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Research Article

Virulence of two entomopathogenic nematodes through their interaction with *Beauveria bassiana* and *Bacillus thuringiensis* against *Pieris brassicae* (Lepidoptera: Pieridae)

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Abstract: *Pieris brassicae* L. is one of the most important pests of Brassicaceae. The insecticidal effect of two entomopathogenic nematodes (EPNs), *Heterorhabditis bacteriophora* and *Steinernema feltiae*, was determined through their interaction with *Beauveria bassiana* and *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) against *P. brassicae*. In the interaction tests, the EPNs were applied at LC_{50} level 0, 12 or 24h after treating the larvae with LC_{10} or LC_{25} of the *B. bassiana* or *Btk*. The interaction between the EPNs and *B. bassiana* was entirely different from the interaction of the EPNs and *Btk*. The interaction with *B. bassiana* was dependant on time intervals, while the interaction of the EPNs with *Btk* was almost additive or synergistic. An antagonistic effect was seen when the EPNs were applied immediately after the *B. bassiana*. However, the application of the EPNs 24h after their treatment with *B. bassiana* caused additive or synergistic effects. The results also showed the best mortality effect when the EPNs were used with *Btk* at 12 h and 24 h time intervals. Based on the results, a simultaneous use of the EPNs and *B. bassiana* is not recommended against *P. brassica*. However, the EPNs could be used simultaneously after *Btk* but it is better to allow a time interval to increase mortality.

Keywords: Biological control, *Heterorhabditis bacteriophora*, IPM, *Steinernema feltiae*

Introduction

Pieris brassicae L. (Lepidoptera: Pieridae) is an economically important pest of Brassicaceae (Cartea *et al*., 2009; Metaspalu *et al*., 2009). This pest has five larval instars, of which the last two can cause significant economic damage to crucifers (Karowe and Schoonhoven, 1992). Currently, the most reliable management of the pest is provided by chemical insecticides. However, they often prove to be the cause of resistance build-up and a source of growing concern about consumer safety and environmental hazard. These factors underline the necessity of developing and using bio-control agents as alternatives to chemical insecticides.

Entomopathogenic nematodes (EPNs) belong to families Heterorhabditidae and Steinernematidae (Order: Rhabditida). Members of these families are obligate parasites of insects. These nematodes are associated with symbiotic bacteria from the family Enterobacteriaceae, empowering them to control pests. *Steinernema* spp. (Steinernematidae) and *Heterorhabditis* spp. (Heterorhabditidae) are associated with

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Xenorhabdus spp. and *Photorhabdus* spp., respectively (Boemare, 2002; Forst and Clarke, 2002). The only free-living stage of these nematodes is that of the non-feeding infective juveniles (IJs), which actively seek out hosts and penetrate the insect body usually through natural openings (Kaya and Gaugler, 1993; Poinar, 1990). These IJs invade the host haemocoel and release their symbiont bacteria, causing septicaemia and, ultimately, killing the host (Akhurst, 1983; Lewis *et al.*, 1993; Forst and Clarke, 2002). No adverse effects of the nematodes have been reported on humans, the environment, and non-target insects (Mbata and Shapiro, 2010).

Another biorational agent for controlling insect pests, especially lepidopteran pests, is *Bacillus thuringiensis* Berliner subsp. *kurstaki* (*Btk*). The virulence of *B. thuringiensis* has been investigated and confirmed in earlier studies (Eilenberg *et al.*, 1998; Helson, 1960; Lecadet and Martoutet, 1987). Koppenhöfer and Kaya (1996), found additive and synergistic effects of *Heterorhabditis bacteriophora* and *Steinernema glaseri* in interaction with *Bacillus thuringienis* subsp. *japonensis* against *Cyclocephala hirta* LeConte and *Cyclocephala pasadenae* Casey.

One group of the most important biopesticides is entomopathogenic fungi. These pesticides are mainly used on insects from the orders hemiptera. Some studies also investigated their virulence on other orders such as Coleoptera and Lepidoptera and found they had a high efficacy in their controlling ability, especially when used as stressors (Ansari *et al.*, 2004; Barbercheck and Kaya, 1990, 1991; Choo *et al*., 2002; Glare 1994).

One of the most important aspects of integrated pest management (IPM) is the integration of multiple safe techniques and materials. The approach would not be useful if the combined effects of these techniques are not understood or determined. Bio-control agents have an important role in the IPM, and investigations on their combined effects could be very helpful in controlling pests. As a matter of fact, the combination of two controlling agents could have three different effects: synergistic, antagonistic or additive (Furlong and Groden, 2001; Robertson *et al.*, 2007). A combination of the EPNs, *B. thuringiensis*, and entomopathogenic fungi would likely produce different actions that could be described under the independent joint action model. In this model, the toxicity of a given component of the combination is not affected by the other components (Robertson *et al*., 2007).

