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Population Development and Influence of *Bursaphelenchus xylophilus* on *Gliocladium virens*[1]

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Abstract: *Gliocladium virens* was isolated from slash pine trees symptomatic and asymptomatic for pine wilt disease with frequencies of 24% and 10%, respectively. Populations of *Bursaphelenchus xylophilus,* the nematode incitant of this disease, reproduced on this fungus and inhibited its growth. Growth inhibition of the fungus was characterized by an absence of sporulation and by the formation of chains of dark, thick-walled, chlamydospore-like cells. Population increase during a 12-day period following infestation of cultures of the fungus with 10,000 nematodes averaged 3-fold at 16 C, 9-fold at 20 C, and 24-fold at 24 C. In greenhouse studies, nematode recovery from slash pine seedlings coinoculated with both organisms was significantly greater than that obtained from seedlings inoculated with the nematode alone.

Key words: pinewood nematode, ecology, mycophagous nematode, *Pinus, Pinus elliotti,* slash pine.

Pine wilt disease has caused major losses in *Pinus thunbergii* Parl. and *P. densiflora* Sieb. et Zucc. in Japan (12). It was first reported in the United States in 1979 (5). Since then, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, the causal agent, has been found in association with conifer species throughout the United States (4,15). In some *Pinus* associations in the United States, the nematode is apparently the primary agent responsible for tree mortality (2,10,11); in others, it apparently is not (17,19,20).

The association between the pinewood nematode and wood inhabiting fungi has been noted since the nematode was first described from *Pinus palustris* Mill. wood (18). In Louisiana, the nematode was present in pine wood samples collected from dead or drying pine trees in 18 of 20 parishes surveyed (3). We reported previously that 28 genera of fungi were isolated from *B. xylophilus*-infested pine trees and tested for their ability to support nematode reproduction (13). On the basis of these tests, the fungus *Gliocladium virens* Miller was rated as an excellent host. Herein we describe 1) the in vitro parasitism of *G. virens* by *B. xylophilus,* 2) the population development of *B. xylophilus* in cultures of *G. virens* maintained at 16, 20, and 24 C, 3) an attempt to determine the frequency of in situ association between *B. xylophilus* and *G. virens,* and 4) attempts to evaluate the importance of this nematode–fungus association in the etiology of pine wilt disease. Portions of this research have been published elsewhere (7).

Materials and Methods

General: *Bursaphelenchus xylophilus* and *G. virens* were originally isolated in 1980 from wood samples collected with an increment borer from a symptomatic slash pine (*P. elliotti* Engelm. var. *elliotti*) tree near Hammond, Louisiana. Samples were surface sterilized, placed in petri dishes (100 × 20 mm) containing 5% water agar, and incubated at room temperature for 48 hours. Portions of emerging fungal colonies on which the nematode was actively feeding were aseptically transferred, with the nematodes, to potato dextrose agar (PDA). This bipartite culture was maintained by serial transfer and provided a source of nematode inoculum for use in subsequent studies. Pure cultures of the fungus were derived from bipartite cultures, and the fungus was identified as *G. virens.*

Two procedures were employed to separate active nematodes from the bipartite stock cultures. In the first procedure, stock cultures maintained in petri dishes (100 × 20 mm) were placed on cheesecloth-lined

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Baermann funnels. After 24 hours, nematode suspensions were pooled and concentrated by passage through a 38-μm-pore sieve. Nematodes were then backwashed through three changes of 25 ml each of a 0.1% $HgCl_2$ solution amended with 10,000 units/ml of penicillin G, followed by three washings in sterile water. Five minutes were allowed between each washing. The number of nematodes in the final wash was determined, and sterile water was added to achieve desired numbers of nematodes per milliliter. The second procedure, which eliminated the use of chemicals to surface sterilize the nematode, involved the establishment of stock cultures in deep petri dishes (100 × 80 mm) and subsequent extraction using the match-stick technique of Adamo et al. (1). Sterile match sticks (2 × 2 × 40 mm) were placed vertically in a circular pattern 10–15 mm outside the leading edge of the fungal mat. After 24 hours, the upper 20 mm of each match stick was clipped off and soaked in 25 ml sterile water. Nematode numbers per milliliter were determined, and the volume was adjusted to achieve the desired density.

