Journal of Entomology 2 (1): 69-76, 2005
ISSN 1812-5670

Role of Entomopathogenic Bacteria, *Photorhabdus luminescens* and its Toxic Secretions Against *Galleria mellonella* Larvae

[1]A.N. Mahar, [2]M. Munir, [1]S.R. Gowen and [1]N.G.M. Hague
[1]Department of Agriculture,
[2]School of Plant Sciences, University of Reading, Reading RG6 6AS, UK

Abstract: The entomopathogenic bacterium, *Photorhabdus luminescens* and its metabolites were found lethal to the *Galleria mellonella* when applied in sand media. Bacterium penetrated quickly in the haemocoele as it got contact with insect body. It was also observed that the toxic metabolites caused more larval death than the bacterial cells. *P. luminescens* cells were recovered from the haemocoele when suspensions containing bacterial cells were applied to the *G. mellonella* indicating that bacterial symbionts do have a free-living existence and can enter the haemocoele in the absence of nematode vector. This bacterium or its toxic secretions can be used for insect control that can be important component of integrated pest management against different insect pests.

Key words: Biocontrol, bacterial symbionts, entomopathogenic nematodes, *Photorhabdus luminescens*, *Galleria mellonella*

INTRODUCTION

Bacterium, *Photorhabdus luminescens* is associated with the entomopathogenic nematode *Heterorhabditis bacteriophora* and belongs to family Heterorhabditidae. The infective juvenile (IJ/IJs) nematode harbors the *P. luminescens* bacterium in its gut and after infecting an insect, the nematode burrows through the intestinal wall of the insect and bacteria is released in haemolymph. The bacteria avoid the immune response of the insect and proliferate rapidly so that within 48 h the insect is dead[1]. When *P. luminescens* is involved in this infection, the insect cadaver is visibly luminous in darkness and has a brick red colour in daylight. Both the luminescence and the pigmentation of the cadaver are the results of the bacterial population in the cadaver[2-4].

Cells when are released by the nematodes into the haemolymph of insect host where they multiply rapidly and kill the host and, in doing so produce conditions that are favourable for nematode development and reproduction[5]. Septicemia becomes established and insect death occurs within 48 h. Although IJ play an important role in insect death by vectoring the bacteria, in most cases the bacteria alone are sufficient to cause insect death following injection into the haemocoele[6,7].

Insect pathogenic bacteria can be classed as either spore formers or non-spore formers. *Bacillus thuringiensis*, a spore former, produces crystal toxins that destroy the epithelial cells lining the insect gut[8]. Non-spore forming bacteria (e.g. *Pseudomonas aeruginosa*, *Serratia marcescens* and *Providencia rettgeri*) are generally pathogenic as a result of extra cellular enzyme production or lipopolysaccharide(s) that destroy the haemocytes and internal organs of the insects once the bacteria have penetrated into haemocoele[9]. The genus *Photorhabdus* and *Xenorhabdus* are highly pathogenic to a variety of insect but their virulence determinants have not yet been characterized. They are non-spore forming bacteria which infect the haemocoele of their insect hosts. Protease, lipases and lecithinase are secreted by both genera[10] and in case of *Photorhabdus* spp. strain Hm produces a single extra cellular protease which has been purified and characterized biochemically[4] while strain K122 produces a lipase enzyme whose gene has also been cloned and sequenced[11]. However the toxicity of these enzymes to insect has not been studied.

Bowen and Ensign[12] have shown that metabolites produced by *P. luminescens* will protect plants from insect attack by direct application of the secretions to plants upon which the larvae of several species of Lepidopterous and other insects. They introduced *P. luminescens* insecticidal toxins used through oral and injection method. Furthermore they reported that virulence of *P. luminescens* for insects is a complex and multifaceted process. The experiments reported

