Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(5): 1550-1553
Received: 10-07-2019
Accepted: 12-08-2019

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Mass production of *Heterorhabditis bacteriophora* on lepidopteran insect pests

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Abstract
A study on the suitability of various lepidopteran insects for the mass production of entomopathogenic nematode *Heterorhabditis bacteriophora* was conducted in Nematology laboratory of Department of Entomology, UHF, Nauni, Solan (H.P) wherein the last instar larvae of rice moth, *Corcyra cephalonica* (Stainton), greater wax moth, *Galleria mellonella* (Linnaeus), silkworm, *Bombyx mori* (Linnaeus) and tobacco caterpillar, *Spodoptera litura* (Fabricius) were used as hosts for the mass production of test nematode. Identical inoculation doses of 10, 20, 40, 80 and 160 infective juveniles (IJs) of test nematode per individual insect larva of test species were used. Among the four insects under reference, *G. mellonella* was found to be the most suitable host for the mass production of the *H. bacteriophora*, with highest production of IJs at all doses (44,673-136,790 IJs/larva), followed by silkworm larva in which 62514 and 93495 IJs/ larva at respective initial inoculum doses of 80 and 160 IJs/ larva were produced. The count of 36,708 - 64,048 IJs per *S. litura* larva were produced at different levels of inocula followed by *C. cephalonica* from which 22,117- 31,430 IJs / larva were harvested. However, despite of good yield of infective juveniles in all the test insects, *G. mellonella* and *C. cephalonica* were found to be the best for the production of this particular nematode.

Keywords: *Heterorhabditis bacteriophora*, mass production, infective juveniles, nematodes, *in vivo*

Introduction
Entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae made an evolutionary leap to parasitism, maintaining their nutritional mutualistic relationship with bacteria, which in turn convert number of proteins into a substrate in which the nematodes develop and reproduce successfully. This capacity of these nematodes led to their acceptance as successful biological pest control agents to manage noxious insects. The vigorous research activities to explore the potential of these nematodes as biocontrol agents over the past two decades have borne fruits, as a result of which, these are being commercially mass produced in six of the seven continents to manage pest problems in agriculture, horticulture veterinary and animal husbandry. The ease of mass production, exemption from registration requirements and their efficacy against number of pests are the reasons for the commercial development of these nematodes as biological control agents of choice. Since the *en masse* production of these nematodes is necessary for their commercial use as biocontrol agents, present investigation was planned to evaluate the suitability of various lepidopteran insect pests for *in vivo* mass production of *Heterorhabditis bacteriophora.*

Materials and Methods
Nematode culture: Pure culture of indigenous strain of entomopathogenic nematode, *Heterorhabditis bacteriophora* maintained in the Nematology laboratory of department of Entomology, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan was used for the study. The nematode was cultured on fully grown *Corcyra cephalonica* larvae as per the method described by Woodring and Kaya [1] and infective juveniles recovered from the white trap were stored in sterilized sponges at 10 ˚C temperature.

Sources of various test insects and their rearing:
Corcyra cephalonica: The eggs of rice moth, *Corcyra cephalonica* Stainton (Lepidoptera: Galleriidae) were obtained from the Biocontrol laboratory, Department of Entomology, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Nauni 173230 (Himachal Pradesh). Rice moth was reared in Nematology laboratory on rearing medium consisting of crushed maize (sterilized at 50 ºC for 3 hrs) and five per cent powdered yeast at controlled conditions of 25 ± 1 ˚C temperature and relative humidity of 68-70 per cent.

The rearing medium consisted about 3-4 kg of rearing medium which was put in three rearing boxes (approx one Kg/box) and inoculated with one cubic centimeter (cc) freshly laid eggs of rice moth per box. After one month, last instar fully grown larvae of *C. cephalonica* obtained from these cages were used for the study.

***Galleria mellonella*:** Larvae of greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleriidae) were collected from old stored honey combs in Apiculture section of Department of Entomology, UHF, Nauni, Solan. These larvae were further reared on artificial diet [2] comprising of following ingredients:

I. Corn flour – 4 parts by weight, whole wheat flour – 2 parts by weight, skimmed milk powder – 2 parts by weight, dried powdered yeast – 1 part by weight, wheat bran – 2 parts by weight

These ingredients were mixed thoroughly and stored in tight container in order to prevent infestation by insect pests.

