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Carbohydrate Catabolism in Populations of *Bursaphelenchus xylophilus* and in *B. mucronatus*[1]

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Abstract: Genotypically different host specific pathotypes of *Bursaphelenchus xylophilus* have been identified. These pathotypes elicit different responses in pines depending on susceptibility, tolerance, or resistance. Continued passage of some of these pathotypes on fungal cultures leads to conversion to nonparasitic populations. These populations metabolize carbon substrates to ethanol by an anaerobic pathway, while operating some level of a phosphoenolpyruvate (PEP)-succinate pathway to excrete succinate–lactate and malate. On the other hand, parasitic populations metabolize glucose to lactate–succinate, mainly by a PEP-succinate pathway, and maintain redox balance through glycerol production. Ethanol and malate are not excreted by parasitic populations.

Key words: alcohol dehydrogenase, *Bursaphelenchus*, carbohydrate metabolism, isocitrate lyase, lactic dehydrogenase, pathotype, phosphoenolpyruvate carboxykinase, *Pinus nigra, P. strobus, P. sylvestris,* pyruvate kinase, susceptibility, tolerance.

Host susceptibility and tolerance, symptom development, and severity of pine wilt disease, following inoculation of pines with *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle vary throughout the range of the pinewood nematode in North America. Infection of *Pinus sylvestris* L. in Missouri, Illinois, and Iowa results in rapid onset of symptoms, and wilting occurs within 2–3 months (17,18,22). Similar symptoms are seen in *P. nigra* Arnold; however, wilting may be extended over two seasons (27). Different symptoms have been described in other areas and in other pine species. A native population of *B. xylophilus* in *P. sylvestris* in Wisconsin is confined to branches at the site of infection, and wilting occurs proximal to this site (36,38). In New Jersey, *B. xylophilus* girdles Austrian pine soon after infection (30,31); however, similar damage has not been observed in infected Austrian pine in some areas (22).

B. xylophilus appears to be the primary cause of wilting of pines planted off site in Missouri, Illinois, Iowa, and New Jersey (22,27,30,36). In other areas, pinewood nematode accelerates the decline of stressed pines (36,38).

Conifer tolerance to pinewood nematode varies (4,9,19,22,25). *P. sylvestris, P. nigra,* and *P. resinosa* Ait. are susceptible hosts for populations of *B. xylophilus* from Missouri and Illinois, and *P. taeda* L. and *P. strobus* L. are resistant (22). Dwinell rated *P. elliottii* Engelm. highly susceptible; *P. strobus, P. taeda,* and *P. serotina* Michx. moderately susceptible; and *P. virginiana* Mill. resistant to *B. xylophilus* isolated from *P. virginiana* in seed orchards in South Carolina, Georgia, and Alabama (19). Nematode populations were largest in *P. elliottii* and smallest in *P. virginiana,* suggesting a correlation between nematode reproduction and pine susceptibility (19). Myers did not see a similar correlation in infections of *P. strobus* in New Jersey, where rapid wilting occurred with only a small number of *B. xylophilus* present (30). In Minnesota, populations of *Bursaphelenchus* from *P. resinosa* and *Abies balsamea* (L.) Mill. are specific to pine and fir, respectively. The population from *A. balsamea* has a modified mucronate tail similar to that of *B. mucronatus* Mamiya and Enda; however, it readily mates with *B. xylophilus* (37). Morphologically different populations of *Bursaphelenchus* also have been reported from pine and fir in Canada (4). Populations from fir in Canada have a modified mucronate tail, and the pine and fir forms are referred to as the R and M forms, respectively (4).