Corresponding Author: Dr. A.N. Mahar, Department of Agriculture, University of Reading, Reading RG6 6AT, UK
E-mail: anmahar4@yahoo.co.uk

by them were designed 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. *Photorhabdus* toxin has caused disruption of the midgut epithelium of *Manduca sexta*. Dunphy and Webester[13] reported that bacteria have not been found in free-living form in nature, raising doubts of their ability to survive and infect an insect host without the help of the symbiont nematodes. It is generally believed by those working with entomopathogenic nematodes[14] that the symbiotic association between the bacteria and the nematode is essential for the survival of both and that the symbiotic bacteria do not have a free-living existence. However, in some preliminary experiments, the fire ant, *Solenopsis invicta*[15] and larvae of the beet army worm, *Spodoptra exigua*[16] were controlled by bacterial suspensions of *X. nematophila* from *Steinernema carpocapsae*. The present study was conducted to test the pathogenicity of bacterial suspension of *P. luminescens* and its metabolites against Lepidopterous insect pest *G. mellonella* larvae when applied directly without nematode vector.

MATERIALS AND METHODS

Larvae of greater wax moth, *Galleria mellonella* were obtained from 'The Mealworm Co. UK' which were infected with IJs of *H. bacteriophora* (HW79 isolate) and cultured at 28°C. IJs suspensions of nematodes were supplied by 'CAB Institute of Parasitology, St. Albans, UK'. Nematodes were cultured in the *G. mellonella* and were stored at 15°C. Later on, the *P. luminescens* bacterium symbiont was isolated from infected cadavers of the larvae.

Isolation of bacterial symbionts and their secretions: *Photorhabdus luminescens* was obtained from the haemolymph of *G. mellonella* infected with IJs of *H. bacteriophora*. Dead *G. mellonella* 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 colony of bacterium was 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 *G. mellonella* 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 solution, 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[17] 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 only toxic secretions from the bacterial symbionts, 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 stated previously and adjusted to 4×10^7 cells mL^{-1}. To obtain cells-free solution of the metabolites from the bacterial symbionts, the bacterial suspensions in broth or water were filtered using a Whatman 25 Mm GD/X filter with a pore size of 0.2 μm. Purity of cells-free toxin solutions were tested on agar plates before application against *G. mellonella* larvae.

Experiment 1. Influence of time interval on the efficacy of bacterial cells and their toxic secretion in response to mortality of *G. mellonella* larvae: This experiment was designed to test the efficacy of the *P. luminescens* suspensions and its toxic secretion in broth and water under sand arena against the larvae of *G. mellonella* at different time intervals. Cells suspension and secretions of *P. luminescens* in broth and water were prepared at concentration of 4×10^7 cells mL^{-1} and 3% Tween-80 was mixed in all treatments in all experiments. In 100 g of sterilised sand 16.4 mL of cells suspension or their secretion was mixed in order to keep 14% moisture content. Late instar of *G. mellonella* larvae of similar age and size were surface sterilised with 2% Hymine for five minutes and then dried under the laminar airflow. Ten larvae were placed in the moist sand in sterilised plastic containers (110×25 mm) with bacterial suspension or secretions. Water and broth alone were also used as control. All containers were incubated at 25°C. The mortality was assessed daily for seven days. Replication was four fold in all experiments. The dead larvae were sterilised in 70% industrial methylated spirit for 5 min to kill the bacteria on the surface of the

G. mellonella larvae. A sample from dead insects 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 2. Influence of filter paper and sand substrates on the efficacy of bacterial cells and their toxic secretion in response to mortality of *G. mellonella* larvae: Efficacy of *P. luminescens* cells suspension and their secretion on the filter paper and sand substrate against the larvae of *G. mellonella* was tested. Similar procedure was adapted to obtain fresh cells suspension and their toxic secretion of *P. luminescens* in broth and water as described in experiment 1. Two Whatman filter papers were placed in 9 cm sterilised petri dish. Two milliliter from each cells suspension and their secretion was sprayed on the filter paper with a hand sprayer. Ten *G. mellonella* larvae were placed in each petri dish and then sealed with Paraflim. In sterilised plastic containers (110×25 mm), 100 g sterilised fine sand was adjusted to 14% moisture content with bacterial suspension and their toxic secretion. Water and broth alone were sprayed as controls. Ten *G. mellonella* larvae were placed on the sand, sealed with Parafilm and incubated at 25°C. The mortality for *Galleria* larvae was assessed after one week. The cause of mortality of larvae was confirmed by taking samples from the haemolymph of the dead larvae and smeared on NBTA agar plates.

