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Carbohydrate Concentration in Pine as Affected by Inoculation with *Bursaphelenchus xylophilus*[1]

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Abstract: Pines responded to inoculation with *Bursaphelenchus xylophilus* by changes in reducing and nonreducing carbohydrate concentrations dependent on the pine species and the pathotype of *B. xylophilus* with which the trees were inoculated. Carbohydrate concentrations, in compatible pine–nematode pathotype combinations, decreased initially after inoculation and then increased slightly before decreasing to approximately 10% of the control levels as the seedlings wilted. In compatible nematode pathotype–pine species combinations, carbohydrate concentrations decreased and then increased as the nematode population densities declined.

Key words: Bursaphelenchus xylophilus, carbohydrate, host response, monoterpene, pathotype, pinewood nematode, *Pinus nigra, P. strobus, P. sylvestris.*

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) Nickle, 1970 (syn. *B. lignicolus,* Mamiya and Kiyohara, 1972) is associated with rapid wilting of several conifers (6). Infection of pines by this nematode is characterized by rapid loss of water movement, decrease or loss of transpiration, decreased resin production at the site of a wound, synthesis of phytotoxic monoterpenes, and rapid onset of leaf chlorosis, eventually leading to complete wilting of the pine (6,7,12). We have described two host specific pathotypes of *B. xylophilus,* MPSy-1 from *Pinus sylvestris* and VPSt-1 from *P. strobus* (2). MPSy-1 induced wilting only of *P. sylvestris.* There was some establishment and reproduction of MPSy-1 in *P. nigra* and *P. strobus,* but these seedlings did not develop wilt symptoms (2). VPSt-1 caused wilting only of *P. strobus,* and although this isolate minimally established in *P. sylvestris,* this pine species did not wilt. VPSt-1 did not establish in either *P. nigra* or *P. taeda,* and MPSy-1 did not establish in *P. taeda* (2). Thus *P. sylvestris* is a compatible host for pathotype MPSy-1 and *P. strobus* is a compatible host for pathotype VPSt-1. Some incompatible pines respond to inoculation with these pathotypes by transient changes in transpiration, water loss, and biosynthesis of unique monoterpenes (2). Wingfield et al. (13) have described *B. xylophilus* pathotypes from pine and fir in Minnesota.

Intermediates of carbohydrate catabolism are used as precursors for synthesis of terpenes, polyphenols, and other organic compounds by many plants as defense chemicals against pathogens (14). Pines synthesize unique monoterpenes in response to inoculation with *B. xylophilus* (10,11). These materials, isolated from compatible, nematode pathotype–pine species combinations, however, do not appear to be successful as defense chemicals for the pine (1,2). Synthesis of these monoterpenes might shunt intermediates of energy metabolism away from pathways of energy production, thereby placing stress on the pine's ability to maintain energy homeostasis through carbohydrate synthesis alone, and mobilization of energy reserves may be required. This stress, in conjunction with leaf chlorosis and decreased photosynthetic capacity, which accompanies *B. xylophilus* infection, might lead to starvation of the pine and might be responsible for the wilting observed in this disease.

Decreases in reducing and nonreducing carbohydrates have been shown in *Meloidogyne javanica*-infected almond and peach rootstocks (8). Similar decreases in carbohydrate concentration was not seen, however, in *M. javanica*-infected rootstocks of

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TABLE 1. Reducing and nonreducing carbohydrate concentration in 2–3-year-old *Pinus sylvestris, P. nigra,* and *P. strobus* control seedlings inoculated with sterile saline.

Days after inoculation	Carbohydrate μg/mg seedling dry weight*		
	P. sylvestris	*P. nigra*	*P. strobus*
	Reducing carbohydrate		
0	42.28 ± 7.40	38.68 ± 6.37	55.17 ± 14.81
15	45.61 ± 5.33	40.29 ± 9.95	48.32 ± 6.66
30	43.22 ± 9.87	36.66 ± 5.78	52.55 ± 9.21
45	46.32 ± 10.33	36.78 ± 8.87	57.34 ± 5.12
60	42.56 ± 9.95	41.42 ± 3.42	59.89 ± 8.32
	Nonreducing carbohydrate		
0	93.24 ± 29.22	90.63 ± 6.51	97.71 ± 22.19
15	105.72 ± 15.78	86.99 ± 24.44	108.88 ± 17.33
30	92.56 ± 6.87	92.42 ± 11.98	106.12 ± 18.56
45	89.44 ± 11.34	90.67 ± 15.66	98.48 ± 11.76
60	91.72 ± 5.71	91.11 ± 7.21	96.33 ± 8.98

* Mean ± standard error, N = 15.

