Antimycotic substances produced by phase one of *Xenorhabdus nematophilus* (two isolates from *Steinernema carpocapsae*), *Xenorhabdus bovienii* (two isolates from *Steinernema intermedia*), and *Photorhabdus luminescens* (from *Heterorhabditis megidis*) and by phase two of *Xenorhabdus nematophilus* (one isolate) were tested for their spectrum of activity against 32 species of fungi from a range of habitats. The plant pathogenic fungi, *Botrytis cinerea*, *Ceratocystis ulmi*, *Ceratocystis dryocosmis*, *Mucor piriformis*, *Pythium coloratum*, *Pythium ultimum*, and *Trichoderma pseudokingii*, were completely inhibited by all phase one variants, whereas the mycorrhizal fungus *Suillus pseudobrevipes* was not inhibited. Wood decay and food spoilage fungi were partially inhibited by the phase one variant of most of the isolates. The degree of antifungal activity of the cell-free filtrates of the isolates of phase one was not significantly different from each other whereas that of phase two was significantly weaker.

**Key Words:** *Xenorhabdus*; *Photorhabdus luminescens*; *Steinernema*; *Heterorhabditis*; antifungal activity; antimycotic substance, plant-pathogenic fungi.

**INTRODUCTION**

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are applied commercially as biological control agents against a range of insect pests (Georgis, 1990). The infective juvenile (IJ) stage of the steinernematids and heterorhabditids is symbiotically associated with the bacteria *Xenorhabdus* and *Photorhabdus*, respectively (Boemare et al., 1993). The IJs enter the insect hemocoel and release the bacteria which kill the insect, establishing conditions for nematode development by providing nutrients (Akhurst and Dunphy, 1993) and initially preventing the growth of other microorganisms (Maxwell et al., 1994). Most species of *Xenorhabdus* and *Photorhabdus luminescens* Boemare, Akhurst, and Mourant (=*Xenorhabdus luminescens*; Boemare et al., 1993) exist as two or more variants that differ in their biochemical and physiological properties (Boemare and Akhurst, 1988, 1990; Hurlbert et al., 1989; Akhurst and Boemare, 1990). The phase one variants produce lecithinase, absorb dyes, contain paracrystalline inclusions, and produce antibiotics, whereas phase two, which occurs after prolonged culture in *vitro*, either lacks or possesses reduced levels of these properties (Akhurst and Boemare, 1990).

Dutky et al. (1964) suggested that the bacterial symbiont of the DD136 strain of *Steinernema carpocapsae* (Weiser) (Neaplectana feltiae Filipjev) produced antibiotics, and Akhurst (1982) reported that phase one of *Xenorhabdus* species and *P. luminescens* produces antibiotics that inhibit the growth of many bacterial and yeast species. The phase one of *Xenorhabdus nematophilus* (Poinar and Thomas) and *P. luminescens* also inhibits the growth of blastospores of the insect pathogenic fungus *Beauveria bassiana* (Barbercheck and Kaya, 1990). Several antibiotics and antimycotics from *Xenorhabdus* and *Photorhabdus* have been isolated and characterized (Paul et al., 1981; Nealon et al., 1990; McInerney et al., 1991a,b), and among these compounds xenocoumacin 1, isolated from *X. nematophilus*, is active against animal and human pathogenic fungi (McInerney et al., 1991b). However, the effects of antimycotics of *Xenorhabdus* species and *P. luminescens* on soil-dwelling fungi and plant symbiotic and pathogenic fungi are unknown. Concern has been raised regarding the impact of antibiotics released from the fragmenting insects on soil bacteria when the nematode–bacterial complex is used as an inundative insecticide against soil-dwelling pest insects (Maxwell et al., 1994). In view of impact of steinernematids and *Heterorhabditis bacteriophora* on commercial mushroom production (Olthof et al., 1991), the present study was done to assess possible impact of the antimycotics of several...
strains of *X. nematophilus* and *Xenorhabdus bovienii* and *P. luminescens* on fungi of importance to agriculture and forestry.