Experiment 3. Influence of moisture contents on the efficacy of bacterial cells and their toxic secretion in response to mortality of *G. mellonella* larvae: *Photorhabdus* cells suspension and their toxic secretion in broth and water were prepared as described already in experiment 1. Moisture contents were adjusted to 10, 14 and 18% adding 10 mL of cells suspension or their toxic secretion. Ten late instar of *G. mellonella* larvae were placed in plastic containers (110×25 mm), sealed with Paraflim and incubated at 25°C. Larval mortality was assessed after seven days and the cause of larval mortality was confirmed by taking samples from their haemolymph and smeared on NBTA agar plates.

Experiment 4. Influence of temperature on the efficacy of bacterial cells and their toxic secretion in response to mortality of *G. mellonella* larvae: Three different temperatures, 20, 25 and 30°C against *G. mellonella* larvae were tested for the pathogenicity of *P. luminescens* cells and their secretion in broth and water. Cells suspension and their secretion from *P. luminescens* in broth and water were prepared at concentration of 4×10^7 cells mL^{-1} as described in experiment 1. Water and broth alone was used as control. Sterilised fine sand (100 g) was adjusted to 14% moisture with bacterial suspension or its secretion. Ten *G. mellonella* larvae were placed in each sterilised plastic containers (110×25 mm). The containers of each treatment were incubated at 20, 25 and 30°C and larval mortality was recorded after one week. The cause of mortality of larvae was confirmed as described in experiment 1.

Experiment 5. Effect of different concentrations of bacterial cells suspension on the mortality of *G. mellonella* larvae: Six concentrations of *P. luminescens* bacterial cells suspension, 4×10^2, 4×10^3, 4×10^4, 4×10^5, 4×10^6 and 4×10^7 cells mL^{-1} in broth and water were prepared (see experiment 1). A 100 g autoclaved fine sand having 14% moisture and the bacterial cells suspension were placed in sterilised plastic containers (110×25 mm). Ten late instar larvae were placed in each container. All containers were incubated at 25°C and mortality of larvae was recorded after 6 days. All dead larvae were confirmed by streaking the haemolymph on agar plates.

Experiment 6. Penetration of bacterial cells into *G. mellonella* larvae at different time interval: The objective of this experiment was to determine the most appropriate time for the bacterial cells to enter into the larvae. Bacterial cells suspension in broth and water at concentration of 4×10^7 cells mL^{-1} were produced as mentioned already (experiment 1). Ten late instar of *G. mellonella* larvae were placed on a Whatman filter paper in sterilised petri dish (9 cm). Two milliliter from each suspension was sprayed with a hand sprayer on the filter paper in petri dish under the laminar airflow cabinet and were kept at 25°C. Results assessing the bacterial penetration to insects whether alive or dead were sampled after 15, 30 min, 1, 2, 4, 8, 16 and 32 h on agar plates.

Experiment 7. Longevity of stored bacterial toxic secretion against *G. mellonella* larvae: Fresh bacterial toxin secretion in broth and water were produced as described in experiment 1. The bacterial secretion of *P. luminescens* having 3% Tween-80 was then stored at the 25°C for 4 weeks. Each stored toxic secretion was then mixed with 100 g sterilised fine sand and was adjusted to 14% moisture. This content was then put into plastic containers (110×25 mm) and ten *Galleria* larvae were placed in each. These containers were then placed at 25°C and mortality of larvae was assessed after one week of application of stored toxin solution.