II. Equal parts of honey and glycerin by volume were mixed and stored at room temperature.

Equal parts of I and II by weight were mixed and left for 24 hours to allow the liquid to penetrate the dry components of food. The clumps of this mixture were placed in screen cages and inoculated with larvae of *Galleria mellonella.* The cages were wrapped externally by a black paper in order to provide dark condition to the insect in order to facilitate the oviposition. The culture was maintained in a BOD at temperature of 27 ± 1 ^{0}C and relative humidity of 60-70 per cent in the laboratory.

***Bombyx mori*:** Fifth instar larvae of *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) obtained from Central silk board, Dehradun, Uttrakhand, India were fed on fresh mulberry leaves until the onset of the experiment and were maintained in rearing boxes in Nematology laboratory at a temperature of 27 ± 1 ^{0}C.

***Spodoptera litura*:** *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) larvae were collected from infested cucumber polyhouse in the Experimental field of Department of Entomology, UHF, Nauni, Solan (H.P). These larvae were fed on the castor leaves collected from university campus. The last instar larvae were evaluated for their suitability as hosts for mass production of *H. bacteriophora.*

***In vivo* mass production**: *In vivo* mass production of *Heterorhabditis bacteriophora* on the last instar fully grown larvae of the referred test insects was followed following the steps given by Singh [2] here under:

1. **Infecting insect larvae:** Fifty sterilized Petri plates of nine centimeter diameter, internally lined with Whatmann filter paper no. 1 were taken for the purpose. The inoculum levels of 10, 20, 40, 80 and 160 IJs of *H. bacteriophora* to be treated as different treatments were taken separately, each in one ml of distilled water. These nematode suspensions in the referred treatment doses were separately and evenly distributed on the filter paper by pipetting. One test insect larva in each treated Petri dish was then placed. The Petri dishes were covered and stored in a poly bags separately (to conserve moisture) and incubated at 25 ± 1 ºC. These treatments were replicated 10 times.
2. **Harvesting**: The insect larvae died (within 24-48 hours of infecting) due to nematode infection were identified on the basis of reddish color of the cadavers that appeared because of *Photorhabdus* bacteria. These cadavers were individually transferred to separate white traps [3] after seven days of infecting, for extraction of juveniles.

White trap: The White trap consisted of an inverted petridish cover (60 mm diameter) placed inside larger Petri dish (90×15 mm). A Whatman filter paper was placed on this Petri dish. The cadavers were placed on the filter paper and the outer Petri dish was filled with 20 ml of sterile 0.1 per cent formalin solution. As the infective juveniles came out of the cadaver, these swam to 0.1 per cent formalin solution.

For harvesting this nematode, suspension from trap was poured into a beaker and 0.1 per cent fresh formalin solution was added to the white trap. The harvesting and counting of the IJs was done on daily basis for 4-5 days or till the nematode production reduced substantially.

Statistical analysis: One way analysis through online statistical software OPSTAT [4] and the significantly different means were separated by Completely Randomized design (CRD) or critical difference (CD) at p = 0.5 was performed for the yield data analysis.

Results and discussions

The suitability of four test insects viz., *C. cephalonica, G. mellonella, B. mori* and *S. litura* was tested for mass production of the IJs of EPN *H. bacteriophora* at various treatment doses of 10, 20, 40, 80 and 160 IJs / insect larva for each insect.

C. cephalonica: The rice moth, *C. cephalonica* larva when inoculated with 10 IJs initially produced mean count of 22117.0 ± 775.8 within a fortnight period, when all the body contents were exhausted by the emerging IJs. The number of harvested IJs increased with increase in the initial inoculum but this increase was not proportional to the level of treatment. The quantity of IJs harvested from the larvae receiving 10(T1), 20(T2) and 40(T3) IJs as initial inoculum remained significantly similar. Consequently, the quantum of IJ harvest increased significantly in T4 where in, averagely, 29323.8 juveniles were harvested from each larva. Highest mean number of 31430.1 IJs was produced by the larva receiving 160 IJs initially. Earlier, Vashishth [5] harvested 26030 IJs/ *C. cephalonica* larva. However, the average yield of *H. bacteriophora* IJs was assessed at 47475 IJs/ larva of *C. cephalonica* by and Singh [2]. In our study,the mean harvest of IJs/ insect was calculated to be 25836.3. The IJ yields when worked out per gram body weight of the *C. cephalonica* remained in the range of 442340 in T1 to 628602 in T5. These observations signified that *C. cephalonica* larvae inoculated with more number of IJs produced significantly more yields of IJs per gram body weight. However, this increase in IJs harvest did not correspond to the level of increase in initial inoculum (Table 1).

