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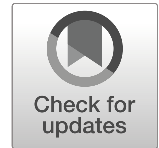

Pathogenicity of ENTOMOPATHOGENIC nematodes against cabbage butterfly (*PIERIS BRASSICAE*) LINNAEUS (LEPIDOPTERA: PIERIDAE) in laboratory conditions

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Abstract

Cabbage butterfly (*Pieris brassicae*) is major pest of cabbage. More than 50% yield losses have been attributed due to this pest annually. Seven insect parasitic nematodes i.e. *Steinerma carpocapsae*, *S. feltiae*, *S. Pakitananese*, *S. asiaticum*, *S. glaseri*, *Heterorhabditis bacteriophora* and *H. indica* were evaluated on 4th larval instar of *P. brassicae* at the different concentrations. Two best performing EPNs (*S. glaseri* and *H. bacteriophora*) were selected and evaluated against 2nd, 3rd and 4th larval instars of *Pieris brassicae* at 1500 infective juveniles/ml. *H. bacteriophora* spp. and *S. glaseri* spp. were sprayed at 1500 IJs/ml on the cabbage leaves alone or in combination (*S. glaseri* + *H. bacteriophora*) and 2nd, 3rd and 4th larval instars were fed on them. Among seven EPNs, hundred percent mortality of *P. brassicae* was recorded in the case of *H. bacteriophora* and *S. glaseri* at1500 IJs/ml concentration after 48 h. Both EPNs spp. were found highly effective against all larval instars of *P. brassicae*. After 48 h of exposure of both EPNs spp. on all larval instar, 100% mortality was recorded. The combined application of *H. bacteriophora* + *S. glaseri* on cabbage leaves resulted into 100% mortality of all larval instars of *P. brassicae.* From present study, it may be concluded that *H. bacteriophora* and *S. glaseri* at 1500 IJs/ml concentration found very effective against *P. brassicae in* laboratory conditions and may be used in the field conditions.

Keywords *Brassica oleracea* · Entomopathogenic nematodes (EPNs) · *Pieris brassicae* · *Heterorhabditis bacteriophora*` · *Steinerma glaseri*

Introduction

Cabbage (*Brassica oleracea var. capitata* Linn.) is one of the essential vegetable crop grown in Pakistan. It is used as a fresh salad, cooked or processed into preserved products such as sauerkraut. It is the cheapest source of useful minerals and food nutrients needed for the balanced human diet (Ashfaq et al. 2018). Successful production of cabbage is facing numerous constraints of fungal, bacterial, viral, insects and nematodes (Krauthausen et al. 2018; Sharma et al. 2018). Among these, infestations of defoliating caterpillars like cabbage butterfly (*Pieris brassicae*) are the major one (Saeed et al. 2017).

P. brassicae is a major pest of cabbage in Pakistan (Saeed et al. 2017). In Khyber Pakhthoon Khwa province, the insect severily damages the cruciferous vegetables throughout the year, single larva can consume 74–80 cm2 leaf area and cause economic losses. In India, the yield losses due to *P. brassicae* ranges from 30 to 40% annualy (Ali and Rizvi 2007; Kular and Kumar 2017). The losses may increase up to 75% percent if susceptible cultivar and favourable climatic conditions prevailes (Chaudhuri et al. 2001; Krishnamoorthy 2004) Larvae of *P. brassicae* feed in such a way leaving the only skeleton of the plant and consumes all the leaves of the plant (Mazurkiewicz et al. 2017). The severe attack resulting damage or deformed cabbage heads are develop which were 100% rejected by the end consumer (Uddin et al. 2007). The severity of the incidence of insect pests is greatly influenced by the prevailing climatic conditions (Meena et al. 2013).

