Use of Entomopathogenic Bacterium *Pseudomonas putida* (Enterobacteriaceae) and its Secretion Against Greater Wax Moth, *Galleria mellonella* Pupae

A.N. Mahar, D.A. Darban, S.R. Gowen, N.G.M. Hague, N.D. Jan, M. Munir and A.Q. Mahar
Department of Agriculture, The University of Reading, P.O. Box 236, Earley Gate, Reading RG6 6AT, UK

Abstract: The bacterium from *Pseudomonas putida* from *Steinernema abbasi* and its metabolic secretions caused the mortality of the *Galleria mellonella* pupae. Experiments were conducted in sand and filter paper on time exposure, temperature, moisture, dose and time of penetration of bacterium in pupae and tested stored or dried toxic metabolites using *G. mellonella* pupae as a test target organism. Death of pupae was probably due to the toxic metabolites. *Pseudomonas putida* cells were recovered from the haemocoele when bacterial cells were applied to the *G. mellonella* pupae indicating that bacterial cells can enter the haemocoele in the absence of nematode vector. Penetration of bacterium was found rapidly after application on *G. mellonella* pupae. *Pseudomonas putida* or its toxic secretions can be used as a microbial control for insect control. The experimental results indicate that there is possibility of using *P. putida* and its toxic secretions as a biopesticide and can contribute in the development of new microbial and biological control against insect pests.

Key words: Bacterial symbionts, entomopathogenic nematodes, *Steinernema abbasi*, biocontrol, *Pseudomonas putida, Galleria mellonella*

INTRODUCTION

Microbial control agents have excellent potential for use in IPM programme. They can be effective and serve as alternatives to broad spectrum chemical insecticides. However, their increased utilization will require increased pathogen virulence and speed of kill, improved pathogen performance under challenging environmental conditions[1]. Greater efficiency in their production, improvements in formulation that enables ease of application, increased environmental persistence and longer shelf life and better understanding of how they will fit into integrated systems and their interaction with the environment and other Integrated Pest Management (IPM) components[2].

Pseudomonas putida, insect pathogenic gram-negative bacterium, belonging to the family Enterobacteriaceae, exhibits swarming motility and form a symbiotic relationship with the entomopathogenic nematode *Steinernema abbasi*[3]. Different species of *Steinernema* carry different bacterial species[4]. The bacterium *P. putida* is a very satisfactory symbiont for infectivity, establishment and reproduction. *P. putida* differs from *Xenrorhabdus* spp. because it is aerobic and has one polar flagellum in contrast to *Xenrorhabdus* spp. which are facultatively anaerobic and have numerous peritrichous flagella[5].

The bacterium is carried in the gut of the infective juveniles of the nematode which, on entry in an insect host, releases the bacteria directly into the haemocoele. The bacteria starts to multiply and septicemia sets in killing the insect within 24-48 h. They support nematode reproduction by producing nutrients and antimicrobial agents that inhibit the growth of a wide range of other organisms[6]. The bacterium can be grown as free-living organisms under standard laboratory conditions. Insecticidal toxin protein secreted by *P. putida* has been found lethal to insects[7,8]. *P. putida* has been shown to control against the beet army worm, *Spodoptera exigua* Hübner[9] and *X. nematophila* against the fire ant, *Solenopsis invicta* Burer (Hymenoptera:Formicidae)[10].

The LD_{50} dose for *X. nematophila* ranged from one to few cells per insect after intrahaemocoelic injection[11] suggesting that *X. nematophila* is a very effective insect pathogen, able to completely resist the antibacterial defense mechanism of insect. Several species of *Xenorhabdus* are also lethal to insects when injected at these low levels. They release a wide variety of virulent factors including high molecular weight toxin complexes, lipopolysaccharides, proteases and a range of different antibiotics[12], which can be assayed in the culture media. Besides insecticidal toxins, *Xenorhabdus* and *Photorhabdus* produce different antibiotics, under both *in vivo* and *in vitro* conditions but generally there is less

---

Corresponding Author: Dr. Ali Nawaz Mahar, Department of Agriculture, The University of Reading, P.O. Box 236, Earley Gate, Reading RG6 6AT, UK E mail: anmahar4@yahoo.co.uk

diversity among the types of antibiotic substances produced by *Photorhabdus* spp. than by *Xenorhabdus* spp.[13].

Recently the pathogenicity of toxic genes from entomopathogenic bacterium *Photorhabdus luminescens* (isolated from *Heterorhabditis bacteriophora*) has been researched extensively[14,15]. Less attention has been paid to the toxin genes from the entomopathogenic bacteria (isolated from various species of *Steinernema*) but recently a patent has been filed in the USA[16] for the use of toxins from *Xenorhabdus* spp. for insect control. Essentially the efficacy of the treatments was similar to the results reported in the present experiments, except that our applications were done in sand and filter paper while their treatments were done on foliage and diet.