Population density (total number of *B. xylophilus*) and composition (numbers of eggs, juveniles, and adults) were determined by placing the contents of a petri dish into a Waring blender containing 100 ml water and agitating for 20 seconds. The nematodes present in a 20-ml subsample of the resultant slurry were identified and enumerated at 24× with the aid of a stereomicroscope. Data were expressed as numbers of *B. xylophilus* per petri dish. Data from all tests were analyzed using standard analysis of variance procedures.

Growth inhibition of G. virens by B. xylophilus: A flamed cork borer was used to aseptically transfer 4-mm-d plugs from 10-day-old cultures of *G. virens* to the center of petri dishes containing 25 ml PDA. Immediately, a sterile syringe fitted with a 16-gauge needle was used to introduce levels of 0, 350, 500, 700, 1,000, 5,000, 10,000, and 15,000 nematodes in a 1-ml volume per dish. Nematode inoculum was prepared using the Baermann funnel sterilization procedure. There were 10 replicates of each nematode level. Dishes were incubated at room temperature (24–26 C) under a bank of fluorescent lights with 16:8 hours of light : dark. Fungal colony area and nematode reproductive factor (14) were determined after 14 days. Mycelium of *G. virens* from control and nematode-infested cultures was examined using bright field and phase contrast light microscopy.

Population development of B. xylophilus on G. virens: Nematodes were separated from stock cultures using the match-stick procedure, and inoculum was introduced into dishes of PDA containing a plug of *G. virens* as described above. Each dish was infested with 10,000 nematodes of which 28% were females, 11% males, and 61% juveniles. A total of 60 dishes, 30 containing the fungus alone and 30 containing the fungus and nematodes, were incubated at each of three temperatures (16, 20, and 24 C). The areas of *G. virens* colonies in five infested and five noninfested dishes at each temperature were measured at 48-hour intervals for 12 days. Similarly, the density and composition of nematode populations in infested cultures were determined.

Association of B. xylophilus and G. virens in nature: Fifty symptomatic and fifty asymptomatic slash pine trees in 11 different field sites were sampled during 1982 and 1983. Trunk samples were collected at the base, 1.5 m, and 3–4 m above ground from each tree. Samples were surface sterilized by flaming and incubated in petri dishes containing 5% water agar. Dishes were examined at 24-hour intervals for 7 days, and the presence or absence of *G. virens* and/or *B. xylophilus* was recorded.

Greenhouse inoculation test: Two-year-old slash pine seedlings were inoculated in quadruplicate with *B. xylophilus* alone, *G. virens* alone, *B. xylophilus* plus *G. virens,* and sterile distilled water and culture filtrate controls. A 30-mm segment of new growth on terminals of pine seedlings was scraped with a flamed scalpel. A 5-ml Teflon scintillation vial with a 24-mm snap cap was then fitted around the scraped area so that the terminal passed through the center of the vial. The vial was stuffed with cotton and sealed with paraffin. One milliliter of spore and/or nematode suspension was introduced through the cap into the vial with a syringe. Inoculum levels for *G. virens* and *B. xylophilus* averaged 30,216 spores and/or 14,717 nematodes (mixed life stages). Controls were handled as above but inoculated with control fluids. Plants were rated weekly for symptoms of pine wilt dis-

ease. The test was terminated after 3 months. Stem sections were collected, surface sterilized, placed on 5% water agar, and examined at 24-hour intervals for 7 days.

