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Studies of Different Application Methods of *Xenorhabdus* and *Photorhabdus* Cells and Their Toxin in Broth Solution to Control Locust (*Schistocerca gregaria*)

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Abstract: Broth culture of *Xenorhabdus nematophila* and *Photorhabdus luminescens* have been observed lethal to the nymphs of locust *Schistocerca gregaria* when injected into the abdomen, applied orally, mixed with bran or applied to the foliage of food plants as compared to broth alone (control). A hyperbolic relationship was observed between different bacterial application methods of cells and their metabolites and time intervals. Bacterial cells and their metabolites caused more or less similar damage to the locust but it is supposed that the insect death was probably due to the toxic metabolites present in the bacterial cells. These bacterial cells were also recovered from the abdominal haemocoele indicating that bacterial symbionts do have a free-living existence and can enter in the haemocoele in the absence of nematode vector. If these bacterial symbionts are to be used for insect control, *X. nematophila* would be the most appropriate as it has never been previously reported from clinical specimens.

Key words: Biological control, bacterial symbionts, *Xenorhabdus nematophila*, *Photorhabdus luminescens*, entomopathogenic nematodes, locust, *Schistocerca gregaria*

INTRODUCTION

The bacteria *Xenorhabdus* and *Photorhabdus* have been used to control a wide range of agriculturally important insect pests[1,2] and are found in genera *Steinernema* and *Heterorhabditis*, respectively. The mechanism by which these nematodes are able to predate and reproduce in the insect host involves a mutualistic relationship between the nematode and these symbiotic bacteria[3,4] as it was first reported in *S. bibionis*[5] but later it was established that a single species of bacterium in the Enterobacteriaceae family is present in the anterior region of the infective juvenile (IJ) of *Steinernema carpocapsae*[6]. Since then it has been known that all *Steinernema* species carry a bacterium of the genus *Xenorhabdus* while nematodes of the genus *Heterorhabditis* harbour a bacterium of the genus *Photorhabdus*[7]. Once an IJ penetrates into the haemocoele the bacterial symbiont is released from the gut of IJ, septicemia becomes established and insect death occurs within hours. Although IJ plays an important role in insect death by vectoring the bacteria, in most cases the bacteria alone are sufficient to cause insect death after entering into the haemocoele[8,9].

Direct application of toxic metabolites produced by *Photorhabdus luminescens* protected plants from larvae of Lepidoptera insects[10]. However, in some preliminary experiments, the fire ant (*Solenopsis invicta*)[11] and larvae of the beet army worm (*Spodoptra exigua*)[12] were controlled by bacterial suspensions of *Xenorhabdus nematophila*. Studies were also conducted to identify the toxic genes from *P. luminescens* so that the gene could be transferred to the plant as a strategy to control insect pests[10]. Keeping in view the importance of these bacteria (*X. nematophila* and *P. luminescens*), experiments have been designed to determine the most appropriate methods of application of bacterial suspensions and their metabolites to control the nymphs of locust.

MATERIALS AND METHODS

Nymphs of the locust were obtained from the Mealworm Co. Sheffield, UK and reared on wheat seedlings at 25°C until required for experiments. Pre-pupae (6th instar larvae) of *Galleria mellonella* also from the same company were infected with IJs of *S. carpocapsae* (All isolate) or *Heterorhabditis bacteriophora* (HW79 isolate) to obtain cadavers containing the bacterial

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symbionts *X. nematophila* and *P. luminescens,* respectively.