This study describes pathogenicity of bacterial cells and secretions from *P. putida* conducted in laboratory experiments considering many factors were used to control the *G. mellonella* pupae which can lead the control of other insect pests.

MATERIALS AND METHODS

Galleria mellonella larvae were obtained from the Mealworm Co., Universal Crescent, Sheffield, S 31 7JJ, UK. Pupae were obtained when larvae were placed in 30°C for the period of one week in incubator. Larvae were infected with IJs of *S. abbasi* and cultured at 30°C. Nematodes IJs suspensions were supplied by CAB Institute of Parasitology, St. Albans, UK. Nematodes were cultured in the greater wax moth, *G. mellonella* and were stored at 15°C. *P. putida* bacterium symbiont was isolated from infected cadavers of *G. mellonella* larvae.

Isolation of bacterial symbionts: Pseudomonas putida was obtained from the hemolymph of *G. mellonella* infected with IJs of *S. carpocapsae*. 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 30°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 *G. mellonella* larvae and streaking the haemolymph of the infected larvae on NBTA plates.

Production of bacterial cell suspensions and its secretion in broth and water: 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 wave length. Based on results obtained[7], 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 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 broth or water were filtered using a Whatman 25 Mm GD/X filter with a pore size of 0.2 μm. Purity of cell-free toxin solutions were tested on agar plates before application against *G. mellonella* pupae. In all experiments fresh pure colonies of bacterium, *P. putida* were used.

Experiment 1. Time exposure to *G. mellonella* pupae: The efficacy of the *P. putida* suspensions and cell-free secretions in broth and water in sand against the pupae of *G. mellonella* at different time intervals was tested. Suspension and secretion of *P. putida* cells in broth and water were prepared at concentration of 4×10^7 mL^{-1} were produced, 1% Tween-80 was mixed in all treatments. In 100 g of sterilized sand 16.4 mL of suspension or secretion was mixed in order to keep 14% moisture content. *G. mellonella* pupae of similar age and size were surface sterilised with 2% Hymine for 5 min and then dried under the laminar airflow. Ten pupae were placed in the moist sand in sterilized plastic containers (110×25 mm) with bacterial suspension or secretions. Control treatments were with water and broth. All containers were incubated at 30°C. The mortality was assessed daily for five days. Replication was 4 fold. The dead pupae were sterilised in 70% industrial methylated spirit for five minutes to kill the bacteria on the surface of the *G. mellonella* pupae (it had been previously determined that 70% industrial methylated spirit was lethal to living bacterial cells). A sample from dead pupae 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. Sand and filter paper substrate test to *G. mellonella* **pupae:** The objective of this experiment was to test the efficacy of cell suspensions and its secretions of *P. putida* on the filter paper and sand substrate on the pupae of *G. mellonella*. Fresh suspensions and secretions of *P. putida* in broth and water were prepared as described already. The concentrations were adjusted to 4×10^7 cells mL^{-1}. 1% Tween–80 was mixed in all suspensions. Replications were four fold. *G. mellonella* pupae were surface sterilized with 2% Hymine for 5 min. Two Whatman filter papers were placed on 9 cm in sterilised petridish. Two milliliter from each suspension was sprayed on the filter paper with a hand sprayer. Ten *G. mellonella* pupae were placed in each petridish and then sealed with Paraflim. In sterilized plastic containers (110×25 mm), 100 g sterilised fine sand was adjusted to 14% moisture content with bacterial suspension and its solutions. Controls were treated with water and broth. Ten *G. mellonella* pupae were placed on the sand, sealed with Parafilm and incubated at 30°C. The mortality for *G. mellonella* pupae was assessed after five days. The cause of mortality of pupae was confirmed by taking samples from the haemolymph of the dead pupae and smeared on NBTA agar plates.

Experiment 3. Moisture test for *G. mellonella* **pupae:** Fresh suspensions and secretion of *P. putida* in broth and water were prepared as described already. The bacterial cell concentration was adjusted to 4×10^7 cells mL^{-1}. *G. mellonella* pupae were washed with 2% Hymine for 5 min. Moisture content was adjusted to 10, 14 and 18% with adding of ten mL of suspension or secretion in each treatment. Ten *G. mellonella* puape were placed in plastic containers (110×25 mm), sealed with Paraflim and incubated at 30°C. Replications were four fold. The mortality for pupae was assessed after five days. The cause of mortality of pupae was confirmed by taking samples from the haemolymph of the dead pupae and smeared on NBTA agar plates.