Results and Discussion

Growth inhibition of G. virens by B. xylophilus: The level of infestation was an important factor in the growth and reproduction of both the fungus and the nematode. Levels of 350, 500, 750, 1,000, and 5,000 *B. xylophilus* introduced simultaneously with a plug of *G. virens* into a dish of PDA increased 0.3–2.5-fold after 14 days and usually died out by 18–20 days. Colonies of *G. virens* appeared macroscopically like axenic cultures of the fungus. However, when the nematode infestation level was increased to 10,000–15,000 per dish, reproductive rates of *B. xylophilus* increased significantly (Table 1). Additionally, colony growth of *G. virens* was severely repressed (Fig. 1), there was virtually no sporulation, and nematode feeding activity resulted in mycelium composed almost exclusively of chains of dark, thick-walled chlamydospore-like cells (Fig. 2). All vermiform stages of the nematode fed on the fungus, although juveniles seemed to feed preferentially on newly formed mycelial strands located near the leading edge of the fungal colony. Adults, however, usually fed on the chlamydospore-like cells (Fig. 3). Copulation occurred anywhere in the petri dish but was frequently confined to the area directly beneath the mycelial mat, at the bottom of the dish. Gravid females almost always moved away from this area and deposited their eggs near the leading edge of the fungus colony. Subculturing of either mycelial or chlamydospore-like fragments from nematode-infested cultures produced colonies that were indistinguishable, macroscopically and microscopically, from pure cultures of *G. virens.* The alternate forms of the third-stage and fourth-stage juvenile of *B. xylophilus,* which have been reported to occur along the dispersal pathway in response to starvation and moisture stress (6,9), were observed in only two of the numerous nematode–fungus cultures examined.

Infestation of fungus cultures with final wash solutions minus nematodes indicated that chemicals used to surface sterilize nematode inoculum did not influence the growth of *G. virens* (Table 1). The possibility, however, that they could influence the physiology of *B. xylophilus* prompted the adoption of the match-stick method of nematode extraction. Masses containing both adults and juveniles of *B. xylophilus* aggregated along the length of the sticks, giving them a pretzel-like appearance (Fig. 4) within 24 hours. Surface sterility of nematodes collected by this method was confirmed by transferring nematode-laden match-stick clippings to PDA and nutrient agar.

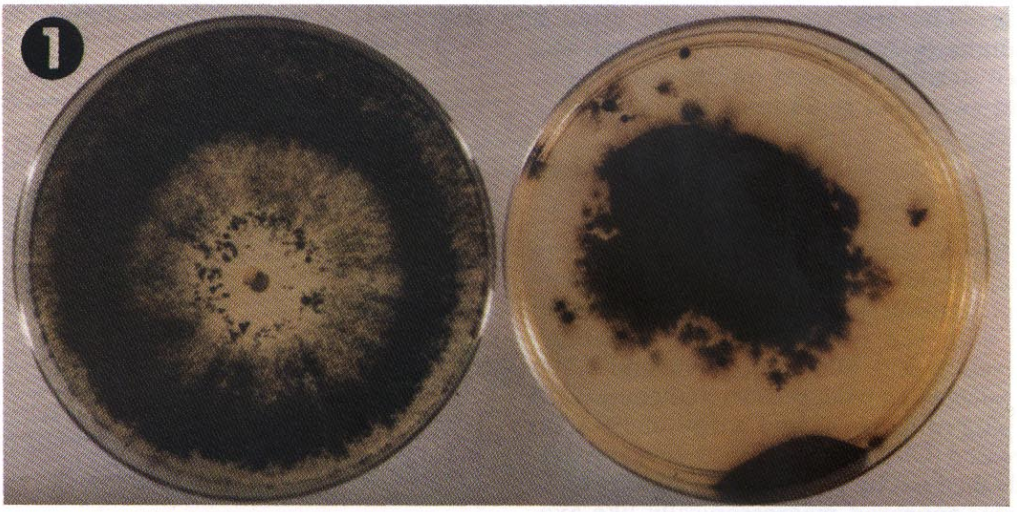
Table 1. Effect of *Bursaphelenchus xylophilus* (Bx) inoculum level on the growth of *Gliocladium virens* (Gv).*

Bx	14-day Bx reproduction (R)† and Gv colony area (mm^2)	
	R	mm^2
0‡		5,024
0§		5,024
500	0.3	5,024
750	0.8	5,024
1,000	1.9	5,024
5,000	2.5	5,024
10,000	11.8	3,117
15,000	16.4	2,826
LSD (0.05)	3.7	591

* Data are means of 10 replicates.
† R = final population divided by initial population.
‡ 0 = sterile distilled water.
§ 0 = final wash solution minus nematodes.

Both the growth of *G. virens* and its degree of inhibition by *B. xylophilus* were temperature dependent (Table 2). In the absence of the nematode, colonies of *G. virens* reached the outer margins of dishes in 12 days at 16 C, 6 days at 20 C, and 4 days at 24 C. Significant inhibition of fungus growth in nematode-infested cultures was first apparent on day 2 at 24 C, day 4 at 20 C, and day 6 at 16 C. Subsequent growth of the fungus in control and nematode-infested cultures remained significantly different at each time interval of each temperature.