Management of *P. brassicae* through the application of hazardous pesticides is an easy approach to minimize the pest

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population but it causes the development of insecticides resistance and presence of pesticide residues in cabbage heads. *P. brassicae* has established numerous resistance against a series of pesticides belongs to carbamates, synthetic pyrethroids and organophosphates (Ullah et al. 2016). It is an alarming threat to consumer health safety, soil and water pollution and biodegradation of pesticides etc. (Anjum 2018). Prevailing conditions stress to find out newly effective options to control *P. brassicae*. EPN enters into the hemolymph of insect pests through anus, mouth, spiracles or cuticle. After successful approaching in the body of insect, EPNs release bacteria already carrying in their intestine (Forst et al. 1997). The symbiotic bacteria quickly multiply and release toxins inside the hemolymph of insects, which primarily destroy the immune system of the host. Due to discharged toxins, the mortality of insects takes place between 24 to 48 h. On the other hand, bacteria convert the body parts of the host into the nutrient soup. EPNs consume this nutrient soup quickly, which ultimately supports the maximum multiplication of nematodes. When the nutrient soup is consumed, the EPNs breakdown the cadaver of insect pests and moves towards the other larvae (Forset and Clarke 2002; Poinar Jr 1990). In Pakistan, there is no systemic work has been done on the efficacy of EPNs against the management of *P. brassicae*. Therefore, keeping in view the efficient performance of EPNs as biocontrol agents, the present study was planned to evaluate environment-friendly nematode species against *P. brassicae* in lab conditions.

Materials and methods

Rearing of *P. brassicae*

For rearing of *P. brassicae* larvae, brassica plants were grown in small disposable cups. Plants were permitted to gain height of 6 to 7 cm which were used for the collection of eggs. 20–25 pupae were acquired from field and placed in cage for adults emergence. Swab of cotton was dipped in nectar and water solutions (1:9) and hanged in cage as nourishment to grow-up *P. brassicae*. When adults appeard, brassica plant were placed inside the cage. Female of *P. brassicae* laid eggs on brassica plants. Eggs hatched between 3 to 4 days. The 1st, 2nd, 3rd and 4th larval instars were allowed to consume the tender leaves. When 50 to 60% leaves were consumed, fresh leaves were provided. The larval stage completed within 12–14 days after this pupation period started which also completed in 6 days. With the help of camel hair brush, pupae were easily dislodged from the leaves. For rearing of *P. brassicae,* 12 h light and dark period with temperature 28 ± 4 °C were provided. In order to avoid any pathogenic contamination the rearing cages and culture room was cleaned with formalin. Furthermore, powdered based pesticide were used around the cages to save them from the attack of ants.

Multiplication of EPNs in *G. mellonela*

G. mellonela larvae were utilized as bait for duplication of EPNs. Seven species of EPNs i.e. *S. carpocapsae*, *S. feltiae*, *S. Pakitananese*, *S. asiaticum*, *S. glaseri*, *H. bacteriophora,* and *H. indica* were utilized during this study. The EPNs species were acquired from Nematological Laboratory, University of Agriculture, Faisalabad. Larvae were disinfected with 0.1% formaline solution to stay away any pathogenic infection after this kept in plastic petri plates already fixed with two whatman filter paper. Approximately, 1000 infective juveniles of each EPNs under study were inoculated to wax moth larvae kept in separate petri plates. EPNs were easily reconfirmed after the mortality of larvae with different colour shades. Larvae inoculated with *Steinernema* spp. confirmed with dark grey shadding and *Heterorhabditis* spp. express brik red shade (Wiesner 1993). Nematodes were harvested using White Trap method (White 1927). In vivo production of EPNs was conducted by the methods described by Poinar (1979). The plates were wrapped with para filim and record of each petri plate was maintained and kept in the incubator at 27 °C.

Counting and storage of EPNs

EPNs obtained through white trap procedure were collected in a different plastic cups and permitted to settle down for three to four hours. In the wake of settling down, the extra water was removed. The concentration of nematodes/ml of suspension was determined by counting the nematodes in a counting dish under stereomicroscope (Olympus 5240). The average of three counts was taken to estimate the final nematode population/ml. When larger nematodes were to be counted (>100/ml), then dilution method was used for this purpose. The EPNs concentration in the original suspension was determined by using the following formula:

$$S = N \times 1/M \times (X + 1)$$

S = Concentration (nematodes/ml) in initial suspension
N = Average No of EPNs per counted sample
M = Number of millilitres per counted sample
$X + 1$ = Dilution

The nematode concentration was standardized between 1500 and 2000 infective juveniles/ ml. EPNs were stored by adjusting the incubator temperature at 10 °C in small plastic cups.