Nemaguard peach, a cultivar resistant to this nematode (8).

Our objective was to compare carbohydrate levels in compatible and incompatible pine–nematode associations in relation to nematode population density changes and wilting of the seedlings.

MATERIALS AND METHODS

B. xylophilus pathotypes MPSy-1 and VPSt-1 were maintained on *Botrytis cinerea* growing on potato dextrose agar (PDA) at 27 C. Nematodes were recovered from cultures by a modified Baermann funnel procedure and allowed to settle for 30 minutes in 13 × 100-mm test tubes containing 500 units penicillin, 500 units streptomycin, 50 μg tetracycline, and 50 μg nystatin per milliliter of sterile distilled water. The nematodes were removed from the bottom of the tube, and this wash procedure was repeated three times. The nematodes were then washed three times by centrifugation through sterile distilled water. An aliquot of the final wash was assayed for microbial contamination by culture in thioglycollate broth and on nutrient agar and PDA.

Two- to three-year-old greenhouse-raised *P. sylvestris, P. strobus,* and *P. nigra* seedlings, ranging from 30 to 50 cm in height and 3 to 8 cm in circumference, were each inoculated with either 5,000 or 25,000 nematodes (2,6). At the same time control seedlings were inoculated with sterile saline. Seedlings were watered twice weekly and were harvested at 15, 30, 45, and 60 days after inoculation. Seedlings were removed from pots, and soil was washed from the roots. Seedlings were weighed and cut into 0.5-cm-long pieces, and nematodes were extracted for 8 hours at room temperature by a modified Baermann funnel procedure (6). The seedling pieces were then frozen in liquid nitrogen and lyophilized. The lyophilized seedling pieces were weighed, then ground in a mortar and pestle; the powder was extracted by reflux boiling in 5 ml distilled water per gram wood for 2 hours. The refluxed mixtures were centrifuged at 500 g for 15 minutes, and the supernatants were assayed for carbohydrates.

Total reducing carbohydrates were assayed by the anthrone and Somogyi microcopper method (5). Nonreducing carbohydrates were determined following reduction of disaccharides and polysaccharides with alkali (3,5). Aliquots of the seedling extracts were adjusted with 12 N KOH to a final concentration of 6 N KOH and boiled for 30 minutes. After cooling, carbohydrate was determined by anthrone assay (5). Solutions of glucose and starch, treated in parallel with the extracts, were used as standards. Three batches of five experimental and five control seedlings of

TABLE 2. Concentration of reducing and nonreducing carbohydrate in 2–3-year-old *Pinus sylvestris, P. nigra,* and *P. strobus,* seedlings inoculated with 5,000 *Bursaphelenchus xylophilus* pathotype MPSy-1.

Days after inoculation	Carbohydrate μg/mg seedling dry weight*		
	P. sylvestris	*P. nigra*	*P. strobus*
	Reducing carbohydrate		
0	42.28 ± 7.48	38.86 ± 6.37	55.17 ± 14.81
15	8.56 ± 0.86	11.88 ± 1.09	16.15 ± 1.33
30	10.84 ± 5.26	16.02 ± 5.97	20.87 ± 3.00
45	4.81 ± 1.27	17.11 ± 7.28	26.34 ± 4.69
60	3.76 ± 2.23	24.36 ± 3.36	39.81 ± 8.82
	Nonreducing carbohydrate		
0	93.24 ± 29.22	90.63 ± 6.51	97.71 ± 22.49
15	7.52 ± 1.17	38.68 ± 1.00	87.01 ± 25.97
30	39.93 ± 7.02	46.49 ± 14.29	105.25 ± 38.48
45	22.65 ± 6.90	35.37 ± 16.19	95.02 ± 25.40
60	6.32 ± 1.26	75.25 ± 11.82	91.31 ± 18.26

* Mean ± standard error, N = 15.

each species were assayed at each experimental point, and the results are reported as mean ± standard error of the combined data. Response differences to inoculation with a pathotype of *B. xylophilus* were determined by analysis of variance.