In the study carried out by Koppenhöfer and Kaya (1996), the synergistic effect of the EPNs and *Bacillus thuringienis* subsp. *japonensis* was reported against two scarab species, *Cyclocephala hirta* and *Cyclocephala pasadenae*. In another research, Oestergaard *et al*. (2006) found that the interaction of *Steinernema feltiae* Filipjev and *Steinernema carpocapsae* Weiser with *B. thuringiensis* subsp. *israelensis* caused additive and synergistic effects on the 1st and 4th instar larvae of the *Tipula paludosa* Meigen. Several studies were done on the interaction of some enthomopathogenic fungi such as *Metharhizium ansopliae* Metchnikoff and *Beauveria bassiana* Balsamo with the EPNs on insect pests. For instance, Barbercheck and Kaya (1990) found that the period of a lethal infection on *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae was shorter when the larvae were exposed to the *B. bassiana* and the EPNs, *S. feltiae* and/or *Heterorhabditis heliothidis* (Khan, Brooks and Hirschmann), than that for larvae exposed to pathogens alone. Kamionek *et al.*, (1974a, 1974b) found that when an EPN and entomopathogenic fungi were applied simultaneously against coleopteran or lepidopteran insects in Petri dishes, the period of lethal infection was shorter than when the fungus was applied singly. Barbercheck and Kaya (1991) also found higher efficacy of *Heterorhabditis bacteriophora* Poinar plus *B. bassiana* than when either the nematode or the fungus was used alone. Ansari *et al.*, (2004) found the combined application of *M. anisopliae* with *Heterorhabditis megidis* Poinar, Jackson and Klein and/or *Steinernema glaseri*

Steiner increased larval mortality either in an additive or a synergistic way.

Accordingly, the objective of our study was to investigate the neglected aspect of research in the above-mentioned area, that is, whether efficient control of *P. brassicae* can be achieved with the EPN, *Btk*, *B. bassiana* or their combinations. Obviously, these results can also show the compatibility of the EPN and *Btk* or *B. bassiana*. In the current study, the interaction of *B. bassiana* and two EPN species, *H. bacteriophora* and *S. feltiae*, were considered for the 4th and 5th instar larvae of *P. brassicae*. Our hypothesis was that *Btk* and *B. bassiana* act as a stressor and increase the efficacy of the EPNs to provide better control against *P. brassicae*. To test our hypothesis, the 4th and 5th instar larvae of *P. brassicae* were used in the experiments.

Materials and Methods

Microbial Provision

Heterorhabditis bacteriophora and *S. feltiae* collected from Kurdistan province (Abdolmaleki *et al.*, 2016) were used in this study. *Bacillus thuringiensis* subsp. *Kurstaki* (24000 IU T. ni mg^{-1}) and *B. bassiana* (10^{10} spore mg^{-1}) were obtained from Intrachem Bio, Grassobbio BG, Italy, and the Sadra Natural Bioproducts Company, Tehran, Iran, respectively.

Preparation of *P. brassicae*

The eggs of *P. brassicae* were collected from the cabbage fields of Urmia (West Azerbaijan province, Iran). The hatched larvae were fed fresh cabbage leaves grown in a research plot at the Iranian Research Institute of Plant Protection, Tehran, Iran, until the 4th and 5th instar larvae appeared.

Bioassays

To determine the interaction of the biocontrol agents, LC_{10} and LC_{25} values of *Btk* and *B. bassiana* were used with LC_{50}s of the EPNs. The pathogenicity of the EPNs, *H. bacteriophora* and *S. feltiae*, has been reported in the published article. The LC_{50} values of *H. bacteriophora* on the 4th and 5th instar larvae of *P. brassicae* were 85.44 and 54.77 IJs per larva, respectively; these values for *S. feltiae* were 96.23 and 68.01 IJs per larvae (Abdolmaleki *et al.*, 2015).

The pathogenicity of *Btk* was investigated on the 4th and 5th instar larvae of *P. brassicae*. Preliminary tests were done to determine the range of concentrations appropriate for the preparation of a dosage response line. Based on preliminary experiments, the ranges of concentrations tested were 120 to 720 µg AI/mL against the 4th and 210 to 950 µg AI/mL against the 5th instar larvae. For the *Btk* pathogenicity investigation, cabbage leaf discs were provided and treated with different concentrations for 30 seconds. Then, the leaf discs were placed in Petri dishes (8 cm in diameter). Finally, 15 larvae were transferred in the Petri dishes. Treatments consisted of five concentrations of the *Btk* and one untreated control.