Pathogenicity of seven EPNs spp. against *P. brassicae* in laboratory

Seven different EPN spp. (*S. carpocapsae*, *S. feltiae*, *S. Pakitananese*, *S. asiaticum*, *S. glaseri*, *H. bacteriophora* and *H. indica*) were assessed against 4th larval instar of *P. brassicae* at different concentrations (250, 500, 1000 and 1500 Ijs/ml). In control treatment, no EPN was applied. Each treatment was five times replicated with five larvae of *P. brassicae* in separate petri plates (9 cm dia) respectively. Larval mortality of the insect pest was recorded at different time intervals.

Pathogenicity of two effective EPNs on different larval instars of *P. brassicae* in laboratory

Two EPN spp. (*H. bacteriophora* and *S. glaseri*) found to be more efficient with the highest mortality percentage of insect pest was utilized against various larval instars with effective concentration i.e. 1500 Ijs/ml. In control treatment, no EPN was applied. Each treatment was five times replicated with five larvae of *P. brassicae* in separate petri plates. Larval mortality was noted after 12, 24, 36 and 48 h time intervals.

Pathogenicity of EPNs on *P. brassicae* feedings on cabbage leaves

Leaves of cabbage obtained from susceptible hybrid (CB-60) were kept in well-sterilized plastic boxes separately. Two best performing spp. of EPNs (*H. bacteriophora* spp. and *S. glaseri* spp.) were sprayed at high concentrations (1500 IJs) of infective juveniles over the leaves alone or in combination (*S. glaseri* + *H. bacteriophora*). Distilled water was sprayed on cabbage leaves, considered as control. Cabbage leaves on which EPNs sprayed allowed to dry for a few minutes and then nourished to testing instars for one day. Before offering, each larva was starved for 3 h and then sprayed on leaves. Each treatment was five times replicated with 10 larvae of *P. brassicae* and kept in separate boxes. Larval mortality was recorded at different time intervals i.e. after 12, 24, 36 and 48 h.

Statistical analysis

Recorded data was analyzed through Analysis of Variance (ANOVA) and treatments means were compared by Fisher's Least Significant Difference (LSD) test. Data was processed statistically through SAS (9.3) software (Inc., 2011–2012) and was represented by Microsoft Excel (2019).

Results

Pathogenicity of seven EPNs spp. against *P. brassicae* in laboratory

In this study seven different spp. of EPNs were evaluated for their pathogenic effects on 4th stage *P. brassicae* larvae in petri plate bioassay. After 12 h of EPNs treatment to 4th larval instar of *P. brassicae* in petri plate bioassays, 5.46, 8.46, 12.53 and 21.46% mortality observed with *S. feltiae* at 250, 500, 1000 and 1500 infective juveniles/ ml.

There was no mortality found with *S. pakistanese* and *S. asiaticum* at 250, 500, 1000 and 1500 infective juvenile concentration after 12 h. There was 20.53%, 48.53%, 68.50% and 88.43% mortality of *P. brassicae* larvae seen in *H. bacteriophora* at a different level of concentrations followed by *S. glaseri* with 80. 46% at 1500 IJs/ml after 24 h of treatment respectively. When time was increased to 36 h there were corresponding increase in mortality which was recorded 65.50, 70.40, 81.43 and 100% with *S. glaseri* followed by *H. bacteriophora* with 41.50, 76.60, 81.43 and 95.50% at 250, 500, 1000 and 1500 infective juveniles/ ml concentration respectively (Table 1). While after 36 h of treatment, there were 70.50, 61.50, 56.36 and 48.40% mortality found in 4th larval instar larvae of *P. brassicae* with *S. feltiae* at different concentrations (1500, 1000, 500 and 250 infective juveniles/ ml) followed by 54.43, 41.46, 31.50 and 21.46% mortality by *S. carpocapsae* with different infective nematodes concentrations respectively.

After 48 h, 77.40, 69.70, 60.50 and 54.36% mortality found in the case of where *S. feltiae* at different concentrations (1500, 1000, 500 and 250 infective juveniles/ ml) followed by 60.50, 57.43, 49.46 and 43.46% mortality by *S. carpocapsae* with different EPNs concentrations respectively.