Population development of B. xylophilus on G. virens: Population densities of *B. xylophilus* increased approximately 3-fold at 16 C, 9-fold at 20 C, and 24-fold at 24 C during the 12-day test (Table 3). During the first 2 days, populations declined approximately 50% at 16 and 20 C, but only slight-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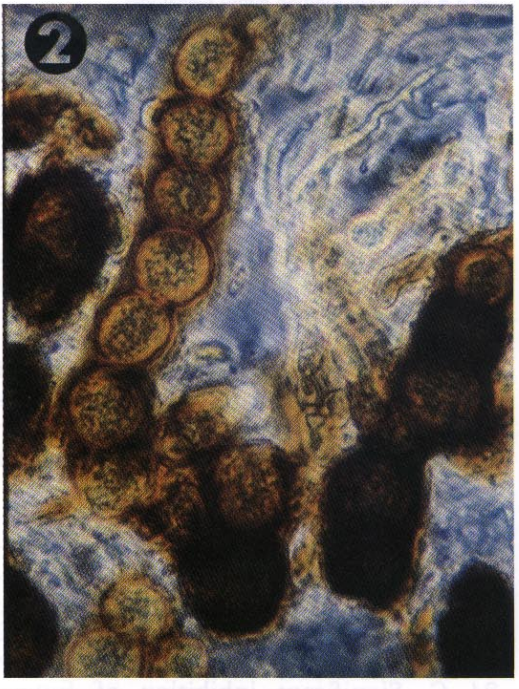
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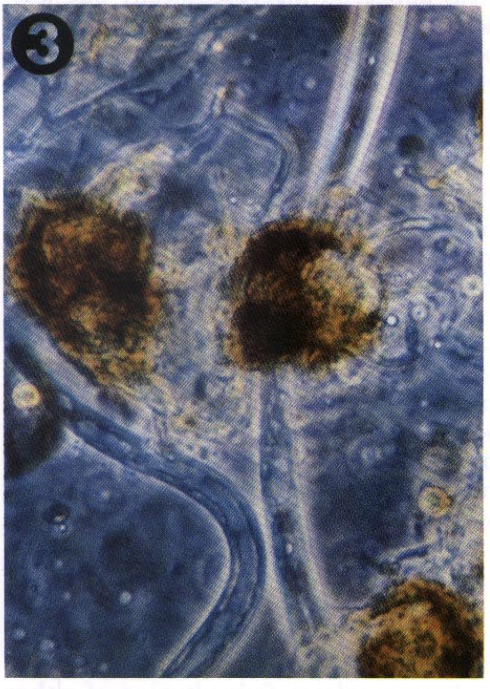
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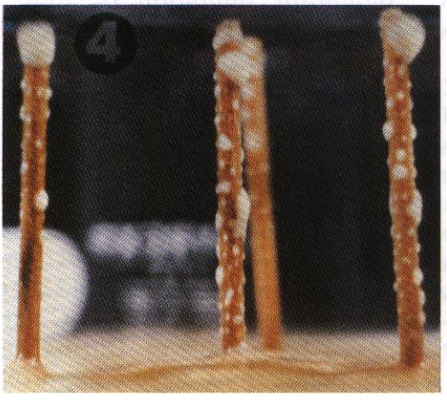
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TABLE 2. Influence of *Bursaphelenchus xylophilus* (Bx) on the colony area (mm^2) of *Gliocladium virens* (Gv) at three temperatures over 0 to 12 days.*

Treatment	Temp. (C)	Days 0†	2	4	6	8	10	12
Gv	16	13	145	485	1,320	2,714	4,126	5,024
Gv + Bx	16	13	67	208	532	964	1,112	1,670
LSD (0.05)		NS	83	317	638	527	811	659
Gv	20	13	1,206	3,940	5,024	5,024	5,024	5,024
Gv + Bx	20	13	742	1,288	1,517	1,605	1,864	1,941
LSD (0.05)		NS	564	735	609	721	847	1,006
Gv	24	13	3,611	5,024	5,024	5,024	5,024	5,024
Gv + Bx	24	13	1,147	1,465	2,001	2,007	2,395	2,519
LSD (0.05)		NS	681	903	577	395	670	415

* Data are means of five replicates.
† Level of infestation was 10,000 nematodes per dish (28% females, 11% males, and 61% juveniles).

ly at 24 C. Thereafter, the density of recovered populations increased and differed significantly at each interval as the incubation temperature was increased.