To prepare the concentrations of *B. bassiana*, it was cultured on potato dextrose agar (PDA) media for 15 days. Then, spore suspension was prepared using distilled water, the spores were determined in 1 ml of the suspension using haemocytometer. Based on preliminary experiments, the range of *B. bassiana* concentrations against the 4th and 5th instar larvae of *P. brassicae* was 10^5 to 10^9 spores per ml. Larvae were placed in *B. bassiana* dilutions for 10 seconds. Then, 15 larvae were transferred to Petri dishes (8 cm in diameter). Treatments included five concentrations of the *B. bassiana* and one control. In all bioassay experiments, Tween-80 was used as surfactant at a concentration of 0.05% (to overcome hydrophobic effect of cabbage leaves). Distilled water, mixed with the surfactant (0.05%), served as the control. To provide humidity, bottom of Petri dishes were covered with filter paper soaked in 1 ml of distilled water. The treatments were replicated four times on different days. Mortality was recorded every 24 hours after each treatment. Also, every 24 hours untreated cabbage leaves were replaced as a food.

Interaction between *B. bassiana* and EPNs

Petri dishes covered by filter paper were used to determine the compatibility of *B. bassiana* with

EPNs. For this approach, larvae were individually treated with either LC_{10} or LC_{25} of *B. bassiana* suspension for 30 seconds. Then, 15 treated larvae were transferred into each Petri dish. The LC_{50} of IJs of either *S. feltiae* or *H. bacteriophora* was applied in a volume of 1 ml to Petri dishes at the same time as *B. bassiana*, or after 12 and 24 hours. Each treatment was repeated thrice. Only distilled water was used as control. Each 24 hours cabbage leaves were provided as food.

Interaction between *Btk* and EPNs

Petri dishes covered by filter paper containing 15 fourth and fifth instar larvae of *P. brassicae* were used to determine the compatibility of *Btk* with EPNs. For this approach, cabbage leaf discs were first treated with either LC_{10} or LC_{25} of *Btk* for 30 seconds. Then, the leaf disks were placed in Petri dishes (8 cm in diameter). LC_{50} of either *H. bacteriophora* or *S. feltiae* were applied to Petri dishes in a volume of 1 ml distilled water at the same time as *Btk*, or after 12 and 24 hours. Each treatment was repeated three times. Only distilled water was used as control. Each 24 hours cabbage leaves were provided as food.

Data Analysis

The LC_{50} values were analysed by probit regression analysis using the SPSS software (SPSS Inc., 2010). Lack of overlap in 95% confidence limits in different treatments was used as criterion for significant differences (Robertson *et al.*, 2007). Results of the interactions were considered, based on a comparison of the expected and observed mortalities, according to the procedure suggested by Koppenhöfer and Kaya (1996). The mortality data correction was done using Abbott's formula (Abbott, 1925). Then the expected mortality (M_E) for the combination of *Btk* and *B. bassiana* with either *S. feltiae* or *H. bacteriophora* was calculated with the formula $M_E = M_C + M_M (1 - M_C)$; M_C: mortalities caused by either *Btk* or *B. bassiana*; M_M: observed mortalities caused by the EPNs. Next, the chi-square (χ^2) values were calculated by the formula $(M_{CM} - M_E)^2/M_E$; M_{CM}: observed mortality for the combination of either *Btk* or *B. bassiana* with either *S. feltiae* or *H. bacteriophora*. If the calculated chi-square were > 3.89 (as specified for df = 1), it would indicate a non-additive effect of the two control agents. The difference $M_{CM} - M_E > 0$ indicated synergism; and the difference $M_{CM} - M_E < 0$ indicated antagonism.

Results

Results of the efficacy of the *Btk* and *B. bassiana* against the 4th and 5th instar larvae of *P. brassicae* are shown in Table 1. At the LC_{50} level, the highest activity was that of *Btk*. The LC_{50} values were considered significantly different in cases where the 95% confidence limits did not overlap. Compared with each other, the toxicities of the *Btk* and *B. bassiana* on the 4th and 5th instar larvae were not significantly different. In the comparison, the slopes of the concentration-response lines were variable and were almost steep for *Btk* but lower for *B. bassiana* (Table 1).

The results of the interaction between the EPNs and *Btk* or *B. bassiana* indicated additive, synergistic, and antagonistic effects, depending on the case and time intervals (Table 2, 3). The interaction between the EPNs and *Btk* showed additive and synergistic effects. Investigations indicated that the simultaneous use of the EPNs and *Btk* caused an additive effect but the use of the EPNs 12 and 24 hours after exposure to *Btk* had a synergistic effect. However, the results showed that the application rate played an important role in the interaction between *H. bacteriophora* and *Btk* when applied simultaneously: synergism was observed when *Btk* was applied at LC_{25}, and an additive effect was seen when this biological agent was applied at LC_{10} (Table 2). The interaction of the EPNs in the experiment with *B. bassiana* was affected by time intervals. The results showed that the simultaneous use of *B. Bassiana* and EPNs caused an antagonistic effect, but the application of the EPNs 12 and 24 hours after exposure to *B. bassiana* showed additive and synergistic effects.

Table 1 Effect of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) *and Beauveria bassiana* against 4th and 5th instars larvae of *Pieris brassicae*.

