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Bioassay of *Heterorhabditis bacteriophora* against various insects

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Abstract
Pathogenicity of *Heterorhabditis bacteriophora* Poinar, was tested against last instar larva of rice moth, *Corcyra cephalonica* Stainton, greater wax moth, *Galleria mellonella* Linnaeus, silkworm, *Bombyx mori* Linnaeus, tobacco caterpillar, *Spodoptera litura* Fabricius, and white grub, *Brahmina coriacea* Hope. Among the test insects, larvae of *C. cephalonica* and *G. mellonella* were found to be the most susceptible to the infective juveniles (IJs) of *H. bacteriophora*, in which 50-100% mortality was incurred at 48 hours of exposure. *B. mori* and *S. litura* were comparatively less susceptible with 30-80 % mortality at exposure of 48 hours. The least susceptible among the insects under reference was *B. coriacea* whose larvae remained alive up to 72 hours at the highest inoculum treatment of 160 *H. bacteriophora* IJs/ larva. Cent per cent larval mortality could be attained only at the highest level of inoculum. Dose - mortality response of the test insects against *H. bacteriophora* also revealed different LD50 values for the referred insects. LD_{50} for *C. cephalonica* and *G. mellonella* at 24 hours was found to be 33.38 and 79.99 IJs/ larva respectively, whereas it was 25.18 and 20.26 IJs/larva for *S. litura and B. mori* respectively at 72 hours after treatment of *H. bacteriophora* at different doses. However, it was 40.28 for *B. coriacea* after 144 hours of treatments. LD_{95} values were 453.18, 527.80, 291.82, 453.17 and 507.92 for *Corcyra, Galleria, Spodoptera, Bombyx* and *Brahmina* respectively at different exposure time.

Keywords: *Heterorhabditis bacteriophora*, pathogenicity, mortality, entomopathogenic nematodes and insects

Introduction
Entomopathogenic nematodes belonging to families Heterorhabditis and Steinernematidae comprise the biological alternatives to chemical based pest management programme against various insect pests. They are lethal obligatory parasites of insects [1], yet pose no threat to plants, vertebrates and most of the other invertebrates. Entomopathogenic nematodes possess many attributes of the excellent biological control agent. They can be easily mass reared and applied, using most standard Equipments. They have a broad host range and being highly virulent, kill their host insect rapidly. Over 200 species of insect pests belonging to different orders have been observed to be infected by these nematodes [2].
These can be applied successfully against soil inhabiting insects (as soil application) as well as above-ground insects (foliar spray) in cryptic habitats [3]. Lepidopterans are considered as the most vulnerable host for Steinernematidae and Heterorhabditidae [4]. White grubs of defoliating beetle *Brahmina coriacea* are the other destructive and widely occurring pests of apple and potatoes in Himachal Pradesh [5]. Fortunately this pest has been reported to be susceptible to the entomopathogenic nematode by various workers [6]. Thus the present investigation was designed to evaluate an indigenous strain of *Heterorhabditis bacteriophora* isolated from Himachal Pradesh against lepidopteran and coleopteran pests so as to incorporate the test nematode in further Bio-intensive Integrated Pest Management (BIPM) programme in the state.

Materials and Methods
Nematode culture
The strain of entomopathogenic nematode, isolated from Raj garh area of district Solan in Himachal Pradesh (Raj garh area) by using the insect baiting method described by Bedding and Akhurst [7] was identified as *Heterorhabditis bacteriophora* in Nematology laboratory of Department of Entomology, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan.

Insect baiting technique

For this method, rice moth (*Corcyra cephalonica*) larvae were used as trap host (bait) for the EPNs. Fully grown five to seven larvae of *C. cephalonica* were placed at the bottom of 250 ml capacity plastic jars and thoroughly mixed 200g soil collected from rhizosphere of the plants was poured over the *Corcyra* larvae. The jars were then covered by muslin cloth and were incubated at 25 ± 1^0 C temperature for seven days. After seven days, the jars were examined for the presence of the nematodes.