**MATERIALS AND METHODS**

**Preparation of Bacterial Inoculum**

The *Xenorhabdus* species and *P. luminescens* used in this study (Table 1) were isolated as described by Woodring and Kaya (1988) and maintained in monoxenic culture. Newly molted last instar of the greater wax moth, *Galleria mellonella* (L.), were exposed to IJs at a rate of 25 IJs/larva. After 24–48 h the dead larvae were surface disinfected by dipping them into 95% ethanol and igniting them. The cadavers were aseptically opened with sterile forceps and hemolymph was streaked onto NETA medium (nutrient agar supplemented with 0.025 g bromothymol blue and 0.04 g 2,3,5-triphenyltetrazolium chloride per liter) and incubated in the dark at room temperature. The resulting phase one [as defined by Boemare and Akhurst (1988)] of each isolate was subcultured at 14-day intervals on NETA. To obtain the phase two of *X. nematophilus*, the phase one form was maintained without subculture for 20 days at 24 °C, during which time many bacterial colonies produced secondary forms. Both phases of the *Xenorhabdus* species and the phase one variant of *P. luminescens* were routinely tested for antibiotic activity against *Bacillus subtilis* Cohen (Maxwell et al., 1994). Inocula of the phase one of *X. nematophilus*, *X. bovienii*, and *P. luminescens* and the secondary form of *X. nematophilus* were grown in 50 ml of tryptic soy broth (TSB) in 100-ml Erlenmeyer flasks on a horizontal, gyratory shaker (80 rpm). The inoculum and shaker conditions kept the bacteria in log growth during which there was no visible bacteriolysis. After 6 days incubation, the pH of the spent medium was adjusted to 7.0 with 6 N HCl, and the medium was centrifuged (12,000g, 20 min, 4 °C) to remove the bacteria and filter-sterilized (0.22 µm; Millipore, Fisher Scientific, Vancouver, Canada). Control medium consisted of uninoculated TSB.

**Activity of Antimycotics Produced in Vivo**

To determine the antimycotic activity of *X. nematophilus in vivo*, final instar larvae of *G. mellonella* were infected either with about 600 IJs of *S. carpocapsae* (DD136 strain) in a petri dish containing 10 insect larvae as described above or by injecting larvae with 5 µl of phase one of *X. nematophilus* cell suspension (4.5 × 10⁶ cells/ml) in 0.9% saline. The infected insects were incubated at 24°C. Insect cadavers were ground with a mortar and pestle 3 days after larval death and mixed with distilled water at a ratio of one larva/ml water, and the suspension was stirred on a magnetic plate for 20 min. As a control, insect larvae were killed by piercing with a

**TABLE 1**

Bacterial Symbionts and Their Nematode Associates Used in This Study

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Isolate</th>
<th>Nematode</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xenorhabdus nematophilus</em></td>
<td>D1</td>
<td><em>Steinernema carpocapsae</em></td>
<td>G. O. Poinar Univ. of California</td>
</tr>
<tr>
<td></td>
<td>DS</td>
<td><em>S. carpocapsae</em></td>
<td>Phase two variant of D1</td>
</tr>
<tr>
<td></td>
<td>BC1</td>
<td><em>S. carpocapsae</em></td>
<td>Soil in British Columbia, Canada</td>
</tr>
<tr>
<td><em>Xenorhabdus bovienii</em></td>
<td>A2</td>
<td><em>Steinernema feltiae</em></td>
<td>Soil in British Columbia, Canada</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td><em>S. feltiae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BC2</td>
<td><em>Steinernema intermediia</em></td>
<td></td>
</tr>
<tr>
<td><em>Photorhabdus luminescens</em></td>
<td>C9</td>
<td><em>Heterorhabdus megaridis</em></td>
<td>Soil in British Columbia, Canada</td>
</tr>
</tbody>
</table>
The inoculated plates were incubated in the dark at 24°C for 1 week, and the fungal macroconidia used as inoculum were suspended in sterile distilled water. The macroconidia were centrifuged (10,000g, 10 min, 24°C), washed twice with sterile, distilled water, and suspended in 0.08% water agar to a final concentration of 3 × 10⁶ macroconidia/ml. One milliliter of the conidial suspension, transferred into petri dishes (9 cm) containing 10 ml of PDA, was spread over the agar surface with a glass spreader and dried under the laminar hood for approximately 20 min. Three 0.5-cm equidistant wells were cut in the agar plates and filled with 50 µl of the test filtrate (sterile TSB was added to a well in the same plate as a control). The inoculated plates were incubated in the dark at 24°C. The size of the inhibition zones of fungal growth,
from the edge of the well to the edge of the inhibition zone, was measured for each of the six replicates after incubating the cultures for 48 h.