Insecticides	Larval stage	LC_{50} (95% CL) (µg a.i/ml)	LC_{25} (95% CL)[1] (µg a.i/ml)	LC_{10} (95% CL)[1] (µg a.i/ml)	χ^2	ρ	Slope ± SE
Btk	4	308.60 (235.26-410.56)	115.19 (54.88-163.97)	47.45 (12.57-84.53)	0.21	0.98	1.58 ± 0.33
	5	450.99 (353.52-577.61)	185.61 (90.69-256.76)	83.48 (23.12-142.65)	0.98	0.81	1.75 ± 0.38
B. bassiana	4	1.52×10^8 (5.06×10^7-7.93×10^8)	3.01×10^6 (6.65×10^5-8.74×106)	8.81×10^4 (4.61×10^3-4.42×10^5)	1.13	0.77	0.46 ± 0.08
	5	6.40×10^8 (1.99×10^8-4.82×10^9)	6.40×10^8 (6.45×10^6-6.29×10^7)	1.09×10^6 (8.94×10^4-4.17×10^6)	0.10	0.92	0.45 ± 0.08

[1] CL: Confidence limits.

Table 2 Interactions between *Heterorhabditis bacteriophora* with *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) and *Beauveria bassiana* against 4th and 5th instars larvae of *Pieris brassicae*.

Insecticides	Larval stage	Application rates (µg a.i/ml)	Interval (h)	Observed mortality (%)	Expected mortality (%)	χ^2	Interaction
Btk	4	LC_{10}	0	71.11	57.28	3.33	Additive
Btk	4	LC_{25}	0	86.67	58.76	13.25	Synergistic
B. bassiana	4	LC_{10}	0	31.11	56.00	11.06	Antagonistic
B. bassiana	4	LC_{25}	0	40.00	58.17	5.68	Antagonistic
Btk	4	LC_{10}	12	82.22	57.28	10.86	Synergistic
Btk	4	LC_{25}	12	84.44	58.76	11.22	Synergistic
B. bassiana	4	LC_{10}	12	37.78	56.00	5.93	Antagonistic
B. bassiana	4	LC_{25}	12	42.22	58.17	4.37	Antagonistic
Btk	4	LC_{10}	24	75.55	57.28	5.82	Synergistic
Btk	4	LC_{25}	24	88.89	58.76	15.44	Synergistic
B. bassiana	4	LC_{10}	24	57.78	56.00	0.05	Additive
B. bassiana	4	LC_{25}	24	62.22	58.17	0.003	Additive
Btk	5	LC_{10}	0	62.22	50.62	2.66	Additive
Btk	5	LC_{25}	0	80.22	53.73	15.11	Synergistic
B. bassiana	5	LC_{10}	0	22.22	48.84	14.51	Antagonistic
B. bassiana	5	LC_{25}	0	51.11	52.00	0.01	Additive
Btk	5	LC_{10}	12	80.00	50.62	17.05	Synergistic
Btk	5	LC_{25}	12	88.89	53.73	23.01	Synergistic
B. bassiana	5	LC_{10}	12	31.11	48.84	6.43	Antagonistic
B. bassiana	5	LC_{25}	12	22.22	52.00	17.05	Antagonistic
Btk	5	LC_{10}	24	62.22	50.62	2.66	Additive
Btk	5	LC_{25}	24	80.00	53.73	12.84	Synergistic
B. bassiana	5	LC_{10}	24	53.33	48.84	0.41	Additive
B. bassiana	5	LC_{25}	24	64.44	52.00	2.98	Additive

Table 3 Interactions between *Steinernema feltiae* with *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) and *Beauveria bassiana* against 4th and 5th instars larvae of *Pieris brassicae*.

Insecticides	Larval stage	Application rates (μg a.i/ml)	Interval (h)	Observed mortality (%)	Expected mortality (%)	χ^2	Interaction
Btk	4	LC_{10}	0	64.44	55.85	1.32	Additive
Btk	4	LC_{25}	0	68.89	57.09	2.44	Additive
B. bassiana	4	LC_{10}	0	35.55	53.92	6.26	Antagonistic
B. bassiana	4	LC_{25}	0	40.00	56.30	4.72	Antagonistic
Btk	4	LC_{10}	12	73.33	55.85	5.47	Synergistic
Btk	4	LC_{25}	12	88.89	57.09	17.72	Synergistic
B. bassiana	4	LC_{10}	12	37.78	54.92	4.83	Antagonistic
B. bassiana	4	LC_{25}	12	26.67	56.30	15.59	Antagonistic
Btk	4	LC_{10}	24	75.55	55.85	6.95	Synergistic
Btk	4	LC_{25}	24	88.89	57.09	17.72	Synergistic
B. bassiana	4	LC_{10}	24	55.55	53.92	0.05	Additive
B. bassiana	4	LC_{25}	24	60.00	56.30	0.24	Additive
Btk	5	LC_{10}	0	68.89	53.92	4.15	Synergistic
Btk	5	LC_{25}	0	84.44	55.41	15.22	Synergistic
B. bassiana	5	LC_{10}	0	33.33	50.96	6.09	Antagonistic
B. bassiana	5	LC_{25}	0	37.78	53.93	4.83	Antagonistic
Btk	5	LC_{10}	12	71.11	53.93	5.48	Synergistic
Btk	5	LC_{25}	12	82.22	55.41	12.98	Synergistic
B. bassiana	5	LC_{10}	12	51.11	50.96	0.0004	Additive
B. bassiana	5	LC_{25}	12	28.89	53.93	11.62	Antagonistic
Btk	5	LC_{10}	24	73.33	53.93	6.98	Synergistic
Btk	5	LC_{25}	24	84.44	55.41	15.22	Synergistic
B. bassiana	5	LC_{10}	24	51.11	50.96	0.43	Additive
B. bassiana	5	LC_{25}	24	71.11	53.93	5.48	Synergistic