Isolation of bacterial symbionts and their metabolites: *Xenorhabdus nematophila* and *P. luminescens* were obtained from the haemolymph of *G. mellonella* infected with IJs of *S. carpocapsae* and *H. bacteriophora.* Dead *Galleria* larvae were surface-sterilised in 70% alcohol for 10 min, flamed and allowed to dry in a laminar airflow cabinet for 2 min. Larvae were opened with sterile needles and scissors, care being taken not to damage the gut and a drop of the oozing haemolymph was streaked with a needle onto nutrient agar (NBTA) plates [37 g nutrient agar (BDH); 25 mg Bromothymol blue powder (Raymond); 4 mL of filtrates of 1% 2,3,5 Triphenyl-tetrazolium Chloride (BDH); 1000 mL distilled water]. The agar plates, sealed with Parafilm, were incubated at 28°C in the dark for 24 h, when single colonies of bacteria were selected and streaked onto new plates of nutrient agar. Sub-culturing was continued until colonies of uniform size and morphology were obtained. The pathogenicity of the isolates was confirmed by inoculating the bacteria into *Galleria* larvae and streaking the haemolymph of the infected larvae on NBTA plates. A single colony of the bacterium was selected and inoculated into 500 mL of nutrient broth solutions, containing 15 g nutrient broth (BDH) and 500 mL of distilled water in a flask stoppered by sterile cotton wool and placed in a shaking incubator at 150 rpm for one day at 28°C. The bacterial concentration of the broth suspension was determined by measuring the optical density using a spectrophotometer adjusted to 600 nm wavelength. Based on results obtained by Elawad[13] the concentration of the bacterial cells used in the present experiments was adjusted to 4×10^7 cells mL^{-1} and 3% Tween 80 was added as an emulsifier.

To obtain solutions containing the bacterial symbionts in water the broth suspension was centrifuged at 4100 rpm for 20 min. A bacterial pellet was formed at the bottom of the centrifuge tube, the supernatant broth solution was drawn off and replaced by distilled water. The concentration of bacterial cells was estimated as previously and adjusted to 4×10^7 cells mL^{-1}. To obtain cell-free solutions of the metabolites from the bacterial symbionts, the bacterial suspensions in water were filtered using a Whatman 25 Mm GD/X filter with a pore size of 0.2 μm.

Experiment 1: Injection method to apply bacterial suspensions or their metabolites into the body of locust adults: The abdomen of thirty two locust adults for each treatment was injected with 30 μmL of broth suspensions of the bacterial symbionts *X. nematophila* or *P. luminescens* or solutions of the metabolites in broth from the bacterial. The control treatment was broth alone plus 3% Tween 80. After each treatment, the locusts were fed on wheat bran in large plastic containers at 28°C and their mortality was determined daily up to 5 days.

Experiment 2: Oral application of bacterial suspensions or their metabolites into the body of locust adults: Broth suspensions of the bacterial cells (4×10^7 cells mL^{-1}) or a solution containing metabolites from the bacterial cells were applied orally to thirty two adult locusts in each treatment using a sterile Pasteur pipette (30 μL per adult). After each treatment, locust adults were fed on wheat bran and mortality was determined daily up to 10 days. The dead locusts were sterilised in 70% industrial methylated spirit for half an hour to kill the bacteria on the surface of the locusts. A sample was then taken from the haemocoele of the abdomen and streaked onto nutrient agar to determine whether or not bacteria were present in the haemocoele.

Experiment 3: Application of bacterial suspensions or their metabolites in wheat bran feed: Ten milliliter of broth suspensions containing 4×10^7 cells of *X. nematophila*, *P. luminescens* or their cell-free solutions containing metabolites were mixed with 50 g of wheat bran. The control treatment was broth alone. Ten *S. gregaria* adults were placed in each container and the mortality was observed for 10 days. The replication was 4-fold. Locust adults were kept hungry one day before fed on each treatments in bran. All dead locusts treated with cell suspensions were sampled to find out whether viable cells of both bacteria could be recovered from the haemocoele.

Experiment 4: Direct application of bacterial suspensions and their metabolites to the intact leaves of host plants: Wheat seedlings were grown in 9 cm plastic pots. When the seedlings were 5 cm high they were sprayed with 10 mL of suspension of either *X. nematophilus* or *P. luminescens* or their metabolites at a concentration of 4×10^7 cells mL^{-1}. Ten locust nymphs per replication were placed on the wheat leaves. The control treatment was 3% Tween 80 in broth. The pots were covered with a small glass bell jar to retain the moisture on the wheat leaves. The mortality percentage of locust nymphs was after 10 days. All dead insects were removed, surface sterilised in 70% industrial methylated spirit and samples taken from the abdominal haemolymph, which were streaked onto nutrient agar to determine whether bacterial cells had entered the haemocoele of the nymphs. Data of all experiments were analysed using the SAS (version 8)

statistical package (SAS Institute Inc., Cary, North Carolina. USA).