Experiment 4. Temperature test to *G. mellonella* **pupae:** The objective of this experiment was to test the pathogenicity of *P. putida* cells and their secretions in broth and water at three different temperatures against *G. mellonella* pupae. Suspensions and secretions from *P. putida* cells in broth and water were prepared at concentration of 4×10^7 mL^{-1} as described in experiment 1. Sterilised fine sand (100 g) was adjusted with 14% moisture with bacterial suspension or its secretion. Ten *G. mellonella* pupae were placed in each sterilised plastic containers (110×25 mm). Control treatments used were water and broth. Replication was four fold. The containers of each treatment were incubated at 20, 25 and 30°C. Pupal mortality was recorded after five days. The

cause of mortality of pupae was confirmed by taking samples from the haemolymph and smearing it on NBTA agar plates.

Experiment 5. Dose concentration against *G. mellonella* **pupae:** Six concentrations of *P. putida* bacterial cell suspensions, 4×10^2, 4×10^3, 4×10^4, 4×10^5, 4×10^6, 4×10^7 cells mL^{-1} in broth and water were prepared. Autoclaved 100 g samples of fine sand with 14% moisture with the bacterial suspension were placed in sterilised plastic containers (110×25 mm). *G. mellonella* pupae of similar age and size were washed with 2% Hymine as a surface sterile for 5 min and then dried under the laminar airflow cabinet. Ten pupae were placed in each container. All containers were incubated at 30°C. Replication was four fold. Mortality of pupae was recorded after five days. All dead pupae were confirmed by streaking the haemolymph on agar plates.

Experiment 6. Penetration of bacterial cells in *G. mellonella* **pupae:** The objective of this experiment was to determine the time for the bacterial cells to enter the pupae. Suspensions in broth and water at concentration of 4×10^7 mL^{-1} were produced as mentioned already. *G. mellonella* pupae were washed and surface sterilised using 2% Hymine for 5 min. Ten *G. mellonella* pupae were placed on a Whatman filter paper in 9 cm petridish. Two mL from each suspension was sprayed with a hand sprayer on the filter paper in sterilized petridish under the laminar airflow cabinet. Containers were placed at 30°C. Replication was four fold. Results assessing the bacterial penetration to insects whether alive or dead were sampled after 15, 30 min, 1, 2, 4, 8, 16, 24 and 30 h on agar plates.

Experiment 7. Stored bacterial secretion to *G. mellonella* **pupae:** The objective of this experiment was to test the efficacy of stored of bacterial toxins on the pathogenicity of *G. mellonella* pupae. Fresh bacterial toxin solutions in broth and water were produced as described before. The bacterial toxin solution of *P. putida* was stored at the 25°C for 4 weeks. Tween-80 was mixed in each solution at the rate of 1%. Sterilized fine sand was adjusted with 14% moisture. Stored toxic solution was mixed in sand. Ten *G. mellonella* pupae were washed with Hymine and placed in each sterilised plastic containers of size (110×25 mm). Containers were placed at 30°C. Replications were four fold. Mortality of pupae was assessed after five days of application of stored toxin solution in all weeks.

Experiment 8. Dried bacterial secretion to *G. mellonella* **pupae:** This experiment was designed to test the effect of drying bacterial toxins on pathogenicity of *G. mellonella* pupae. Fresh bacterial toxin solution in broth and water was produced in same manner as mentioned already.

Bacterial toxin solutions were dried in the sterilized containers at the 25°C for two days under laminar airflow. Dried toxins were rewetted with either broth or water solution. Tween-80 was mixed in each solution at the rate of 1%. Sterilized fine sand (100 g) was adjusted with 14% moisture with each toxin solution. Ten *G. mellonella* pupae were washed with Hymine and placed in each sterilised plastic containers of size (110×25 mm). Containers were placed at 30°C. Experiment was designed in four replications. Mortality of pupae was recorded after five days of treatment.

Statistical analysis: Data were analysed by using a factorial analysis of variance, statistical technique of GENSTAT-6, Release 5.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Error bars on histograms represent the least significant difference at the 5% level of probability.

RESULTS

Time exposure: Mortality of the *G. mellonella* pupae increased as the time was increased (Fig. 1). The maximum mortality of 100% found when treated with bacterial suspensions or secretions of *P. putida* in broth after five days. Secretions in water caused 80% mortality. Bacterial suspensions in water caused 75% mortality. In the controls only 15 and 12.5% mortality was found with broth and water. Statistical analysis showed that time has highly significant effect (p<0.001) on the mortality of *G. mellonella* pupae. There was no significant effect of the broth treatments as compared with water treatments.