Interaction of Antimycotics with Fungi in Soil

Soil (pH 5.7; organic matter, 2.7%; clay, 4%; silt, 18%; sand, 78%) collected from the top 10 cm of grassland (Simon Fraser University, Burnaby, British Columbia) was air dried and sieved (No. 30 sieve, 600 µm) prior to use. Larvae previously injected with 5 µl of the phase one of X. nematophilus BC1 [one of the more potent producers of antimycotics (this study)] or infected with the corresponding IJ and which had been dead for 48 h were punctured with a needle to mimic the emergence of IJs from the cadavers and the subsequent release of antimycotics. The insects were encased in dialysis tubing at a density of five larvae per bag and buried in 10 g of soil that had been saturated with 7.5 ml of distilled water in a petri dish. Control groups consisted of larvae killed prior to use (representing the effects of nutrient augmentation) and 500 µl of distilled water in dialysis tubing (representing nutrient dilution). The level of insects represented numbers routinely encountered for soil dwelling pest insects (Jansson et al., 1992). To further assist antimycotic diffusion into the soil, distilled water (100 µl/insect) was added to each bag. The petri dishes were sealed with Parafilm and incubated at 24 °C for 10 days, and the levels of fungi were determined at designated times. Aliquots from each of nine samples per treatment of soil were diluted and plated on acidic glucose-peptone agar (Wollum, 1982).

RESULTS AND DISCUSSION

Antimycotic substance(s) inhibited fungal growth to an extent that varied with the fungal species and bacterial isolate (Table 2). The growth of seven species of plant pathogenic fungi, Botrytis cinerea Pers., Ceratocystis ulmi Buisman, Ceratocystis dryocoeidis Kendri and Molar, Mucor piriformis Fischer, Pythium coloratum Vaartaja, Pythium ultimum Trow and Trichoderma pseudokoningii Oud., was completely inhibited by the phase one variants of all the test bacteria. Considerable resistance to the antimycotic(s) was demonstrated by Aspergillus niger von Tiegh, Cephaloascus fragrans Hansawa, by the insect pathogenic fungi B. bassiana Vuill and Metarhizium anisopliae Sorok. and by the mycorrhizal fungi Oidiodendron griseum Robak and Suillus pseudo-brevipes Smith and Thiers. The isolates of Xenorhabdus differed in their spectrum of activity against the fungal species tested; the BC1 isolate of X. nematophilus had the widest activity spectra and the phase two variant of D1 had a more restricted range. It is not known, however, if the different activity spectra are due to quantitative or qualitative differences in the antifungal substances produced by each species, strain, and phase type of the bacteria. The mechanisms of antagonistic interaction between microorganisms include parasitism, direct competition and antibiosis (Singh and Faull, 1988). The latter two possibilities could explain the results of the initial inhibition studies (Table 2), but the inhibition zones on Fusarium solani plates (Table 3) caused by bacteria-free filtrates of the Xenorhabdus and Photobacterium and insect cadaver extracts show that the inhibitory effect of bacteria on the fungi was caused by antimycotic activity rather than by direct microbial competition.

