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# Efficacy of single and combined applications of entomopathogenic fungi and nematodes against the pupae of colorado potato beetle (*Leptinotarsa decemlineata* [Say]), (Coleoptera: Chrysomelidae)

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## Abstract

**Background:** The lethal effects of different entomopathogenic fungi (EPF) and nematodes (EPN) on the pupae of the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) were investigated in a two-step study when used separately or together. In the first step, Nostalgist (*Beauveria bassiana* strain Bb-1, Agrobrest Co.), *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae), *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) (Bioglobal Co.), 2 Turkish isolates of *Beauveria bassiana* BIM-001 and BY2 were applied separately to the pupal stage of pest in the soil. In the experiments, 3 different concentrations (150, 200, and 250 million infective juveniles (IJs)/100 l water) of EPN and a single concentration ( $1 \times 10^8$  spores/ml for and maximum recommended field dose  $1 \times 10^8$  CFU/ml) of EPF were used. In the second step, BIM-001 and BY2 isolates of *B. bassiana* were combined with both EPN species; *S. feltiae* + *B. bassiana* BIM-001, *S. feltiae* + *B. bassiana* BY2, *H. bacteriophora* + *B. bassiana* BIM-001, *H. bacteriophora* + *B. bassiana* BY2 were applied to pupae. At this stage, 150 million IJs/100 l water which was the maximum recommended field concentration for EPN and  $1 \times 10^8$  spores/ml for local EPF isolates were used.

**Results:** According to the study results, pupae deaths were significantly higher in Nostalgist (74%) than those of *B. bassiana* BIM-001, *B. bassiana* BY2, *S. feltiae*, and *H. bacteriophora* ( $P < 0.05$ ). While both EPN caused higher mortality than *B. bassiana* BY2, *B. bassiana* BIM-001 had the highest mortality rates. The combination of *S. feltiae* + *B. bassiana* BIM-001 at the highest mortality rate (80%) was found statistically different and significant from other EPF + EPN treatments on pupae. Moreover, there were non-statistical differences between the other EPF and EPN combinations.

**Conclusion:** It was found that the combined use of EPF and EPN was more effective on the pupae of *L. decemlineata* than a single application. According to the results of the study, it may be promising to use EPN and EPF together for the control of the Colorado Potato Beetle pupae.

**Keywords:** *Beauveria*, Biological control, *Heterorhabditis*, *Steinernema*

A part of this study was presented at the 8th Plant Protection Congress with International Participation, Turkey.

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## Background

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) is an important pest that causes significant economic losses in plants belonging to Solanaceae (Balaško et al. 2020). Colorado potato beetle spends its pre-pupal stage on the soil surface and

then enters 4–8 cm below the soil and starts its the pupal stage after approximately the 2nd day (Kekillioğlu and Yılmaz, 2018). It has been known that the entomopathogenic nematodes (EPNs) exist as natural microorganisms in a complex environment such as the soil.

The capability of these EPNs is to actively seek and infect their hosts and also survive in the soil for a long time under optimal conditions, and also their absence of negative effects on non-target organisms provides significant advantages in pest control. In recent years, the importance of EPNs has been standing out due to the aforementioned features in the biological control of harmful species found under the soil surface, especially in certain life periods (Özdemir and Bayram 2017). The 2 genera, *Steinernema* and *Heterorhabditis* spp., are actively used commercially in pest control (Özdemir and Bayram 2017). EPFs have a large share in the world biopesticide market today (Bugti et al. 2020). There are an estimated 750 species of EPF in about 90 genera (Abd El-Ghany, 2015). Numerous EPF species belonging to *Beauveria*, *Metarhizium*, *Lecanicillium*, and *Isaria* are widely used commercially due to their easier mass production (Rai et al. 2014). In 2 decades, insecticides containing neonicotinoids have been widely used for controlling of the Colorado potato beetle around the world. The resistance to neonicotinoids and the negative effects of these compounds on other organisms in the agro-ecosystem reduced the chance of sustainable management of the pest (Oberemok et al. 2018). The use of entomopathogenic organisms in pest control has become inevitable for the many reasons presented above.

As known, the lethal effects of EPF and EPN on *L. decemlineata* are higher, especially in pre-adult stages (Ropek and Kołodziejczyk 2018). Use of the entomopathogenic organisms on the pupal stage of this pest has not been studied. Therefore, the lethal effects of EPF and EPN on pupae of the Colorado potato beetle, separately or combined, were investigated in the present study.

