Journal of Nematology 19(3):304–310. 1987.

Characterization of a Nonparasitic Isolate of *Bursaphelenchus xylophilus*[1]

R. I. Bolla,[2] C. Weaver,[2] P. Koslowski,[2] K. Fitzsimmons,[2] and R. E. K. Winter[3]

Abstract: *Bursaphelenchus xylophilus* isolate MPSy-1av was subcultured from pathotype MPSy-1. MPSy-1av is nonparasitic and does not establish in *Pinus sylvestris, P. strobus, P. nigra,* or *P. taeda.* This isolate produces ethanol as an end product of carbohydrate metabolism, whereas its parent pathotype, MPSy-1, does not. Alcohol dehydrogenase activity was easily detectable in homogenates of MPSy-1av but barely detectable in some homogenates of MPSy-1. Genomic differences were seen between MPSy-1 and MPSy-1av by restriction endonuclease analysis of total nematode DNA, and hybridization of DNA fragments to the alcohol dehydrogenase gene from *Drosophila.*

Key words: alcohol, alcohol dehydrogenase, *Bursaphelenchus mucronatus, Bursaphelenchus xylophilus,* metabolism, pathotype, pinewood nematode, *Pinus sylvestris, P. strobus, P. nigra, P. taeda.*

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934; Nickle, 1970) is associated with wilting of various conifers in the United States, Canada, and Japan (13). Differences in host preference and in response of pines to nematode inoculation have led to the suggestion that pathotypes exist (4,9,18–20).

Large populations of *B. xylophilus* develop rapidly in the laboratory on *Botrytis cinerea* and several other fungi grown on potato dextrose agar (PDA). These cultures are maintained by serial passage at 1–2-month intervals (13), which circumvents maintaining the nematodes in compatible pine species and facilitates obtaining sufficient numbers for biochemical studies.

Tylenchid nematodes include insect parasites, mycophagous nematodes, and phytoparasites; as a consequence, different strategies for energy metabolism might be expected (3,7,14,17). Some tylenchids switch from typical aerobic pathways to anaerobic pathways when placed in environments where oxygen and (or) nutritional substrates are limited (7,14,17). Other members of this order, such as *Ditylenchus dipsaci* and *D. triformis,* retain most of the enzymes for typical aerobic energy metabolism; however, a fumarate reductase pathway, similar to that described for some microaerobic animal parasitic nematodes, seems to be used (1,3,14).

Bursaphelenchus xylophilus lives as a fungal feeder or as a phytoparasite within the resin canals of conifers. Pathotype MPSy-1, an isolate of *B. xylophilus* from *Pinus sylvestris* in Missouri, induces wilting of *P. sylvestris* but not of *P. strobus, P. nigra,* or *P. taeda* (4). A second pathotype, VPSt-1, isolated from *P. strobus* in Vermont, causes wilting of *P. strobus* but not of *P. sylvestris, P. nigra,* or *P. taeda* (4). During the course of our studies, a nonparasitic isolate of *B. xylophilus* was subcultured from a population of the *P. sylvestris* specific pathotype MPSy-1 (4). Our objectives were to describe this nonparasitic subpopulation, designated MPSy-1av to indicate its origin from pathotype MPSy-1 (4), and to compare one aspect of its energy metabolism with that of MPSy-1, VPSt-1, *B. mucronatus,* and *Caenorhabditis elegans.*

Materials and Methods

Culture of nematodes: Bursaphelenchus xylophilus was surface sterilized with antibiotics and cultured at 25 C on *B. cinerea*

Received for publication 29 September 1986.

[1] This research was supported in part by grants CRGO-84-CRCR-1-1423 and USDA-86-CRCR-1-1984 from the U.S. Department of Agriculture and by a grant from the Weldon Springs Fund, University of Missouri–St. Louis.

[2] Department of Biology, University of Missouri–St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121.

[3] Department of Chemistry, University of Missouri–St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121.