RESULTS

Injection of bacteria and their metabolites: Statistical analysis showed a significant difference ($p<0.05$) between treatments and time interval. However, there was no significant effect of bacterial treatments was observed after third day (Fig. 1). One day after injecting the *X. nematophila* cells and their metabolites in broth, mortality of adult locust was 56 and 59%, respectively. Similarly, when *P. luminescens* cells and their metabolites in broth were injected into the locust body, the insect mortality was recorded as 69 and 72%, respectively. Insect mortality increased afterward and on fifth day after injection, 100% of the locust adults treated with cells and their metabolites of either of the bacteria were found dead.

Oral application of bacteria and their metabolites: Bacterial cells and their metabolites in broth showed a significant difference ($p<0.05$) at different time intervals when applied orally (Fig. 2). After two days of bacterial application only 9-19% locust mortality was observed in *X. nematophila*, whereas application of *P. luminescens* (cells and metabolites) killed 16-22% insects. Locust mortality was gradually increased with the passage of time and after 10 days 72-78% mortality was recorded when cells and metabolites of *X. nematophila* were applied. However, 78 and 82% locust mortality was observed when

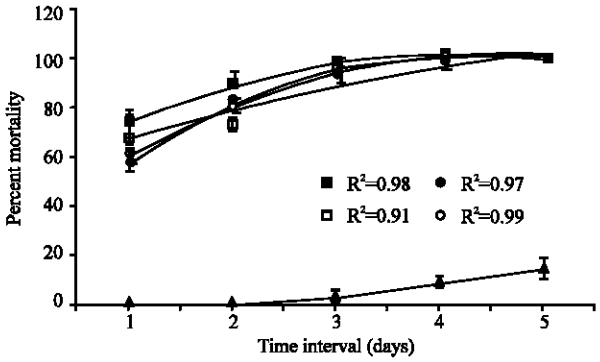

Fig. 1: Effects of bacterial cells suspension and their metabolites on mortality percentage of locust adults as applied by injection. Figures symbols are denoted as: metabolites (▪) and cells (▫) of *Photorhabdus luminescens*, metabolites (○) and cells (●) of *Xenorhabdus nematophila* and (Δ) broth alone (control). Vertical bars (where larger than the points) represent the standard error (s.e.) of variability

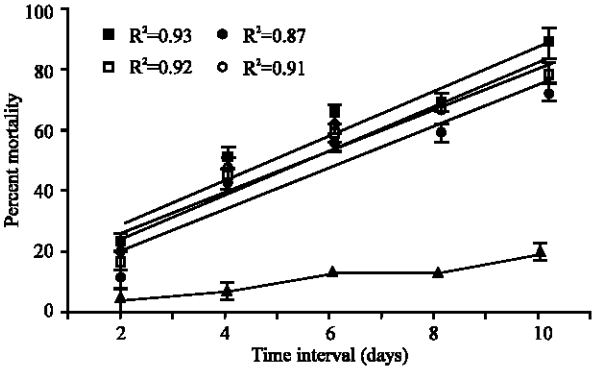

Fig. 2: Effects of bacterial cells suspension and their metabolites on mortality percentage of locust adults as applied orally. Figures symbols are denoted as: metabolites (▪) and cells (▫) of *Photorhabdus luminescens*, metabolites (○) and cells (●) of *Xenorhabdus nematophila* and (Δ) broth alone (control). Vertical bars (where larger than the points) represent the standard error (s.e.) of variability