Extraction of EPNs

The soil jars were emptied into the plastic pan after seven days of baiting method, out of which dead insects were collected and checked for the nematode infection. The nematode infection was identified by the red coloration of the insect cadavers. Dead, intact insect larvae were removed and surface-sterilized in 1.0 % Sodium hypochlorite solution for 3 min, then washed three times in sterile distilled water [8]. Cadavers were placed on white trap [9] for extracting the emerging nematode.

White trap method

The White trap method comprised of an inverted petridish cover (60 mm diameter) placed inside larger Petri dish (90×15 mm) having 0.1 per cent formalin solution. A What man filter paper was placed on inverted Petri dish upon which the nematode infected cadavers were placed. The infective juveniles of the nematodes came out of the cadavers and swam through the moist filter paper into the formalin solution. The isolated infective juveniles of *H. bacteriophora* were mass cultured on *Corcyra cephalonica* larvae as per the procedure suggested by Singh [10] in Biocontrol laboratory of the department.

Mass culturing

a) **Infecting *Corcyra* larvae:** One ml of the suspension (containing 400 IJs) collected from the white trap was spread evenly on what Mann no. 1 filter paper lined inside a 9 cm Petri dish. Twenty full grown larvae of rice moth were placed in the Petri dish, with the goal to achieve infection by at least 20 nematodes per larva. The Petri dishes were covered and stored in a poly bags separately (to conserve moisture) and incubated at 25 ± 1^0 C.
b) **Harvesting:** After seven days, infected *Corcyra* larvae were transferred to white trap for extraction of juveniles. The harvesting was done daily for 4-5 days or till the nematode production reduced substantially.
c) **Storage of nematodes:** The infective juveniles of the test nematode collected in 0.1% formalin solution were stored in sterilized synthetic sponge pieces (2×2×1 cm) at 10ºC.

The fresh culture of nematode was used for bioassay of five test insects the details of which have been presented vide Table 1.

Table 1: Details of insect pests tested for their susceptibility to *Heterorhabditis bacteriophora*

Scientific name	Common name	Order: Family	Host/ Commodity	Source	Status
Corcyra cephalonica	Rice meal moth	Lepidoptera: Pyralidae	Stored grains, maize	Biocontol Lab, Department of Entomology, UHF, Nauni, Solan, H.P	Pest
Galleria mellonella	Greater wax moth	Lepidoptera: Pyralidae	Honey comb	Apiculture field, Department of Entomology, UHF, Nauni, Solan , H.P	Pest
Spodoptera litura	Tobacco cutworm	Lepidoptera: Noctuidae	Tobacco, tomato, cucumber	Entomology field, UHF, Nauni, Solan	Pest
Bombyx mori	Silkworm	Lepidoptera: Bombycidae	Mulberry	Silkworm rearing unit, Dehradun, Uttarakhand	Beneficial insect
Brahmina coriacea	White grubs	Coleoptera: Scarabaeidae	Potato	Khedadhar, Agriculture research station, Sirmour, H.P	Pest

Larval mortality bioassay

The bioassay of all the test insects was carried out in 9 cm diameter Petri plates internally lined with Watt Mann filter paper no.1 except for white grubs for which small plastic cups of 5 cm diameter filled with autoclaved soil were used. All the test insects were separately placed at the rate one fully grown larva/ replicate. All the Petri plates/ cups were further inoculated with different population counts of the test nematode *H. bacteriophora* separately. The inocula used were 10, 20, 40, 80, and 160 IJs of test nematode per insect. All the inoculated Petri plates/cups were maintained in the culture room of biocontrol laboratory at 25 ± 2ºC temperature. Each treatment was replicated ten times. Pathogenicity of *H. bacteriophora* against the test insects was worked out by recording mortality up to five days or till per cent mortality was achieved in at least one treatment. Observations regarding per cent mortality were recorded every 24 hours.

Statistical analysis

Data on mortality of the test insects were converted to corrected per cent mortality using Abbott's formula [11]. The data thus obtained were analyzed by using one way analysis of variance after square root transformation through online statistical software OPSTAT [12] Dose - response of the larvae of test insects to *H. bacteriophora* was worked out by calculating their LD_{50} and LD_{95} values individually by Probit Analysis software [13]. Dosage mortality curve was also obtained.