Differences between *Bursaphelenchus*

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populations throughout North America suggest evolution of host-specific pathotypes. We compared a population of *B. xylophilus* from *P. sylvestris* in Missouri (MPSy-1) to a population from *P. strobus* in Vermont (VPSt-1) (9). Populations of MPSy-1 were largest in 3-year-old *P. sylvestris* seedlings 30 days after inoculation. The seedlings wilted within 22 to 28 days, and from 30 to 60 days the nematode population decreased. *P. nigra* and *P. strobus* seedlings were tolerant to the nematode and did not wilt; *P. taeda* was resistant (9). VPSt-1 caused wilting of 3-year-old *P. strobus* seedlings within 28–32 days. Maximum populations were reached by 15–30 days at 10 and 200 times the original inoculum in seedlings inoculated with 25,000 and 5,000 nematodes, respectively. *P. sylvestris* was tolerant and did not wilt; *P. nigra* and *P. taeda* were resistant (9).

Distinct host responses are associated with host susceptibility and tolerance (9,11). Monoterpene synthesis, which initially increased in all inoculated seedlings, returned to control levels by 30–45 days in resistant or tolerant seedlings. Resins from these seedlings were not phytotoxic, and 10-hydroxyverbenone and carvone hydrate, known to be associated with *B. xylophilus* infections (33), were not present. In susceptible seedlings, monoterpene synthesis continued through seedling wilting, resins were phytotoxic, and both 10-hydroxyverbenone and carvone hydrate were present.

Changes in host carbohydrate concentration reflect pine susceptibility and appear to be associated with changes in monoterpene synthesis. That is, decreased carbohydrate concentration was associated with increased monoterpene synthesis, and vice versa (9,11). This association suggests use of intermediates of carbohydrate catabolism for production of monoterpenes.

Populations of *Bursaphelenchus* also differ genotypically. This can be shown by restriction fragment length polymorphisms (RFLP) and DNA:DNA hybridization (14,15). RFLP and DNA:DNA hybridization differences are greatest between *B. xylophilus* and *B. mucronatus*. Under stringent hybridization conditions, DNA:DNA hybridization intensity between these two species is weak and RFLP can be identified (10). DNA:DNA hybridization intensity does not differ between MPSy-1 and VPSt-1, indicating these are populations of *B. xylophilus*. RFLP, however, are apparent (10). Hybridization intensity in DNA:DNA hybridizations of total genomic DNA were compared for *Bursaphelenchus* populations from *P. sylvestris* in Ontario, Canada, and Missouri; *P. strobus* in Vermont; *P. nigra* in New Jersey; *A. balsamea* in Quebec, Canada; and *P. halepensis* Mill. in Arizona. These comparisons showed that the New Jersey population is unrelated to the other populations; the populations from Arizona, Missouri, and Vermont are similar; and the population from *A. balsamea* in Quebec, Canada, is more similar to *B. xylophilus* than to *B. mucronatus* (10). None of these populations is closely related to *B. mucronatus*.

Development and stability of host specific pathotypes are presently unknown. MPSy-1 and VPSt-1 have remained host specific for 2 years when grown on fungal cultures with passage every 3 months through susceptible seedlings. Response of pine cultivars to these pathotypes is unclear. Seedlings used for our studies were from a single seed source.

Increased monoterpene synthesis immediately after inoculation of noncompatible seedlings, the subsequent decline, and nonphytotoxicity may be a host resistance response (5,35). Phytotoxic monoterpenes synthesized in susceptible seedlings have limited biological effect against *B. xylophilus* (8). Monoterpenes synthesized following nematode inoculation must be assumed to be of host origin because nematodes are unable to condense mevalonic acid residues into bicyclic monoterpenes (6). The nematode may elicit this host response.

Disease resistance in plants can involve alteration of any cellular system, but specific de novo synthesis of an abnormal metabolite at the site of infection presupposes host recognition of a particular pathogen

(5,16,20,35). Elicitation of phytochemical responses, including phytoalexins, by plant-parasitic nematodes may involve a relationship between the plant taxonomic family and the type of response produced, as well as qualitative and quantitative composition of nematode secretions and excretions (16,20,35).

