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Use of *Photorhabdus* as a biopesticide a- Cell suspension from *Photorhabdus* sp. against *Galleria mellonella* insect

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ABSTRACT

Integrated Pest Management (IPM) is today a widely accepted strategy for reducing overdependence on chemical insecticides and to reduce their potentially negative environmental and economic effects. *Photorhabdus* is a gram-negative enteric bacterium that is found in association with entomopathogenic nematodes of the family Heterorhabditidae. The nematodes infect a variety of soil insect pests. Upon entering an insect host, the nematode releases *Photorhabdus* spp. cells from its intestinal tract, and the bacteria quickly establish a lethal septicemia. When grown in yeast salt broth, in the absence of the nematodes, the bacteria produce a toxin protein that is lethal when fed to the hemolymph of several insect species. Broth cultures of five isolates of *Photorhabdus* (A, B, C, D, E) were lethal to the nymphs of *Galleria mellonella* when mixed different concentrations (5,10 and 20 ml) from suspension bacterial cell with two kind of media (wheat bran and fine sand) as compared to broth alone (control). Results obtained showed that, after one day the mortality reached 100 % on sand inoculated with 20 ml cells suspension of the isolates A, C and D. A hyperbolic relationship was observed between different isolates, type of media, doses and time intervals. 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.
Keywords: *Photorhabdus, Galleria,* Biopesticide.

INTRODUCTION

Recent concerns about the development of the insect control by microbial agent, such as some species of *Bacillus,* directed the focus of research toward isolation of novel toxin molecules from other microorganisms in the soil (Khandelwal *et al*., 2004). Steinernematidae and Heterorhabditidae are two families of entomopathogenic nematodes which are strongly virulent against a wide range of insects. Gaugler and Kaya (1990) showed that they are non-pathogenic to mammals and suggested that they could be raised for the use in biological control of insect pests. All *Steinernema* spp. carry, in their gut, symbiotic bacteria of the genus *Xenorhabdus* (Akhurst and Boemare, 1988; Thomas and Poinar, 1979), and all *Heterorhabditis* spp. carry symbiotic bacteria of the genus *Photorhabdus* (Akhurst *et al*., 1996; Fischer-Le Saux *et al*., 1999). Poinar and Thomas (1966) found that a single species of bacterium in the family Enterobacteriaceae was present in the anterior region of the infective juvenile (IJ/IJs) of *Steinernema* spp. Once an IJ penetrates the haemocoele of the insect, the bacterial symbiont is released from the nematode gut, and septicemia occurs following by insect death within 48 h. In most cases the bacteria alone are sufficient to cause insect mortality when injected into the haemocoele (Gotz *et al*., 1981). Recently, successful laboratory and field evaluations proved the insecticidal virulence of the bacterium when applied alone as a biopesticide (Mohan and Sabir, 2005). The major objective of the present investigation was to determine the effect of different bacterial isolates alone on mortality of *Galleria mellonella* larvae under different substrates, doses, and time intervals.

MATERIALS AND METHODS

Larvae of greater wax moth, *Galleria mellonella* infected with the IJs of *Heterorhabditis* (five isolates) were obtained from Nematology laboratory, Phytopathology Department, National Research Center. Nematodes were cultured in the *G. mellonella* and then stored at 7 °C.

Isolation of bacterial symbionts

Five isolates of *Photorhabdus* were obtained from the haemolymph of *G.mellonella* infected with IJs of *Heterorhabditis* sp. Dead *G. mellonella* larvae were surface sterilized in 70% ethyl 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 on MacConkey agar. The agar plates, sealed with parafilm, were incubated at 28 °C in the dark for 24 h, then a single colony of bacterium was selected and streaked onto new plates of MacConkey agar. Sub-culturing was continued until colonies of uniform size and morphology were obtained. The pathogenicity of the isolates was confirmed by injecting the bacterial cells into the body of *G. mellonella* larvae and, later on, streaking the haemolymph of the infected larvae on new MacConkey agar plates.

