analysis of spherules described above. On the other hand, moon rocks are rich in Ti. Detailed work to establish the chemical nature of the material recovered from Barmer basin is now in progress.

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Biosafety concerns on the use of *Photorhabdus luminescens* as biopesticide: experimental evidence of mortality in egg parasitoid *Trichogramma* spp.

Sharad Mohan[1,*] and Naved Sabir[2]

[1]Division of Nematology, Indian Agricultural Research Institute, and [2]National Centre for Integrated Pest Management, IARI Campus, New Delhi 110 012, India

***Photorhabdus luminescens*, a symbiotic bacterium associated with the entomopathogenic nematode *Heterorhabditis indica*, has recently been shown to exhibit biopesticidal potential against important pests, independent of its host nematode. The bio-ecological compatibility of the bacterium was tested *in vitro*, against two species of the common biocontrol agent *Trichogramma* in their hyperparasitized form inside the host eggs of the rice grain moth, *Corcyra cephalonica*. In 65% of the eggs exposed to *P. luminescens* cells alone or their toxins, the *Corcyra* egg-shells became flaccid and there was significant reduction of up to 84% in the emergence of *Trichogramma* adults. The possible access of bacterial cells or their secreted toxins to the *Trichogramma* embryo sheltered inside the *Corcyra* eggshell is discussed. The nematode *H. indica* carrying the bacterium within its gut had no effect on the emergence. The results point to the bio-ecological hazards of indiscriminate use of *P. luminescens* as a biopesticide. Due to its wide host range, the inclusion of *P. luminescens* in any integrated pest management programme would be suspect, until proven safe for natural enemies and non-target organisms.**

Keywords: Biopesticide, biosafety, *Heterorhabditis indica*, *Photorhabdus luminescens*, *Trichogramma*.

THE motile Gram-negative bacterium *Photorhabdus luminescens* Thomas & Poinar (Enterobacteriaceae) found in symbiotic association with the entomopathogenic nematode

*For correspondence. (e-mail: srivastavasharad@yahoo.com)

Heterorhabditis spp. (Nematoda: Heterorhabditidae) is a potent insect pathogen[1,2]. Recently, successful laboratory and field evaluations proved the insecticidal virulence of the bacterium when applied alone as a biopesticide, independent of its nematode symbiont, against the cabbage white butterfly, *Pieris brassicae* (L.)[3], mango mealy bug, *Drosicha mangiferae* (Green)[4] and the pupae of the diamondback moth, *Plutella xylostella* (L.)[5]. The bacterium is reported to be non-toxic to humans and mammals and differs genetically from the human clinical isolate *P. asymbiotica* (Fischer-Le Saux)[6].

Studies on the insecticidal-complex produced by this bacterium have revealed that several extracellular macromolecules such as proteases, lipases, broad-spectrum antibiotics confer the insecticidal ability[7,8]. The insecticidal toxin proteins secreted by *Photorhabdus* during its growth, into the culture medium, have been purified[9,10] and their genes cloned[11]. In the wake of such advances, *P. luminescens* or its formulations are being considered as potential inputs in pest management strategies. However, the wide-ranging entomopathogenicity gives rise to doubts regarding their ecological safety against beneficial/non-target fauna in general, and insects in particular.

Trichogramma Ashmead (Hymenoptera: Trichogrammatidae) wasps are the most widely used natural enemies of insect eggs[12]. They occur naturally in nearly every terrestrial habitat and cause efficient destruction of the eggs of over 200 insect species through hyperparasitization. *Trichogramma* are successful components of many an integrated pest management (IPM) programme, where they are used as 'Trichocards' (hyperparasitized eggs of *Corcyra cephalonica* Stainton [Lepidoptera: Pyralidae] by *Trichogramma* pasted on small paper strips). However, they exhibit variable sensitivity to the broad spectrum insecticides and biopesticides commonly in use: parathion wafting from treated cotton fields was found to kill *Trichogramma* adults up to a mile away[13,14], whereas the application of *Bacillus thuringiensis* Berliner (*Bt*) insecticide had no impact on adult emergence or survival[15].