Sand and filter paper substrates: The maximum mortality of *G. mellonella* pupae 100% occurred when treated with cell suspension or secretions in broth *P. putida* and applied in sand but in filter paper only 40 and 47.5% mortality was observed for same treatments. Bacterial suspensions in water caused 82.5% and cell secretion in water caused only 77.5% mortality in sand media. Same mortality of 32.5% was observed when *P. putida* suspension or secretions in water were applied using filter paper (Fig. 2). Greater mortality of *G. mellonella* pupae was obtained in sand compared to filter paper. The mortality on sand was higher than the treatment on filter paper (p<0.001). There was no significant difference in the mortality of *G. mellonella* pupae either cells or cell-free secretions.

Moisture effect: The maximum mortality of *G. mellonella* pupae of 100.0% was observed at 14% moisture in cell secretions in broth treatments as compared to 10 and 18%
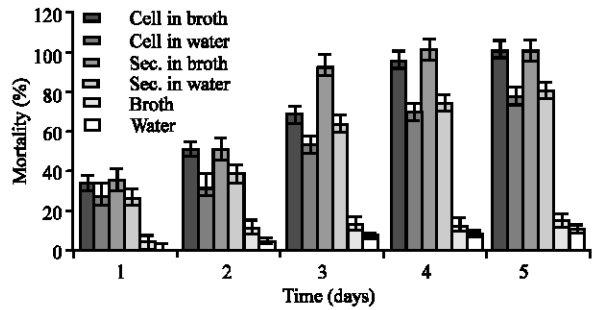

Fig. 1: Percent mortality of *G. mellonella* pupae when treated with *P. putida* cells and its secretions in broth and water at different days. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates
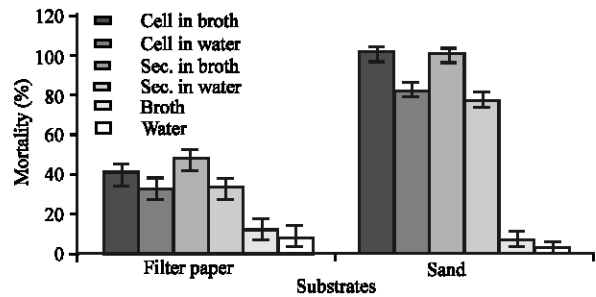

Fig. 2: Percent mortality of *G. mellonella* pupae when treated with *P. putida* cells and its secretions in broth and water using sand and filter paper. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates
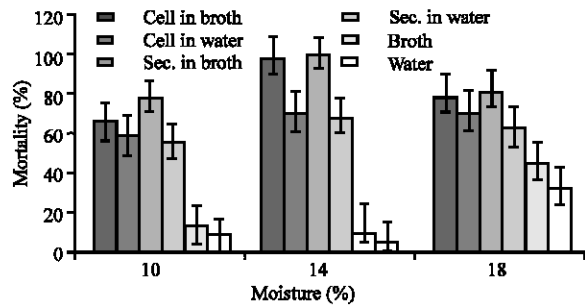

Fig. 3: Percent mortality of *G. mellonella* pupae when treated with *P. putida* cells and its secretions at different moisture levels. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates

moisture where only 77.5 and 82.5% mortality occurred (Fig. 3). In case of cell suspension in broth the maximum
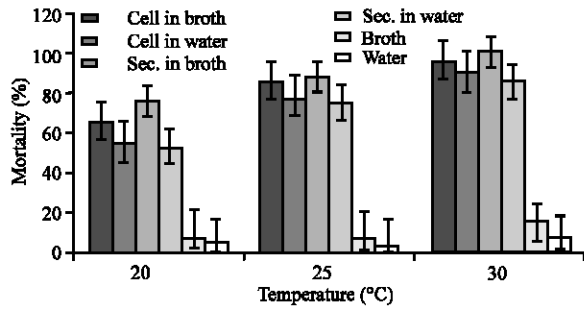
Fig. 4: Percent mortality of *G. mellonella* pupae when treated with *P. putida* cells and its secretions at different temperatures. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates
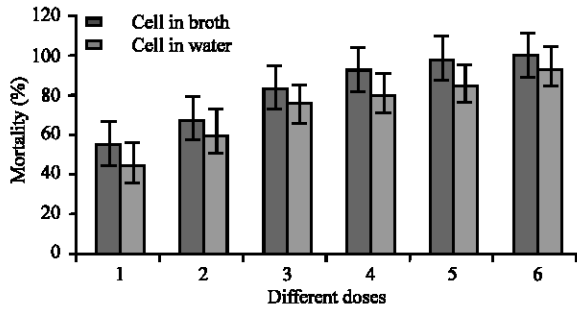