Resin composition varies interspecifically and intraspecifically in pines (29). In *B. xylophilus*–pine interactions this variation could affect host specificity. Pine species producing phytotoxins in response to pinewood nematode have turpentines composed mainly of the bicyclic monoterpenes α- and β-pinene, whereas turpentine of *B. xylophilus*-resistant *P. jeffreyi* is 90% n-heptane (29). Because synthesis of unique terpenes may be elicited by the nematode feeding on parenchymal cells of the resin canals, host specificity could involve variation of the available precursor for terpene biosynthesis. *A. balsamea*, which is infected by a host-specific population of *B. xylophilus* but does not wilt, has no resin canals, and its resin acid composition differs greatly from that of pine (23,29).

Rapid cellular damage following nematode inoculation may cause release of host cell hydrolases which, via a damage cascade, could compromise cellulose biosynthesis (23,31). In support of this, Odani et al. (32) demonstrated that resin canal damage and oleoresin leakage initially occurred in *P. densiflora* Sieb. seedlings inoculated with either *B. xylophilus* or *B. mucronatus*. There was no further disease development in *B. mucronatus*-inoculated seedlings, and reproduction and migration of this nematode were restricted. *B. xylophilus*-inoculated seedlings wilted, however, and the nematode population increased progressively and became systemic. They suggested that these differences were related to differences in cellulase isozymes between these nematodes.

Energy Metabolism

Metabolic studies with plant-parasitic nematodes have been difficult because of the small size of the nematode and problems with in vitro culture (7). Amino acids, amines, ammonia, 1,2-dicarboxylic acids, and aldehydes are excreted by *Ditylenchus triformis* Filipjev. This nematode and *D. dipsaci* (Kuhn) Filipjev have several enzymes associated with anaerobic glycolysis and the tricarboxylic acid cycle, but they probably metabolize carbohydrate via a phosphoenolpyruvate-succinate pathway similar to that of several animal-parasitic nematodes (2,6,21,24,34).

Nematode energy metabolism seems to be characterized by a variety of pathways reflecting adaptability to oxygen availability and other environmental factors. Nematodes catabolize carbon substrates completely to CO_2 and H_2O or incompletely to a diverse group of reduced organic molecules (2,3,6,13,21,28,34). Adult animal-parasitic nematodes often incompletely metabolize carbohydrates, whereas their free-living juvenile stages may show complete metabolism and synthesize energy stores via gluconeogenesis (2,34). The nutrient-rich medium in which some parasitic stages live often precludes the need to completely catabolize carbohydrates or to consume energy for carbohydrate synthesis. Energy is conserved by running minimally efficient metabolic pathways which do not require synthesis and maintenance of enzymes of complete and highly efficient energy pathways. Although carbohydrate is the main carbon source of most nematodes, several species can use ethanol, acetate, and short-chain fatty acids (2,6,13,34).

Glucose is catabolized to phosphoenolpyruvate (PEP) via the Embden-Meyerhof pathway. Some nematodes then metabolize PEP anaerobically to lactate or ethanol using pyruvate kinase (PK), lactate dehydrogenase (LDH), and alcohol dehydrogenase (ADH) to maintain redox balance (2,3), whereas others use an alternate energy-yielding microanaerobic pathway (2,6,34). In this pathway, PEP is converted to oxaloacetate (OAA) by CO_2 fixation catalyzed by phosphoenolpyruvate carboxykinase (PEPCK). OAA is reduced to malate via cytoplasmic malate dehydrogenase (MDH) coupled to the oxidation of NADH,