Table 1: Infective juveniles of *H. bacteriophora* produced by *C. cephalonica*

Treatment	Yield (IJs) /larva Mean ± SD	Yield (IJs)/g body weight
T1 (10IJs/insect)	22,117.000 ± 775.794	442340
T2(20IJs/insect)	22,563.000 ± 430.640	451260
T3(40IJs/insect)	23,747.400 ± 664.218	474948
T4(80IJs/insect)	29,323.800 ± 503.688	58647.6
T5(160IJs/insect)	31,430.100 ± 732.825	62860.2
Average yield	25,836.3	

CD (0.05) = 1,816.070
CV = 7.778

G. mellonella: The larvae of greater wax moth, *G. mellonella* when infected with various inocula separately produced significantly different yields of *H. bacteriophora* IJs. The specifics relating to IJ production in this insect have been presented in Table 2. The larvae infected by 10 IJs produced the mean harvest of 44,673 IJs. Subsequently, mean harvested quantity of 54,609.5 and 56,006.5 IJs produced per larva receiving initial treatment of 20 and 40 IJs respectively were significantly at par. The mean number of 1,36,790 IJs per larva harvested from the insects infected with 160 IJs was significantly high with an average yield of 5,47,160 IJs/gram body weight. The mean yield per gm larva was assessed to be 71201.6 *H. bacteriophora* IJs/ larva. In a study [6], accessement of the suitability of three insects viz., *Helicoverpa armigera*, *G. mellonella* and *Corcyra cephalonica* for mass production of EPNs was performed where it was found that *G. mellonella* was the best host for the mass production of *S.seemae* IJs which yielded 2.1 x 10^5 IJs/larva. In another experiment, some workers [7] harvested about 50,000- 2,00,000 IJs of *H. indica* from *G. mellonella* larva. Mwaniki [8] also used *G. mellonella* for mass production of different EPN species and recorded production of 60,680 IJs, 43,780 IJs and 43,470 IJs of *S. yirgalemense*, *S. karii* and *H. indica* respectively.

Table 2: Infective juveniles produced by *G. mellonella*

Treatment	Yield (IJs) /larva Mean ± SD	Yield (IJs)/g body weight
T1 (10IJs/insect)	44,673.000 ± 507.865	17,8692
T2(20IJs/insect)	54,609.500 ± 629.078	2,18,436
T3(40IJs/insect)	56,006.500 ± 717.134	2,24,026
T4(80IJs/insect)	63,929.000 ± 638.295	2,55,716
T5(160IJs/insect)	136,790.000 ± 4,958.190	5,47,160
Average yield	71,201.6	

CD (0.05) = 6,536.575
CV= 10.159

B. mori: Silkworm larvae are often used for mass production of EPNs. The infection of *B. mori* larvae by various inocula of IJs of *H. bacteriophora* produced significantly different quantities of juveniles. The data have been presented in Table 3. Perusal of data revealed an interesting picture of IJ production where in, the larvae receiving the lower infection rate of 10 and 20 IJs produced respective mean yields of 8,180.5 and 9,917 IJs/larva; both the values being significantly similar to each other. Thereafter, the infective juvenile production increased significantly and averagely 28,666.5 IJs were produced by each larva inoculated with 40 IJs initially. The mean quantum of harvest was appreciably high at 62,514.0 and 93,495.0 juveniles/ larva in respective T4 and T5 treatments. The mean IJ yield of 2,820.9 per gram body weight of *B. mori* larva receiving initial infection of 10 IJs was extremely low, it enhanced to 32,239.7 per gram body weight in the larvae receiving initial infection of 160 IJs. However, a large number of *H. bacteriophora* IJs were harvested from last instar of silkworm (4,00,000 - 6,45,000 IJs/ larva) with an average yield of 5,46,840 IJs/larva by Vashishth [5]. Similarly, Prabhuraj and his co-workers [9] and Mwaniki [8] also mass produced *Heterorhabditis* sp. on silkworm larvae and harvested 1,32,104.2 and 60,000 IJs/ insect larva respectively. Our results are contrary to these findings, because we could not harvest good amount of IJs of the test EPN out of the silkworm larvae due to non suitability of the environmental conditions to the test insect.

