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Control of Insects with Entomopathogenic Bacterium *Xenorhabdus nematophila* and its Toxic Secretions

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ABSTRACT

Direct application of either cell solutions or cell free-filtrates from symbiotic bacterium *Xenorhabdus nematophila* can control insects on leaves and in soils. Larvae of the beet armyworm (*Spodoptera exigua*), diamondback moth (*Plutella xylostella*), black vine weevil (*Otiorhynchus sulcatus*) and nymphs of desert locust (*Schistocerca gregaria*) were killed by both cell treatments and cell-free filtrates, which were equally effective. Therefore, the toxin present in both treatments was responsible for the lethal effects. Cells of *X. nematophila* recovered from the haemocoele of all treated insects, indicating that cells were able to move from the external environment and enter insects in the absence of the nematode vector. The toxicity of cell suspensions and cell-free filtrates, as assessed by *Galleria mellonella* mortality, persisted for up to 5 months in soil indicating that soil treatments with either cells or cell-free filtrates controlled *O. sulcatus* in potted plants.

Key Words: *Xenorhabdus nematophila;* Cell treatments; Cell-free filtrates; Toxins

INTRODUCTION

Species of the genus *Xenorhabdus* are gram-negative entomopathogenic bacteria, which live symbiotically with nematodes of the genus *Steinernema* (Boemare, 2002). The nematode-bacterium association is highly toxic to many insect species and in most cases the bacteria alone are highly virulent when they enter the insect haemocoele (Forst & Nealson, 1996). The life cycle of bacteria and nematodes is complex particularly at pathogenic and symbiotic stages. Infective 3rd stage juvenile nematodes of *Steinernema carpocapsae*, which carry the bacterium *Xenorhabdus nematophila* in their gut, enter insect larvae via the mouth, anus or spiracles (Georgis & Hague, 1981). The combined actions of the nematode and bacterium kill the insect usually within 48 h. The bacteria reproduce causing septicemia, which break down the host. Nematodes develop in insect cadaver and can produce 2 - 3 generations when there is a re-association between the bacteria and the non-feeding infective juvenile (J3) nematodes. They emerge from the insect cadaver to find new hosts.

Many strains of *Xenorhabdus* and *Photorhabdus* bacteria produce a variety of exoenzymes. Lecithinase is produced by *X. nematophila* and *X. bovienii* but is absent in several strains of *Photorhabdus*. Enriched fractions of lecithinase are involved in the breakdown of insect phospholipids thereby providing a lipid source for the growth of *Steinernema*. Throughout their life cycle, the bacteria and the nematodes produce a variety of metabolites to enable them to colonize and reproduce in the insect host. These metabolites often have overlapping functions, a strategy that is likely to contribute to the success of the nematode–bacteria association against a variety of insect hosts. The metabolites produced help to evade the insect immune system, enzymes such as proteases, lipases and phospholipases to maintain a food supply during reproduction (Thaler *et al.*, 1998) and antifungal and anti-bacterial agents to prevent degradation or colonization of the insect carcass, while bacteria and nematode reproduce (Akhurst & Dunphy, 1993).

The entomopathogenic bacterium *Photorhabdus luminescens*, the symbiont of *Heterorhabditis bacteriophora* has been shown to express complex toxin (*tc*) genes, which is lethal to insects (Bowen & Ensign, 1998). Ffrench-Constant and Bowen (1999) have indicated that *Xenorhabdus* spp. also secrete toxins similar to those found in *P. luminescens*. The theme of research on *tc* genes was the transfer of toxins to plants for insect control, but so far the methodology are not commercially feasible. The details of the modes of action of the various toxins of *Xenorhabdus* and *Photorhabdus* are still elusive. Injection of the *Tca* complex of *P. luminescens* strain W14 or ingestion by *Manduca sexta* larvae damaged the midgut cells, resulting in shedding of the midgut epithelium (Blackburn *et al.*, 1998). Injection of A24tox into lepidopteran larvae caused the larvae to cease feeding almost immediately. Preliminary histological studies indicated that the main site of action for the toxin was insect midgut (Brown *et al*., 2004).

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In this study, we describe the direct effect of applying solutions containing cells of *X. nematophila* or its cell-free filtrates to control larvae of the beet armyworm (*S. exigua*), diamondback moth (*P. xylostella*), black vine weevil (*O. sulcatus*) and the nymphs of the desert locust (*Schistocerca gregaria*). The persistence of *X. nematophila* and its cell-free filtrates in soil were also investigated.