RESULTS

Concentrations of reducing and nonreducing carbohydrates did not vary in saline-inoculated *P. sylvestris, P. nigra, or P. strobus* control seedlings over the 60-day experimental period (Table 1). Significant variations occurred, however, in seedlings of these species inoculated with *B. xylophilus.* The magnitude and rate of carbohydrate concentration change were dependent on the species of pine, the nematode pathotype, and the amount of the inoculum.

MPSy-1 inoculation: Reducing carbohydrate concentration in *P. sylvestris* inoculated with 5,000 MPSy-1 decreased to 20% of the control at 15 days after inoculation, and then decreased gradually to 10% of the control by 45 days after inoculation (Table 2). These seedlings wilted completely by 28–32 days after inoculation. Nonreducing carbohydrate concentration decreased to 8% of the control by 15 days after inoculation, then increased fivefold by 30 days, and decreased again through 60 days (Table 2).

P. nigra and *P. strobus* showed an initial decrease in the concentration of reducing carbohydrates after inoculation with *B. xylophilus* followed by an increase reaching 62% and 73% of the control value, respectively, by 60 days after inoculation (Table 2). MPSy-1-inoculated *P. nigra* showed decreased concentrations of nonreducing carbohydrates until 45 days after inoculation, but then the levels increased to approach the control by 60 days. Nonreducing carbohydrate concentration was not affected in *P. strobus* seedlings inoculated with 5,000 MPSy-1. Neither *P. nigra* nor *P. strobus* seedlings wilted when inoculated with MPSy-1.

When the *B. xylophilus* inoculum was increased to 25,000 MPSy-1, the concentration of reducing carbohydrates in *P. sylvestris* decreased to 12% of the control by 15 days after inoculation and then remained constant through 60 days (Table 3). These seedlings wilted by 25–30 days after inoculation. The concentration of nonreducing carbohydrates was comparable and followed a similar pattern to that in *P. sylvestris* inoculated with 5,000 MPSy-1.

The concentration of reducing carbohydrates in *P. nigra* and *P. strobus* inoculated with 25,000 MPSy-1 was 25–50% of that in saline-inoculated controls by 15–30 days after inoculation, but it rose to equal

TABLE 3. Reducing and nonreducing carbohydrate concentration in 2–3-year-old *Pinus sylvestris, P. nigra,* and *P. strobus* seedlings inoculated with 25,000 *Bursaphelenchus xylophilus* pathotype MPSy-1.

Days after inoculation	Carbohydrate μg/mg seedling dry weight*		
	P. sylvestris	*P. nigra*	*P. strobus*
	Reducing carbohydrate		
0	42.28 ± 7.40	38.68 ± 6.37	55.17 ± 14.81
15	25.37 ± 1.07	18.79 ± 0.87	13.57 ± 1.22
30	3.68 ± 0.61	3.88 ± 0.39	12.42 ± 0.82
45	7.53 ± 0.71	22.69 ± 5.72	68.72 ± 1.95
60	5.71 ± 0.88	45.62 ± 8.96	63.43 ± 9.37
	Nonreducing carbohydrate		
0	93.24 ± 29.22	90.63 ± 6.51	97.71 ± 22.19
15	9.50 ± 0.93	62.92 ± 13.62	57.10 ± 11.23
30	24.47 ± 11.38	30.52 ± 13.12	40.58 ± 4.89
45	19.64 ± 2.94	23.28 ± 2.57	28.26 ± 5.87
60	7.76 ± 1.26	58.32 ± 11.81	46.36 ± 6.96

* Mean ± standard error, N = 15.

control levels by 60 days (Table 3). The initial decrease, as well as the recovery to control levels, was greatest in *P. strobus* seedlings. Changes in nonreducing carbohydrates in seedlings inoculated with 25,000 MPSy-1 were similar in both *P. strobus* and *P. nigra*; that is, nonreducing carbohydrate concentrations were approximately 60% of the control by 15 days after inoculation, they decreased approximately 50% by 45 days, and they increased toward control levels by 60 days. Seedlings of neither of these species wilted when inoculated with 25,000 MPSy-1, although there was some initial establishment of the nematode in the seedlings.