Results and Discussions

The interpretation of data was indicative of highly variable mortalities due to *H. bacteriophora* among the test insects. The Per cent mortality of the five test insects when inoculated with inocula of 10, 20 40, 80 and 160 larvae of *H. bacteriophora*, separately, has been presented through Tables 2-6.

Mortality of *C. cephalonica* larvae: Perusal of data pertaining to the mortality of *C. cephalonica* (Table 2) reveals mortality to the level of twenty per cent within 24 hours even at the minimum initial inoculum of ten *H. bacteriophora* juveniles. The mortality increased significantly with progression of time and 80 per cent insect larvae were killed by 96 hours at this level of inoculum. Interestingly, the per cent mortality remained same at 120 hours also, at this level of inoculum. All the *Corcyra* larvae were killed within 120

hours when inoculated with 20 infective juveniles of test nematode per larva. Cent per cent mortality of *Corcyra* larvae was attained at 96 hours when inoculated with 40 juveniles and at 72 hours when inoculated with 80 juveniles. The initial inoculum of 160 juveniles incurred mortality of 90 per cent *Corcyra* larvae within 24 hours and all the larvae were dead by 48 hours. The results were indicative of high susceptibility of *C. Cephalonica* larvae to *H. bacteriophora.* Probit analysis of the data (Table 7, Fig: 1) after Abbott's correction revealed LD_{50} and LD_{95} values of *C. cephalonica* to *H. bacteriophora.* The Table revealed that 33.38 (fiducial limit: 19.35 and 57.56) individuals of *H. bacteriophora* per *Corcyra* larva were able to cause mortality of 50 % population of the insect, whereas, 453.18 (fiducial limit: 84.82 and 1407.60) IJs/ *Corcyra* killed 95% population, when exposed to the test nematode for 24 hours. Whereas, in a study, 100 per cent mortality in *C. cephalonica* was observed after 48-72 hours of treatment with *H. bacteriophora* (isolated from Kashmir, India) [14] and LC_{50} value of 10.17 IJs/larva was recorded at 48 hours. Similarly, in another study, *C. cephalonica* was found as best host among various other test insects in which 10-95% mortality was observed at 24 hrs of exposure to *H. indica* [15].

Table 2: Mean per cent mortality of *C. cephalonica* at different inocula of *H. bacteriophora* infective juveniles (IJs)

Treatments	24hours	48hours	72hours	96hours	120hours
T1 (10 IJs/larva)	20 (4.49)	50 (7.12)	70 (8.41)	80 (8.99)	80 (8.99)
T2 (20IJs/larva)	40 (6.37)	60 (7.12)	80 (8.99)	90 (9.53)	100 (10.05)
T3 (40 IJs/larva)	50 (7.12)	80 (8.99)	90 (9.53)	100 (10.05)	100 (10.05)
T4 (80 IJs/larva)	70 (8.41)	90 (9.53)	100 (10.05)	100 (10.05)	100 (10.05)
T5 (160 IJs/larva)	90 (9.53)	100 (10.05)	100 (10.05)	100 (10.05)	100 (10.05)
CD (T x h)	0.83				

Figures in parenthesis are square root transformed values

Mortality of *G. mellonella* larvae

Statistically scrutinized data relating to analysis of susceptibility of *G. mellonella* to *H. bacteriophora* as placed in Table 3 revealed that the initial inoculum of ten juveniles per larva of *G. mellonella* could kill one out of ten larvae by 24 hours, but at 120 hours, six out of ten (60%) larvae were deceased. The inoculum of 40 juveniles caused mortality of all the inoculated larvae at 120 hours. Similar results were achieved at 96 hours when the inoculum of 80 juveniles parasitized each larva of *G. mellonella.* All the larvae were killed at 48 hours when 160 juveniles of the test nematode were inoculated per larva. Table 7 (Fig 2) revealed that 80.0 (fiducial limits of 42.28 and 119.19) IJs of *H. bacteriophora* per individual of *G. mellonella* caused mortality of 50 per cent individuals within 24 hours. However, 527.8 IJs (fiducial limits of 133.56 and 2085.76) of test nematode were required to incur mortality of 95 % population within this period. Cent per cent mortality of *G. mellonella* was achieved by *H. bacteriophora* (Kashmir isolate) within 48-72 hours of nematode exposure and LC_{50} value of 28.72 IJs/ larva in a lab experiment[14] and 10-95 per cent mortality[15] was also incurred by *H. indica* at 24 hours of nematode treatment.