At each time interval, the mortality with the combination of *H. bacteriophora* and *Btk* was significantly greater than in the treatments with *H. bacteriophora*, *Btk* or *B. bassiana* alone and also these values were greater than combination of *H. bacteriophora* and *B. bassiana* ($F_{8, 18} = 92.22$, $F_{8, 18} = 101.07$, $F_{8, 18} = 107.28$ at $P < 0.05$ for treatments of the 4th instar larvae at 0, 12 and 24 h intervals, respectively; however, these values on the 5th instar larvae were $F_{8, 18} = 100.35$, $F_{8, 18} = 169.42$, $F_{8, 18} = 138.92$ at $P < 0.05$, respectively). Similarly, the effect of a combination of *S. feltiae* and *Btk* was greater than other treatments at each time interval ($F_{8, 18} = 154.12$, $F_{8, 18} = 162.36$, $F_{8, 18} = 140.18$ at $P < 0.05$ for treatments on the 4th instar larvae at 0, 12 and 24 h intervals, respectively; however, these values for the 5th instar larvae were $F_{8, 18} = 169.66$, $F_{8, 18} = 118.52$, $F_{8, 18} = 165.96$ at $P < 0.05$, respectively) (Figs 1 and 2).

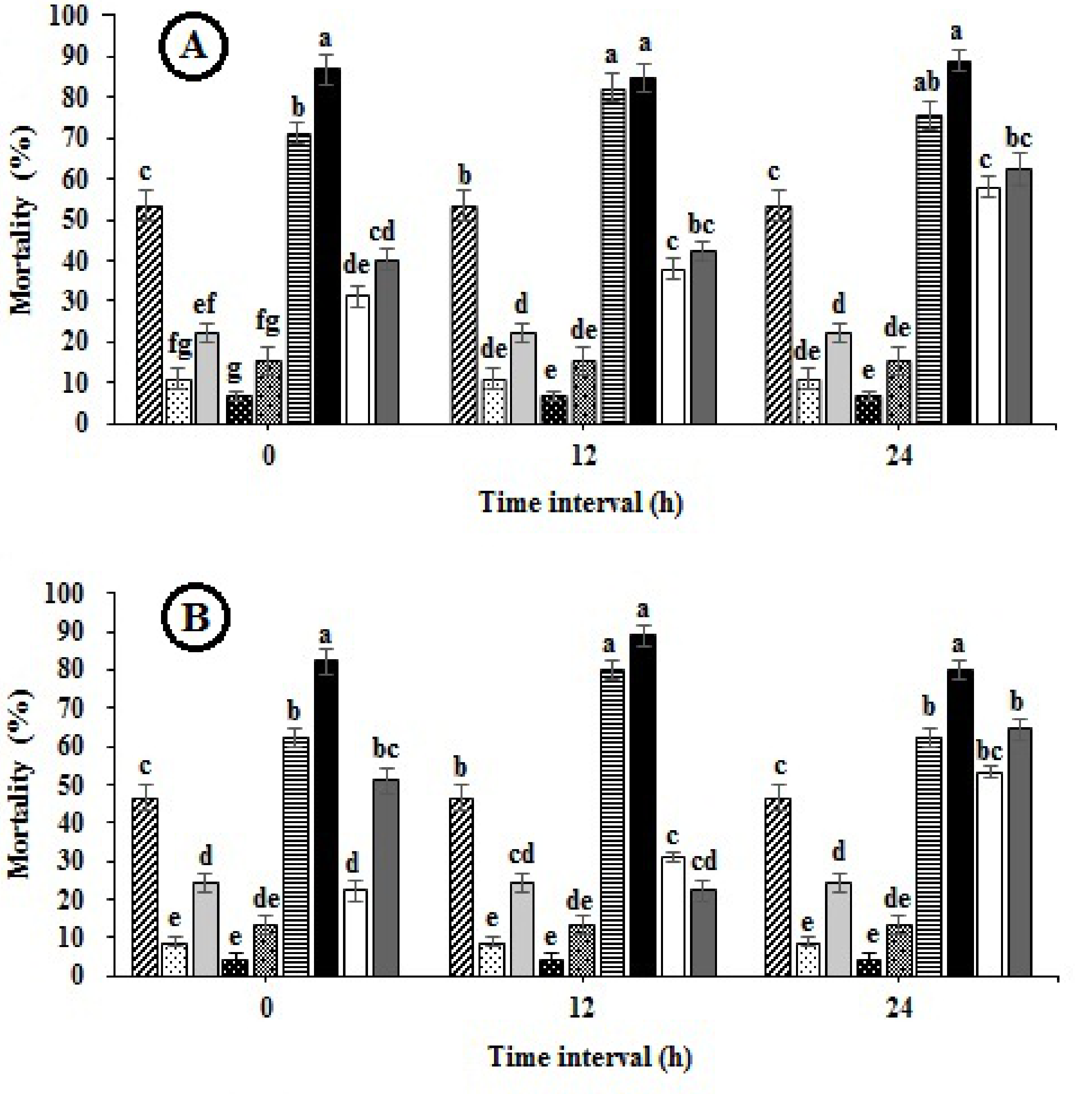

Figure 1 Mortality of 4th (A) and 5th (B) instar larvae of *Pieris brassicae* after exposure to the *Heterorhabditis bacteriophora*, *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) and *Beauveria bassiana* alone and in combinations of *H. bacteriophora* and either *Btk* or *B. bassiana*. Note: The values shown are the percent mortality ± SE (Means followed by the same letters are not significantly different ($p < 0.05$) within each time interval).