MATERIALS AND METHODS

Larvae of the beet armyworm, *Spodoptera exigua* were reared in the laboratory on an artificial diet (Elawad *et al.*, 2001). The diamondback moth, *Plutella xylostella* was cultured on Chinese cabbage cv. Wong Bok at 26 ± 2°C in a growth room. The locust, *Schistocerca gregaria*, was obtained from a commercial source and maintained on wheat seedlings at 25°C. Late instar larvae of the black vine weevil were obtained from infected polyanthus.

Isolation of *X. nematophila*. The entomopathogenic nematode, Steinernema carpocapsae (ALL isolate) was cultured in late instar larvae of the greater wax moth *Galleria mellonella* (Woodring & Kaya, 1988). Larvae of *G. mellonella* infected with J_3 *S. carpocapsae* died after 48 h when they were sterilised, opened with sterile needles and a drop of the oozing haemolymph was streaked onto nutrient agar, the agar plates were incubated at 28°C in the dark for 24 h. The bacterial colonies were sub-cultured until pure colonies of uniform morphology were obtained. A single colony was selected and inoculated into a 50 mL solution of nutrient broth and placed in shaking incubator for 2 days at 28°C. The concentration of bacterial cells in the broth suspension was estimated using a spectrophotometer and the number of cells for use in experiments was adjusted to 4×10^7 cells mL^{-1}, a concentration that had been shown to be effective against several insect larvae (Elawad, 1998). To obtain cell-free solutions containing only toxic metabolites, the cell suspensions were filtered through a bacterial filter (pore size 0.2 µm).

The beet armyworm, *S. exigua*. The stalks of single detached leaves of cotton were put in distilled water in a small plastic container so that the leaves remained fresh and moist throughout the experiment. Both top and bottom leaf surfaces were sprayed with 1 mL of cells in broth suspension or cell-free filtrate with 3% w/v of Tween 80 (polyoxyethylene (20) sorbitan mono-oleate) as emulsifier. Control leaves were sprayed with broth and Tween emulsifier only. After spraying, one-third instar larva of *S. exigua* was put on each leaf, which was placed inside a larger plastic container to maintain moisture. Mortality was assessed after 72 h.

The diamondback moth, *P. xylostella*. Both the top and bottom of leaves of single detached leaves of chinese cabbage were sprayed with 1 mL of either the cells in broth or the cell-free filtrate from *X. nematophila*, with 3% w/v of Tween 80 as emulsifier, control was broth alone. Ten 3rd instar larvae of *P. xylostella* were placed on each leaf, the stalks of the leaves placed in water in a small plastic container inside a larger sealed container at 25°C. Mortality was determined after 48 h.

The desert locust, *S. gregaria*. Wheat seedlings were grown to 7 cm height in plastic pots and they were sprayed with 10 mL of either the cells in broth or the cell-free filtrate, using 3% w/v of Tween 80 as emulsifier; the control treatment being broth alone. Tween-80 (2%) was added as emulsifier for leaf surface. Twenty *S. gregaria* nymphs were placed on each pot in a ventilated cage at 25°C, replication was threefold. The behaviour of the locust nymphs was observed and mortality was determined after 8 days.

Black vine weevil, *O. sulcatus*. Strawberry plants cv Elsanta were grown singly in a peat based potting compost in 9 cm plastic pots. Ten late instar *O. sulcatus* larvae were placed around the roots of each strawberry plant and one week later they were treated with 20 mL of either cells in broth or the cell-free filtrate, with 3% w/v of Tween 80 as emulsifier. The plants were grown in a glasshouse at 15 ± 3°C. Mortality of *O. sulcatus* larvae was determined 14 days after application.

Fate of *X. nematophila* cells. To determine whether *X. nematophila* cells were able to penetrate into insects treated with cell treatments, small sample was taken from the haemolymph of all dead insects and streaked onto nutrient agar. Bacterial growth from the sample was compared with that from the original culture. Identification of the bacterial colonies was done by examining their colour, morphology and growth at the margin of the developing culture.

Persistence of *X. nematophila* in soil. Ten ml of the broth solution containing cells or the cell-free filtrates was mixed thoroughly into 50 g of a peat-based potting compost and stored at 20°C in controlled environment chambers the untreated control was broth alone. Last instar larvae of the *G. mellonella* were used to assess the toxicity of the cells and filtrates over a period of five months. Immediately after setting up the experiment and then every month, containers from each treatment were removed from storage and 10 last instar larvae of *G. mellonella* were placed in each container to assess the presence of bacterial cells and to evaluate the efficacy of toxins in the filtrates. Mortality was determined after 7 days. A small sample was taken from the insect's haemolymph and streaked onto the nutrient agar to determine whether bacterial cells had penetrated into the insect. The growth of *X. nematophila* was assessed qualitatively to measure the viability of the cells.