The entomopathogenic attributes of *P. luminescens* qualify it equally as a potential biopesticide against insect pests. However, due to lack of information on its bio-ecological safety, it was important to investigate the toxicity of *Photorhabdus* to *Trichogramma* parasitized insect eggs, in case both were to be recommended simultaneously in any IPM programme. Therefore, an *in vitro* trial was conducted for testing the ecological compatibility of *P. luminescens* cells alone or their secreted toxins against the hatching of the two species of the commercially recommended biological control agent, viz. *T. chilonis* (Ishii) and *T. japonicum* (Ashmead) on the hyperparasitized eggs of the stored grain moth, *C. cephalonica.*

P. luminescens isolated from *Heterorhabditis indica* (IARI strain) was used in these studies. Cultures were grown overnight on nutrient broth at 28°C to obtain a concentration of 10^5 colony forming units (CFU) per ml. The bacterial suspension was pelleted by centrifuging at 15,000 *g* at 4°C and the pellets were re-suspended in sterile water yielding approximately 1×10^5 cells per ml. The supernatant was passed through a 0.22 µm filter under aseptic conditions. Cardboard strips carrying *C. cephalonica* eggs freshly parasitized by *T. chilonis* and *T. japonicum* were obtained from the National Centre for Integrated Pest Management (NCIPM), New Delhi. Whatman No. 1 filter paper was cut into 3 cm^2 size and placed at the centre of 2″ plastic petri plates and moistened with 100 µl sterile water. Strips carrying the parasitized eggs of either of the two *Trichogramma* species were cut into 1 cm^2 size, each carrying approximately a hundred parasitized eggs, and were placed on the moist filter paper. Each strip was individually exposed to the following treatments:

T1. Egg strips with only moist filter paper base and no treatment.
T2. Freshly emerged *H. indica* infective juveniles @1000 IJ/50 µl sterile water.
T3. Nutrient broth @ 50 µl.
T4. *P. luminescens* cells (PC) alone in 50 µl sterile water (from 1×10^5 cells/ml stock).
T5. *P. luminescens* supernatant (PS) @ 50 µl.
T6. Sterile water @ 50 µl.

The petri plates were sealed with parafilm to prevent the escape of the emerging adult wasps. Each treatment was replicated four times and incubated in a BOD incubator maintained at 28°C. A progressive and cumulative count of adult emergence was made daily up to four days. Prior to counting, the petri plates were exposed to 4°C for 3–4 min, which immobilized the adult wasps and facilitated easy counting. Observations on egg distortion were taken under a stereo-microscope. The per cent distortion of eggs refers to the mean per cent of eggs exhibiting distortion in the four replicates. The distortion was observed in the form of protrusion of the red-eyeballs of the *Trichogramma* embryo through the partially dissolved *Corcyra* egg-shell (Figure 1 *c*). Observations on per cent hatching and egg distortion were transformed using arcsine transformation and subjected to repeated measurement analysis using SAS software version 8.1. Treatments were compared for their statistical parity and coded accordingly, as shown in Tables 1 and 2.

The secreted toxins of *P. luminescens* (PS) had an adverse effect on the emergence of the adult wasps of both *Trichogramma* species, which was statistically at par with the bacterial cells alone (PC). The mean per cent emergence of *T. chilonis* and *T. japonicum* was 6 and 8.25 respectively, for PS-treated eggs, and 13 and 16.25 respectively, for PC-treated eggs after four days of exposure (Table 1 and Figure 2). This indicates that both the treatments resulted in more than 84% mortality of the *Trichogramma* embryo sheltered in the *C. cephalonica* egg-shell.

Interestingly, the PC and PS treatments caused flaccidity in the *Corcyra* egg-shell, which collapsed around the *Trichogramma* embryo inside, while the unparasitized eggs were completely degenerated (Figure 1 *a*). The eggs remained unaffected and maintained their integrity in other treatments where the adult wasps emerged through a neatly cut hole (Figure 1 *b*). The infected eggs exhibited a peculiar characteristic where the eyes of the un-emerged *Trichogramma* adults were seen protruding out of the partially dissolved egg-shell (Figure 1 *c*). The percentage of such eggs was more in PS-treated eggs (65.5 in *T. chilonis* and 48.75 in *T. japonicum*), suggesting the activity of chitinase (Table 2).

