only at concentrations above 100 μg/ml (Table 2).

Short-term effect of phytotoxin on survival of B. xylophilus: As early as 1.5 hours after inoculation of *B. xylophilus* into soy-peptone/yeast extract medium supplemented with 250 to 2,000 μg of partially purified pinewilt phytotoxin, 40–50% of the nematodes appeared dead (Table 3); that is, they lost motility and body flexure, the esophageal bulb did not move, and the intestinal cells were highly vacuolated. Approximately 20–30% of the remaining nematodes appeared to be dying. These nematodes were somewhat straightened, movement of the esophageal bulb was only periodic, and the only body movement was slight flexure and straightening of the posterior end. These effects were dependent on phytotoxin concentration. At the 10–100-μg/ml concentrations of partially purified phytotoxin, "dead" nematodes appeared at 3 hours and few additional "dead" nematodes were seen by 6 hours. Again 20–30% of the "living" nematodes showed symptoms of "paralysis." At concentrations of 250–2,000 μg/ml, the number of apparently dead nematodes increased significantly by 3 hours, and the percentage of dying or paralyzed nematodes increased to 30–40%. In the phyto-

TABLE 2. Effect of phytotoxin extracted from *Bursaphelenchus xylophilus*-infected *Pinus sylvestris* on dry weight (mg) of *Ceratocystis ips*.

Extract	Concentration (μg/ml)	Days after inoculation†		
		3	6	9
Phytotoxin‡	10	8.3 ± 1.4*	11.2 ± 3.1*	17.4 ± 3.0*
	100	6.2 ± 2.1*	9.3 ± 2.5*	12.1 ± 1.7*
	500	5.1 ± 1.7*	7.1 ± 1.4*	9.7 ± 1.1*
Noninfected‡	10	11.7 ± 2.0	14.3 ± 0.6	20.7 ± 0.9
	100	12.4 ± 1.6	15.5 ± 1.0	23.5 ± 1.1
	500	10.8 ± 2.4	14.8 ± 1.7	24.2 ± 1.7
Control‡ (KOH/Tween 80)		10.2 ± 3.1	14.3 ± 0.2	25.5 ± 0.3

* Differences are significant at $P \leq 0.05$ as determined by Students's *t*-test.
† Results are reported as mean ± standard error of 10 separate determinations.
‡ Phytotoxin: extract from *B. xylophilus*-infected *P. sylvestris*. Noninfected: similarly prepared extract from noninfected *P. sylvestris*. Control: PDA supplemented with KOH/Tween 80.

TABLE 3. Effect of pinewilt phytotoxin on the short-term survival of *B. xylophilus*. A similarly prepared extract from noninfected *P. sylvestris* was used as a control.

Extract	Concentration (μg/ml)	Nonparalyzed *B. xylophilus* (%)† Time (hours after inoculation) 1.5	3	6	24
Phytotoxin	10	100 ± 1.6	76.6 ± 13.7	77.6 ± 20.8	90.2 ± 7.3
	25	100 ± 3.1	80.7 ± 10.0	81.2 ± 11.5	87.8 ± 10.1
	50	95.1 ± 4.3	82.4 ± 14.4	84.0 ± 9.8	89.6 ± 8.3
	100	100 ± 2.0	65.4 ± 19.8	73.0 ± 14.6	83.7 ± 12.7
	250	65.7 ± 5.5	58.9 ± 9.8	43.1 ± 15.5	77.7 ± 14.3
	500	58.3 ± 7.2	42.3 ± 14.6	22.0 ± 12.5	78.4 ± 14.2
	1,000	52.4 ± 12.2	54.6 ± 11.8	27.7 ± 7.3	86.2 ± 12.4
	2,000	51.6 ± 8.5	48.1 ± 9.9	31.7 ± 6.4	78.5 ± 14.4
Noninfected wood extract	10	100 ± 10.5	97.8 ± 3.9	90.0 ± 9.4	93.3 ± 8.0
	25	100 ± 0.8	92.3 ± 6.8	90.7 ± 9.5	93.5 ± 8.0
	50	100 ± 10.0	93.8 ± 8.2	87.1 ± 9.3	90.4 ± 9.1
	100	100 ± 3.0	92.7 ± 9.2	85.6 ± 10.0	87.4 ± 8.7
	250	100 ± 5.0	95.3 ± 7.2	83.1 ± 8.8	91.2 ± 11.4
	500	100 ± 3.6	89.8 ± 10.0	87.4 ± 8.2	89.2 ± 11.3
	1,000	90.4 ± 3.6	90.4 ± 4.1	89.4 ± 6.9	86.7 ± 3.7
	2,000	87.6 ± 2.9	94.2 ± 7.2	89.5 ± 5.6	87.8 ± 2.7
KOH/Tween 80		100 ± 9.0	92.4 ± 8.3	90.3 ± 6.4	92.0 ± 11.3

† Values are reported as mean ± standard error of 25 separate determinations. Differences significant at $P \leq 0.05$ by the Studentized range test and two-way ANOVA.

toxin range of 500–2,000 μg/ml, the percentage of active nematodes reached a plateau. The overall trend was an increase in nematode mortality dependent on time and phytotoxin concentration. At 1.5–6 hours and phytotoxin range of 250–2,000 μg/ml, the percentage of active nematodes was significantly less than in the control cultures, and by 3–6 hours it appeared as if all concentrations of phytotoxin may have had an effect on the nematodes (Table 3).