We thank D. Hartl, Department of Genetics, Washington University Medical School, for the *Drosophila* alcohol dehydrogenase gene probe, and the Missouri Department of Conservation for pine seedlings. We also thank Dr. J. Starling, Department of Biology, University of Missouri–St. Louis, for advice on enzyme assays.

growing on PDA (4). Nematodes were subcultured monthly. The nematodes were removed from fungal cultures and washed once with sterile phosphate buffered 0.9% NaCl (PBS, pH 7.4), and ca. 100,000 nematodes were transferred to fresh 2-week-old fungal cultures. Infectivity was tested by inoculation of 3-year-old *P. sylvestris* seedlings with 5,000 nematodes at each subculture; the seedlings were observed for wilt symptoms, and nematode population size was estimated (4,13). At the time of subculture, an aliquot of nematodes from each culture was fixed in Heckler's solution and examined microscopically. *C. elegans* was cultured on *Escherichia coli* OP-50 as described (7).

Seedling inoculations: Three-year-old *P. sylvestris, P. strobus, P. nigra,* and *P. taeda* field-grown seedlings, 30–50 cm tall and 5–8 cm in circumference, were placed in a greenhouse at 25 ± 5 C with a 12-hour photoperiod. After growth of new shoots occurred, 20 seedlings of each species were inoculated with either 5,000, 25,000, or 60,000 MPSy-1 or MPSy-1av through a scraped area at the midpoint on the stem (4,13).

Energy metabolism: Nematodes were removed from fungal cultures with sterile PBS containing 50 μg/ml streptomycin sulfate, 1,000 U/ml penicillin, 50 U/ml tetracycline, and 50 μg/ml nystatin (AB buffer). They were collected by filtration through a 20- × 5-cm column of 2-mm-d sterile glass beads and washed five times by settling through AB buffer and twice by settling through sterile PBS. An aliquot of the final wash solution was analyzed for microbial contamination by standard microbiological procedures. Approximately 5×10^6 nematodes were suspended in 5 ml *Caenorhabditis* minimal salts medium (5) containing 10 mM glucose and (or) 10 mM $NaHCO_3$ in cotton plugged test tubes (150 × 10 mm) and incubated for 10 hours at 25 C. Nematodes were recovered from the incubation medium by centrifugation at 500 *g* for 5 minutes, and the supernatant was saved. The nematode pellet was homogenized in H buffer (50 mM imidazole, 100 μg/ml bovine serum albumen, 2 mM dithiothreitol, pH 7.6). A supernatant fraction (S-10) was prepared by centrifugation for 30 minutes at 10,000 *g*. Aliquots of the S-10 and incubation medium were tested on thioglycollate broth, PDA, and nutrient agar for microbial contamination. Preparations showing contamination were discarded.

Ethanol excreted into the incubation medium by nematodes was determined spectrophotometrically by changes in absorbance at 340 nm using the alcohol dehydrogenase (ADH) catalyzed oxidation of ethanol to acetaldehyde coupled to the reduction of NAD to NADH + H^+ at pH 9.0 (6,10). Results are reported as pmoles ethanol produced per nematode. Concentrations of ethanol retained in nematodes were determined by the same reaction using aliquots of the S-10 homogenate as substrate.

ADH activity in the S-10 homogenate of the nematodes was quantified by adding homogenate (enzyme) to a 3.0-ml reaction mixture containing 200 mM glycine buffer (pH 9.0), 310 mM ethanol, and 7.8 mM NAD (substrate). Changes in absorbance at 340 nm were measured with a Gilford recording spectrophotometer. Results are reported as specific activity of ADH (μmoles ethanol oxidized per minutes per nematode). Lactic dehydrogenase (LDH) activity was measured in S-10 nematode homogenates by standard methods based on the reduction of pyruvate to lactate coupled to oxidation of NADH + H^+ to NAD recorded by changes in absorbance at 340 nm (6). Results are reported as LDH specific activity (μmoles NADH + H^+ oxidized per minute per nematode).

C. elegans, which synthesizes ethanol when incubated as described (1), was used as a positive control.

Five separate incubations were established, and the medium and nematode homogenate from each incubation were assayed separately for ethanol and enzyme activities.

Isolation of DNA and analysis of the ADH gene: Nematode total genomic DNA was isolated and digested with restriction en-

TABLE 1. Ethanol production of *Bursaphelenchus xylophilus* isolates MPSy-1, MPSy-1av, and VPSt-1 and by *B. mucronatus* and *Caenorhabditis elegans* in pmoles per nematode.