VPSt-1 inoculation: When *P. strobus, P. sylvestris,* and *P. nigra* were inoculated with 5,000 VPSt-1, reducing carbohydrate concentrations decreased 40–50% by 15 days after inoculation and then decreased further by 30 days to 15–30% of the control concentration (Table 4). The concentration remained unchanged at 10–15% of the control in VPSt-1-inoculated *P. strobus,* from 30 through 60 days. VPSt-1-inoculated *P. strobus* seedlings wilted by 30–32 days after inoculation. In the incompatible

TABLE 4. Reducing and nonreducing carbohydrate concentration in 2–3-year-old *Pinus sylvestris, P. nigra,* and *P. strobus* seedlings inoculated with 5,000 *Bursaphelenchus xylophilus* pathotype VPSt-1.

Days after inoculation	Carbohydrate μg/mg seedling dry weight*		
	P. sylvestris	*P. nigra*	*P. strobus*
	Reducing carbohydrate		
0	42.28 ± 7.40	38.68 ± 6.37	55.17 ± 14.81
15	12.31 ± 0.61	20.23 ± 3.22	17.58 ± 2.75
30	5.61 ± 1.57	14.13 ± 5.41	8.36 ± 0.94
45	23.91 ± 3.69	22.50 ± 7.05	9.87 ± 2.22
60	33.42 ± 7.62	37.21 ± 9.96	6.21 ± 4.28
	Nonreducing carbohydrate		
0	93.24 ± 29.22	90.63 ± 6.51	97.71 ± 22.49
15	30.20 ± 5.62	56.14 ± 11.66	8.75 ± 2.88
30	34.58 ± 9.35	76.68 ± 14.70	29.16 ± 5.87
45	89.01 ± 19.56	83.72 ± 20.42	17.91 ± 7.57
60	76.43 ± 11.96	79.49 ± 14.31	9.81 ± 6.21

* Mean ± standard error, N = 15.

TABLE 5. Reducing and nonreducing carbohydrate concentration in 2–3-year-old *Pinus sylvestris, P. nigra,* and *P. strobus* seedlings inoculated with 25,000 *Bursaphelenchus xylophilus* pathotype VPSt-1

Days after inoculation	Carbohydrate μg/mg seedling dry weight*		
	P. sylvestris	*P. nigra*	*P. strobus*
	Reducing carbohydrate		
0	42.28 ± 7.40	38.68 ± 6.37	55.17 ± 14.81
15	24.56 ± 6.53	36.77 ± 4.80	18.52 ± 4.00
30	25.99 ± 3.54	25.65 ± 2.99	30.65 ± 5.40
45	67.39 ± 10.70	28.35 ± 4.38	4.85 ± 0.88
60	62.42 ± 11.43	43.71 ± 9.76	3.62 ± 1.73
	Nonreducing carbohydrate		
0	93.24 ± 29.22	90.63 ± 6.51	97.71 ± 22.19
15	48.49 ± 6.17	50.20 ± 4.43	7.55 ± 3.61
30	56.11 ± 19.96	66.04 ± 22.08	23.84 ± 4.11
45	43.48 ± 6.68	45.84 ± 5.31	3.91 ± 0.92
60	49.32 ± 9.22	51.42 ± 8.72	2.06 ± 0.86

* Mean ± standard error, N = 15.

pine species, *P. sylvestris* and *P. nigra,* the reducing carbohydrate concentration increased beginning 45 days after inoculation; by 60 days it was 80–100% of the control.

Nonreducing carbohydrate concentration was also decreased in all three pine species at 15 days after inoculation with 5,000 VPSt-1. Whereas this decrease was 90% in the compatible host, it was only 40–70% in the incompatible seedling species. Nonreducing carbohydrate then was increased in all seedling species by 30 days after inoculation. The concentration of nonreducing carbohydrate continued to increase through 60 days in the incompatible seedlings, *P. nigra* and *P. sylvestris,* but the concentration decreased to 18% of the control in the compatible *P. strobus* seedlings by 60 days after inoculation (Table 4).