Statistical procedures. All sets of experiments were laid out in completely randomized design with four replications. Data were analyzed for variance using GenStat for Windows (Release 8.1), Lawes Agricultural Trust, Rothamsted Experimental Station, UK.

RESULTS

Treatments with solutions containing cells of *X. nematophila* and cell-free filtrates had significant ($P < 0.001$) effect on the mortality of all insects as compared to

Table I. The percentage mortality of larvae of *Spodoptera exigua, Plutella xylostella* and *Otiorynchus sulcatus* and nymphs of *Schistocerca gregaria* treated with cells and cell-free filtrates of *Xenorhabdus nematophila*

Treatments	*S. exigua*	*P. xylostella*	*O. sulcatus*	*S. gregaria*
X. nematophila cells	100.00 ±4.03	100.00 ±2.04	92.50 ±4.00	100.00 ±1.92
Cell-free filtrates	95.00 ±4.03	97.50 ±2.04	100.00 ±4.00	100.00 ±1.92
Untreated, Broth alone	5.00 ±4.03	7.50 ±2.04	10.00 ±4.00	18.33 ±1.92

Values are means ±SE

Table II. The percentage mortality of *Galleria mellonella* larvae used to assay soil treated with cells and cell-free filtrates from the bacterium *Xenorhabdus nematophila* applied at 20°C, assessed monthly (0-5) after application to soil

Treatments	Months					
	0	**1**	**2**	**3**	**4**	**5**
X. nematophila Cells	93.33 ±3.33	90.00 ±5.77	76.67 ±3.33	53.33 ±8.82	40.00 ±5.77	40.00 ±5.77
Cell-free filtrates	90.00 ±10.00	76.67 ±3.33	73.33 ±8.82	63.33 ±3.33	40.00 ±5.77	33.33 ±8.82
Untreated, Broth alone	3.33 ±3.33	3.33 ±3.33	3.33 ±3.33	6.67 ±6.67	6.67 ±3.33	10.00 ±5.77

Values are means ±SE

control. Solutions containing cells of *X. nematophila* caused 100% mortality of *S. exigua* larvae on cotton leaves, 100% mortality of *P. xylostella* larvae on Chinese cabbage and 100% mortality of *S. gregaria* on wheat seedlings (Table I). Treatments with cell-free filtrates were relatively less effective against *P. xylostella* and *S. exigua*. *S. gregaria* nymphs feeding on wheat seedlings treated with either cells or cell-free filtrate were repelled by the treatments and did not feed on the host plant. The nymphs on treated seedlings were also found well down in the leaf canopy compared to nymphs in the controls, which fed actively on the top of the leaves. Larvae of the black vine weevil, *O. sulcatus*, were effectively controlled by both cell (92.5%) and cell-free (100%) treatments in soil. When evaluated 14 days after treatment the strawberry plants in the control pots were severely damaged, while controls were undamaged. Cells of *X. nematophila* were recovered from the haemocoele of all insects treated with cells.

In case of persistence of treatments with solutions containing cells of *X. nematophila* and cell-free filtrates had significant ($P < 0.001$) effect on the mortality of *G. mellonella* larvae as compared to control. The toxicity of both the cell solution and cell-free filtrate stored at 20°C, declined over a period of 5 months as evaluated by the mortality of *G. mellonella* larvae (Table II). Similar results (not illustrated) were obtained for storage at 25°C. The percentage mortality of *G. mellonella* larvae was 93.33% immediately after the experiment was set up and declined to 40% after 4 and 5 months, when *X. nematophila* cells were applied. The decline in effectiveness of the cell-free filtrates being less than that of the cell treatment. Cells of *X. nematophila* were recovered from all *G. mellonella* larvae, which were used to assess the cell treatment. There was a gradual decline in the number of viable cells recovered from the *G. mellonella* larvae. Until after 5 months, very few colonies of *X. nematophila* developed on the agar plates, which indicated the persistence of the treatments.