Fig. 5: Percent mortality of *G. mellonella* pupae when treated with different doses of *P. putida* cells in broth and water. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates
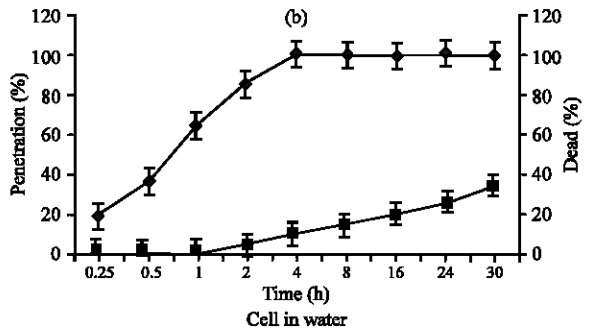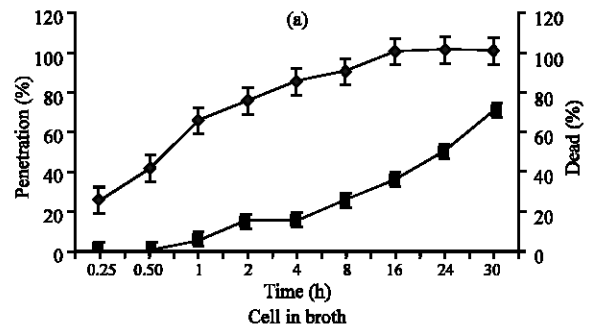

Fig. 6: Percent penetration (♦) of *P. putida* cells in broth (a) and water (b) in *G. mellonella* pupae and percent dead (■) at different time (h). Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates.

mortality of 97.5% was observed at 14% moisture as compared to 10 and 18% where only 65 and 80% mortality of pupae was noticed. Cell suspension or secretion in water caused lower mortalities than suspended in broth solution. Control mortality in 18% was observed only 47.5% in broth and 35% in water. So 14% moisture showed the better mortality than 10 and 18% moisture.

Temperature effect: Temperature has significant effect (p<0.001) on the mortality of *G. mellonella* pupae. Results showed that the higher mortality from 85 to 100% of the *G. mellonella* pupae was recorded when these were exposed at 30°C as compared to 25 and 20°C (Fig. 4). There was no difference in the mortality caused by either cell of *P. putida* suspension or cell free secretions. Bacterial secretions were found more effective and caused 100% dead insects when suspended in broth as compared to water (95%) at 30°C. Minimum mortality was found only 52.5 to 55% at 20°C when treated with cell secretions in water and cell suspensions in water.

Bacterial dose concentration: Cells in broth treatments were found effective than cells in water even at the lowest

concentration of 4×10^2 also caused 45 to 55% mortality when cell were applied in water or broth suspensions (Fig. 5). Mortality was found increased when dose concentration was increased. The maximum mortality of 100% was found when 4×10^7 dose in broth was used. Statistical analysis showed that there was a significant difference in the different dose concentrations of the bacterial suspensions for *G. mellonella* pupae. Mortality for bacterial suspensions in broth was significantly different from the suspensions in water (Fig. 5).

Time of penetration of bacterial cells: Fig. 6a and b indicated the time of penetration when cells in broth suspension and cells in water entered the pupae of *G. mellonella*. In 1 h time 65% cells in broth were entered but only 50% cells in water were found inside of *G. mellonella* pupae in 1 h. All *G. mellonella* pupae were infected with bacterial cells in broth and water with in 16 h. Only 70% pupae were found dead when treated with cells in broth suspension but cells in water caused only 35% mortality of *G. mellonella* pupae with in 32 h of time observed.

Stored secretions: Fresh toxin secretions were found more effective to *G. mellonella* pupae (Fig. 7). Mortality
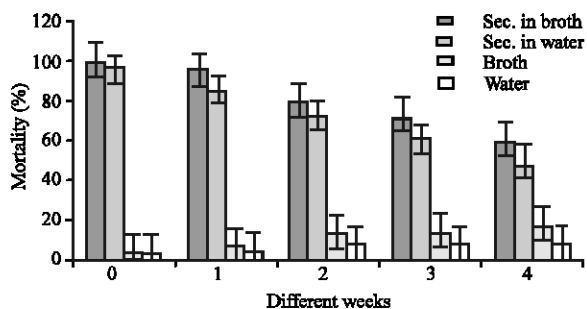
Fig. 7: Percent mortality of *G. mellonella* pupae when treated with stored secretions in broth and water from *P. putida* cells. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates
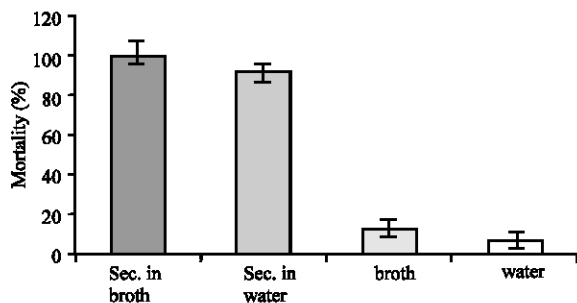

Fig. 8: Percent mortality of *G. mellonella* pupae when treated with dried secretions in broth and water from *P. putida* cells. Error bars represent the Standard Error of Difference (SED) within means. Values are means of four replicates.

effect degraded from 100 to 60% mortality with in four weeks when stored secretions of *P. putida* were applied against *G. mellonella* pupae. The mortality rate decreased in secretions in water from 95 to 47.5% in four weeks time. Statistical analysis showed that there is significant effect of stored toxin on the *G. mellonella* pupae in different weeks and there was also significance difference between the stored secretions of *P. putida* in broth and water solutions.