Production of bacterial cell suspensions containing metabolites

A single colony of each bacterium was selected and inoculated into 500 ml of yeast salt (YS) broth in a flask and placed in a shaking incubator at 150 rpm for

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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 (1998), the concentration of the bacterial cells used in the present experiments was adjusted to 4×10^7cells/ml with addition of 3% Tween- 80 as an emulsifier.

Application of different isolates of *Photorhabdus* against *G. mellonella*

Fresh cells suspensions of five different isolates (A, B, C, D and E) in YS broth were prepared at concentration of 4×10^7 cells/ml with 3% Tween-80. These suspensions were mixed with either sand or wheat bran at different concentrations (5, 10 and 20ml) to determine how fast is the mortality of larvae.

Response of *G. mellonella* to different dose of bacterial suspensions on different substrates

To determine the appropriate bacterial dose, different values (5, 10 and 20ml) of cells suspension of each isolate *Photorhabdus* were prepared at concentration of 4×10^7 cells/ml with 3% Tween-80 as an emulsifier and mixed with different substrate. Each dose of all isolates (5, 10 and 20 ml cell suspensions) was mixed with 50 g of sterilized wheat bran or with 100 g of sterilized fine sand. Replication was three fold in all experiments. Non inoculated broth medium was used as control (5, 10, 20 ml). *G. mellonella* larvae, of similar age and size, were kept hungry for one day before feeding on any treatment in wheat bran or sand. Ten larvae were placed in sterilized Petri dishes (9 cm) containing wheat bran or fine sand. All plates were incubated at 28 ^{o}C.

Effect of different time intervals on mortality of *G. mellonella*

After each treatment, *G. mellonella* which survived on wheat bran or sand were counted. The number of dead larvae was determined after 1, 2, 3, 4, 6 and 10 days.

RESULTS

Statistical analysis showed a significant difference ($P < 0.05$) between different isolates, bacterial doses and time interval, whereas there was no significant different within isolates after the third day. Table (1) shows that the percentage of mortality of *G. mellonella* after one day reached 100 % on sand inoculated with 20 ml cells suspension of the isolates A, C and D. On wheat bran, mortality was 63.33, 43.33 and 36.67%, respectively. Table (2) showed an increasing in insect mortality after 48 hrs on wheat bran inoculated with the isolates A, C and D (63.33, 50.00 and 53.33% respectively). After the third day, the second dose (10 ml) gave results approximately resemble that of the final dose (20 ml), whereas, 90% and 80% mortality was recorded with the isolate A and C on sand (Table 3). The mortality increased with the isolates A, C and E, (60, 83 and 60 %, respectively) after four days with the first dose (5 ml), while the second dose caused 93, 86 and 90 % mortality. The mortality of insect reached 100 % with the isolates A, C, D and E in dose 20 ml on sand, and only with the strain D on wheat bran (Table 4).

With elapsing of time (6 days) no significant different was seen between media, Table (5) also shows no significant difference between different isolates, as mortality reached 100% in most isolates. At the end of this experiment, percentage of nematode mortality in the control treatment ranged between 13.33 to 20 % at different concentration (Table 6).

Control treatments of broth showed poorest effect on insect mortality when compared to the bacterial treatments, in the end of experiment Table (6).

Table (1): Percent of mortality after 24h, 48h, 72h, 4 days, 6 days, 10 days by *Photorhabdus*. **h:** hours, **d:** days, and **nd**: no detect.