Experiment 8. Effect of dried bacterial toxic secretion (powder) on mortality of *G. mellonella* larvae: Bacterial toxin solution in broth and water was produced (experiment 1) and then dried in the sterilised containers at the 25°C for two days under laminar airflow. The dried toxins were rewetted with either broth or water solution and 3% Tween-80 was mixed within each solution. Fine sterilised sand (100 g) was adjusted to 14% moisture and dried toxin was mixed in that. Ten *G. mellonella* larvae were washed with Hymine and placed in each sterilised plastic containers (110×25 mm) which were placed at 25°C. The mortality of larvae was recorded after one week of treatment. Data of all experiments were analysed using the SAS (version 8) statistical package (SAS Institute Inc., Cary, North Carolina. USA).

RESULTS

Statistical analysis showed that time has highly significant effect ($p<0.01$) on the mortality of *G. mellonella* larvae. There was significant effect of the broth treatments as compared to water. Mortality of the *G. mellonella* larvae increased linearly as the time of exposure increased (Fig. 1). Maximum mortality of 100% was found when bacterial secretion in broth was applied after 7 days whereas toxic secretion in water caused 80% mortality. Similarly, 95% mortality was observed when cells of *P. luminescens* in broth were applied after 7 days whereas bacterial suspension in water caused 75% mortality after similar period of time. In the controls only 15 and 13% mortality was found with broth and water respectively. Statistically ($p<0.01$) greater mortality of *G. mellonella* larvae was obtained in sand as compared to filter paper (Fig. 2). Maximum mortality of *G. mellonella* larvae (100%) was occurred when treated with secretions of *P. luminescens* in broth followed by bacterial cells in broth (95%) and bacterial secretions in water (90%) using sand bioassay after 7 days. On the other hand, insect mortality on filter paper was 20 to 35% when treated with bacterial secretions in water and broth, respectively. However, there was non-significant difference between the bacterial cells or cell-free secretions.

Figure 3 showed the effect of three moisture content levels on the pathogenicity of *P. luminescens* bacterium and its secretions. The higher mortality (100%) was observed at 14% moisture content than 10 and 18% when *G. mellonella* larvae were treated with bacterial secretions in broth. Mortality at 10% moisture was 67.5 and 75% when treated with bacterial secretions in broth and water. Temperature has significant effect ($p<0.01$) on the mortality of *G. mellonella* larvae (Fig. 4). However, there was non-significant difference in the mortality caused by

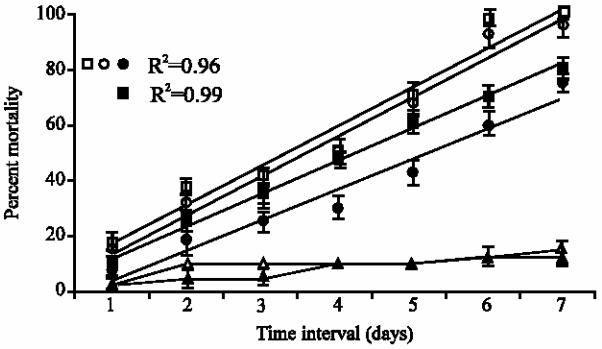

Fig. 1: Mortality response of *Galleria* larvae to *Photorhabdus* cells in broth (○) and water (•), *Photorhabdus* secretion in broth () and water (■), broth alone (△) and water alone (▲) after different time intervals. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

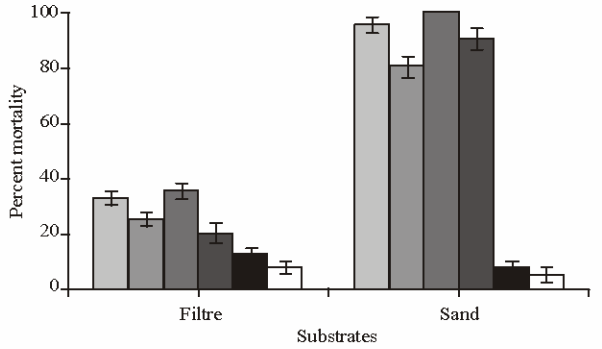

Fig. 2: Effect of *Photorhabdus* cells in broth (▭) and water (▭), *Photorhabdus* secretion in broth (▭) and water (▭), broth alone (▭) and water alone (▭) on mortality of *Galleria* larvae using filter and sand substrates. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