A significant change in the cultures was noted at 24 hours when the percentage of active nematodes increased over those present at 6 hours. In addition, dying or "paralyzed" nematodes were no longer observed. The nematodes appeared to be actively feeding, their esophageal bulbs were pumping rapidly, and their intestinal cells were no longer vacuolated. At this time there were no significant differences in nematode mortality among any of the cultures. The increase in percentage of active nematodes at 24 hours did not occur in the control cultures and was not due to an increase in number of juveniles, as the juvenile composition of the various populations remained at 32–40% throughout the 24 hours incubation.

Long-term effect of phytotoxin on B. xylophilus: Addition of 100 or 250 μg/ml phytotoxin to *B. xylophilus* cultures suppressed the population growth rate of the nematode by 3–4-fold compared to control cultures (Fig. 1, Table 4). Populations decreased in control cultures at 8 days but increased at 10 days. On the other hand, populations peaked in the phytotoxin-supplemented cultures at 6 days and declined at 8 days, after which there was no change.

In an attempt to define these changes in population growth, the juveniles and adults were counted (Table 4). During the first 4 days of culture the numbers of adults and juveniles in all cultures increased, although this rate was suppressed in the phytotoxin-supplemented cultures. Between 4 and 6 days the adult population decreased in the presence of 250 μg of phytotoxin while the juvenile population continued to increase; in the noninfected wood extract control cultures both juvenile and adult numbers increased during this period. Between day 6 and day 10 the numbers of adults did not change significantly in any culture, although the numbers of juveniles fluctuated. In the noninfected wood extract control cultures the numbers of adults decreased between day 6 and day 8, then increased to day 10; the population of juveniles did not change significantly during this period.

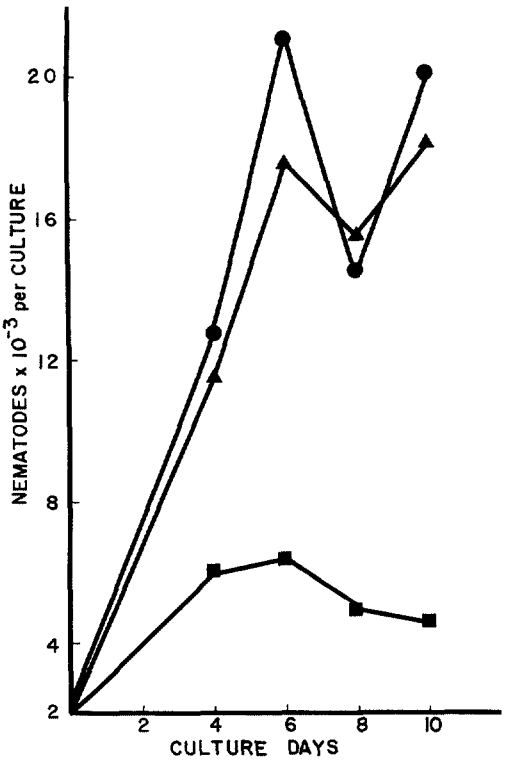

FIG. 1. Growth of *Bursaphelenchus xylophilus* on fungal mat cultures of *Botrytis cinerea* initiated with 2,000 nematodes and supplemented with 100 or 250 μg/ml of partially purified extract from *B. xylophilus*-infected (■) or noninfected (▲) *Pinus sylvestris*, or with 0.1 M KOH plus 0.1% Tween 80 (●).

Neither the phytotoxin nor the extract from noninfected *P. sylvestris* had any effect on the rate of growth of *B. cinerea* during the 10 days of culture. The dry weights of the fungal mats recovered from the various cultures did not differ significantly.

DISCUSSION

Shaheen et al. (22) and Oku et al. (19) have presented evidence for involvement of a phytotoxin in the mechanism of wilting caused by *B. xylophilus* in *P. sylvestris*, *P. thunbergii*, and *P. resinosa*. This toxin appears shortly after nematode infection, seems to be of tree origin, may contain oxygenated monoterpenes not normally present in pine resin, and could be synthesized from normal resin components of the pine (22, and our unpublished results). We investigated the possibility that this phytotoxin might be a host defense mechanism

TABLE 4. Effect of phytotoxin on long-term growth and development of *B. xylophilus* on fungal mats of *Botrytis cinerea*. Results† presented as populations (× 10^{-3}) of active adults (A) and juveniles (J). Control cultures contained a comparably prepared extract from noninfected *P. sylvestris*.