Figures 7–9 illustrate the composition (developmental stages) of *B. xylophilus* populations recovered from *G. virens* cultures at 2-day intervals at the three temperatures. During the first 2 days, females decreased by 40–50% at all temperatures and eggs per dish averaged 729 at 16 C, 990 at 20 C, and 2,917 at 24 C. Juvenile counts declined after 2 days from the original infestation level of 6,100/dish to 2,656 at 16 C and 2,475 at 20 C. Juveniles at 24 C averaged only 1,848/dish after 2 days as a result of maturation rather than mortality, since there was a concomitant 3-fold increase in the numbers of males at 24 C versus a 40–50% decrease at 16 and 20 C.

The greatest increases in nematode populations, 3–5-fold, occurred between 2 and 4 days at each temperature. The increase in females was almost equal, approximately 4-fold, at each temperature. However, rates of both egg deposition and eclosion increased markedly with temperature. Accordingly, between days 2 and 4, juveniles increased by factors of 1.7 at 16 C, 3.6 at 20 C, and 9.7 at 24 C and males increased by factors of 6.9 at 16 C, 4.5 at 20 C, and 6.4 at 24 C.

Values of R (= rate of nematode population growth) increased from 1.5 to 2.2 at

TABLE 3. Average populations of *Bursaphelenchus xylophilus* per culture of *Gliocladium virens* at intervals between 0 and 12 days after infestation with 10,000 nematodes per dish.*

Temp. (C)	Days 0†	2	4	6	8	10	12
16	10,000	5,208	15,238	22,046	20,815	30,004	33,472
20	10,000	5,500	21,419	45,670	81,275	87,500	90,673
24	10,000	9,725	48,511	101,233	154,276	188,251	236,593
LSD (0.05)	NS	6,218	4,905	9,311	19,208	24,914	43,306

* Data are means of five replicates.
† Composition of inoculum was 28% females, 11% males, and 61% juveniles.

←

FIGS. 1–6. Influence of *Bursaphelenchus xylophilus* on the growth of *Gliocladium virens* and *Pinus elliotti*. 1) Growth of *G. virens* on potato dextrose agar 12 days after introduction of 1 ml of wash solution minus nematodes or 1 ml of wash solution containing 10,000 nematodes of *B. xylophilus*. 2) Chlamydospore-like cells of *G. virens* produced in *B. xylophilus*-infested cultures. × ca. 750. 3) Individuals of *B. xylophilus* embedded in chlamydospore-like cells. × ca. 750. 4) "Pretzel-like" appearance of match sticks covered with masses of *B. xylophilus*. 5) Two-year-old control *Pinus elliotti*. 6) *B. xylophilus*-inoculated *Pinus elliotti*.

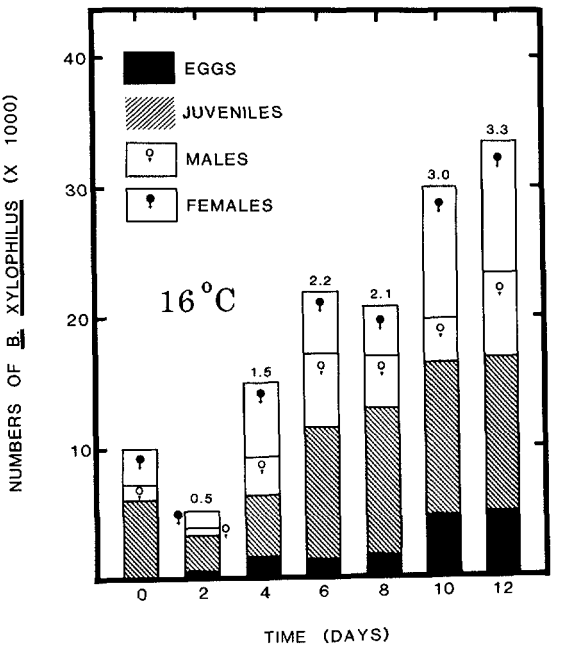

FIG. 7. Population development of *Bursaphelenchus xylophilus* in cultures of *Gliocladium virens* maintained at 16 C. (Data are means of five replicates. Values of R, final population divided by initial population, are given at the top of each bar. Initial population was 28% female, 11% male, and 61% juvenile.)