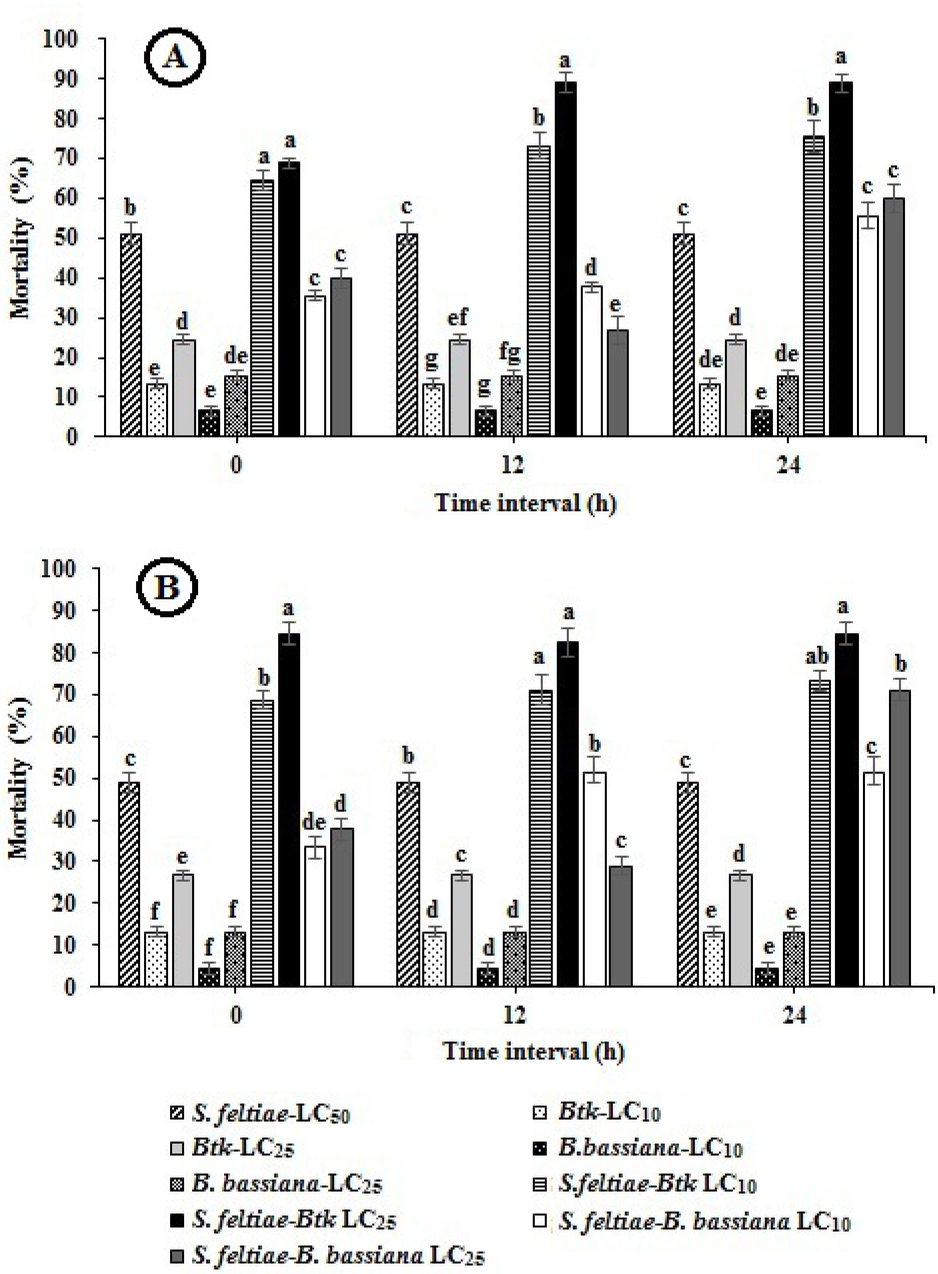

Figure 2 Mortality of 4th (A) and 5th (B) instar larvae of *Pieris brassicae* after exposure to the *Steinernema feltiae*, *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) and *Beauveria bassiana* alone and combinations of *S. feltiae* and either *Btk* or *B. bassiana*. Note: The values shown are the percent mortality ± SE (Means followed by the same letters are not significantly different ($p < 0.05$) within each time interval).

Discussion

This study investigated the effect of *H. bacteriophora*, *S. feltiae*, *Btk*, and *B. bassiana* each singly and the combinations of these EPNs with *Btk* and *B. bassiana* in controlling *P. brassicae*. In the previous publication, 5th instar larvae were reported as more susceptible than 4th instar larvae (Abdolmaleki *et al*., 2015). However, in this study of *Btk* and *B. bassiana* results showed that 4th instar larvae were more susceptible than 5th instar.

The effects of *Btk* on *P. brassicae* were reported in previous studies (Eilenberg *et al*., 1998; Lecadet and Martoutet, 1987). In this study, the estimated LC_{50} values of the 4th (308.60 µg AI/mL) and 5th instar larvae (450.99 µg AI/mL) exceeded the values calculated in the earlier studies (Eilenberg *et al*., 1998; Lecadet and Martouret, 1987). This may suggest a lower susceptibility of our population, lower virulence of *Btk* used in this study or may be due to different experimental conditions.

Beauveria bassiana was also investigated against the 4th and 5th instar larvae of *P. brassicae*. Several studies have investigated fungi virulence, especially of *B. bassiana* and *M. anisopliae* on lepidopteran pests (Arand *et al*., 2009; Asi *et al*., 2013; García-Gutiérrez *et al*., 2010; Hatting, 2012; Nguyen *et al*., 2007; Wraight *et al*., 2010). García-Gutiérrez *et al*. (2010) experimented with *B. bassiana* and *M. anisopliae* on *P.rapae* in the field and noted the mortality effect of them. Nguyen *et al*. (2007) investigated virulence of some entomopathogenic fungi on *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in laboratory conditions and found *B. bassiana* to have greater virulence than *M. anisopliae*. Compared to the earlier mentioned studies on lepidopteran pests, our results showed higher LC_{50}s of *B. bassiana* on *P. brassicae* larvae. This could possibly be due to a difference in the resistance of the experimented species to the fungi and also in the virulence of fungi used in the earlier studies. However, the main aim of using *B. bassiana* on P. brassica was to determine the role of this fungus as a stressor in combination with the EPNs. The low virulence of *B. bassiana* when used alone is not of great concern, as we emphasized a combination of *B. bassiana* and EPNs.