DISCUSSION

Application of cells of *X. nematophila* in broth solution and cell-free filtrates were lethal to larvae of *S. exigua* and *P. xylostella* and nymphs of *S. gregaria* feeding on treated leaves. Similar treatments in soil controlled larvae of the black vine weevil, *O. sulcatus*. Both treatments contained the same toxic metabolites secreted by the bacterium and, therefore, indicated that the toxins secreted by the bacterium (not the bacterium itself) were responsible for the observed lethal effects. The toxic metabolites from another entomopathogenic bacterium *Photorhabdus luminescens* (a symbiont of *Heterorhabditis bacteriophora*) were shown to be highly toxic to insects (Bowen & Ensign, 1998). The toxin complex (*tc*) genes have been identified but attempts to transfer the toxins genes to plants have not yet been commercialized. Ffrench-Constant and Bowen (1999) reported that *Xenorhabdus* spp produce metabolites containing *tc* genes similar to those of *P. luminescens.*

This research has shown that *X. nematophila* can be recovered from all larvae treated with cells under moist conditions either on leaves or in the soil. This indicated that the bacterium can enter the host in the absence of the nematode vector, which is an interesting finding because Morgan *et al*. (1997) showed very restricted survival of bacteria both in water and in soil. In the present study, viable *X. nematophila* cells were recovered 5 months after application, confirming the reports by Elawad (1998) and Akhurst and Smith (2002). The motility of *Xenorhabdus* cells is dependent on moisture (Givaudan *et al*., 1995) and their numerous pertrichous cilia (Forst & Clarke, 2002). Cells of entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus* are normally only found in the nematode J3 or in infected insects (Poinar, 1979) but we have shown that they can be free-living either on leaves or in soil. The means of entry into the haemocoele was not observed but it seems likely that they enter via the same orifices as the nematode J_3 i.e., mouth, anus or spiracles, the latter being the most likely as cells of both *X. nematophila* (Elawad, 1998) and *P. luminescens* (Mahar, 2003) have been recovered from pupae, which have only one organ, the spiracle, open to the external environment. This study has shown that the toxins produced by *X. nematophila* can be applied directly to leaves or soil to control insect pests. The control of *O. sulcatus* by soil application of bacterial toxins

is of particular interest, because this technique could replace entomopathogenic nematodes or the persistent insecticides, choropyrifos or imidiachlorpyrid. The bacterial toxins are sufficiently resistant to control vine weevil larvae in winter when much of the damage occurs to plants (Gwynn, 1993).

Toxins from entomopathogenic bacteria have a very wide spectrum of activity against soil organisms (Webster *et al.*, 2002) including plant parasitic nematodes (Samaliev *et al.*, 2000) and soil fungi (Chen *et al.*, 1994). Patents for the use of toxins from *X. nematophila* against various insect pests have been issued i.e., for the control of fire ants (Dudney, 1997), for *Pieris brassicae* (Jarrett *et al.*, 1997) and for several insect species by various species of *Xenorhabdus* (Ensign *et al.*, 2002). Brown *et al*. (2004) showed that *X. nematophila* had an active secretion system and others (Ffrench-Constant *et al*., 2000; Duchaud *et al*., 2003) have shown that genome of *P. luminescens* contains many proteins predicted to be involved in secretion systems. The Tc toxins in *P. luminescens* have a high level of redundancy, where knockout of any one of the *tc* gene products causes a reduction but not a loss of toxicity (Bowen *et al*., 1998; Duchaud *et al*., 2003). Brown *et al.* (2006) reported Txp40, a ubiquitous insecticidal toxin protein from *Xenrhabdus* and *Photorhabdus* bacteria, which occurs widely and highly conserved toxin in these bacteria. Txp40 is important for the broad insecticidal activity and is a significant component of the extensive array of toxins that the bacteria and nematodes use to destroy their insect hosts.

Brown *et al.* (2004) described that there is still some debate on the species classification of *Xenorhabdus* and *Photorhabdus*, but because the invertebrates evolved before vertebrates, it is possible that theses insect pathogens are the ancestors of the animal pathogen. It is also clear that *Xenorhabdus* may be potentially rich resource for the discovery of additional virulence mechanisms and proteins with other novel bioactivities. *Steinernema carpocapsae* carries the bacterium *X. nematophila* into insects was exempted from normal pesticide legislation (Kaya & Gaugler, 1993). The toxins produced by entomopathogenic bacteria are essentially insecticides and probably require full Tier 1 acute toxicity studies before they can be used commercially (Akhurst & Smith, 2002). Therefore, treatments with *X. nematophila* bacterial cells or cell-free filtrates require further studies before being used in the field.

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