

Pathotypes of the Pinewood Nematode

Bursaphelenchus xylophilus

R. I. Bolla, R. E. K. Winter, K. Fitzsimmons, and M. J. Limit

Abstract: An isolate of Bursaphelenchus xylophilus from Pinus sylvestris in Missouri infected and reproduced in 2–3-year-old seedlings of P. sylvestris and to some extent in seedlings of P. nigra. Wilting, however, occurred only in P. sylvestris. B. xylophilus isolated from P. strobus in Vermont infected and reproduced only in P. strobus seedlings. P. taeda seedlings were resistant to both of these isolates. Phytotoxin production was seen only in susceptible seedling species–nematode combinations. Significant water loss occurred only in those seedlings that were wilted because of infection by a compatible nematode isolate. Our results suggest that these isolates are pathotypes of B. xylophilus.

Key words: nematode pathotypes, pathology, pinewood nematode, phytotoxin, Pinus nigra, Pinus strobus, Pinus sylvestris, Pinus taeda, resistance, susceptibility.

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934; Nickle, 1970; syn. B. lignicolus, Mamiya and Kiyohara, 1972) is indigenous to the United States and has been reported in 27 pine and 7 nonpine species (14). Pinewilt disease has been reported from southern Canada to Texas, from the Missouri River basin to the East Coast, and from Vermont to Florida (5,8,14,20–22). Pinewood nematode is also found in California and southern Nevada. The species of pine showing highest susceptibility to infection and expression of disease pathology varies regionally (8,12,14,22). In some regions of the United States, B. xylophilus appears to be a primary pathogen responsible for wilting of the tree; in other areas it appears to be a secondary infection in disease stressed trees (20,21). Geographical isolation or feeding preference of transport insects may lead to evolution of specific gene pools within this nematode species, and as a consequence, it is possible that genes involved in pathogenicity may vary from one area of the United States to another.

Wingfield et al. (22) have compared the infectivity of three B. xylophilus populations, one isolated from P. sylvestris in Missouri and one each from P. nigra and Abies balsamea in Minnesota. The pine isolates infected and caused wilting of only pine seedlings, whereas the A. balsamea isolate infected only A. balsamea seedlings. In vitro reproductive success of these isolates on fungal cultures of Botrytis cinerea and Cer-
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atocystis ips also differed (22). Such differences suggest the possible existence of pathotypes of B. xylophilus. We have investigated this possibility by comparing the susceptibility of four pine species to infection by B. xylophilus isolated from P. sylvestris in Missouri (MPSy-1) and from P. strobus in Vermont (VPSt-1).

MATERIALS AND METHODS

Source and culture of isolates: MPSy-1 was recovered from P. sylvestris in Columbia, Missouri, by Dr. V. H. Dropkin and was supplied on a culture of B. cinerea (5,8). VPSt-1 was isolated from P. strobus in Vermont by Dr. D. Bergdahl and was supplied on a culture of Pyrenochaeta sp. by Dr. R. F. Myers.

The isolates were maintained at 26 C on B. cinerea or Pyrenochaeta sp. on potato dextrose agar (PDA), subcultured monthly, and routinely assayed for contaminating bacteria and fungi. Nematode growth was comparable on both fungi.

Seedling inoculation: Two- to three-year-old field-grown seedlings of P. sylvestris, P. strobus, P. nigra, and P. taeda were brought into the greenhouse, maintained at 26 ± 5 C with a 12-hour photoperiod and watered by spraying twice weekly. When substantial new shoot growth had occurred, seedlings were inoculated with either MPSy-1 or VPSt-1 recovered from cultures by a modified Baermann procedure and axenized (2). Either 5,000 or 25,000 nematodes were applied to a wounded area made by scraping off a 2-cm-long strip of bark at the midpoint of the stem as described previously (15). This area was wrapped with sterile absorbent cotton and kept moist. Noninoculated wounded seedlings served as controls. Ten seedlings were used for each assay.

Field inoculations: In September 1984, four P. sylvestris and four P. strobus (20–30 years old) located in a pine plantation near Ashland, Missouri, were each inoculated with 60,000 VPSt-1 as previously described (8). The trees were examined monthly for wilt symptoms and for signs of insect feeding.