Dried secretions: Dried toxin secretions have induced high mortality to *G. mellonella* pupae (Fig. 8). Secretions of *P. putida* in broth resulted the maximum mortality of 100% than 90% secretions in water when observed after five days. There was no significance difference between the dried secretions from either in broth or water solution of *P. putida.*

DISCUSSION

The results of present experiments suggest that *G. mellonella* pupae can be controlled by *P. putida* bacterium or its secretions at proper environmental conditions in the laboratory. Optimal temperature, moist sand, soil or foliar conditions can increase the effectiveness of this bacterium against any insect pests. It is possible that these entomopathogenic bacteria or their secretions could be used to reduce the pest damage. The present results are similar with the findings that have been reported for the control of the fire ant, *Solenopsis invicta*[10]. The efficacy of *X. nematophila* (*S. carpocapsae*) and *P. putida* (*S. abbasi*) using the same moisture content 14% and same dose of bacterial cells against the beet army worm *Spodoptera exigua* larvae and pupae in sand media bioassay produced 100% mortality of larvae and 80% mortality in pupae after 5 days using 28°C[9].

The pathogenicity of *G. mellonella* pupae and diamondback moth (DBM) pupae using *X. nematophila* cells and its secretions in broth and water in sand media resulted that there was highly significant difference in values of LC_{50} and LT_{50} values for *G. mellonella* pupae as well as for DBM pupae. He applied the same dose of cells of *X. nematophila* in broth and water suspension against diamondback moth larvae and pupae. He observed the 100% penetration of cells with in 4-8 h and 100% mortality occurred with in 48-36 h for DBM larvae and pupae. In case of *G. mellonella* larvae and pupae 100% penetration of cells occurred with in 8-16 h and 100% mortality o f *G. mellonella* larvae was observed after 6 days of *X. nematophila* cells application but all pupae died with in 4 days after application[17].

Bacterium *Pseudomonas putida* and their secretions resulted 80% mortality in pupae of *S. exigua* when they used bacterial suspension in broth using sand bioassay. In an other experiment they concluded that 91% mortality of *G. mellonella* larvae was found in 14% moist sand but 100% pupae were found dead after 6 days of treatment of *P. putida* cells in broth[18]. The pathogenicity of *G. mellonella* larvae using *X. nematophila* cells in broth and water in sand media resulted that there was highly significant difference in values of LC_{50} and LT_{50} values for *G. mellonella* larvae[19].

The differential toxicity of the entomopathogenic bacteria could be attributed to the produced exoenzymes that affect the virulence of the bacteria towards their hosts. Phospholipase C is the best-described bacterial lipase toxin and this enzyme damages *G. mellonella.*

Non-spore forming bacteria toxic to insects also, contain toxic proteinase and chitinases[20]. *P. putida* is also non-spore forming bacterium and possibly contain a high degree of these enzymes which include the toxic components in different degrees. Lipopolysaccharide (LPS) is a component of the outer membrane of the bacterial cells and has been shown to damage haemocytes and inhibit activation of the humoral immune system. Findings of secretion are more or less similar[21] when applied the metabolite secretions extracts of *X. nematophila* against the *G. mellonella* larvae. It caused the 100% mortality with in 24 h of treatment. They studied the optimal conditions for the *in vitro* production of the entomotoxin.

The cells of *X. nematophila* (All) and other four strains of different bacteria were found highly pathogenic when injected into the *Spodotera littoralis*. They recorded the complete mortality of insect after 64 h exposure[22]. Their findings are similar to the present[23]. However interaction between insect immunity and an insect-pathogenic nematode with symbiotic bacteria using the diapause pupae of *Hyalophora cecropia* remained as a model system. They found that in normal pupae LD$_{50}$ (the injected dose that cause death of 50% of the population) of the bacteria *X. nematophila* were about 500 cells; for immunized pupae it was 5×10^5 cells. *Cecropia* pupal immunity did not affect the nematodes. Fewer than ten nematodes with bacteria were lethal, while LD$_{50}$ of axenic nematodes was about 500. Immune hemolymph caused lysis of *X. nematophila*. Immune proteins P9 A and P9 B were identified as the active components in pupae of *Cecropia* immunity against *X. nematophila*.