Table 3: Mean per cent mortality of *G. mellonella* at different inocula of *H. bacteriophora* infective juveniles (IJs)

Treatments	24hours	48hours	72hours	96hours	120hours
T1 (10 IJs/larva)	10 (3.32)	30 (5.52)	40 (6.37)	50 (7.12)	60 (7.79)
T2 (20IJs/larva)	10 (3.32)	40 (6.37)	60 (7.79)	70 (8.41)	90 9.53)
T3 (40 IJs/larva)	30 (4.49)	50 (7.12)	70 (8.41)	80 (8.99)	100 (10.05)
T4 (80 IJs/larva)	50 (7.12)	70 (8.41)	90 (9.53)	100 (10.05)	100 (10.05)
T5 (160 IJs/larva)	80 (8.99)	100 (10.05)	100 (10.05)	100 (10.05)	100 (10.05)
CD (T x h)	0.92				

Figures in parenthesis are square root transformed values

Mortality of *Bombyx mori*

Table 4 revealed that *Bombyx mori* larvae were not affected by the inoculum of ten nematodes till 48 hours of treatment. The rate of mortality remained low at this inoculum with mortality of only 30 per cent treated larvae at 120 hours of treatment. Mortality rate of 50 and 70 per cent were recorded at respective inocula of 20 and 40 juveniles per larva at 120 hours of application. Despite the fact that 90 per cent *B. mori* larvae were killed at 120 hours of treatment when infected by 80 juveniles per larva, complete kill was observed only at the inoculum level of 160 juveniles at exposure period of 96 hours. The population of *B. mori* required minimum exposure of 72 hours to infective juveniles at the rate of 36.15 (fiducial limits: 20.26 and 64.49) for death of 50 per cent of its population (Table 7, Fig: 3). Mortality of 95 per cent population was recorded when 453.17 IJs per insect (fiducial limits: 88.07 and 2331.94) infected *B. mori* population. Whereas, in one of the experiment conducted on the efficacy of *H. bacteriophora,* 100 per cent of the *B. mori* larvae died when exposed for 48-72 hours to IJs of the nematode [14]. Contrary to this, silkworm larval mortality within 24-72 hours at inocula of 100 and 200 IJs/ larva was achieved in a study [16].

Table 4: Mean per cent mortality of *B. mori* at different inocula of *H. bacteriophora* infective juveniles

Treatments	24hours	48hours	72hours	96hours	120hours
T1 (10 IJs/larva)	0 (1.00)	0 (1.00)	20 (4.58)	30 (5.52)	30 (5.52)
T2 (20 IJs/larva)	0 (1.00)	0 (1.00)	40 (6.37)	40 (6.37)	50(7.12)
T3 (40 IJs/larva)	0 (1.00)	0 (1.00)	50 (7.12)	60 (7.79)	70(8.41)
T4 (80 IJs/larva)	0 (1.00)	30 (5.52)	60 (7.79)	70 (8.41)	90 (9.53)
T5 (160 IJs/larva)	0 (1.00)	50 (7.12)	90 (9.53)	100 (10.05)	100 (10.05)
CD (T x h)	0.89				