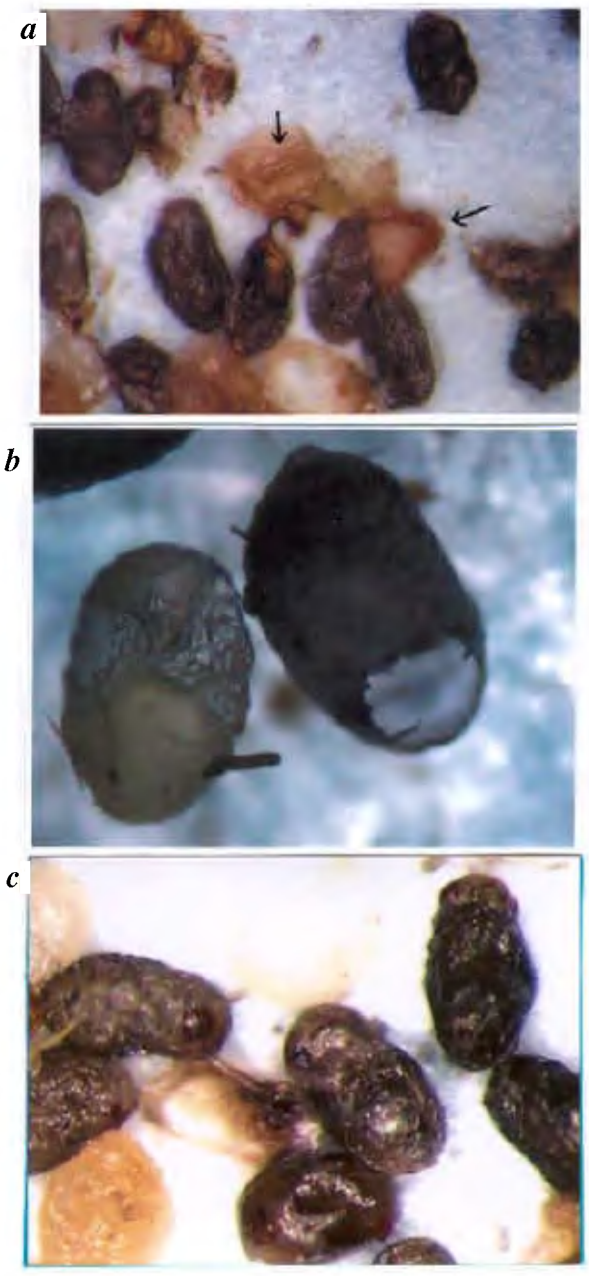

Figure 1. ***a***, Hyperparasitized eggs of *Corcyra cephalonica* by *Trichogramma chilonis* treated with *Photorhabdus luminescens* showing flaccidity of egg-shells Arrow shows completely degenerated unparasitized eggs. ***b***, Untreated eggs of *C. cephalonica* showing neatly cut hole by adult *Trichogramma* wasps for emergence. ***c***, Eyes of unemerged dead *T. chilonis* adults seen protruding out of the partially dissolved *Corcyra* egg-shell treated with *P. luminescens*.

The emergence of healthy adults was observed in the remaining treatments over a period of four days in both the species (Figure 2 *a*). A significantly suppressed emergence for the first couple of days was observed in *T. chilonis* treated with the nematode *H. indica*. However, no nematode developmental stage or bacterial colony was isolated from the parasitized eggs, negating the role of the nematode towards pathogenicity.

It is speculated that the secreted toxins or the motile bacterial cells could have accessed the *Trichogramma* embryo as follows:

(i) Entry could possibly have been through the micropyles present at the anterior pole of the *Corcyra* eggs. Four or more micropyles are known to exist on the lepidopteran eggshell along with several minute aeropyles, which enable continuity between the external and the internal environment[16].

(ii) Parasitism by *Trichogramma* also weakens the egg shell as the female drills a hole through the chorion to insert its eggs, which could act as another passage for the bacterial cells or toxins.