assuring redox balance. One molecule of malate is then oxidized in a mitochondrion to pyruvate and CO_2 by NAD^+-malic enzyme. The reducing power of NADH is used to reduce fumarate, formed from a second molecule of malate via fumarate hydratase, to succinate via fumarate reductase. No net reducing power is generated by this reaction couple; however, it produces a site I-coupled anaerobic synthesis of ATP and provides a regulatory couple by which the NAD^+/NADH ratio can be manipulated (2). Further catabolism of pyruvate and succinate provides nematodes with a flexibility of regulation based on oxidation and reduction of malate. Succinate may be excreted or metabolized to branched-chain fatty acids (2,6,34). Lactate could also arise independently of traditional glycolysis from mitochondrial pyruvate via an intramitochondrial LDH (2). Ethanol production and ADH activity have been reported in free-living and mycophagous nematodes. The ethanol produced is remetabolized via the glyoxylate cycle for synthesis and storage of glycogen (6,13). Ethanol probably is synthesized from acetaldehyde via pyruvate decarboxylase or from acetyl -CoA via pyruvate dehydrogenase. *Panagrellus redivivus* Goodey uses a mitochondrial pyruvate decarboxylase associated with the pyruvate dehydrogenase complex to decarboxylate pyruvate anaerobically to acetaldehyde in an NAD^+-dependent manner. ADH then catalyzes acetaldehyde reduction to ethanol (3). Pyruvate dehydrogenase activity has not been identified in nematodes (2,34).

Glycerol synthesis in nematodes appears to be via reduction of dihydroxyacetone phosphate catalyzed by a NAD^+-linked glycerol phosphate dehydrogenase coupled to ATP production (2,6).

Investigation of metabolism of *B. xylophilus* and *B. mucronatus* is important because excreted metabolic end products may elicit phytochemical host responses. In addition, CO_2 fixation via PEPCK might be important for pinewood nematode survival in resin canals where pO_2 is probably low and pCO_2 high. Furthermore, excretion of excessively acidic end products by the nematode might adversely affect the host's physical environment through tissue destruction and alteration of internal pH. There also are ramifications for the chemical environment of host cells and for host survival if the glyoxylate cycle operates in *B. xylophilus*. This cycle would allow use of the host's supply of acetyl CoA for nematode energy production which, coupled with diversion of acetyl CoA for terpene synthesis and decreased photosynthesis, could put the host into energy stress and accelerate wilting.

When *B. xylophilus* pathotypes MPSy-1 and VPSt-1 were cultured on *Botrytis cinerea*, Pers. Fr. nonparasitic populations, MPSy-1av and VPSt-1av, respectively, evolved. These populations do not establish in pine seedlings (12).

Carbohydrate catabolism was studied in adults and juveniles of MPSy-1, VPSt-1, MPSy-1av, VPSt-1av, and *B. mucronatus* recovered from fungal cultures and axenized with antibiotics (7). Nematodes were washed with *Caenorhabditis briggsae*, Dougherty and Nigon-minimal salts (CbMM-ms, 7), pH 5.0 potassium phosphate buffer (P buffer), or homogenization buffer (H buffer, 50 mM imidazole pH 7.6, 20 mM dithiothreitol, 100 μg/ml bovine serum albumen) for determining metabolic end products or enzyme activities. All nematode suspensions were analyzed for bacterial or fungal contamination by standard microbiological procedures. To determine metabolic end products, 2.5×10^5 nematodes/ml were incubated at 26 C in CbMM-ms containing 50 μCi/ml of either D[U^{14}C]-glucose (sp. act. 224 mCi/mmol), or [U^{14}C]-$NaHCO_3$ (sp. act. 56 mCi/mmol). Respired $^{14}CO_2$ was collected on KOH-saturated filter paper. After incubation for 2 or 18 hours, nematodes were collected by centrifugation at 500 *g*. Excreted products were determined in the incubation medium by thin layer chromatography (TLC), descending paper chromatography, and gas-liquid chromatography (GC) (39). Nematodes were homogenized in sterile water to determine the presence of meta-

TABLE 1. Ethanol and CO_2 production by *Bursaphelenchus xylophilus* populations and by *B. mucronatus*.