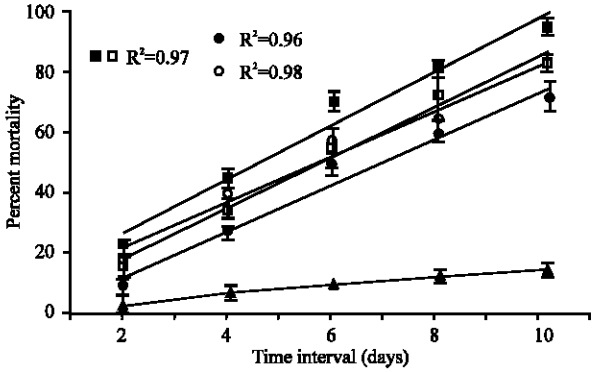

Fig. 3: Effects of bacterial cells suspension and their metabolites on mortality percentage of locust adults as applied in wheat bran feed. Figures symbols are denoted as: metabolites (▪) and cells (▫) of *Photorhabdus luminescens*, metabolites (○) and cells (●) of *Xenorhabdus nematophila* and (Δ) broth alone (control). Vertical bars (where larger than the points) represent the standard error (s.e.) of variability

cells and their metabolites of *P. luminescens* were applied. The mortality caused by oral application of both bacterial solutions containing metabolites in broth was slightly more effective than the cells. However, no bacterial cells were recovered from the abdomen of adult locust fed orally with cell suspensions of either bacterial symbiont.

Application of bacteria and their metabolites in wheat bran feed: Mortality percentage of *S. gregaria* adults

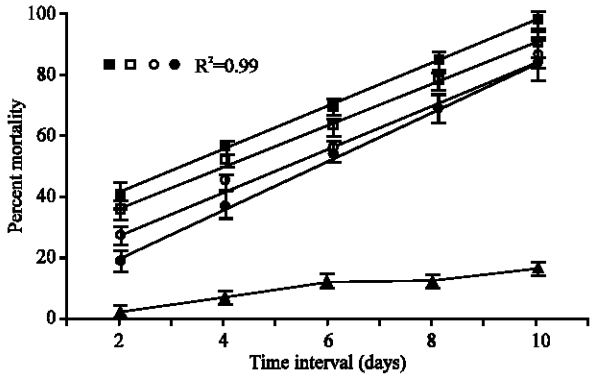

Fig. 4: Effects of bacterial cells suspension and their metabolites on mortality percentage of locust adults as applied to the leaves of host plant. Figures symbols are denoted as: metabolites (▪) and cells (▫) of *Photorhabdus luminescens*, metabolites (○) and cells (●) of *Xenorhabdus nematophila* and (Δ) broth alone (control). Vertical bars (where larger than the points) represent the standard error (s.e.) of variability

significantly ($p<0.05$) increased with the increase in time after feeding wheat bran containing bacterial cells and their metabolites (Fig. 3). Cells suspension of *Xenorhabdus* when mixed with wheat bran feed slowly killed locust after 2 days (8%), however mortality percentage was 50 and 70% after 6 and 10 days, respectively. Similarly, when metabolites of the same bacterium were applied through feed, the mortality was 18% after 2 days but it was increased up to 58 and 83% after 6 and 10 days, respectively. Similar trend was observed when cells and their metabolites of *Photorhabdus* were applied and 83-95% mortality was observed after 10 days of cells and their metabolites application. No cells of *X. nematophila* or *P. luminescens* were recovered from the locust abdomen treated with bacterial suspensions.

Direct application of bacteria and their metabolites to the intact leaves of host plants: Again a linear but significant ($p<0.05$) increase in mortality percentage was observed as the time passed on after applying cells and their metabolites on wheat leaves (Fig. 4). After 8 days of *Xenorhabdus* cells and their metabolites 70% locust nymphs were killed whereas *Photorhabdus* cells and metabolites killed 78 and 85% locust nymphs, respectively. Similarly, after 10 days of treatments, 85-88% nymphs were found dead when *Xenorhabdus* cells and their metabolites were applied, respectively whereas *Photorhabdus* cells and metabolites killed 90-98% nymphs, respectively. Bacterial cells were recovered from the haemocoele of all dead nymphs treated with cells suspensions of bacterial symbionts.