Table 3: Infective juveniles produced by *Bombyx mori*

Treatment	Yield (IJs) /larva Mean ± SD	Yield (IJs)/g body weight
T1 (10IJs/insect)	8,180.500 ± 184.747	2,820.86
T2(20IJs/insect)	9,917.700 ± 216.257	3,419.89
T3(40IJs/insect)	28,666.500 ± 472.245	9,885
T4(80IJs/insect)	62,514.000 ± 1,480.626	21, 556
T5(160IJs/insect)	93,495.000 ± 4,704.249	32,239.67
Average yield	56,954.74	

CD (0.05) = 6,342.166
CV=17.305

S. litura: *S. litura* is another lepidopteran pest used for mass production of EPNs. As evident in Table 4, minimum and significantly poor mean IJ production of 36,708 and 41,042 was recorded in T1 and T2; both quantities being significantly similar to each other. The mean count of infective juvenile harvest (50,208) recorded in T3 was significantly high and statistically at par with 56,262.7 IJs harvested / larvae receiving initial inoculum of 80 IJs (T4).Highest mean number of IJ yields (64,047.5) were harvested from *S. litura* larvae infected by 160 IJs/ insect. The number of IJs yielded per gram body weight of *S. litura* larva showed significant variations in different treatments and ranged from 44,885 in T1 to 80,059.4 IJs / gram in T5. In a study [7], *S. litura* produced 10,000- 30,000 IJs of *H. indica.* Another worker [10] harvested 1, 66, 000 IJs of EPNs from *Spodoptera exigua* larvae. *Spodoptera litura* [11] produced 6.29x10^3 - 1.94x10^4 IJs of *S. carpocapsae.*

Table 4: Infective juveniles produced by *Spodoptera litura*

Treatment	Yield (IJs) /larva Mean ± SD	Yield (IJs)/g body weight
T1 (10IJs/insect)	36,708.000 ± 2,332.782	45,885
T2 (20IJs/insect)	41,042.000 ± 2,839.417	51,302.5
T3 (40IJs/insect)	50,208.000 ± 1,516.285	62,760
T4 (80IJs/insect)	56,262.700 ± 3,215.706	70,328.4
T5 (160IJs/insect)	64,047.500 ± 2,139.251	80,059.4
Average yield	49,653	

CD (0.05) = 7,083.587
CV = 15.786

Careful examination of the figures presented in Tables 1-4 relating to the suitability of the insects under reference for mass production of *H. bacteriophora* juveniles revealed highest quantum of IJ production at the highest inoculum and *vice versa.* This signified that the yield of IJ harvests were dependent on initial nematode inoculum. The lower initial inoculum resulted in to low host mortality. This meant that insect larvae needed to be appropriately infected to achieve the maximum IJ harvests. The larvae of *G. mellonella* produced highest IJ yields as compare to other test insects at all the respective rates of initial inocula. This was followed by the yields harvested from infected *S. litura* larvae at lower three levels of initial inoculum. However, IJ production was significantly high in *B. mori* larvae receiving initial infection of 80 and 160 IJs. In fact the mean yield of 93,495 IJs/larva of B. m receiving initial inoculum of 160 IJs (T5) was second only to the quantum of harvest achieved in *G. mellonella* larvae receiving this initial inoculum. However, mean IJ yields were very poor in *B. mori* larva at lower initial infection rates. At lower levels of infection (T1 and T2), the mean number of IJs harvested per larva in *B. mori* was minimum and even lower to the IJ yields harvested in the smallest test insect *C. cephalonica* In general, minimum number of IJs/ larva were yielded by *C. cephalonica* larvae. However, IJ yields when worked out per gram body weight, *C. cephalonica* proved to be the best with mean production of 6,28,602 IJs/ gram (T5) followed by *G. mellonella* in which mean yield of 5,47,160 IJs per gram body weight was attained (T5). Generally, the susceptible insect larvae are preferred over the smaller ones for *in vivo* production of EPNs. But number of other factors viz., ease and success rate of rearing the insect, vunerability of other secondary infections, environmental factors etc need to be considered before selecting any insect for mass production of EPNs. During present investigations, keeping in mind all these factors, *G. mellonella* was rated as the most suitable insect followed by *C. cephalonica* and *S. litura. B. mori*, despite being biggest in size and susceptibility to the test nematode, was found to be prone to secondary infections which hampered the multiplication of IJs in its body.

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