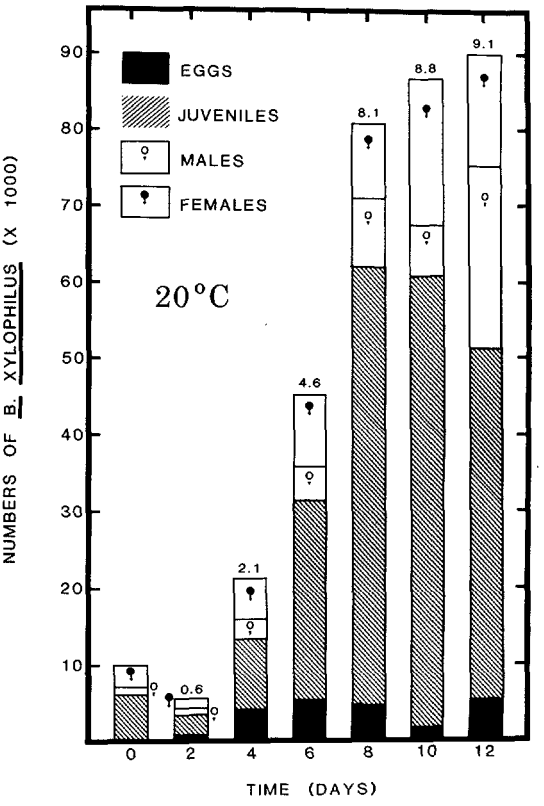

FIG. 8. Population development of *Bursaphelenchus xylophilus* in cultures of *Gliocladium virens* maintained at 20 C. See legend for Figure 7.

16 C, from 2.1 to 4.6 at 20 C, and 4.9 to 10.1 at 24 C between 4 and 6 days. The rate of increase in the numbers of adults produced during this time was similar at each temperature and ranged from 1.6 to 2.0, except for females at 16 C and males at 24 C. The 18% decrease in the numbers of females at 16 C, coupled with a 15% decrease in egg counts, was the first indication that the population at 16 C was approaching the ceiling level or saturation point (8). Conversely, the 58% decline in the number of males at 24 C, along with corresponding increases of 3.3-fold and 6.3-fold in juvenile and egg numbers, respectively, indicated the population remained in a state of active growth.

Between days 6 and 8, populations of *B. xylophilus* decreased slightly at 16 C, but increased by 78% at 20 C and 53% at 24 C. Numbers of females declined 27% at 16 C and increased by factors of 0.1 and 2.6 at 20 and 24 C, respectively. Similarly, males decreased by 27% at 16 C and increased 0.8-fold at 20 C and 2.4-fold at 24 C. Juvenile counts increased at each temperature during this interval: 11% at 16 C and by factors of 2.2 at 20 C and 1.3 at 24 C. Egg counts averaged 1,873 per dish at 16 C, 4,877 at 20 C, and 13,885 at 24 C.

During the remainder of the test period, days 8–12, the R value for *B. xylophilus* increased only from 2.1 to 3.3 at 16 C and from 8.1 to 9.1 at 20 C. The population at 24 C, however, continued to increase rapidly, and R values increased from 15.4 to 23.7. By day 12 of the test, populations at 16 and 20 C had essentially reached equilibrium (16). Accordingly, the increase in total populations between days 10 and 12 at both 16 and 20 C was due almost entirely to the accumulation of males.