either cells of *P. luminescens* or cell-free secretions. Bacterial secretions were found more effective and caused 100% mortality when applied with the broth rather than water (82.5%) at 25°C. Minimum mortality (57.5% at 20°C) was found when larvae were treated with cell suspension in water as compared to broth (62.5%) at same temperature. Statistical analysis showed that there was a significant ($p<0.01$) difference among various concentrations of the bacterial suspensions against *G. mellonella* larvae (Fig. 5). Similarly, mortality for bacterial suspensions in broth was significantly different from the suspensions in water. Maximum mortality (97.5%) was found when 4×10^7 bacterial concentration in broth was used whereas minimum mortality (35%) was observed when 4×10^2 bacterial cells in water were applied.

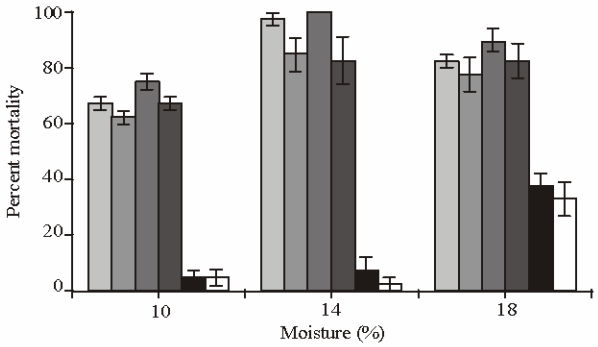

Fig. 3: Effect of *Photorhabdus* cells in broth (▭) and water (▭), *Photorhabdus* secretion in broth (▬) and water (▬), broth alone (■) and water alone (□) on mortality of *Galleria* larvae at three moisture contents. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

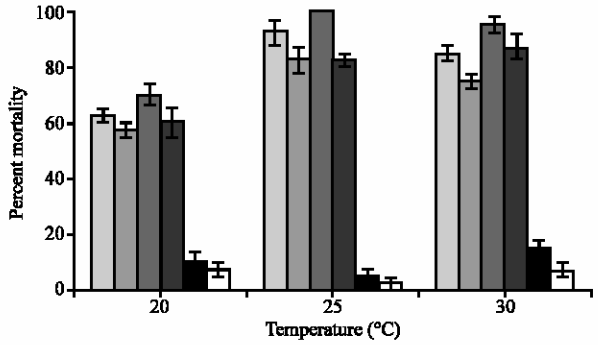

Fig. 4: Effect of *Photorhabdus* cells in broth (▭) and water (▭), *Photorhabdus* secretion in broth (▬) and water (▬), broth alone (■) and water alone (□) on mortality of *Galleria* larvae at three temperature regimes. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

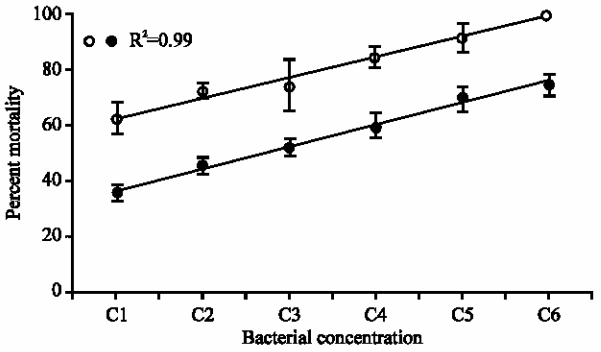

Fig. 5: Effect of different bacterial cell concentrations (4×10^2, 4×10^3, 4×10^4, 4×10^5, 4×10^6 and 4×10^7 cells mL^{-1}) in broth (○) and water (•) on mortality percentage of *Galleria* larvae. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