Media/ concentration/ No. isolate	Time	Sand / Concentration			Wheat bran/ Concentration ml/L		
		5	10	20	5	10	20
A	24 h	46.67	75.00	100.00	00.00	33.33	63.33
	48 h	53.33	83.33	100.00	00.00	43.33	63.33
	72 h	53.33	90.00	100.00	00.00	43.33	73.33
	4 d	60.00	93.33	100.00	70.00	86.67	83.33
	6 d	90.00	100.00	100.00	83.33	100.00	100.00
	10 d	100.00	100.00	100.00	100.00	100.00	100.00
B	24 h	00.00	26.67	nd	10.00	13.33	13.33
	48 h	00.00	33.33	nd	13.33	23.33	26.67
	72 h	00.00	53.33	56.67	13.33	33.33	76.67
	4 d	50.00	73.33	66.67	56.67	63.33	93.33
	6 d	56.67	93.33	96.67	63.33	93.33	100.00
	10 d	56.67	96.67	96.67	86.67	93.33	100.00
C	24 h	43.33	46.67	100.00	00.00	20.00	43.33
	48 h	56.67	50.00	100.00	00.00	26.67	50.00
	72 h	76.67	80.00	100.00	53.33	56.67	80.00
	4 d	83.33	86.67	100.00	53.33	56.67	96.67
	6 d	83.33	86.67	100.00	53.33	86.67	96.67
	10 d	83.33	86.67	100.00	56.67	96.67	100.00
D	24 h	13.33	10.0	100.00	00.00	13.33	36.67
	48 h	16.67	36.67	100.00	00.00	13.33	53.33
	72 h	30.00	63.33	100.00	00.00	13.33	100.00
	4 d	40.00	66.67	100.00	10.00	16.67	100.00
	6 d	56.67	96.67	100.00	10.00	56.67	100.00
	10 d	80.00	100.00	100.00	100.00	100.00	100.00
E	24 h	12.33	43.33	43.33	00.00	30.00	30.00
	48 h	33.33	46.67	50.00	00.00	40.00	40.00
	72 h	50.00	63.33	66.67	00.00	43.33	53.33
	4 d	60.00	90.00	100.00	00.00	43.33	73.33
	6 d	60.00	100.00	100.00	00.00	63.33	100.00
	10 d	76.67	100.00	100.00	00.00	80.00	100.00
Cont.	24 h	0.000	00.00	00.00	00.00	00.00	00.00
	48 h	00.00	00.00	00.00	00.00	00.00	00.00
	72 h	00.00	00.00	00.00	00.00	00.00	00.00
	4 d	00.00	00.00	00.00	00.00	00.00	00.00
	6 d	6.67	10.00	00.00	03.33	00.00	00.00
	10 d	20.00	13.33	16.67	13.33	16.67	20.00

Figure (1) shows the effect of different doses of *Photorhabdus* (isolate A) on mortality percentage of *G. mellonella* as applied to sand and wheat bran media. The results indicated that isolate (A) had a harmful

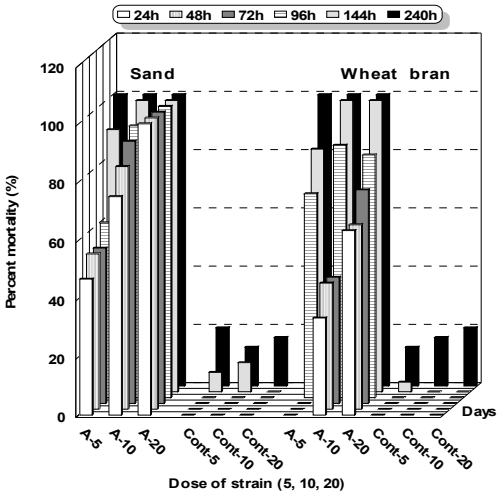

Figure (1): Effect of different doses of *Photorhabdus* sp., isolate (A) on mortality of *Galleria mellonella*, on different media.

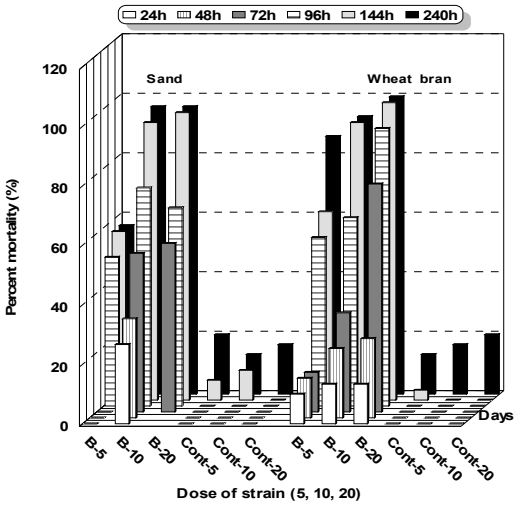

Figure (2): Effect of different doses of *Photorhabdus* sp., isolate (B) on mortality percentage of *Galleria mellonella* on different media.