Nematode isolate	Ethanol	
	In incubation medium	In S-10
MPSy-1	0.419 ± 0.324	0.083 ± 0.056
MPSy-1av	3.413 ± 1.092	3.89 ± 1.24
VPSt-1	0	0
B. mucronatus	2.099 ± 0.960	2.97 ± 0.89
C. elegans	2.950 ± 1.450	Not done

Mean ± standard error. Five separate replicates were assayed in duplicate.

donucleases Hae III, Xho I, and Mbo I at 1 μg DNA per five units of enzyme for 3 hours at 37 C (8,16,19). Loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, and 100 mM EDTA in 50% glycerol) was added to the digestion mixture, and DNA fragments were separated by electrophoresis in 1% agarose gels containing 50 μg/ml ethidium bromide. DNA fragments were visualized at 310 nm and transferred to nitrocellulose paper (16) for hybridization to a 1.0-kilobase fragment of DNA containing a 650-base pair Xba I fragment of the *Drosophila* ADH gene. This gene fragment, inserted into the unique Xho restriction site of plasmid pPA-1, was transfected into *Escherichia coli,* strain HB-101 (11). For use as a hybridization probe, this gene fragment was digested from the plasmid with Xho I and isolated after electrophoresis on 1% low melting point agarose (16). The purified fragment was radioactively labelled by nick translation with [^{32}P]dCTP and [^{32}P]TTP (16). Hybridization was for 24 hours at 42 C using standard procedures (16,18). After washing twice for 15 minutes each with 0.1% SDS, 1× SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) at 22 C and twice for 30 minutes with 0.5% SDS, 0.1× SSC at 42 C, the hybridizations were exposed to X-ray film for autoradiography. Autoradiograms were analyzed by densitometry, and fragment sizes were estimated by comparison to standard sized DNA fragments separated on the same gel. This procedure resolves DNA fragments with differences of 100 base pairs or greater (17).

RESULTS

Identification of a nonparasitic isolate of B. xylophilus: Subpopulation MPSy-1av appeared during serial passage of *B. xylophilus* pathotype MPSy-1 on *B. cinerea* cultures. Three-year-old *P. sylvestris, P. strobus, P. nigra,* and *P. taeda* seedlings inoculated with 5,000, 25,000 or 60,000 MPSy-1av did not develop wilt symptoms through at least 240 days after inoculation. *P. sylvestris* seedlings inoculated with 5,000 MPSy-1 often wilted in 10–15 days, and all wilted by 32 days after inoculation. MPSy-1 did not cause wilting of *P. nigra, P. strobus,* or *P. taeda* seedlings.

Only 3 ± 2 large, apparently dead, or dying females per seedling (mean ± SEM, N = 20 seedlings) were recovered 15 days after inoculation of *P. sylvestris* seedlings with either 25,000 or 60,000 MPSy-1av. No nematodes were recovered from seedlings 30–90 days after inoculation. When 5,000 MPSy-1av were inoculated into *P. sylvestris* seedlings, no nematodes were re-

TABLE 2. Activities of alcohol dehydrogenase (ADH), lactic dehydrogenase (LDH), pyruvate kinase (PK), and phosphoenol pyruvate carboxykinase (PEPCK) in homogenates of *Bursaphelenchus xylophilus* isolates MPSy-1, MPSy-1av, and VPSt-1 and in *B. mucronatus.*

Nematode isolate	ADH	LDH	PK	PEPCK	PK/PEPCK
MPSy-1av	0.595	0.416	0.653	0.155	4.21
MPSy-1	0.182	2.20	0.316	3.42	0.093
VPSt-1	0.131	1.14	0.068	1.00	0.068
B. mucronatus	0.577	0.366	0.129	0.03	4.20

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

covered 5–120 days after inoculation. In comparison, *P. sylvestris* seedlings inoculated with 5,000 MPSy-1 yielded 130,000 ± 27,580 juveniles and adults per seedling (N = 20 seedlings) 15 days after inoculation. No nematodes were recovered from *P. nigra, P. strobus,* or *P. taeda* seedlings inoculated with 5,000, 25,000, or 60,000 MPSy-1av through 240 days after inoculation. MPSy-1 and MPSy-1av were morphologically identical, and the microscopic characteristics were consistent with identification of these isolates as *B. xylophilus.*