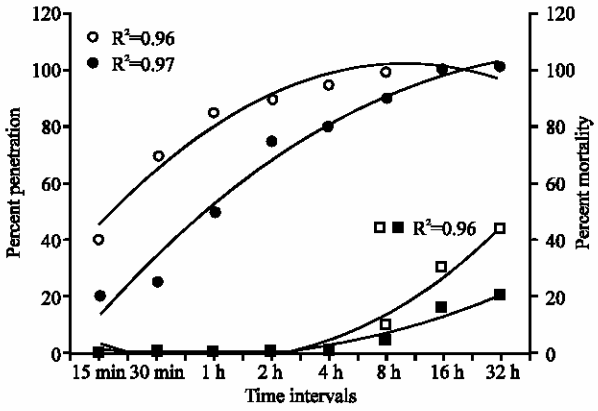

Fig. 6: Percent penetration of *Photorhabdus* cells in broth (○) and water (•), on primary axis and mortality of *Galleria* larvae caused by *Photorhabdus* cells in broth (▫) and water (▪), on secondary axis after different time intervals. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

Bacterial cells in broth suspensions entered in the larval body after 8 h while in water they took 8-16 h (Fig. 6). In 1 h, 85% cells in broth were entered but 50% cells in water were found inside the body of *G. mellonella* larvae. Similarly, in 16 h, 30% larvae were found dead when treated with cells in broth suspension but in water 15% mortality of *G. mellonella* larvae was noticed (Fig. 6). Fresh toxin secretions were found more effective to *G. mellonella* larvae as compared to stored toxin (Fig. 7). Mortality rate decreased with the increase in storage time. Toxin when applied afresh with broth and water caused 95 and 80% mortality, respectively. The mortality was then declined with the storage time and after 4 weeks storage at 25°C the same toxin caused 48 and 28% mortality when applied with broth and water, respectively. Dried toxin of *P. luminescens* when applied with broth induced maximum mortality (100%) to *G. mellonella* larvae after 7 days whereas the application of dried toxin when dissolved in water caused 90% mortality (Fig. 8). However, a non-significance difference was observed between the dried secretions dissolved either in broth or water.

DISCUSSION

Bacterium, *P. luminescens* and its free-cell secretions (metabolites) were proved lethal when applied against

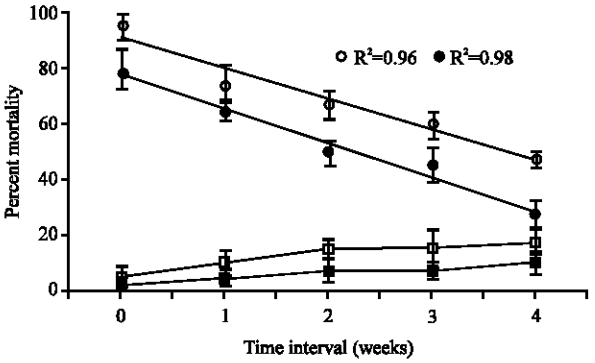

Fig. 7: Effect of stored bacterial secretion in broth (○) and water (•), broth alone (▫) and water alone (▪) on mortality percentage of *Galleria* larvae after different time intervals. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

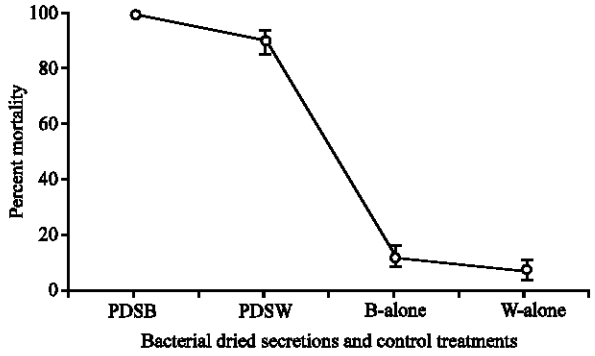

Fig. 8: Effect of *Photorhabdus* dried secretion in broth (PDSB) and water (PDSW), broth alone (B-alone) and water alone (W-alone) on mortality percentage of *Galleria* larvae. Vertical bars (where larger than the points) represent the Standard Error (SE) of variability