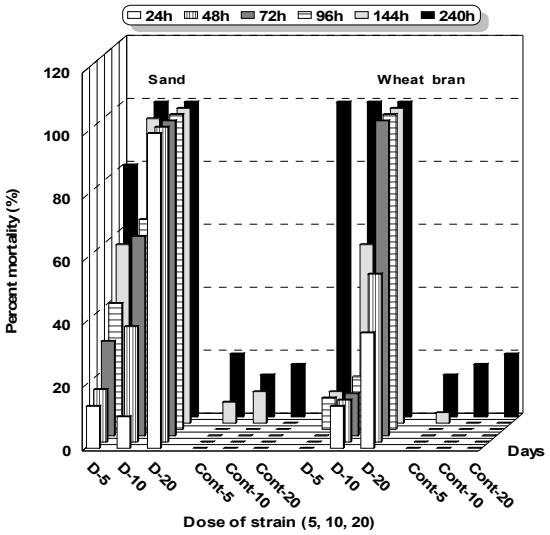

Figure (3): Mortality percentage of *Galleria mellonella* after treatment with the isolate (C) of *Photorhabdus* sp. on different media.

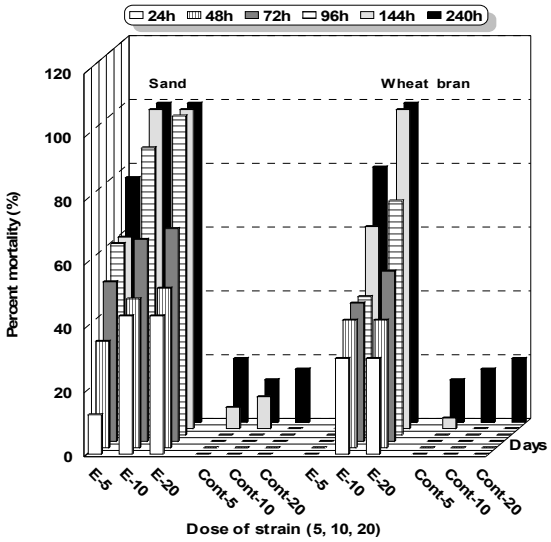

Figure (4): Effect of treatment with the isolate (D) of *Photorhabdus* sp. on mortality of *Galleria mellonella* on Sand and Wheat bran as a different

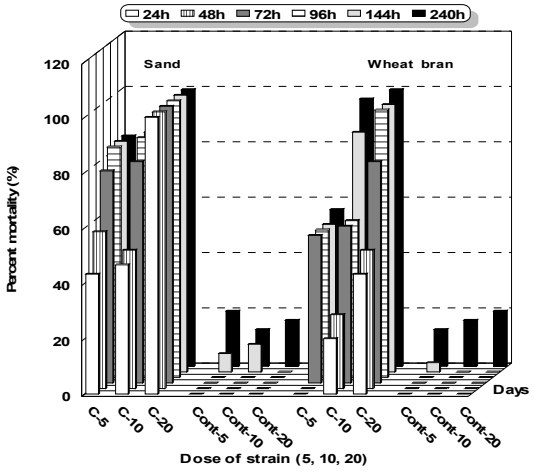

Figure (5): Effect of the isolate (E) of *Photorhabdus* sp. at different concentrations on *Galleria mellonella*.

effect from the beginning of the experiment to its end, where mortality reached 100% with the three used concentrations.

Gradual increase of mortality was observed with the elapsing of time; after 10 days mortality was 96.67 % at the concentrations 10 and 20 ml on fine sand inoculated with the isolate (B). Whereas on wheat bran mortality was 86.67, 93.33 and 100 % with the doses 5, 10 and 20ml respectively (Fig. 2). The final dose (20 ml) of the isolate C had more harmful effect on insect in sand media after one day, whereas on wheat bran, the effect increased gradually with time (Fig. 3). Similar result was observed with the isolate (D) in the case of the dose 20 ml from the beginning of the test on sand, while on wheat bran the effect appeared after the third day **(**Fig. 4).

In second dose of the isolate (E), the highly killing effect was observed after the third day on sand compared to wheat bran (Fig 5).