This is the first study to evaluate the combined effects of entomopathogenic nematodes with either *Btk* or *B. bassiana* on the 4th and 5th instar larvae of *P. brassicae*. We did not find any report on the combined application of EPNs with *Btk* or *B. bassiana* against *P. brassicae*. However, some experiments were performed with combinations of EPNs and fungi or *Btk* against other insects (Ansari *et al*., 2004; Barbercheck and Kaya, 1990, 1991; Choo *et al*., 2002; Kamionek *et al*., 1974 a,b; KoppenhÖfer and Kaya, 1996; Oestergaard *et al*., 2006). The most important limitation of using bio-control agents in the IPM is the lack of knowledge about their biotic interactions. Hence, studies on combinations of biological control factors seem necessary and could be helpful in IPM.

There are some studies on the effect of combinations of biological factors in controlling insects. In several studies, double infection by different types of bio-control agents increased virulence when used sequentially (Dubois and Dean, 1995; Jaques and Morris, 1981; Morris *et al*., 1996; Tanada, 1985).

Results of *Btk* and EPN combinations showed additive and synergistic effects in the different time intervals. There were no differences in the time intervals in the case of EPN and *Btk* combinations. The result of a combination of the EPNs and *Btk* was not stage-dependent and had a similar pattern in both experimented larval stages. We can only speculate about the mechanisms explaining the interaction between the EPNs and *Btk* on *P. brassicae*. The fact that *P. brassicae* are better controlled if they are first exposed to *Btk*. It seems that *Btk* as a stressor cause a synergistic effect and make the larvae more susceptible. Oestergaard *et al*. (2006) found that the larvae of *Tipula paludosa* Meigen that ingested even a small inoculum of *Bacillus thuringiensis* subsp. *israelensis*, lost their typical behaviour of contracting themselves in response to mechanical stimuli. Also, bacterial infestation could cause a loss of defence reactions like suppression of encapsulation against the invading EPNs (Peters and Ehlers, 1994).