Supplement	Concentration (μg/ml)	Days of culture‡ 4 A	4 J	6 A	6 J	8 A	8 J	10 A	10 J
Phytotoxin	250	6.5 ± 3.2	1.8 ± 1.6	3.1 ± 1.7	5.4 ± 2.5	3.8 ± 2.3	1.5 ± 1.3	1.9 ± 1.0	3.4 ± 1.8
Control	250	10.8 ± 4.1	4.8 ± 1.5	13.6 ± 2.3	9.4 ± 1.3	9.0 ± 1.8	7.7 ± 2.2	13.5 ± 1.5	8.5 ± 3.8
0		9.9 ± 5.1	3.9 ± 2.8	9.2 ± 3.2	10.6 ± 2.1	8.9 ± 1.7	8.9 ± 2.6	12.1 ± 4.2	8.2 ± 2.2

† Results are reported as mean ± standard error of 10 separate determinations.
‡ Cultures were initiated with 4,000 *B. xylophilus*.

against either *B. xylophilus* or *C. ips* which has been proposed to be associated with *B. xylophilus*-caused pinewilt (8,10,12). In many plant-pathogen interactions the pathogen induces a resistance mechanism in which the host plant synthesizes phytoalexins (1,2,6,24). Such compounds have been associated with nematode infection of several plant species (2,24). In addition, production of monoterpenes with biological activity against spore germination and germ tube growth of *Diplodia pinea* in *D. pinea*-infected *P. radiata* and production of biologically active polyphenols in *Ceratocystis minor*-infected *P. taeda* has been reported (4,7). In order to be classified as phytoalexins, materials synthesized in response to infection must show a time–site–effect relationship (1,24).

Synthesis of the phytotoxin in *B. xylophilus*-infected *P. sylvestris* can be detected 3 days after infection of 2-year-old seedlings (22) and thus fulfills the phytoalexin time requirement. In addition, the phytotoxin partially suppresses the growth of *C. ips* and the growth, development, and reproduction of *B. xylophilus* in culture.

The initial and immediate effect of the phytotoxin on *B. xylophilus* is a "paralysis" which is dependent on time and toxin concentration. "Paralysis" is manifested by total loss or severe impairment of motility, irregular contraction or complete loss of contraction of the esophageal bulb, and by emptying of the intestinal cells. These effects disappear after 24 hours of exposure to the phytotoxin, and the recovered nematodes apparently can continue growth, development, and reproduction. Such recovery suggests that the nematodes may metabolically detoxify the phytotoxin. Metabolic detoxification of phytoalexins by plant pathogens has been suggested from investigations of several plant/pathogen interactions (1,2,9). The phytotoxin may also undergo autodegradation by 24 hours in the culture medium. Autodegradation would seem unlikely, as growth and development of *B. xylophilus* was suppressed over 10 days in the presence of phytotoxin as compared to the noninfected wood extract supplemented controls. In addition we have found that the phytotoxin remains active when stored for 6 to 9 months either in 20% aqueous acetone or in $CHCl_3$ (our unpublished results).

In vitro effects of the phytotoxin on *C. ips* and *B. xylophilus* may be minimal. In a natural infection the concentration of phytotoxin increases with time (22) so that the organisms are exposed to continually increasing levels of phytotoxin. However, *B. xylophilus*, being a transient nematode, may be able to move from the site of elicitation of the phytotoxin and thereby avoid its effects. The transient nature of this nematode could result in continued elicitation of phytoalexin at multiple sites of cellular damage throughout the tree, and possible accumulation of what might be synthesized initially as a phytoalexin may increase to concentrations that become secondarily phytotoxic. Phytotoxicity caused by excessive production of phytoalexins has been reported (23). It should be noted that the most successful nematode-elicited phytoalexins so far reported are those elicited only at the site of infection by nematodes which become sedentary after infection (24). It is also possible that in this disease the phytotoxin becomes systemic as the infection develops in the infected pine.

Based on our results, it appears that phytotoxins from *B. xylophilus*-infected *P. sylvestris* satisfy one criterion but not a second for being classified as phytoalexins. That is, they are synthesized shortly after infection, but their activity against *B. xylophilus* or *C. ips* does not impart resistance to infection of *P. sylvestris* by either organism. Studies are in progress to determine the site of synthesis of these compounds and their relation to cellular damage caused by *B. xylophilus*.

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