Figures in parenthesis are square root transformed values

Mortality of *Spodoptera litura* larvae

In *Spodoptera litura* (Table 5) the infection of larvae was delayed and none of the treated larva was killed till 48 hours at the treatment inoculum of 10 and 20 juveniles of *H. bacteriophora.* The per cent mortality in the range of 10 to 70 per cent was recorded at 72 hours of exposure to different concentrations of *H. bacteriophora.* However, total mortality was obtained at 120 hours at the dosage of 80 infective juveniles and at 96 hours, at the treatment dose of 160 juveniles. Probit analysis of the data (Table 7, Fig:4) indicated that the mortality to the tune of 50 per cent population of *S. litura* was attained when exposed to *H. bacteriophora* IJs for 72 hours @ 25.18 nematodes/ insect (fiducial limits: 13.75 and 46.10). LD_{95} for this insect was worked out to be 291.82(fiducial limits: 71.56 and 1190.02). In a similar study [15], LC_{50} value to 4th instar *S. litura* larvae were found to be 4.46, 6.20, 5.56 and 4.21 and LC_{90} 13.80, 10.62, 12.40 and 7.74 for four different species of *Steinernema.* Earlier, also *S. litura* could not be killed within 24 hours and at least 48 hours were required to achieve 12.5- 60 per cent mortality at all doses of EPN (10-500IJs/larva) [15]. Working with another EPN *Steinernema,* some workers [17] revealed that inoculum level of 100 IJs *of Steinernema* sp. per 20 larvae of *S. litura,* caused mortality of later in the range of 83 to 88 per cent within 24 hours. Similarly, cent per cent mortality of *Spodoptera litura* was also achieved with the inoculum of 100 IJs of *S. masoodi, S. mushtaqi* and *S. seemae* within 48 hours, but, *S. carpocapsae* and *Oscheius amsactae* could incur *S. litura* larvae mortality after the exposure time of 96 hours at the same inoculum rate [18]. *Steinernema thermophilium @* 100 IJs/ larva incurred 100 per cent mortality to *S. litura* larvae within 24 hours of exposure [19].

Table 5: Mean per cent mortality of *S. litura* at different inocula of *H. bacteriophora* infective juveniles (IJs)

Treatments	24hours	48hours	72hours	96hours	120hours
T1 (10 IJs/larva)	0 (1.00)	0 (1.00)	10 (3.32)	40 (6.37)	50 (7.12)
T2 (20IJs/larva)	0 (1.00)	0 (1.00)	20 (4.49)	60 (7.81)	70 (8.41)
T3 (40 IJs/larva)	0 (1.00)	30 (5.52)	40 (6.37)	70 (8.41)	80 (8.99)
T4 (80 IJs/larva)	20 (4.49)	50 (7.14)	50 (7.12)	90 (9.53)	100 (10.05)
T5 (160 IJs/larva)	40 (6.59)	60 (7.79)	70 (8.43)	100 (10.05)	100 (10.05)
CD(T x h)	0.96				

Figures in parenthesis are square root transformed values

Mortality of *Brahmina coriacea*

The results pertaining to *B. coriacea* as shown in Table 6 revealed this insect to be the least prone to infection by *H. bacteriophora* among all the test insects. The insect larvae were not infected till 72 hours in any of the treatments. Thereafter, mortality of a few insect larvae was observed at higher inocula of 40, 80 and 160 juveniles which killed 10, 20 and 30 per cent of insect larvae respectively, at 96 hours of application. The per cent mortality varied from 10 to 70 per cent at 120 hours and 10 to 80 per cent at 144 hours of exposure to different concentrations of nematode juveniles. The complete kill could be attained only at the highest concentration of 160 IJs/ larvae after the exposure period of 192 hours. Probit analysis of the *Brahmina* mortality data could be worked out after 144 hours of EPN exposure. LD_{50} and LD_{95} for *B. coriacea* grub were recorded to be 40.28 (fiducial limits; 22.53 and 72.02) and 507.92 (fiducial limits; 94.19 and 2739.07) respectively (Table 7, Fig 5). These findings match with the revelation of a report [5] which indicated 86.7 per cent mortality of third instar white grubs of *B. coriacea* after 21 days of inoculation of 500 IJs of *H. indica.*

Table 6: Mean per cent mortality of *B. coriacea* at different inocula of *H. bacteriophora* infective juveniles (IJs)