Nematode population	Ethanol†		CO_2‡
	Excreted	Retained	
B. xylophilus			
MPSy-1	0.42 ± 0.32	0.08 ± 0.05	$12 \times 10^6 \pm 8 \times 10^5$
MPSy-1av	3.41 ± 1.09	3.89 ± 1.24	140 ± 146
VPSt-1	0	0	$8 \times 10^6 \pm 8 \times 10^5$
VPSt-1av	2.20 ± 1.01	1.68 ± 0.97	
B. mucronatus	2.10 ± 0.96	2.97 ± 0.89	266 ± 224

Five separate replicates were assayed in duplicate.
† Values are reported as the mean pmoles per nematode ± standard error of the mean.
‡ Values are reported as cpm of $^{14}CO_2$ evolved per 1×10^6 nematodes per hour ± standard error of the mean.

bolic intermediates and end products. Perchlorate was added to the homogenate to a final concentration of 0.5% to precipitate protein. The samples were neutralized with KOH, and after centrifugation at 10,000 *g* for 10 minutes to remove the protein precipitate, the supernatant fractions were acidified with 0.1 N HCl and analyzed by TLC, GC, or paper chromatography. Thin layer chromatograms were analyzed by autoradiography and paper chromatograms by radiometric scanning or by colorimetric methods (39). Ethanol was determined spectrophotometrically based on ADH catalyzed reduction of NAD^+ coupled with the oxidation of ethanol. NADH production was measured by a change in absorbance at 340 nm (1). Scintillation spectrometry of the KOH-saturated filter paper was used for $^{14}CO_2$ determination. Glycerol was determined chromatographically (39).

Nematodes were homogenized in H buffer, and the homogenate was centrifuged at 30,000 *g* for 1 hour (S-30). Activities of ADH, LDH, PK, PEPCK, and isocitrate lyase (ICL) were determined by adding an aliquot of S-30 supernatant to an appropriate reaction mixture. Reactions were initiated by adding substrate, and reaction rate was recorded on a Gilford recording spectrophotometer. ADH activity, in the direction of ethanol production, was determined by changes in absorbance at 340 nm associated with reduction of NAD^+ (1,3). LDH activity in the direction of pyruvate reduction was measured by changes in absorbance at 340 nm from oxidation of NADH coupled to reduction of pyruvate. PK activity was estimated spectrophotometrically in a coupled reaction in which phosphoenolpyruvate was dephosphorylated to pyruvate coupled with the LDH-catalyzed oxidation of NADH (1). PEPCK activity was measured in a reaction couple in which OAA, formed by the carboxylation of pyruvate by PEPCK, was reduced to malate by malate dehydrogenase coupled with the oxidation of NADH (1). ICL-catalyzed glyoxylate formation was measured spectrophotometrically at 520 nm as an increase in a glyoxylate-phenylhydrazone-potassium ferricyanide complex (26). All enzyme activities are reported as μmoles product formed per minute per 10^6 nematodes. All experiments were replicated five times, and each replicate was assayed in triplicate.

MPSy-1av and *B. mucronatus* excreted ethanol as the primary end product of carbohydrate catabolism, whereas MPSy-1 and VPSt-1 produced either no ethanol or only insignificant amounts (Table 1). In nonparasitic populations, the concentration of ethanol excreted appeared to be in equilibrium with the concentration retained. Interestingly a subpopulation of VPSt-1 (VPSt-1av), which initially produced no ethanol, began ethanol production after 4 months on fungal cultures. Ethanol production was associated with a decrease in virulence. After 6 months on fungal culture, 25,000 VPSt-1av failed to infect white pine seedlings. Another subpopulation of

TABLE 2. Activities of alcohol dehydrogenase (ADH), lactic dehydrogenase (LDH), pyruvate kinase (PK), phosphenolpyruvate carboxykinase (PEPCK), and isocitrate lyase (ICL) in populations of *Bursaphelenchus xylophilus* and *B. mucronatus*.