H. bacteriophora and *S. glaseri* showed maximum mortality (100%) at higher concentrations of infective juveniles i.e. 1500 and 1000 IJs/ml. Results show that 9.46, 19.56, 27.43 and 38.36% mortality of 4th larval instar larvae of *P. brassicae* was found in *S. carpocapsae* treatment which was minimum as compared to the other treatments after 48 h (Table 1).

Pathogenicity of two effective EPNs on different larval instars *P. brassicae* in laboratory

In the 2nd larval instar of *P. brassicae*, 60.50% mortality was recorded where *H. bacteriophora* was applied while 25.56% mortality of larvae was recorded due to *S. glaseri* after 12 h. Mortality percentage increased with the increase in the time interval. After 24 h, 100% mortality was seen where *H. bacteriophora* was applied and 60.53% larval mortality was observed in *S. glaseri* treatment. After 36 and 48 h of

Table 1 Biocontrol potential of EPNs against *P. brassicae*

Entomopathogenic nematodes spp.	EPN Conc. (No.of IJs)	Mortality % after hours			
		After 12 h	After 24 h	After 36 h	After 48 h
S. carpocapsae	250	00.00zz	03.50yy	21.46jj	43.46x
	500	00.00zz	5.40ww	31.50ee	49.46u
	1000	11.40nn	29.56ff	41.46y	57.43p
	1500	15.46qq	41.43y	54.43 s	60.50o
S. feltiae	250	05.46ww	07.60uu	48.40v	54.36 s
	500	08.46tt	11.40qq	56.36q	60.50o
	1000	12.53 pp	35.50dd	61.50n	69.70 k
	1500	21.46jj	41.70y	70.50j	77.40 h
S. pakistanese	250	00.00zz	00.00zz	00.00zz	9.46ss
	500	00.00zz	00.00zz	00.00zz	19.56ll
	1000	00.00zz	00.00zz	07.73uu	27.43gg
	1500	00.00zz	00.00zz	17.46 ll	38.36bb
S. asiaticum	250	00.00zz	00.00zz	10.46rr	22.50ii
	500	00.00zz	00.00zz	15.56 mm	31.46ee
	1000	00.00zz	12.50 pp	20.53kk	41.76y
	1500	00.00zz	17.43 mm	27.70gg	43.46x
S. glaseri	250	20.43kk	40.53z	65.50 m	77.46 h
	500	38.50bb	49.53u	70.40j	85.46e
	1000	48.53v	61.63n	81.43f	95.50b
	1500	55.36r	80.46 g	100.0a	100.0a
H. bacteriophora	250	04.40xx	20.53kk	41.50y	81.53f
	500	15.53nn	48.53v	76.60i	88.53d
	1000	25.53 hh	68.50 l	81.43f	92.50c
	1500	40.50z	88.43d	95.50b	100.0a
H. indica	250	00.00zz	00.00zz	10.46rr	27.46 gg
	500	00.00zz	00.00zz	17.40 mm	37.83 cc
	1000	4.46xx	14.46oo	21.46jj	45.56w
	1500	15.46nn	27.43gg	39.53az	53.53 t
Control (Water only)		00.00z	00.00z	00.00z	0.00z
		LSD = 0.3857			

*Mean values in a column having comparable letters contrast significantly as determined by the LSD test ($P \leq 0.05$)

the interval, 100% mortality was recorded in both treatments. No mortality of larva was seen in the control treatment.

In the 3rd larval instar, *S. glaseri* and *H. bacteriophora* exhibited 10.50 and 30.50% mortality respectively after 12 h of application. After 24 h interval, 80.53 and 50.46% mortality recorded in case *H. bacteriophora* and *S. glaseri* respectively. Hundred percent mortality was recorded in the case of *H. bacteriophora* while 90.46% due to *S.*

glaseri respectively after 36 h. After 48 h, 100% mortality of *P. brassicae* larvae recorded in both treatments after 48 h. No mortality was seen in control treatment at all time intervals.