Analysis of host response: Greenhouse seedlings were examined twice weekly for onset of wilt symptoms including loss of resin flow to the site of a wound and leaf stomate closure, determined on 10 leaves from each seedling (15). At days 15, 30, 45, 60, and 90 postinoculation, the seedlings were removed from the pots and the soil was washed from the roots. Seedling fresh weights were determined gravimetrically. The seedlings were cut into 5-mm pieces, and nematodes were recovered overnight by a modified Baermann procedure (2) and counted. The seedling pieces were then frozen in liquid nitrogen and lyophilized, and the seedling dry weights were determined. Resin flow was estimated visually based on the amount of resin that collected at a cut site when the seedlings were cut for nematode extraction. Similarly infected seedlings were cut into small pieces which were autoclaved in five volumes of distilled water for 3 hours at 121 C, 15 psi. The resulting aqueous phase was made alkaline and extracted with CHCl₃ as previously described (3,15,17). Phytotoxicity of the extracts was estimated by bioassay on 2–3-month-old P. strobus and P. sylvestris seedlings (3,15,17). Measurements from 10 seedlings were averaged to produce each experimental point. Data are reported as mean ± SE.

Nematode mating: B. xylophilus isolates VPSt-1 and MPSy-1 and B. mucronatus were aseptically recovered from fungal cultures and used for reciprocal matings of VPSt-1 x MPSy-1 and VPSt-1 x B. mucronatus. Two fourth-stage female juveniles and five males were used for each mating. These nematodes were transferred to fresh fungal cultures and incubated for 21 days at 26 C, and juveniles in each culture were counted. Ten replications were used for each mating for a total of 40 cultures.

RESULTS

Nematode population dynamics in inoculated seedlings: When 2–3-year-old P. sylvestris seedlings were inoculated with 25,000 MPSy-1, the nematode population increased to 210,000 ± 26,000 nematodes per seedling by 15 days postinoculation and then declined throughout the further course of infection (Fig. 1A). This population decline paralleled the progression of wilt symptoms, as subsequently described. The percentage of juveniles present in the total population increased from 62.6 ± 7.0 at 15 days postinoculation to 82.4 ± 8.4 at
30 days and then declined. In *P. sylvestris* seedlings inoculated with 5,000 MPSy-1, nematodes increased to a peak of 148,000 ± 37,600 nematodes per seedling (80% were juveniles) at 30 days postinoculation and then declined (Fig. 1B). Although a slightly greater nematode population density was reached earlier in the 25,000 nematode inoculation series, the total population increase was only 6-fold, compared with 28-fold in seedlings inoculated with 5,000 MPSy-1. All inoculated seedlings wilted at 28–32 days postinoculation independent of initial inoculum size.

MPSy-1 also infected *P. nigra* seedlings; however, the maximum population size reached was significantly less than in *P. sylvestris* seedlings (Fig. 1). There was no significant difference in population growth between the two inoculum sizes. Juveniles accounted for about 80% of the total nematodes in inoculated seedlings, and peak population density was reached at 30 days postinoculation. At 90 days postinoculation, when the seedlings were still not wilted, only about 100 nematodes were recovered from each seedling. This population contained 92.0 ± 6.0% juveniles, of which 50.8 ± 18.0% were dauer juveniles. Adults recovered at this time were apparently dying, as indicated by minimal movement, irregular pumping of the esophageal bulb, empty intestine, and highly vacuolated intestinal cells.

When *P. strobus* seedlings were inoculated with 25,000 MPSy-1, the population increased slightly by 15 days postinoculation and then declined rapidly (Fig. 1A). By 30 days postinoculation, no adults were recovered from the seedlings and 35.8 ± 6.3% of the juveniles recovered were dauer juveniles. With 5,000 MPSy-1, infection did not establish in *P. strobus* seedlings, and, although some adults were recovered 15 days postinoculation, by 30 days no nematodes were isolated from seedlings (Fig. 1B). Adults recovered appeared to be dying as indicated by the lack of motility, irregular pumping of the esophageal bulb, empty intestine, and highly vacuolated intestinal cells.

The MPSy-1 isolate did not establish in *P. taeda* (Table 1).

Results from seedling inoculation with VPSt-1 suggest that this nematode isolate has a specificity or preference for infection of *P. strobus*. When 2–3-year-old *P. strobus* seedlings were inoculated with either 5,000 or 25,000 VPSt-1, the population size increased through 30 days postinoculation and then declined (Fig. 2). All seedlings wilted at 32–40 days postinoculation. In *P.
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Table 1. Reaction of four pine species to the Missouri (MPSy-1) and Vermont (VPSt-1) isolates of *Bursaphelenchus xylophilus*.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>P. sylvestris</th>
<th>P. strobus</th>
<th>P. nigra</th>
<th>P. taeda</th>
</tr>
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<tbody>
<tr>
<td>Reproduction</td>
<td></td>
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<tr>
<td>5,000 nematodes</td>
<td>++</td>
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<td>25,000 nematodes</td>
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<tr>
<td>Wilt</td>
<td>++</td>
<td>-</td>
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<tr>
<td>Phytotoxin</td>
<td>++</td>
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<tr>
<td>Stomate closure</td>
<td>++</td>
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<tr>
<td>% dry weight</td>
<td>++</td>
<td>#</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

Observed reactions: ++ = definite recorded reaction of seedlings sustained throughout infection until time seedling wilted and did not reverse during presence of nematode in seedling. + = slight or moderate reaction of seedlings seen only at high inoculation levels. # = reaction of seedlings seen only during initial period following inoculation of seedling with nematode, induced to a limited extent, reversed late in study period. - = no reaction of seedlings.