Nematode populations	ADH	LDH	ADH/ LDH	PK	PEPCK	PK/ PEPCK	ICL
B. xylophilus							
MPSy-1av	0.60 ± 0.02	0.42 ± 0.07	1.43	0.65 ± 0.04	0.16 ± 0.02	4.21	0.80 ± 0.05
MPSy-1	0.18 ± 0.03	2.20 ± 0.06	0.08	0.32 ± 0.03	3.42 ± 0.15	0.093	0.13 ± 0.07
VPSt-1	0.13 ± 0.05	1.14 ± 0.05	0.11	0.07 ± 0.08	1.00 ± 0.12	0.068	
VPSt-1av	0.41 ± 0.07	1.30 ± 0.07	0.316	0.35 ± 0.04	0.07 ± 0.01	5.28	0.63 ± 0.05
B. mucronatus	0.58 ± 0.07	0.37 ± 0.04	1.58	0.13 ± 0.02	0.03 ± 0.01	4.16	0.12 ± 0.08

Values are reported as mean μmoles of substrate hydrolyzed per min per nematode. Five separate replicates were assayed in triplicate for each enzyme.

VPSt-1, cultured alternately on fungus and through white pine seedlings, retained pathogenicity and did not produce ethanol. Nonparasitic populations consistently produced minimal quantities of CO_2, whereas parasitic populations produced significantly higher amounts (Table 1). MPSy-1av and *B. mucronatus* also excreted some malate and succinate-lactate. Populations MPSy-1 and VPSt-1 excreted succinate-lactate, but not malate, and glycerol as metabolic end products.

These observations suggest significant differences in energy metabolism in the parasitic and nonparasitic populations of *Bursaphelenchus*. To verify this, activities of crucial metabolic enzymes were determined. LDH, PK, PEPCK, and ICL were present in all populations. ADH activity was about four times greater in MPSy-1av and *B. mucronatus* than in MPSy-1, whereas LDH activity was 3–5 times greater in MPSy-1 and VPSt-1 than in MPSy-1av and *B. mucronatus* (Table 2). These results support the difference in pathways for pyruvate metabolism in the parasitic and nonparasitic populations. ADH activity was also present in VPSt-1av (Table 2). PK activity was high in ethanol-producing populations, whereas PEPCK activity was high in populations not producing ethanol. The PK/PEPCK ratio supports the hypothesis that populations not producing ethanol use an alternate PEP-succinate pathway of metabolizing carbohydrates (2,6,34).

ICL activity was about five times higher in nonparasitic populations of *B. xylophilus* than in parasitic populations (Table 2). Metabolism of acetyl CoA through this cycle could produce succinate and malate excreted by these nematodes. The parasitic populations could use this pathway, not only for the production of succinate and malate, but also to derive precursors for glycerol biosynthesis.

Enzyme activities in, and metabolite production by, VPSt-1av are not entirely consistent with nonparasitism (Tables 1, 2), but suggest that this isolate was in transition at the time of study.

Energy metabolism in *Bursaphelenchus* is similar to that of other nematodes (2,6,34), and there is a metabolic difference between the parasitic and nonparasitic forms. The presence of PK, LDH, and ADH activities coupled with the production of lactate, ethanol, and CO_2 from glucose confirms traditional anaerobic glycolysis via the Embden-Meyerhof pathway. PEPCK activity in the parasitic populations coupled with the production of malate and succinate from glucose or sodium bicarbonate indicates a PEP-succinate pathway (2,34). ICL activity indicates the presence of the glyoxylate cycle (2,25). ADH activity and ethanol production only in MPSy-1av and VPSt-1av indicate that an alteration in metabolism occurs in these populations when they are consistently maintained on *B. cinerea*. In nonparasitic populations, redox balance could be maintained and energy conserved by production of 1–2 moles of ATP per mole of glucose hydrolyzed. Redox balance could be maintained in the

parasitic populations when glycerol is produced from pyruvate by a glucose dismutation and 1 mole of ATP per mole of glucose is generated.

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