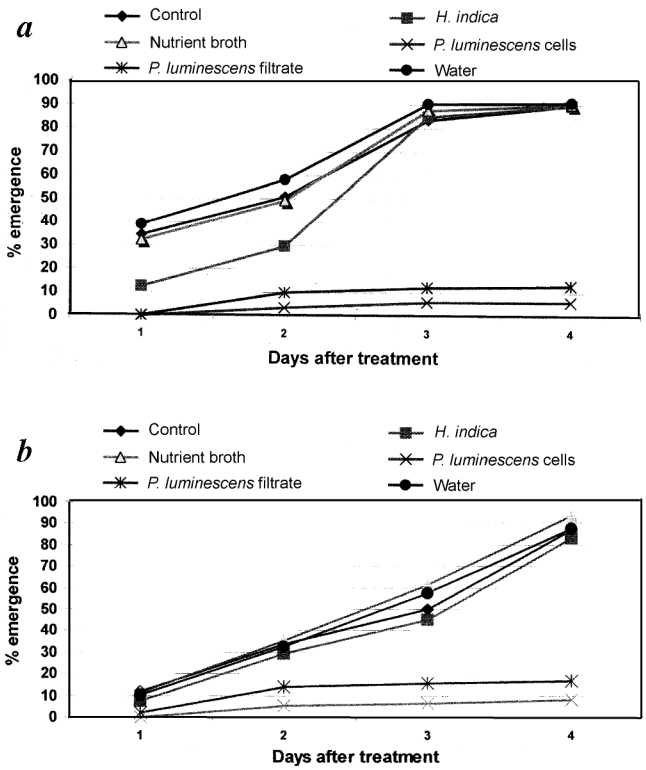

Figure 2. Per cent emergence of *T. chilonis* (***a***) and *T. japonicum* (***b***) as affected by different treatments of *P. luminescens*.

Table 1. Effect of different treatments on per cent emergence of *Trichogramma chilonis* (Tc) and *Trichogramma japonicum* (Tj) from parasitized eggs of *Corcyra cephalonica*

	Day 1		Day 2		Day 3		Day 4	
	Tc	Tj	Tc	Tj	Tc	Tj	Tc	Tj
Control	34.5^{a}	12^{a}	51^{a}	33.75^{a}	83.5^{a}	50^{ab}	90^{a}	87^{a}
Heterorhabditis indica	12.5^{b}	7.5^{ab}	29.75^{b}	29.25^{a}	85^{a}	45.25^{b}	89.75^{a}	83.25^{a}
Nutrient broth	32.25^{a}	11.5^{ab}	49.25^{a}	35.25^{a}	87.75^{a}	61.25^{a}	90.25^{a}	93.5^{a}
Photorhabdus luminescens cells alone	0.25^{c}	2^{bc}	10^{c}	14.25^{b}	12.25^{b}	15.75^{c}	13^{b}	16.75^{b}
P. luminescens culture filtrate	0^{c}	0^{c}	3.5^{c}	5.25^{b}	6^{b}	6.75^{c}	6^{b}	8.25^{b}
Water	39^{a}	10.5^{ab}	58.25^{a}	32.5^{a}	90.5^{a}	57.5^{ab}	91^{a}	87.25^{a}

Values indicate mean hatch (%) based on progressive repeated observations; Number of replications = 4; Treatments with the same letter code are not significant (at 5%) within the same column.

Table 2. Effect of different treatments on per cent distortion of *T. chilonis* (Tc) and *T. japonicum* (Tj) from parasitized eggs of *C. cephalonica*

	Day 1		Day 2		Day 3		Day 4	
	Tc	Tj	Tc	Tj	Tc	Tj	Tc	Tj
Control	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
H. indica	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
Nutrient broth	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
P. luminescens cells alone	18.25^{b}	10^{b}	25^{b}	15.5^{b}	31.25^{b}	23.5^{b}	35.75^{b}	31.5^{b}
P. luminescens culture filtrate	23^{b}	13.25^{b}	44.75^{c}	21.25^{b}	52.5^{c}	37.75^{c}	65.5^{c}	48.75^{c}
Water	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Values indicate mean number of eggs distorted (%) based on progressive repeated observations; Number of replications = 4; Treatments with the same letter code are not significant (at 5%) within the same column.

(iii) The host yolk that oozes out of the oviposition hole due to the internal pressure[17], could be a direct source of nutrient for the bacterial cells to grow and subsequently pervade the internal environment. The recovery of viable bacterial colonies from the infected eggs on nutrient agar plates suggests the activity of the *Photorhabdus* cells.

The mortality to the *Trichogramma* embryo could be due to the direct action of enzymatic secretions of the bacteria. The activity of lipase[18], protease[19] and chitinase[20] is already established, and either or all could be responsible for the dissolution and weakening of the *Corcyra* egg-shell chitin (Figure 1 *a*, *c*). Subsequently, the *Trichogramma* embryo, whose embryonic membranes contain lipids and proteins, could easily have been digested by the bacterial metabolites. The activity of toxins on the *Corcyra* yolk surrounding the *Trichogramma* embryo could also have resulted in the loss of gaseous exchange vital for the growth of the embryo. A possibility of physiological alteration in the molecular cues, affecting the physiology of development and/or hatching, cannot be ruled out. Some directions to further investigations can be drawn from these findings, to prove the mode of action of the bacteria.

This study brings to the fore, the concerns of biosafety in connection with the use of *P. luminescens* as a biopesticide. The virulence exhibited by *P. luminescens* towards a beneficial insect point to the possible ecological perils of the indiscriminate application of *P. luminescens* as a broad-spectrum biopesticide.

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