*strobus* seedlings inoculated with 25,000 VPSt-1, maximum population size of 290,000 ± 38,000 nematodes per seedling (a 12-fold increase) was reached at 30 days postinoculation. Reproduction was greatest in *P. strobus* seedlings challenged with 5,000 VPSt-1 where the resultant maximum nematode population reached 1,020,000 ± 280,000 nematodes per seedling, with juveniles and adults present in equal numbers (Fig. 2B). The population of both adults and juveniles decreased rapidly between 30 and 90 days postinoculation. The decrease was greater where 5,000 nematodes were inoculated than where 25,000 nematodes were inoculated (Fig. 2). At both inoculum levels more than 40% of the juveniles recovered in the later stages of infection were dauer juveniles. Adults remained active and appeared to be feeding. By 90 days postinoculation, fewer than 1,000 nematodes were recovered from the seedlings inoculated with either 5,000 or 25,000 VPSt-1, 726 ± 86 and 942 ± 56 nematodes per seedling, respectively.

VPSt-1 reproduced in *P. sylvestris* challenged with 25,000 nematodes, but the population did not reach the densities reached in *P. strobus* and the seedlings did not wilt. The nematode population in these seedlings declined 15-fold during the first 15 days postinoculation and then increased to 59,000 ± 12,800 nematodes per seedling by 30 days postinoculation. The populations then began to decline until by 90 days postinoculation there were only 5,600 ± 3,160 nematodes per seedling, of which 80.4 ± 12.2% were juveniles, including 60.0 ± 9.7% dauer juveniles (Fig. 2A). Fifteen days after inoculation of *P. sylvestris* seedlings with 5,000 VPSt-1, 6,200 ± 1,320 nematodes per seedling were recovered. Nematodes increased to 30,000 ± 9,600 per seedling by 30 days postinoculation and then decreased until by 90 days postinoculation there were only 485 ± 163 nematodes per seedling, of which 81.4 ± 4.6% were juveniles, including 72.6 ± 18.0% dauer juveniles. Adults recovered at this time were either dead or dying.

The VPSt-1 isolate did not establish in either *P. nigra* or *P. taeda* (Table 1).

Wilt symptom onset: *P. sylvestris* seedlings infected with MPS-1 ceased resin flow to the site of a wound as early as 3 days postinoculation and remained closed throughout the course of the experiment. Leaf chlorosis was first noted 7–10 days postinoculation and complete wilting was observed by 28–32 days. Neither *P. strobus*, *P. nigra*, nor *P. taeda* wilted through 120 days, or more, postinoculation although 10–20% of the leaves showed some chlorosis. Resin flow to the site of a wound ceased 3–5 days postinoculation, and 100% sto-
Fig. 2. Establishment and reproduction of Bursaphelenchus xylophilus isolate VPSt-1 in 2-3-year-old Pinus strobus and P. sylvestris seedlings. A) 25,000 initial nematode inoculum. B) 5,000 initial nematode inoculum. ■ = number of adults per seedling. □ = number of juveniles per seedling. Values are reported as the mean number of nematodes per seedling x 10^4 ± SEM, N = 10. Neither P. nigra nor P. taeda seedlings were infected by VPSt-1; thus these species are not shown in the figure.

Phytotoxin production: Extensive production of CHCl₃-extractable material (15) was seen only in seedlings of those pine species inoculated with a compatible isolate of B. xylophilus (Fig. 3). Thus after inoculation with MPSy-1, P. sylvestris showed a large increase in production of this material reaching a plateau, which coincided with complete wilting, at about 30 days. Seedlings of the other pine species inoculated with MPSy-1 showed either no increase or a transient insignificant increase in CHCl₃-extractable material.

When P. strobus was inoculated with 25,000 VPSt-1, CHCl₃-extractable material increased through 30 days postinoculation then declined after 45 days postinoculation. CHCl₃-extractable material increased in P. strobus inoculated with 5,000 VPSt-1 to a plateau at 30 days postinoculation and remained unchanged thereafter (Fig. 3). Concentration of CHCl₃-extractable material in susceptible seedlings depended on the initial inoculum level.