Treatments	24hours	48hours	72hours	96hours	120hours	144hours	168hours	192hours
T1 (10 IJs/larva)	0 (1.00)	0 (1.00)	0 (1.00)	0 (1.00)	10 (3.32)	10 (3.32)	20 (4.49)	20 (4.58)
T2 (20IJs/larva)	0 (1.00)	0 (1.00)	0 (1.00)	0 (1.00)	10 (3.32)	40 (6.37)	40 (6.40)	40 (6.37)
T3 (40 IJs/larva)	0 (1.00)	0 (1.00)	0 (1.00)	10 (3.32)	40 (6.37)	60 (7.79)	60 (7.79)	70 (8.41)
T4 (80 IJs/larva)	0 (1.00)	0 (1.00)	0 (1.00)	20 (4.58)	60 (7.79)	60 (7.79)	60 (7.74)	60 (7.79)
T5 (160 IJs/larva)	0 (1.00)	0 (1.00)	0 (1.00)	30 (5.52)	70(8.37)	80 (8.99)	90(9.54)	100 (10.05)
CD (T x h)	0.81							

Figures in parenthesis are square root transformed values

Table 7: Dose response of different insect larvae to *H. bacteriophora*

Insects tested	Exposure time (Hours)	LD_{50}	Fiducial limits (LD_{50})		LD_{95}	Fiducial limits (LD_{95})		Regression equation	R^2
			LL	UL		LL	UL		
C. cephalonica	24	33.38	19.35	57.56	453.18	84.82	1407.60	Y = 2.47 + 1.67 X	0.96
G. mellonella	24	80.0	42.28	119.20	527.80	133.56	2085.76	Y= 1.61 + 1.84 X	0.94
S. litura	72	25.18	13.75	46.10	291.82	71.56	1190.02	Y = 2.81 + 1.56 X	0.99
B. mori	72	36.15	20.26	64.49	453.17	88.07	2331.94	Y = 2.56 + 1.58 X	0.93
B. coriacea	144	40.28	22.53	72.02	507.92	94.19	2739.07	Y = 2.43 +1.58X	0.89

Graphs

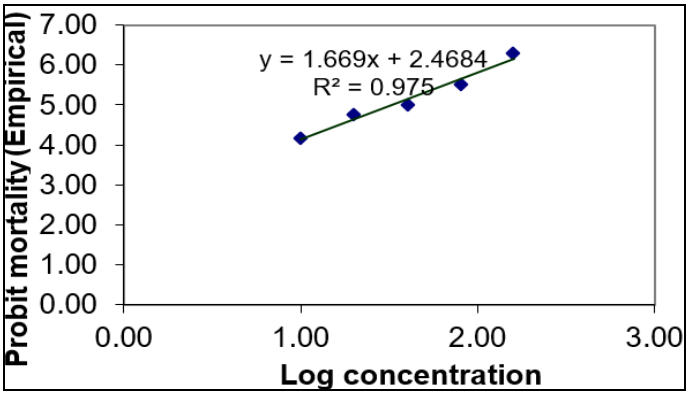

Fig 1: Dose mortality response of *C. cephalonica* to lJs of *H. bacteriophora* (24hours)

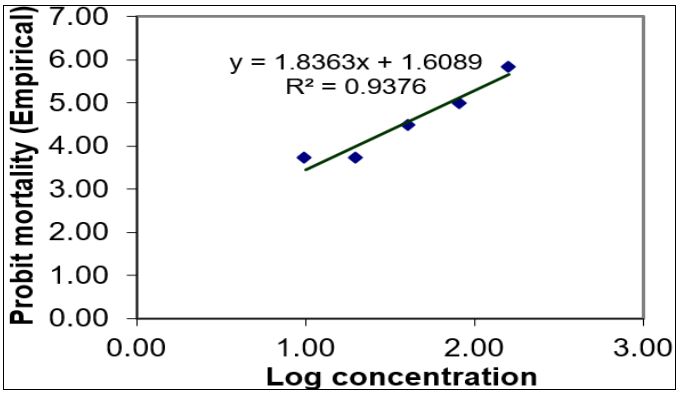

Fig 2: Dose- response mortality of *Galleria mellonella* to lJs of *H. bacteriophora* (24hours)