Ethanol production: When axenic MPSy-1av were incubated for 10 hours in *Caenorhabditis* minimal salts medium containing glucose and $NaHCO_3$, 3.413 ± 1.09 pmoles ethanol per nematode (Table 1) was excreted into the medium. Analysis of the S-10 fraction from these nematodes indicated that 3.89 ± 1.24 pmoles ethanol per nematode was retained inside the nematode (Table 1). MPSy-1 incubated under identical conditions excreted only 0.419 ± 0.324 pmoles of ethanol per nematode, and there was only a trace of ethanol in the

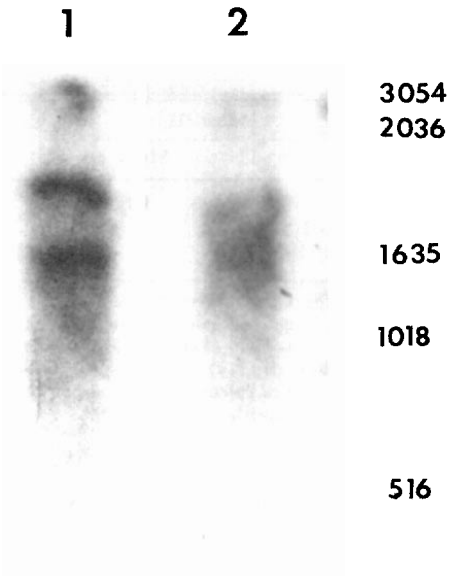

FIG. 1. Autoradiogram of hybridized blot showing differences in the alcohol dehydrogenase (ADH) codon from isolates MPSy-1 (lane 1) and MPSy-1av (lane 2) of *Bursaphelenchus xylophilus* total genomic DNA detected in Hae III-generated DNA fragments probed with the ADH gene from *Drosophila.* Position of marker fragments are indicated to the right of the autoradiogram.

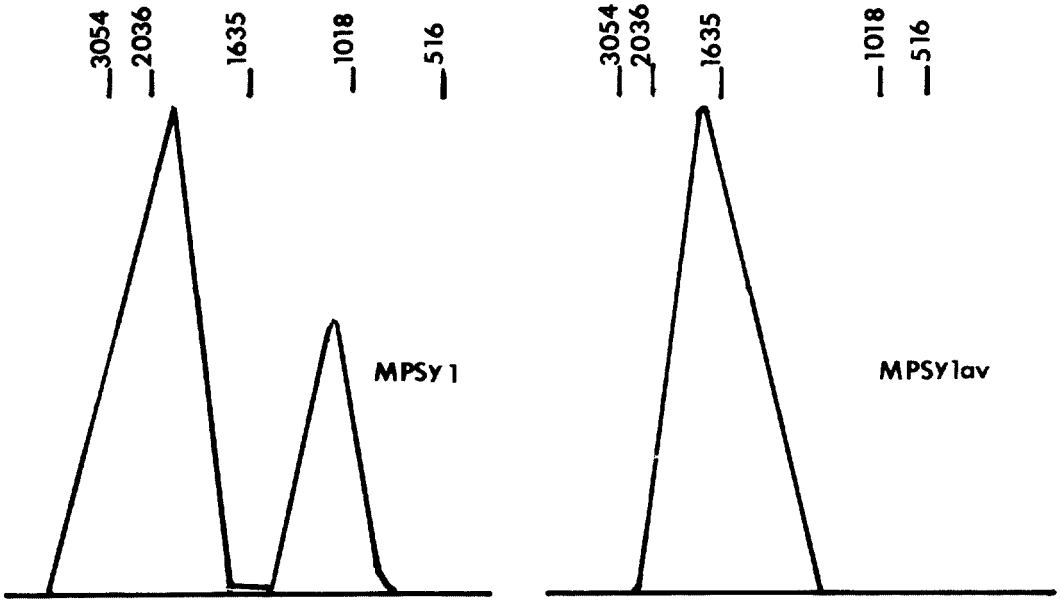

FIG. 2. Densitometric tracing of autoradiogram shown in Figure 1. Mobility and size of marker fragments are indicated.

TABLE 3. Fragment-size† differences between the alcohol dehydrogenase codons of *Bursaphelenchus xylophilus* pathotype MPSy-1 and isolate MPSy-1av.