When beet armyworm pupae were exposed to 200 infective juveniles per pupae of the (All) strain of *S. carpocapsae*, they were the most susceptible of the soil pupating Lepidopteraous tested with 63% mortality but our bacterial application showed better mortality than nematode application[24]. Resistance of beet armyworm pupae to the *S. carpocapsae* (All strain) was reported with the LC$_{50}$ value of 1.129 per pupae[25]. Two entmopathogenic nematodes were tested against the pupae of three insects, pink bollworm, *Pectinophora gossypiella* (Saunders), Cabbage looper, *Trichoplusia ni* and beet armyworm *Spodoptera exigua* in laboratory conditions. Completely formed or uninjured pink bollworm pupae were not susceptible to infection by *S. riobravis* or *S. carpocapsae* nematode. However cabbage looper were susceptible with 43.5 and 83.3% nematode-related mortalities for *S. riobravis* and *S. carpocapsae*, respectively. Beet armyworm pupae were resistant to both nematode species with no significant difference in nematode-related mortality[26]. These results are

contrasting, as *G. mellonella* pupae were found most susceptible in the present when *P. putida* and its secretions were used.

Different levels of susceptibility of lepidopterous pupae were reported due to different strains, nematode viability or especially with fragility of pupae that may receive handling injuries[27]. Pupae were not susceptible to *S. carpocapsae* unless the pupal integument was punctured with a pin whereas in case of bacterium *P. putida* or its secretion has caused rapid penetration in the pupae of *G. mellonella* pupae[28]. Prepupal and pupal *S. riobravis* parasitism were found in corn earworm, *Helicoverpa zea* and fall armyworm, *Spodoptera frugiperda*[29].

We have proved the lethality of *P. putida* bacterium and its secretions against the *G. mellonella* pupae. Less attention has been paid to test the pupae of other insect pests. Recently a patent has been filed in the USA[16] for the use of toxins from *Xenorhabdus* spp. for insect control.

Further studies are needed to identify the characteristics of this bacterium or its toxic secretions to enhance the potentiality of *P. putida* as a biocontrol agent which can be used against insect pests.

REFERENCES

1. Akhurst, R. J.1990. Safety to Non-target Invertebrates of Nematodes of Economically Important Pests. In: Safety of Microbial Insecticides. (Eds. M. Laird, L.A. Lacey and E.W. Daidson). CRC Press, Boca Raton, FL., pp: 233-240.

2. Akhurst, R.J., 1990. Bacterial Symbionts of Entomopathogenic Nematodes. The Power Behind the Throne. In: Nematodes and the Biological Control of Insect Pests. (Eds. R. Bedding, R. Akhurst and H. Kaya). CSIRO Publication, East Melbourne, pp: 127-135.

3. Elawad, S.A., M.S. Abbas and N.G.M. Hague, 1996. The establishment and pathogenicity of a new species of *Steinernema* from the Sultanate of Oman in *Galleria mellonella*. Afro-Asian J. Nematol., 6: 40-45.

4. Akhurst, J. and N.E. Bomeare, 1988. A numerial taxonomic study of the genus *Xenorhabdus* and proposed elevation of the subspecies *X. nematophilus* to species. J. Gen. Microbiol., 134: 1835-1845.

5. Bergey, D.H., 1994. Bergey's Manual of Determinative Bacteriology. 9th Edn., Holt, J.G. *et al.*, (Eds.), pp: 787.

6. Akhurst, R.J., 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. Gen. Microbiol., 128: 3061-3065.

7. 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, U.K.

8. Mahar, A.N., S.A Elawad, S.R. Gowen, N.G.M. Hague and M. Munir, 2004. Biological control of diamondback moth *Plutella xylostella* (Lepidoptera:Yponomeutidae) with bacteria from entomopathogenic nematodes. Pak. J. Nematol., 22: 189-198.

9. Elawad, S.A., Gowen, S.R. and Hague, N.G.M., 1999. Efficacy of bacterial symbionts from entomopathogenic nematodes against the beet army worm (*Spodoptera exigua*), Tests of Agrochemicals and Cultivars, No. 20, (Ann. Applied Biol., 134 Supplement), pp: 66-67.

10. Dudney, R.A., 1997. Use of *Xenorhabdus nematophilus* Im/l and 1906/1 for fire and control. US patent, No. 5616318.

11. Dunphy, G.B. and J.M. Webster, 1988. Virulence mechanisms of *heterorhabditis, heliothidis* and its bacterial associate, *Xenorhabdus luminescens*, in non-immune larvae of greater wax moth, *Galleria mellonella*. Intl. J. Parisitol., 18: 729-737.