In the 4th larval instar, *S. glaseri* and *H. bacteriophora* showed 10.66 and 18.50% mortality respectively of *P. brassicae* larvae after 12 h of application. After 24 h, 35.53% mortality was seen due to *S. glaseri* application and 75.33% moratlity was observed where *H. bacteriophora* was applied. Hundred percent mortality was recorded in the case of *H. bacteriophora*, while 81.50% by *S. glaseri* after 36 h. After 48 h of application, 100% mortality was noted in the 4th larval instar of *P. brassicae* in both treatments. In control treatment, 0% mortality was recorded irrespective to the time intervals (Table 2).

Pathogenicity of EPNs on *P. brassicae* feedings on cabbage leaves

In 2nd larval instar, maximum mortality was seen when EPNs were applied in combination (*H. bacteriophora* + *S. glaseri*) as compared to alone (*S. glaseri* and *H. bacteriophora*) after

Table 2 Evaluation of EPNs against different larval instars of *P. brassicae*

Larval instars	Entomopathogenic nematodes spp.	Mortality percentage after hours			
		After 12 h	After 24 h	After 36 h	After 48 h
2nd	***S. glaseri***	25.56j	60.53f	100.00a	100.0a
	H. bacteriophora	60.50f	100.00a	100.00a	100.0a
	Control	00.00o	00.00o	00.00o	0.00o
3rd	***S. glaseri***	10.50 l	50.46 g	90.46b	100.0a
	H. bacteriophora	30.50i	80.53d	100.00a	100.0a
	Control	00.00o	00.00o	00.00o	00.00o
4th	***S. glaseri***	10.66 l	35.53 h	81.50c	100.0a
	H. bacteriophora	18.50 k	75.53e	100.00a	100.0a
	Control	00.00o	00.00o	00.00o	00.00o
LSD = 0.5933					

*Mean values in a column having comparable letters contrast significantly as determined by the LSD test ($P \leq 0.05$)

12 h. After 24 h, 100% mortality was seen when EPNs were appllied in combination (*H. bacteriophora* + *S. glaseri*), *S. glaseri* and *H. bacteriophora* exhibited 95.46 and 65.50% mortality of *P. brassicae* larvae respectively when apllied separately. After 36 h of interval, 100% mortality of *P. brassicae* larvae was recorded in case of *H. bacteriophora* + *S. glaseri* and *S. glaseri* treatments except *H. bacteriophora* where 80.53% mortality was recored. After 48 h inoculation, 100% mortality of *P. brassicae* was recorded in all EPNs treatments.

In 3rd larval instar, 35.53% mortality of *P. brassicae* larvae noted due to the application of *H. bacteriophora* after 12 h. After 24 h of inoculation, 70.46% mortality recorded with *S. glaseri*. Similarly, 36 h after pathogenic EPNs spray, 90.06% mortality was seen in case of combine application of EPNs (*H. bacteriophora* + *S. glaseri*). After 48 h of exposure, 90.04% and 80.46% mortality assessed in the case of *S. glaseri* and *H. bacteriophora* respectively while 100% mortality was seen because of the combine application of EPNs treatments (*H. bacteriophora* + *S. glaseri*). In control, no mortality was seen.

In 4th larval instar, 49.46% mortality was recorded where EPNs were applied in combination (*H. bacteriophora* + *S. glaseri*) while 25.50% mortality was recorded with *S. glaseri* after 12 h of application. After 24 h of exposure, 55.46 and 45.43% mortality was assessed in the case of *S. glaseri* and *H. bacteriophora* respectively. After 36 h of nematodes spray, 64.43% was seen in case of *H. bacteriophora* and 80.46% mortality of *P. brassicae* larvae observed where EPNs (*H. bacteriophora* + *S. glaseri*) @ 1500 infective juvenile/ml. After 48 h of exposure, 90.02 and 80.46% mortality was recorded with *S. glaseri* and *H. bacteriophora* treatments while 100% mortality recorded by the application EPNs (*H. bacteriophora* + *S. glaseri*). No mortality was seen in control treatment at all the time intervals (Table 3).