G. mellonella larvae. It confirmed the reports of Bowen and Ensign[12], Bowen *et al.*[18] who injected or fed orally the toxic metabolites isolated from *P. luminescens* to the larvae of *Manduca sexta* and others. Insecticidal toxin proteins secreted by *P. luminescens* have also been purified by Rajagopal and Bhatnagar[19]. Similarly, Mohan *et al.*[20] tested the pathogenicity of *P. luminescens* bacteria under natural conditions on the plant foliage against cabbage butterfly. They reported significant larval mortality (100%) using the concentration of 10^8 CFU mL^{-1} within 24 h. Present findings are in line with these studies. In another reports, suspensions containing cells of the bacterial symbionts *X. nematophila*[6] and *P. luminescens*[21] were established lethal against the larvae of *G. mellonella* when injected into the haemocoele. Similarly, Jackson *et al.*[22] isolated *Providencia rettgeri* from *Heterorhabditis* spp. which killed the *G. mellonella* larvae when injected in the insect body. This specie was found as pathogenic as *Photorhabdus* sp. K122. In another study, Clarke and Dowds[23] assessed the virulence of *Photorhabdus* sp. K122 by injecting their cells into haemocoele of *G. mellonella* larvae. They indicated that virulence correlated with the growth rate of cultures and all larvae died after the cells entered the stationary phase. Further more they reported that maximal production of protease and lipase exoenzymes occurred at the stationary phase and the extra cellular fraction was found to be toxic to the insects. Part, but not all, of that toxicity was attributed to secreted lipase. Total lysates of K122 were also found toxic to *G. mellonella* larvae.

The results of present experiments suggest that *G. mellonella* larvae and other lepidopterous insect pests can be controlled by bacterial symbionts or their secretions at temperatures between 25 and 30°C, which is the temperature range of most tropical cropping pattern. Optimum moist sand conditions can increase the effectiveness of these bacteria against any insect pests. It is possible that these entomopathogenic bacteria or their secretions could be used to reduce the pest damage in field conditions. It has always 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[14].

In the present study and those by Dudney[15] and Elawad *et al.*[16] it has been shown that these symbiotic bacteria are able to penetrate into the haemocoele of the hosts in the absence of the nematode vector, but the method 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[17,24,25]. It was noted that bacterial cells were able to penetrate into the haemocoele when applied and so through 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. Further evidence is required to define the exact mode of entry of these bacteria into the haemocoele.

The purpose of the present experiments was to demonstrate that it would be possible to use these bacterial symbionts or their secretions directly to control insect pests. In order to use these bacteria or their toxic secretions in the field it would be necessary to carry out normal toxicology tests but it is relevant to point out that these bacteria and their normal nematode hosts are wide spread in the soil. It will also be necessary to find

ways of easy mass production, persistence of these symbiotic bacteria in the general environment, particularly with respect to desiccation on foliage and use of bacterial metabolites which release toxins from *P. luminescens* for the species to fulfil its potential in insect pest control.