The growth and suitability in culture of *G. virens* as a host for *B. xylophilus* was significantly influenced by temperature. Nematode population density increased significantly with each incremental increase in incubation temperature. At 12 days after infestation, nematode popula-

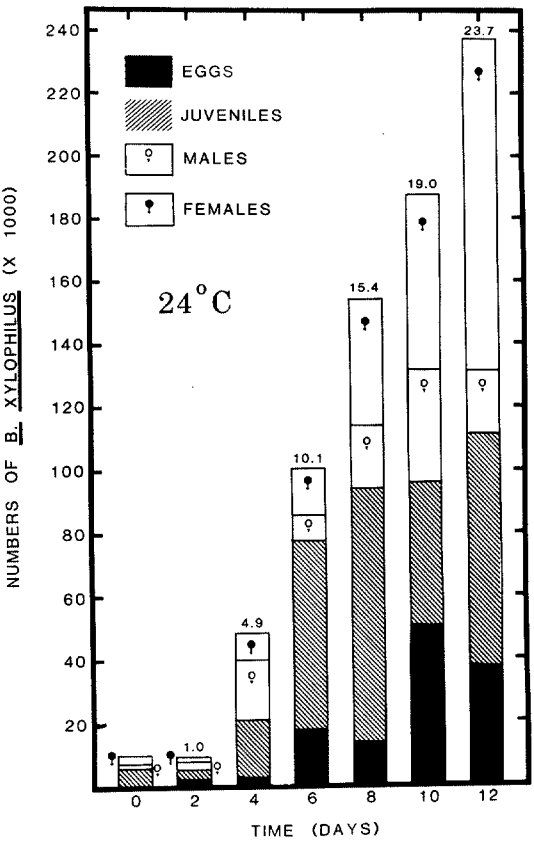

FIG. 9. Population development of *Bursaphelenchus xylophilus* in cultures of *Gliocladium virens* maintained at 24 C. See legend for Figure 7.

tions per square millimeter of *G. virens* colony area averaged 20 at 16 C, 47 at 20 C, and 94 at 24 C. Growth rate of *G. virens* in association with *B. xylophilus*, however, was least at 16 C, greatest at 24 C, and intermediate at 20 C.

Association of B. xylophilus and G. virens in nature: Both the nematode and fungus were isolated from symptomatic and asymptomatic slash pine trees. The frequency of detection for both organisms was greater in symptomatic than in asymptomatic trees (Table 4). Moreover, 12% of the symptomatic and none of the asymptomatic trees contained both *B. xylophilus* and *G. virens*. In the 12% that contained both organisms, with only one exception, they were isolated from all three sample collection points on each tree.

TABLE 4. Percentage of symptomatic vs. asymptomatic *Pinus elliotti* from which *Bursaphelenchus xylophilus* (Bx) and/or *Gliocladium virens* (Gv) were isolated.*

	Bx	Gv	Both
Symptomatic	34	24	12
Asymptomatic	6	10	0

* Fifty each of symptomatic and non-symptomatic trees sampled.

Greenhouse inoculation test: Seedlings inoculated with *B. xylophilus* alone or in combination with *G. virens* displayed symptoms of pine wilt within 21–30 days and died within 35–56 days. An average of 3,732 nematodes/10 g stem tissue were recovered from seedlings inoculated with the nematode alone and a significantly greater number, 16,295/10 g stem tissue, were recovered from plants inoculated with both organisms (Table 5). Plants inoculated either with the fungus alone or with sterile water remained healthy. The fungus was recovered from all inoculated plants. This experiment was repeated three times with similar results.

Our field and greenhouse data indicate

TABLE 5. Influence of *Gliocladium virens* (Gv) on symptom expression, plant mortality, and numbers of *Bursaphelenchus xylophilus* (Bx) recovered from slash pine seedlings inoculated with 10,000 nematodes per seedling.*

Treatment	Time (days) after inoculation symptoms first apparent	Plants killed/ plants inoculated	Organism recovered from inoculated plant		No. Bx/10 g stem tissue
			Bx	Gv	
Bx alone	21–28	3/4	+	−	3,732
Gv alone	No symptoms	0/4	−	+	0
Bx + Gv	16–30	4/4	+	+	16,295**
Sterile water	No symptoms	0/4	−	−	0

* Data are means of four replicates.
** $P = 0.01$.

that *Gliocladium virens* is able to colonize and survive in apparently healthy pine seedling tissues, as was observed with *G. roseum* in Delaware (2). Additionally, isolation of a *Gliocladium* spp. from *Bursaphelenchus xylophilus*-infested pine tissue was reported in Japan (9). *Gliocladium virens* and perhaps other wood-inhabiting fungi may serve not only to enhance nematode survival in drying pine tissues (2) but may also function as an important substrate for nematode population increase.

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