P. sylvestris, P. nigra, and P. taeda all responded to inoculation with VPSt-1 by a transient increase in CHCl₃-extractable materials. The increase in P. sylvestris was nearly linear through 60 days postinoculation, declining thereafter. Peak production of this material in P. nigra and P. taeda
occurred at 15 days postinoculation, but by 45 days the extract concentration returned to baseline levels (Fig. 3). Noninoculated seedlings produced 0.96 ± 0.36 mg of CHCl₃-extractable material per gram fresh weight throughout the experiment.

Phytotoxicity of the CHCl₃ extracts from all inoculated and noninoculated seedlings was tested by treatment of 25 2–3-month-old seedlings with 500 µg of the extract as previously described (15). The extracts from *P. sylvestris* seedlings inoculated with MPSy-1 and from *P. strobus* seedlings inoculated with VPSt-1 induced wilting of all test seedlings within 10 days. Extracts from noncompatible host–nematode inoculations and extracts from noninoculated seedlings did not induce wilting in any test seedlings.

Seeding water loss: Dehydration of seedlings of the four pine species after inoculation with the isolates of *B. xylophilus* was monitored by comparing the seedling fresh weight with dry weight.

Dry weight increased to approximately 80% of wet weight through 90 days postinoculation in *P. sylvestris* seedlings inoculated with MPSy-1 and in *P. strobus* inoculated with VPSt-1. In both cases dry weight was independent of the initial inoculum level (Figs. 4, 5). The increase in dry weight percentage began by 15 days postinoculation in *P. strobus—VPSt-1* and by 35–45 days postinoculation in *P. sylvestris—MPSy-1* combinations. At the 25,000 VPSt-1 inoculum level there was an initial increase in dry weight of *P. sylvestris* followed by a decrease between 60 and 90 days postinoculation (Fig. 4). *P. strobus* and *P. nigra* inoculated with 25,000 MPSy-1 showed an increase in dry weight until 45 days postinoculation followed by a decrease to noninoculated control levels by 90 days postinoculation. The dry weight of
noninoculated control seedlings was 35.0 ± 12.0% of the fresh weight throughout the experiment.

Field studies: Four 20–30-year-old P. sylvestris and P. strobus were inoculated in September 1984 with 60,000 VPSt-1 as previously described (8). By December 1984 one infected P. strobus had wilted and nematodes were recovered from the branches of two other P. strobus. As of May 1985 no nematodes had been recovered from the branches of inoculated P. sylvestris and the trees showed no wilt symptoms. No indication of insect attack was seen on the inoculated trees.

Nematode mating: No juveniles were recovered from reciprocal matings of VPSt-1 with B. murrayi; however, an average of 9,340 ± 3,840 nematodes per culture (range 2,550–12,460 nematodes per culture) were recovered from reciprocal matings of VPSt-1 × MPSy-1, indicating that these isolates were the same species.

Discussion

Maximum infection success of two isolates of B. xylophilus and full development of disease pathology occurred only in seedlings of the host species from which the isolate was originally obtained. This pattern, apparent in both the greenhouse and field, suggests the occurrence of two nematode pathotypes. These results on intragenic host specificity are similar to the intergeneric results of Wingfield et al. (22) who reported that an isolate of B. xylophilus from A. balsamea infected and caused wilting only of A. balsamea seedlings and that isolates from pine infected only pine seedlings. Host specificity of the isolates used in the present study and of those used by Wingfield et al. (22) was maintained after several passages in vitro on fungal cultures.

The initial establishment and immediate reproductive success of isolates MPSy-1 and VPSt-1 in pine seedlings were dependent on the challenge inoculum amount. At 5,000 nematodes MPSy-1 established and reproduced in P. sylvestris and P. nigra seedlings but only P. sylvestris seedlings wilted. At 25,000 nematodes VPSt-1 appeared to establish, but the nematodes did not survive and wilting did not occur. VPSt-1 established and reproduced in, and caused wilting of, only P. strobus seedlings. At 25,000 nematodes VPSt-1 appeared to reproduce in P. sylvestris, but the seedlings did not wilt.

Maximum population size of MPSy-1 in P. sylvestris was reached earlier in seedlings inoculated with 25,000 than in those inoculated with 5,000 nematodes. Comparable population sizes were eventually attained, however, with both inoculum levels. Greatest population size of VPSt-1 was achieved in P. strobus seedlings inoculated with 5,000 nematodes. Inoculum level had no effect on the time of onset of wilting symptoms or on the rate of wilting.