REFERENCES

1. Poinar, G.O., G.M. Thomas, M. Haygood and K.H. Nealson, 1980. Growth and luminescence of the symbiotic bacteria associated with terrestrial nematode *Heterorhabditis bacteriophora*. Soil Biol. Biochem., 12: 5-10.
2. Akhurst, R.J., 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. General Microbiol., 128: 3061-3065.
3. Richardson, W.H., T.M. Schmidt and K.H. Nealson, 1988. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Applied Environ. Microbiol., 54: 1602-1605.
4. Schmidt, T.M., B. Bleakley and K.H. Nealson, 1988. Characterisation of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. Applied Environ. Microbiol., 54: 2793-2797.
5. Boemare, N.E., R.J. Akhurst and R.G. Mourant, 1993. DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus*. Intl. J. Systematic Bacteriol., 43: 249-255.
6. Gotz, P., A. Boman and H.G. Boman, 1981. Interactions between insect immunity and an insect-pathogenic nematode with symbiotic bacteria. Proceedings Royal Society London, 212: 333-350.
7. Balcerzak, M., 1991. Comparative studies on parasitism caused by entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. The roles of the nematode-bacterial complex and of the associated bacteria alone, in pathogenesis. Acta Parasitologica Polonica, 36: 175-181.
8. Van-Rie, J., S. Jansens, H. Hofte, D. Degheele and H. Van-Mellaert, 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Applied Environ. Microbiol., 56: 1378-1385.
9. Lysenko, O., 1985. Non-spore forming bacteria pathogenic to insects: Incidence and mechanisms. Ann. Rev. of Microbiol., 39: 673-695.
10. Boemare, N.E. and R.J. Akhurst, 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). J. General Microbiol., 134: 751-761.
11. Wang, H. and B.C.A. Dowds, 1993. Phase variation in *Xenorhabdus luminescens*: Cloning and sequencing of the lipase gene and analysis of its expression in primary and secondary phases of the bacterium. J. Bacteriol., 175: 1665-1673.
12. Bowen, D.J.J. and C. Ensign, 1998. Purification and characterisation of the high-molecular-weight insecticidal protein complex produced by the entomopathogenic bacterium, *Photorhabdus luminescens*. Applied and Environ. Microbiol., 64: 3029-3035.
13. Dunphy, G.B. and J.M. Webester, 1988. Virulence mechanisms of *heterorhabditis, heliothidis* and its bacterial associate, *Xenorhabdus luminescens*, in non-immune larvae of greater wax moth, *Galleria mellonella*. Intl. J. Parasitol., 18: 729-737.
14. Poinar, G.O., 1979. Nematodes for Biological Control of Insects. C.R.C. Press. Boca Raton, Florida.
15. Dudney, R.A., 1997. Use of *Xenorhabdus nematophilus* Im/1 and 1906/1 for fire ant control US Patent, No. 5616318.
16. Elawad, S.A., S.R. Gowen and N.G.M. Hague, 1999. Efficacy of bacterial symbionts from entomopathogenic nematodes against the beet army worm (*Spodoptera exigua*), Tests of Agrochemicals and Cultivars. No. 20, Annals of Applied Biology (Supplement). 134: 66-67.
17. Elawad, S.A., 1998. Studies on the taxonomy and biology of a newly isolated species of *Steinernema* (Steinernematidae: Nematoda) from the tropics and its associated bacteria. Ph.D. Thesis, University of Reading, UK.
18. Bowen, D., T.A. Rocheleau, M. Blackburn, O. Andreev, E. Golubeva, R. Bhartia and R.H. ffrench-Constant, 1998. Insecticidal toxins from the Bacterium *Photorhabdus luminescens*. Science, 280: 2129-2132.
19. Rajagopal, R. and R.K. Bhatnagar, 2002. Insecticidal toxic proteins produced by *Photorhabdus luminescens akhurstii*, a symbiont of *Heterorhabditis indica*. J. Nematol., 34: 23-27.
20. Mohan, S., R. Raman and H.S. Gaur, 2003. Foliar application of *Photorhabdus luminescens*, symbiotic bacteria from entomopathogenic nematode *Heterorhabditis indica*, to kill cabbage butterfly *Pieris brassicae*. Current Sci., 84: 1397.
21. Milstead, J.E., 1979. *Heterorhabditis bacteriophora* as a vector for introducing its associated bacterium into the haemocoel of *Galleria mellonella* larvae. J. Invertebrate Pathol., 33: 324-327.

22. Jackson, T.J., H. Wang, M.J. Nugent, C.T. Griffi, A.M. Burnell and B.C. Dowds, 1995. Isolation of insect pathogenic bacteria, *Providencia rettgeri* from *Heterorhabditis* spp. J. Applied Bacteriol., 78: 237-244.
23. Clarke, D.J. and B.C.A. Dowds, 1995. Virulence mechanisms of *Photorhabdus* sp. Strain K 122 towards wax moth larvae. J. Invertebrate Pathol., 66: 149-155.
24. Forst, S. and K. Nealson, 1996. Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiological Rev., 60: 21-43.
25. Boemare, N.E., A. Givaudan, M. Brehelin and C. Laumond, 1997. Symbiosis and pathogenicity of nematode bacterium complexes. Symbiosis, 22: 21-45.