11. 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.

12. Forst, S. and K. Nealson, 1996. Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiol., Rev., 60: 21-43.

13. Webster, J.M., 2001. The Role and Potential of Secondary Metabolites of the Bacterial Symbionts. In COST 819 Virulence Factors and Secondary Metabolites from Symbiotic Bacteria of Entomopathogenic Nematodes, (Eds. N.E. Boemare), pp: 67-74.

14. Bowen, D.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.

15. French-Constant, R. and D. Bowen, 1999. *Photorhabdus* toxins: Novel biological insecticides. Current Opinion in Microbiol., 2: 284-288.

16. Ensign, J.C., D.J. Bowen, J.L. Tenor, T.A. Ciche, J.K. Petell, J.A. Strickland, G.L. Orr, R.O. Fatig, S.B. Bintrim and R.H. Ffrench-Constant, 2002. Proteins from the genus *Xenorhabdus* are toxic to insects on oral exposure. US Patent Application No. 0147148 A1.

17. Mahar, A.N., 2003. The efficacy of bacteria isolated from entomopathogenic nematodes against the Diamondback moth *Plutella xylostella* L. (Lepidoptera:Yponomeutidae). Ph.D. Thesis, University of Reading, UK.

18. Mahar, A.N., S.A. Elawad, S.R. Gowen and N.G.M. Hague, 2003. Efficacy of bacterial cells suspensions from entomopathogeic nematodes *Stienernema abbasi* against larvae and pupae of *Spodoptera exigua* and *Galleria mellonella*. Pak. J. Nematol., 21: 103-108.

19. Mahar, A.N., S.A. Elawad, S.R. Gowen and N.G.M. Hague, 2000. Pathogenicity of bacterial symbionts from entomopathogeic nematodes to larvae of *Galleria mellonella*. The BCPC Conference. Pests and Diseases, 2000. Brighton UK.

20. Lysenko, O., 1985. Non-spore forming bacteria pathogenic to insects: Incidence and mechanisms. Ann. Rev. Microbiol., 39: 673-695.

21. Palha, A.F., P. Oliveira and N. Simoes, 1996. *In Vitro* Obtention of an Entomotoxin (Insecticidal Protein) from the *Steinernema carpocapsae- Xenorhabdus nematophilus* Complex. In: Entomopathogenic Nematodes. Pathogenicity of Entomopathogenic Nematodes Versus Insect Defence Mechanism: Impact on Selection of Virulent Strains. (Eds.): N. Simões, N., N. Boemare and R.-U. Ehlers, Proceedings of a Workshop Held at Universidade dos Açores 17 to 20 March 1996, Ponta Delgada, Açores, Portugal. Brussels, pp: 85-91.

22. Glazar, I., S. Galper and E. Sharon, 1991. Virulence of the nematode (Steinernamatids and Heterorhabditis)-bacteria (*Xenorhabdus* spp.) Complex to the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera:Noctuidae). J. Invertebrate Pathol., 57: 94-100.

23. 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.

24. Kaya, H.K. and A.H. Hara, 1981. Susceptibility of various species of *Lepidopterous* pupae to the entomogenous nematode *Neoplectana carpocapsae*. J. Nematol., 13: 291-294.

25. Thurston, G.S and H.K. Kaya, 1994. Physical stressors affecting interactions of *Spodoptera exigua* (Hubner) (Lepidoptera:Noctuidae) and an entomopathogenic nematode. The Canadian Entomol., 126: 261-267.

26. Henneberry, T.J., J.E. Lindegren L.J. Forlow Jech. and R.A. Burke, 1995. Pink bollworm (Lepidoptera: Gelechiidae), Cabbage looper and Beet armyworm (Lepidoptera: Noctuidae) pupal susceptibility to Steinernematid nematodes (Rhabditida: Steinernematidae). J. Econ. Entomol., 88: 835-839.

27. Kaya, H.K. and A.H. Hara, 1980. Differential susceptibility of Lepidopterous pupae to infection by the nematode *Neoplectana carpocapsae*. J. Invertebrate Pathol., 36: 389-393.

28. Lindegren, L.E., K.F. Meyer, T.J. Henneberry, P.V. Vail, L.J. Forlow Jech and K.A. Valero, 1993. Susceptibility of pink bollworm (Lepidoptera: Gelechiidae) soil associated stages to the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida: Steinernematidae). Southwest. Entomol., 18: 113-120.

29. Raulston, J.R., S.D. Pair, J. Loera and H.E. Cabanillas, 1992. Prepupal and pupal parasitism of *Helicoverpa zea* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) by *Steinernema* sp. in corn fields in the lower Rio Grande Valley. J. Econ. Entomol., 85: 1666-1670.