	Hae III		Mbo I		Xho I	
Fragments	MPSy-1	MPSy-1av	MPSy-1	MPSy-1av	MPSy-1	MPSy-1av
1	1,968	1,834	4,823	2,969	5,408	6,642
2	1,381		2,277	2,350	1,062	
3				1,998		

DNA from MPSy-1 and MPSy-1av was cleaved with the restriction endonucleases Hae III, Mbo I, and Xho I and electrophoresed on 1% agarose gels. Fragments were blotted to nitrocellulose paper (16) and hybridized to a fragment of the ADH gene from *Drosophila* (11). Hybridized blots were exposed to X-ray film; after development, autoradiograms were analyzed by densitometry (17).

† Fragment sizes were calculated in base pairs by comparison to migration of known DNA fragments separated electrophoretically in parallel with nematode DNA.

S-10 homogenates. Pathotype VPSt-1 did not produce or excrete detectable amounts of ethanol; however, *B. mucronatus* excreted and retained ethanol in nearly equal amounts. *C. elegans,* used as a control, produced moderate amounts of ethanol.

Alcohol dehydrogenase (ADH) activity was ca. fivefold greater in homogenates of MPSy-1av and *B. mucronatus* than in MPSy-1 and VPSt-1, whereas lactic dehydrogenase (LDH) activity was greater in MPSy-1 and VPSt-1 (Table 2). Pyruvate kinase (PK) activity was ca. twofold greater in MPSy-1av than in MPSy-1, and phosphoenol pyruvate carboxykinase (PEPCK) activity was nearly 20-fold greater in MPSy-1 than in MPSy-1av.

Differences in the ADH gene: Cleavage of MPSy-1av genomic DNA with Hae III yielded a single 1,834-base pair DNA fragment with sequence complementarity to the ADH gene fragment from *Drosophila,* whereas fragments of 1,968 and 1,381 base pairs were identified from MPSy-1 (Figs. 1, 2; Table 3). Xho I cleavage of MPSy-1 total genomic DNA generated fragments of 5,408 and 1,062 base pairs with sequence complementarity to the *Drosophila* ADH gene, whereas only one fragment, 6,642 base pairs, with ADH sequence complementarity was seen in MPSy-1av DNA (Table 3). Differences were also seen when Mbo I was used to restrict nematode DNA.

DISCUSSION

The nonpathogenic population of *B. xylophilus,* MPSy-1av, could have arisen by random selection if the original population was a mixture of MPSy-1 and MPSy-1av, or by random mutation within cultures of MPSy-1 which was subsequently isolated by selection. Selection might occur from differences in nutritional substrates available to the nematode feeding on fungi as compared with feeding as a phytoparasite on parenchyma cells in resin canals of pine trees. Kiyohara (12) reported mixed populations of *B. xylophilus,* varying in pathogenicity and virulence, from Japan and isolated a naturally occurring avirulent population of *B. xylophilus* from *Pinus thunbergii* in Kagawa prefecture. This isolate appears, however, to differ from MPSy-1av in that it can reproduce on pine. MPSy-1av does not survive when inoculated into *P. sylvestris* which is a good host for MPSy-1. On the basis of these observations, however, it is seemingly possible that an isolate such as MPSy-1av could arise in a natural population where it would survive as a mycophagous nematode probably not associated with pines.

Metabolic differences between MPSy-1 and MPSy-1av are suggested by differences in their glucose catabolism pathways. Ethanol production by MPSy-1av assures redox balance and continued metabolism. Additionally, ethanol production in the mitochondria may serve as an electron sink, effectively removing a proton and preventing tissue acidification (2). MPSy-1av apparently metabolizes carbon substrates to pyruvate which then may be decarboxylated to acetaldehyde by pyruvate decar-

boxylase activity possibly associated with the ADH enzyme complex, as recently demonstrated in *Panagrellus redivivus* (2). ADH would then catalyze the reduction of acetaldehyde to ethanol. Since MPSy-1av was incubated in a nutritionally rich environment, energy derived from this pathway may be sufficient to sustain the nematode. It appears that *B. mucronatus* uses the same metabolic pathway. MPSy-1 does not produce ethanol as a metabolic end product suggesting that carbohydrate metabolism in this population is via a different pathway.

Additional metabolic differences between MPSy-1 and MPSy-1av are suggested by comparison of the activity ratios of ADH to LDH (1). The ratios observed support the concept that ethanol is produced by nonparasitic forms but not by parasitic forms. The PK to PEPCK ratios further support this interpretation (1).