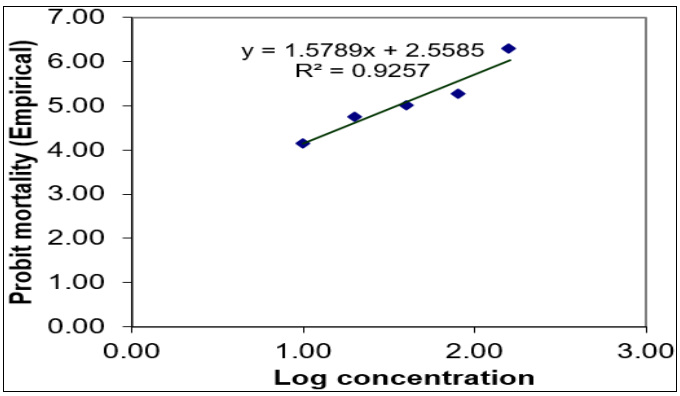

Fig 3: Dose- mortality response of *Bombyx mori* to LJS of *H. bacteriophora* (72hours)

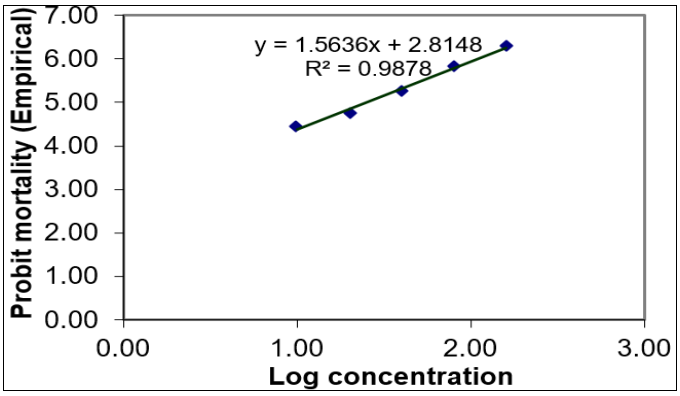

Fig 4: Dose – mortality response of *Spodoptera litura* to lJs of *H. bacteriophora* (72hours)

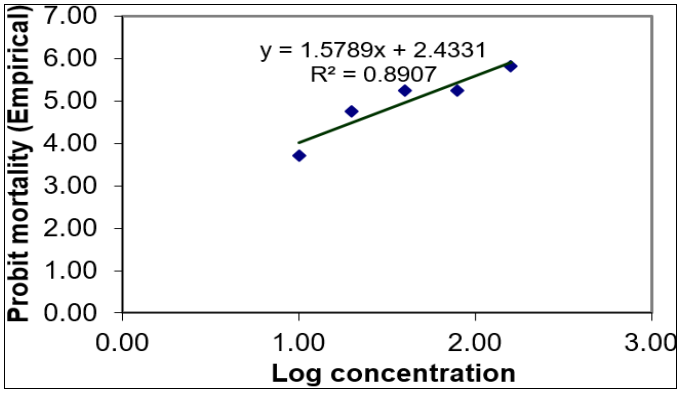

Fig 5: Dose mortality response of White grubs of *Brahmina coriacea* to LJS of *H. bacteriophora* (144 hours)

The present investigation revealed the pathogenicity of *H. bacteriophora* to all the test insects at different doses. However, the inocula of test nematode required to kill these insects were different for all the insects. *Corcyra* was found to be the most susceptible; its larvae catching infection very fast even at lower levels of inoculum in contrast to white grubs, which caught infection after exposure to nematode treatment for long duration. As *H. bacteriophora* is indigenous to Himachal Pradesh, its role as a successful bio-insecticide against various insect pests needs to be ascertained by undertaking further investigations. The efficacy of the test nematode needs to be tested under field conditions before incorporating in IPM as bio - pesticide.

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