DISCUSSION

Entomopathogenic nematode, *Heterorhabditis* carries specific symbiotic bacterium, *Photorhabdus luminescens* which is pathogenic to a wide range of agriculturally important insect pests (Poinar, 1979). *Photorhabdus* in broth caused 100 % mortality after 24h in the case of the isolates A, C and D at the dose of 20 ml cell suspension on sand, whereas on wheat bran the maximum mortality was 63.33 % after 24h with the isolate (A) at the same dose. The difference between the two treatments could be due to the direct effect of the kind of the substrate.

It has been assumed that the association between entomopathogenic nematodes and their symbiotic bacteria is mutualistic and that this relation is essential for their survival (Poinar, 1979). In the present and some previous experiments it has been shown that these symbiotic bacteria are able to penetrate the haemocoele of the insect in the absence of the nematode vector. However the mechanism by which this bacteria gain entry to the haemocoele is unclear. *P. luminescens* exhibit swarming motility when grown on suitable solid media (Elawad, 1998). The symbiont bacterian *Photorhabdus* was lethal to *Gallaria mellonella* larvae when applied to sand media rather than wheat bran, which confirming the results of Gotz *et al*. (1981). Similar results were also reported with fire ant (Dudney, 1997) and with the beet army worm (Elawad *et al*., 1999) when *X. nematophila* bacterium was applied to sand media in order to control these pests. Mahar *et al*., (2005) demonstrated that bacteria produce toxins which break down the immunity of the insect. In the present study bcterial concentration of 4×10^7 cells/ml in broth caused 100% mortality of *Galleria* in 80% of isolates on sand substrates after 24 hrs. This result indicated the harmful effect of *Photorhabdus* containing nematode (*Heterorhabditis*), that penetrate into the haemocoel of the insect, when added to sand rather than to wheat bran.

The purpose of the present experiments was to demonstrate the possibility of using these bacterial symbionts for direct application on plants to control insect pests such as *Galleria*. In order to use these bacteria in the field, it would be necessary to carry out normal toxicology and human hygen tests.

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إستخدام بكتريا الفوتورابدس كمبيد حيوى
أ-إستخدام المعلق الخلوى لبكتريا الفوتورابدس ضد حشرة الجلاريا ميلونيلا

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الملخص العربـــى

يعد التوسع في أساليب إستخدام المكافحة الحيوية للحد من التأثير السالب على البيئه والناتج من زيادة إستخدام المبيدات الكيميائية بأنواعها من أهم ما تتجه أنظار العالم إليه اليوم. لذا تم إختبار 5 عزلات من بكتريا الفوتورابدس وهى بكتريا معزولة من النيماتودا من نوع الهيتيرورابدس وهو نوع محلى يصيب أنواع كثيرة من حشرات الأرض. ومن خلال دخول هذه النيماتودا إلى الحشرة تنطلق البكتريا الموجودة فى جوفها مسببه تسمم ثم موت الحشرة.

نميت هذه البكتريا على بيئة خميره وأملاح سائله فى غياب النيماتودا، وقدُوجد أن البكتريا ُتنتج أيضاً السم وتقضى به على خلايا دم الحشرة أثناء التغذية. كما تم إختبار هذه البكتريا ضد حشرة دودة الشمع العظمى (جلاريا ميلونيلا) حيث تم خلط البيئه السائله والمحتويه على خلايا هذه البكتريا بنوعين من البيئه - ردة القمح والرمل الناعم – وباستعمال ثلاث اضافات من البكتريا (5، 10، 20مل) بالمقارنة بالبيئة السائلة بمفردها. وجد أن هناك اختلافات بين أنواع السلالات المختبرة ونوع البيئة والكميات المضافة وأيضا اختلاف الوقت المأخوذ عنده العينة. أثبتت النتائج أن ثلاث عزلات من البكتريا عندما اضيفت بكمية 20 مل أعطت نتائج 100 % موت عندما خلطت بالرمل الناعم. هذه البكتريا تم عزلها من دم العائل مره أخرى وثبت أنها من الممكن أن تعيش في غياب النيماتودا وتفرز مواد قاتله من الممكن استخدامها في برامج المكافحة الحيوية.