When P. sylvestris and P. resinosa seedlings were inoculated with a P. nigra isolate of B. xylophilus from Minnesota, 27% of the inoculated P. resinosa seedlings and 40–60% (dependent on inoculum level) of the P. sylvestris seedlings were visibly wilted within 4 months (22). This amount of wilting compared with 100% wilting within 28–35 days of directly compatible seedlings inoculated with MPSy-1 or VPSt-1. In the
ences are particularly notable when pine species (6,7,11,13,19). Resin differ-
pene resin composition among the various
fection success between MPSy-1 and VPSt-1
ally 50% ~- and t3-pinene and as high as 10%
monoterpene resin contains approximate-
are subject to natural inoculations as high
vation that agrees with our earlier findings
phytotoxic material was produced only in
inoculated seedlings that wilted, an obser-
compatible isolate-host combinations were not
phytotoxic to 45-day-old
or VPSt-1, it is possible that the CHCls-
extractable resins in susceptible
seedlings inoculated with 25,000 nematodes may be higher than in those inoculated with 5,000 nematodes. Furthermore, since
P. taeda did not respond to either MPSy-1
or VPSt-1, it is possible that the CHCls-
extractable material recovered from these
seedlings after inoculation is a successful
phytoalexin. The extracts from noncom-
patible isolate-host combinations were not
phytotoxic to 45-day-old P. sylvestris, P. stro-
bus, P. nigra, or P. taeda seedlings. The
phytotoxic material was produced only in
inoculated seedlings that wilted, an obser-
ervation that agrees with our earlier findings
(15). It is unlikely that pines in the field
are subject to natural inoculations as high
as those used for the present studies (8-
10).

Another possibility for differences in in-
fec tion success between MPSy-1 and VPSt-1
might lie in differences in the monoter-
pene resin composition among the various
pine species (6,7,11,13,19). Resin differ-
ences are particularly notable when P. stro-
bus and P. taeda are compared. P. strobus
monoterpene resin contains approximately
50% 3- and 3-pinene and as high as 10%
3-carene (19), whereas 3- and 3-pinene
constitute 80–90% of the monoterpene
resin of P. taeda and 3-carene accounts
for only 0–2% (6,7). Other differences also exist in monoterpene resin composition
(19). Resin composition is somewhat simi-
lar in P. nigra and P. sylvestris, yet signifi-

cant minor differences occur, and the resin
composition of these species differs greatly
from that of either P. strobus or P. taeda
(13,19). The similarities in resins in P. nigra
and P. sylvestris may be the reason that
MPSy-1 was initially able to establish and
survive in P. nigra seedlings. The effect of
resin composition on susceptibility to B. xy-
lophilus infection may be even more strik-
ing if differences in resin acids are consid-
ered (13). The resin acids of A. balsamea
are vastly different from those of either P.
nigra or P. resinosa and pines in general,
and may be responsible for differences in
infectivity among the B. xylophilus isolates
reported by Wingfield et al. (22).

Physical space limitations in the resin can-
als may also limit the number of nema-
todes that can develop and survive in an
infected pine (9,10).

Water flow and retention may be a major
contributing factor for disease pathology
in pine wilt caused by B. xylophilus (16).
Changes in water content of pine seedlings
are reflected in the present study by a time-
dependent increase in the dry weight per-
centage in inoculated susceptible seed-
lings. Although there was a slight increase
in the dry weight percentage in inoculated
nonsusceptible seedlings, this increase was
transient, returning to noninoculated con-
trol levels as the nematode population de-
creased. Changes in water content of in-
fected seedlings could result from physical
or chemical blockage of the water trans-
port system. It is possible that the resin
components synthesized in response to B.
xylophilus infection (15), the nematode it-
self, or damage caused by the nematode
(9,10,12) could disrupt water flow and
maintenance of water balance in infected
susceptible pine seedlings. Other plant
pathogens have been observed to cause
physical blockage of water transport in sus-
cceptible hosts (1,4). In nonsusceptible seed-
lings the closure of some leaf stomates soon
after nematode inoculation might reflect
an attempt by the seedling to maintain
water balance while it mounts other de-
fenses against the nematode. Stomate closure is reversed with time, parallelizing the return of the seedling water content to noninoculated control levels.

Based on our studies with isolates MPSy-1 and VPSt-1, on the successful mating of the two isolates, and on preliminary restriction enzyme mapping of DNA from these isolates (C. Weaver and R. I. Bolla, unpubl.), it appears that these isolates may be genetically distinct pathotypes of _B. xylophilus_ which may have arisen because of a feeding specificity of the carrier insect on pines (18) or geographical isolation.

**LITERATURE CITED**