DISCUSSION

Cells suspension of *X. nematophila* and *P. luminescens* were found lethal to the larvae of *Galleria* when injected into the haemocoele[9,14]. Present research findings of locust are in line with the reported results of *Galleria* when both bacterial cells injected into the nymphs. Similarly, bacterial solutions containing toxic metabolites were also found lethal when injected into locust nymphs, thus confirmed the reports of Bowen and Ensign[10] who injected the toxic metabolites of *P. luminescens* into the larvae of *Manduca sexta*. In another study, the intrahaemocoelic injection of bacterium *X. japonicus* (isolated from *Steinernema kushidai*) has caused 100% mortality of Cupreous Chafer larvae (*Anomala cuprea*) within 2-3 days[15].

Bacterial cells suspension or metabolites solution of both species were lethal to locust adults when fed orally indicated that the metabolites secreted by the bacteria are probably responsible for the death of the locust nymphs. However, in the present experiments the bacterial cells were not recovered from the haemocoele when fed orally showed that they were unable to penetrate the gut wall. Whereas, IJs of *Steinernema feltiae* and *Heterorhabditis megidis* were able to penetrate through the gut wall of locust into the haemocoele when fed orally[16].

Bacterial cells or their toxic metabolites of *X. nematophila* and *P. luminescens* when applied on wheat leaves as feed were proved lethal to nymphs of locust. In a similar study, the bacterial cells and their associated metabolites of *X. nematophila* have also been shown to be effective against the fire ant[11] and beet army worm when they were applied on cotton leaves[12]. In the present study, it was observed that bacterial cells of both *X. nematophila* and *P. luminescens* were able to penetrate into the haemocoele of the targeted insect (locust) in the absence of the nematode vector, which normally carries the lethal bacteria into the insect host.

It has been assumed that the association between entomopathogenic nematodes and their symbiotic bacteria is mutualistic and that the symbiosis is essential for the survival of both the nematode and the bacterium[2]. In the present and some previous experiments[11,12] it has been shown that these symbiotic bacteria are able to penetrate into the haemocoele of insect hosts in the absence of the nematode vector, but the mechanism by which the bacteria gain entry to the haemocoele is unclear. Both *X. nematophila* and *P. luminescens* exhibit swarming motility when grown in suitable solid

media[4,13,17]. It was noted that bacterial cells were unable to penetrate into the haemocoele when applied orally and so an alternative point of entry into the haemocoele for motile bacterial cells under moist conditions could be either be directly through the cuticle or through the spiracle, the only organ in the insect cuticle, other than the mouth and anus, open to the external environment. The pupae of the beet army worm have also been reported susceptible to cells of both *X. nematophila* and *P. luminescens*[13]. It is therefore emerged that the spiracle of pupae is the only organ open to the external environment through which these bacteria enter into the haemocoele. However, further evidence is required to define the exact mode of entry of these bacteria into the haemocoele.

The results obtained here confirm the findings of Bowen and Ensign[10] that metabolites from bacterial symbionts are toxic to insect pests feeding on plants to which the toxic secretions have been applied. The toxin complexes from *P. luminescens*, which have a high oral toxicity to *M. sexta*, could be used for transgenic deployment into plants[18]. The purpose of the present experiments was to demonstrate that it would be possible to use formulations of these bacterial symbionts directly applied to plants to control insect pests such as locusts. In order to use these bacteria or their toxic secretions in the field, it would be necessary to carry out normal toxicology tests. However, it is relevant to point out that these bacteria and their normal nematode hosts are widespread in the soil, often in very large numbers and have not been reported as damaging to human health though *P. luminescens* has been reported from clinical specimens[19]. Therefore, *X. nematophila* is judged to be the most appropriate bacterium for field application because unlike *P. luminescens* it has never been reported from clinical specimens.

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