In the current study, a combination of EPNs and *B. bassiana* indicated that the results were entirely depended on the time interval. Combination of both the experimented EPNs and *B. bassiana* caused additive and synergistic effects on the 4th and 5th instar larvae of *P. brassicae*. However, to achieve additive or synergistic effects, the larvae should be exposed to *B. bassiana* for at least 24 h before the addition of the EPNs. However, the simultaneous use of the EPNs and *B. bassiana* and even 12 hours after being exposed to *B. bassiana* caused marked antagonistic effect.

We can only speculate on the mechanism that explains the interaction between the EPNs and *B. bassiana* against *P. brassicae* larvae. Additive and synergistic effects of using the EPNs and *B. bassiana* could be due to *B. bassiana* acting as a stressor. Ansari *et al*. (2004) found that the use of *M. anisopliae* and either *H. megidis* or *S. glaseri* led to synergistic effects on *Hoplia philanthus* Fuessly. They suggested that *M. anisopliae* acted as a stressor. They hypothesized that *M. anisopliae* had a pernicious effect on the grubs by reducing food intake. Owing to insufficient food, the homeostasis of the grubs could be disturbed, affecting the behavioural, morphological, and even physiological mechanisms, thus rendering the grubs more susceptible to nematode penetration and establishment. The current study conforms to the finding of Barbercheck and Kaya (1990) that the period of lethal infection for *G. mellonella* larvae infected with the EPNs, *S. feltiae* and *H. heliothidis*, and *B. bassiana* was shorter compared to larvae treated with the EPNs or fungi alone.

In conclusion, it can be said that the effect of a combination of these EPNs with *Btk* and *B. bassiana* on *P. brassicae* must be investigated under field conditions. If the additive or synergistic effect of the EPNs in combination with *Btk* and *B. bassiana* is confirmed under field conditions, they may offer a practical and safe method of controlling *P. brassicae*.

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بیماری‌زایی دو نماتد بیماری‌زای حشرات از طریق تداخل با *Beauveria bassiana* و *Bacillus thuringiensis* علیه *Pieris brassicae* (Lepidoptera: Pieridae)

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چکیده: سفیده بزرگ کلم .*Pieris brassicae* L یکی از مهم‌ترین آفات Brassicaceae می‌باشد. تأثیر کشندگی دو نماتد بیمارگر حشرات (EPNs)- *Heterorhabditis bacteriophora* و *Steinernema feltiae*- از طریق تداخل با *Beauveria bassiana* و *Bascillus thuringiensis* subsp. *Kurstaki* (*Btk*) علیه *P. brassicae* بررسی گردید. در آزمایشات تداخلی نماتدهای بیمارگر حشرات در غلظت LC_{50} هم‌زمان، ۱۲ یا ۲۴ ساعت پس از تیمار لاروها با LC_{10} یا LC_{25} قارچ *B. bassiana* یا باکتری *Btk* مورد استفاده قرار گرفت. تداخل بین نماتدهای بیمارگر حشرات با *B. bassiana* کاملاً با تداخل نماتدهای بیمارگر حشرات و *Btk* متفاوت بود. نتیجه تداخل با *B. bassiana* کاملاً وابسته به فواصل زمانی استفاده شده بود درحالی‌که تداخل بین نماتدهای بیمارگر حشرات و *Btk* تقریباً همیشه افزایشی یا سینرژیستی بود. اگرچه کاربرد نماتدهای بیمارگر حشرات ۲۴ ساعت پس از تیمار با *B. bassiana* سبب ایجاد اثرات افزایشی یا سینرژیتی شد. هم‌چنین نتایج نشان داد زمانی‌که نماتدهای بیمارگر حشرات با فواصل زمانی ۱۲ و یا ۲۴ ساعت پس از *Btk* مورد استفاده قرار گرفتند باعث بیش‌ترین اثر کشندگی شدند. براساس نتایج، استفاده هم‌زمان نماتدهای بیمارگر حشرات و *B. bassiana* علیه *P. brassicae* توصیه نمی‌شود. اگرچه نماتدهای بیمارگر حشرات می‌توانند بلافاصله بعد از *Btk* مورد استفاده قرار بگیرند اما بهتر است یک فاصله زمانی به‌منظور افزایش مرگ‌ومیر ایجاد گردد.

واژگان کلیدی: کنترل بیولوژیک، *Heterorhabditis bacteriophora*، کنترل تلفیقی آفات، *Steinernema feltiae*