With inoculation of 25,000 VPSt-1, both reducing and nonreducing carbohydrate concentrations decreased in *P. strobus* seedlings 15 days after inoculation, increased significantly by 30 days, and decreased to ca. 5% of the control level by 60 days after inoculation (Table 5). These seedlings wilted by 28–32 days after inoculation. The concentration of reducing carbohydrate was unaffected at 15 days after inoculation of *P. sylvestris* with 25,000 VPSt-1, it decreased slightly from 15 to 45 days, and then it increased to equal the control concentration by 60 days. A similar trend was observed in *P. nigra* inoculated with 25,000 VPSt-1. Nonreducing carbohydrate concentration in *P. sylvestris* and *P. nigra* seedlings was reduced about 50% by 15 days after inoculation and remained unchanged through 60 days.

DISCUSSION

Stress induced by changes in temperature, oxygen or nutritional substrate availability, drought, or pathogen infection can affect metabolic activity of plant tissues (4). Pine seedlings respond to the stress imposed by *B. xylophilus* by an initial decrease in both reducing and nonreducing carbohydrates in compatible and incompatible plant–pathotype combinations. The decrease continues in compatible combinations but not in incompatible combinations. We suggest that these changes are the result of several alterations associated with nematode parasitism of the pine host. First, *B. xylophilus* locates in, and feeds on, the parenchyma cells lining the resin canals (6). These cells and adjacent cells may be destroyed by the action of nematode-released cellulase (9). Because the nematode is transient in the host, plant damage may be extensive and may result in disruption of nutrient movement throughout the infected pine. Second, *B. xylophilus* disrupts

water movement within the tree (7,12), again suggesting that nematode activity may disrupt systemic nutrient flow. Third, leaf chlorosis resulting from *B. xylophilus* infection places a further stress on photoassimilation of nutrients by the tree and thereby further stresses energy homeostasis. These stresses on nutrient assimilation and energy synthesis are further compounded by the induction of synthesis of unique monoterpenes in *B. xylophilus*-infected pines (10,11). Synthesis of these monoterpenes may require diversion of intermediates of carbohydrate metabolism (e.g., phosphoenol pyruvate, acetate, or mevalonate) away from pathways of biosynthesis of storage carbohydrates and energy production and toward synthesis of monoterpene resins (11,14). Such effects, alone or in combination, could cause the reduction in carbohydrate concentration seen in the compatible host–pathotype combinations. The initial carbohydrate decrease may reflect induction of phytotoxic monoterpene synthesis, which begins as early as 3 days after inoculation (11), and the later declines may reflect the reduction of photosynthetic activity in these seedlings. In compatible host–pathotype inoculations, the unique monoterpenes synthesized in response to *B. xylophilus* are phytotoxic but have little noticeable biological effect on the nematode (11).

The initial tree response in incompatible pine–pathotype combinations, in which some establishment of the nematode may occur (2), may reflect diversion of products of energy metabolism toward synthesis of monoterpene defense chemicals. Soon after inoculation, there is an initial transient increase in nonphytotoxic monoterpenes which parallels the initial decrease in carbohydrate concentration (2). Carbohydrate concentration then appears to be restored toward noninfected seedling levels as synthesis of monoterpene resin decreases and the nematode population density declines (2). Changes in carbohydrate concentration and their recovery in incompatible combinations are dependent on the number of nematodes inoculated into the seedlings.

In summary, it appears that the compatible pine response to *B. xylophilus* infection is an attempt to mount a defense against the nematode. This occurs when nematode infection interrupts nutrient, and hence energy flow through the apoplastic and symplastic systems of the pine, and when photosynthetic activity is declining from progressive infection-induced leaf chlorosis. The ultimate result may be starvation of the tree. In incompatible seedlings, however, the pine appears to be able to mount a defense response to reject the nematode, and even though this response places a temporary stress on nutrient movement and energy metabolism, the seedling is able to recover.

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