Xenocoumacin 1, isolated from X. nematophilus, was shown to be mycoidal against A. niger, Aspergillus fumigatus Fres., Trichophyton mentagrophytes Blanchard, and Trichophyton rubrum Semon (Mcinerney et al., 1991b). Xenorhabdus nematophilus and P. luminescens have been shown to be antagonistic to the insect pathogenic fungus B. bassiana (Barbercheck and Kaya, 1990). Antifungal activity against B. bassiana was confirmed here, but, only for phase one of a X. nematophilus isolate. In our experiments B. bassiana showed more resistance to the antimycotic(s) than did most of the other fungal species tested. This discrepancy may reflect differences in fungal isolate susceptibility. It is also possible that the different media used in the present and previous studies (Barbercheck and Kaya, 1990) and the different cultural history (Maxwell et al., 1994) of the

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>Inhibition zone (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>3.6 ± 0.1c</td>
</tr>
<tr>
<td>D2</td>
<td>1.8 ± 0.2d</td>
</tr>
<tr>
<td>BC1</td>
<td>3.2 ± 0.6e</td>
</tr>
<tr>
<td>BC2</td>
<td>3.4 ± 0.3c</td>
</tr>
<tr>
<td>A2</td>
<td>2.6 ± 0.2d</td>
</tr>
<tr>
<td>A3</td>
<td>4.3 ± 0.5c</td>
</tr>
<tr>
<td>C9</td>
<td>3.1 ± 0.6c</td>
</tr>
<tr>
<td>GMSC</td>
<td>3.0 ± 0.6c</td>
</tr>
<tr>
<td>GMXN</td>
<td>3.1 ± 0.2c</td>
</tr>
<tr>
<td>GM</td>
<td>0.0 ± 0.0e</td>
</tr>
<tr>
<td>TSB</td>
<td>0.0 ± 0.0e</td>
</tr>
</tbody>
</table>

*The sources of the bacterial isolates are given in Table 1. GMSC, water extract from Galleria killed with S. carpopcapsae (DD136). GMXN, water extract from Galleria killed with X. nematophilus D1 alone. GM, water extract from Galleria killed by needle puncture.

Note: Activity is expressed as the size of the zone of inhibition of Fusarium solani growth on agar plates after 48 h.

TABLE 3

Antimycotic Activity of Cell-Free Aqueous Filtrates of Spent Culture Media of Xenorhabdus Isolates and P. luminescens and of Crushed Larval Galleria mellonella Cadavers, Compared with Tryptic Soy Broth

Means ± standard error of the mean (n = 6). Means with the same letter are not significantly different (P > 0.05).

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Note: Activity is expressed as the size of the zone of inhibition of Fusarium solani growth on agar plates after 48 h.
isolate to the different levels of antifungal(s) required to kill B. bassiana. Antifungal substances were produced by Xenorhabdus during its growth in vivo. The present study shows (Table 3) that extracts from insect cadavers killed by the IJ-bacterium complex or by phase one X. nematophilus alone have antifungal activity, and that the level of antifycotic, as evident by the size of the zones of inhibition, is similar. Phase two of X. nematophilus has significant, albeit diminished, antifungal activity. However, the phase two variant had no antibiotic activity when tested against B. subtilis which may reflect the presence of different levels of xenocoumacins.

Many important plant pathogenic fungi, such as Fusarium sp., Pythium sp., Rhizoctonia sp., and Verticillium sp. under laboratory conditions were inhibited by the antifungal activity of Xenorhabdus species and P. luminescens, while the mycorrhizae, O. griseum and S. pseudobrevipes, were little or not affected. The study of antifungal release of the fungus-feeding nematodes in the soils support the proposal of reduced impact on soil-dwelling fungi in that in 4 days after the antifungal treatment, the level of fungi returned to the level in distilled water (soil nutrient dilution) control treatment (Table 4). That does not mean that all beneficial or nonpathogenic fungi are unharmed since Stenhof et al. (1991) found that when Steinernema feltiae and H. bacteriophora were undissipately applied to control a sciarid fly infestation on mushrooms in greenhouses the mycelial development slowed.

In summary it is tentatively proposed that the antifungal activity of X. nematophilus, X. brownii, and P. luminescens would have limited if any direct impact on soil fungi, insect pathogenic fungi used in biological control, or soil mycorrhizae. They do, however, offer promise in the control of many plant pathogenic fungi.