Discussion

EPNs associated with symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) converts the body parts of the host into a nutrient soup on which nematodes feed. In the first part of the present study, minimum mortality of *P. brassicae* with exposure of *S. pakistanese*, *S. asiaticum* spp. and *H. indica* spp. maybe due to their weak association or slow releasing of mutualistic bacteria into the haemocoel of the insect. On the other hand, highly virulent EPNs like *H. bacteriophora* spp. and *S. glaseri* spp. after entering into the host immediately released bacteria. Bacteria release broad-spectrum antibiotics responsible for the mortality of the insect pest in minimum time by breaking down of the tissues of insect and rapidly converts the body parts of host into the nutrient soup and provide food for the development and multiplication of EPN. *H. bacteriophora* spp. and *S. glaseri* spp. consume the nutrient soup quickly which ultimately supports the maximum production of nematodes due to competition of food and space *H. bacteriophora* spp. and *S. glaseri* spp. breakdown of the dead body of *P. brassicae* and move towards the other larvae is one of major the reasons regarding higher mortality percentage.

H. bacteriophora spp. and *S. glaseri* spp. during 2nd and 3rd part of the present study were found to be very virulent with maximum mortality of various larval instar after 48 h. The duration of nematode emergence was shorter in 2nd larval stage as compared to other larval instars. The sound reason regarding the increase in mortality percentage with an increase in time is due to the presence of the defensive system in *P. brassicae*. Insect pest defense himself against EPN with two progressions i.e. haemocytic and humoral response. Haemocytic process comprising of celluar encapsulation, nodule formation and phagocytosis while the humoral response

Table 3 Evaluation of EPNs on *P. brassicae* feedings on cabbage leaves in lab

Larval Instar	Entomopathogenic nematodes spp.	Mortality % after hours			
		After 12 h	After 24 h	After 36 h	After 48 h
2nd	***H. bacteriophora***	40.46n	65.50 g	80.53e	100a
	S. glaseri	60.46i	95.46b	100a	100a
	H. bacteriophora + S. glaseri	80.46e	100a	100a	100a
	Control	0.00u	0.00u	0.00u	0.00u
3rd	***H. bacteriophora***	35.53o	50.46 k	70.46f	80.46e
	S. glaseri	40.46n	70.46f	82.16d	90.4c
	H. bacteriophora + S. glaseri	60.46i	70.46f	90.06c	100a
	Control	0.00u	0.00u	0.00u	0.00u
4th	***H. bacteriophora***	20.46q	45.43 m	64.43 h	80.46e
	S. glaseri	25.50p	55.46j	70.50f	90.2c
	H. bacteriophora + S. glaseri	49.46 l	65.50 g	80.46e	100a
	Control	0.00u	0.00u	0.00u	0.00u
		LSD = 0.6313			

*Mean values in a column having comparable letters contrast significantly as determined by the LSD test ($P \leq 0.05$)

including melanotic encapsulation and production of antimicrobial peptides (Khush and Lemaitre 2000). EPNs release bacteria inside the host within minutes to hours, depending upon the strain. In any case, haemolymph of host detects EPNs as well as bacteria quickly and tries to restrict their pathogenic activities. The bacteria multiply inside the nodule and again enter into the haemolymph but this time suppress the insect immune system with releasing of lethal toxin after that mortality of insect takes place (Dunphy and Bourchier 1992). This may be the reason behind this increase in mortality % with the increase in time. After penetration of EPNs into the haemocoel, the insect immune system alarms immediately. Insect pest instantly produced phenoloxidase that deposit a layer of melanin around the EPNs as well as lysozyme and antibacterial peptides against bacterial infection (Khush and Lemaitre 2000).

The second reason might be that in small-sized larvae of *P. brassicae* the food for EPNs depleted quickly and nematodes were compelled to exit from the dead body of insects earlier. This situation does not exist in 4th larval stage because larger sized larvae food did not deplete quickly and multiplication of EPNs inside the dead body continues until the nutrient soup is totally exhausted. These outcomes of the present study are supported by the work of Pal and Prasad (2012) who find maximum mortality of fourth instars of *P. brassicae* larvae with higher infective juvenile concentration. Abdolmaleki et al. (2017a); Abdolmaleki et al. (2017b); Gorgadze et al. (2018); Zolfagharian et al. (2016) concluded that with increased nematode concentration and time, mortality percentage of *P. brassicae* larvae was also increased.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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