Restriction endonuclease analysis of the ADH gene from MPSy-1av and MPSy-1 suggests possible genetic differences between the populations. These differences may be either within the coding sequences of the ADH gene, in introns, or in regulatory sequences upstream or downstream from the coding sequences. Several upstream and downstream sequences have been shown to be involved in regulation of gene transcription and processing (15). The effect of such differences in the ADH gene on synthesis and activity of ADH depends upon the location of the alterations within the codon. If these differences are within regulatory regions, the type and concentration of mRNA transcribed would be affected as would the transcriptional rate (15). And if the genomic differences are within the ADH coding sequence, the protein translated would be affected, which might be reflected in the pattern and activity of ADH isozymes transcribed.

Literature Cited

1. Barrett, J. 1984. The anaerobic end-products of helminths. Parasitology 88:179–198.

2. Barrett, J., and P. E. Butterworth. 1984. Acetaldehyde formation by mitochondria from the free-living nematode *Panagrellus redivivus*. Biochemical Journal 221:535–540.

3. Bolla, R. I. 1980. Nematode energy metabolism. Pp. 165–192 *in* B. M. Zuckerman, ed. Nematodes as biological models, vol. 2 New York: Academic Press.

4. Bolla, R. I., R. E. K. Winter, K. Fitzsimmons, and M. J. Linit. 1986. Pathotypes of the pinewood nematode, *Bursaphelenchus xylophilus*. Journal of Nematology 18:230–238.

5. Buecher, E. J., E. L. Hansen, and E. A. Yarwood. 1970. Growth of nematodes in defined medium containing hemin and supplemented with commercially available proteins. Nematologica 16:403–409.

6. Clark, J. M., Jr., and R. L. Switzer. 1977. Experimental biochemistry. San Francisco: W. H. Freeman Co.

7. Cooper, A. F., and S. D. Van Gundy. 1971. Ethanol production and utilization by *Aphelenchus avenae* and *Caenorhabditis* spp. Journal of Nematology 3: 205–214.

8. Curran, J., D. L. Baillie, and J. M. Webster. 1985. Use of genomic DNA restriction fragment length differences to identify nematode species. Parasitology 90:137–144.

9. Dwinell, L. D. 1985. Relative susceptibilities of five pine species to three populations of the pinewood nematode. Plant Disease 69:440–442.

10. Geraci, G., and Q. H. Gibson. 1967. The reaction of liver alcohol dehydrogenase with reduced diphosphopyridine nucleotide. Journal of Biological Chemistry 242:4275–4286.

11. Goldberg, D. A., J. W. Posakony, and T. Maniatis. 1983. Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germ line. Cell 34:59–73.

12. Kiyohara, T. 1985. Induced resistance in pine wilt disease. Pp. 178–184 *in* V. H. Dropkin, ed. Proceedings of the United States–Japan Seminar: Resistance mechanisms of pines against pine wilt disease. Columbia, Missouri: University of Missouri Press.

13. Kondo, E., A. Foudin, M. Linit, M. Smith, R. Bolla, R. E. K. Winter, and V. H. Dropkin. 1982. Pinewilt disease: Nematological, entomological, and biochemical investigations. Bulletin SB-372 University of Missouri Experiment Station, Columbia, Missouri.

14. Krusberg, L. R. 1971. Chemical composition of nematodes. Pp. 213–234 *in* B. M. Zuckerman, W. F. Mai, and R. A. Rhode, eds. Plant parasitic nematodes, vol. 2. New York: Academic Press.

15. Lewin, B. 1984. Gene II. New York: John Wiley and Sons.

16. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

17. Mendis, A. H. W., and A. A. F. Evans. 1984. Substrates respired by mitochondrial fractions of two isolates of the nematode *Aphelenchus avenae* and the effects of electron transport inhibitors. Comparative Biochemistry and Physiology 78B:373–378.

18. Weaver, C., and R. I. Bolla. 1986. Pathotypes

of *Bursaphelenchus xylophilus* show genetic differences. Journal of Nematology 18:635 (Abstr.).

19. Wingfield, M. J., R. A. Blanchette, and E. Kondo. 1983. Comparison of the pine wood nematode *Bursaphelenchus xylophilus* from pine and balsam fir. European Journal of Forest Pathology 13:360–372.

20. Wingfield, M. J., P. J. Bedker, and R. A. Blanchette. 1986. Pathogenicity of *Bursaphelenchus xylophilus* on pines in Minnesota and Wisconsin. Journal of Nematology 18:44–49.