## Methods

Colorado potato beetle *L. decemlineata* adult individuals were collected from Isparta central potato production areas (37°50'16.77"N 30°32'17.61"E, 1017m, 05.06.2020). These adult individuals were cultured and produced under laboratory conditions. The main material of the study was the pest pupae which was fed until the 4<sup>th</sup> larval stage and separated into other boxes during this period reared in a climatic chamber (26 ± 1°C, 65 ± 5% RH, and 16:8 L:D photoperiod). The other materials used in the study were BIM-001 isolate of *Beauveria bassiana* obtained from *L. decemlineata* in potato production areas of central province of Isparta (37°50'16.77"N 30°32'17.61"E, 1017m, 23.08.2018), BY2 isolate of *B.*

*bassiana* isolated from Phlaeothripidae species in Burdur-Yeşilova wheat production areas (37° 30' 52,3" N 29° 45' 45" E, 1191 m, 14.06.2017), and commercial preparations Nostalgist (*Beauveria bassiana* strain Bb-1, Agrobrest Co.), *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae), and *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) (Bioglobal Co.). The study was carried out in two separate stages, experiment 1 and experiment 2.

### Experiment 1

The EPF and EPN were applied separately to the pupae of *L. decemlineata*, and their lethal effects were determined. For this purpose, individuals in the 4th larval stage of the Colorado potato beetle were transferred to plastic boxes (500 ml, 8 × 12 × 8.5 cm), which had a standard soil substrate (Zemek et al. 2017) moistened with sterile distilled water (12.5 ml). These boxes were divided into 5 separate groups with sterile cardboard. Only one 4th instar larva was left in each compartment (total 5 larvae for one box), and it was observed whether these individuals penetrated into the soil after 24 hrs. It is known that the 4th instar larvae of the Colorado potato beetle usually enter the pupal stage approximately 48 hrs after they throw themselves into the soil (Kekillioğlu and Yılmaz 2018). Therefore, fungi and nematode applications were carried out 3 days after these larvae were transferred to the plastic boxes at 5 ml (pre-pupa) per compartment (Table 1). Each compartment was moistened with 5 ml of sterile distilled water before 24 h from the applications.

### Experiment 2

In experiment 2, lethal effects were detected in the pupal stage, as a result of the combined use of BIM-001 and BY2 local isolates of *B. bassiana* and EPN. Experiment 2 was also designed the same as in experiment 1, and the EPF and EPN applications were made (Table 2).

Both experiments were carried out with 10 replications according to the randomized plot design. All experiments were carried out in the climatized chambers at 25 ± 1 °C, 75 ± 5% humidity, and 16:8 photoperiod conditions. The pupal development process can vary between 5 and 8 days under the stated conditions (Kekillioğlu and Yılmaz 2018), and accordingly, the dead–alive counts were done on the 7th day after the applications, and the mortality rates were calculated (Choo et al. 2002). To figure out whether the pupae were dead poked with a fine brush. Those wiggled ones were taken into different boxes and the adult emergence was awaited. Pupae that did not wiggle were kept in Petri dishes with a moist filter paper for re-isolation of EPF. The re-isolation was performed on all pupae that died in EPF applications, according to Meng et al. (2017). Those containing EPF spores were

**Table 1** Application doses and concentrations of entomopathogenic fungi and nematodes in experiment 1

Treatments	Concentrations (per pupa/ 5ml)
Nostalgist	1x10 <sup>8</sup> CFU/ml
<i>Beauveria bassiana</i> BIM-001	1x10 <sup>8</sup> spores/ml
<i>B. bassiana</i> BY2	1x10 <sup>8</sup> spores/ml
<i>Heterorhabditis bacteriophora</i>	150 million IJs/100 l water
	200 million IJs/100 l water
	250 million IJs/100 l water
<i>Steinernema feltiae</i>	150 million IJs/100 l water
	200 million IJs/100 l water
	250 million IJs/100 l water
Control	Sterile distilled water

recorded as dead due to EPF. In addition, each cadaver in EPN treatments was taken into the White Trap to re-isolate of EPNs. Nematode emergence was observed after 15 days in the White Traps (Kaya and Stock 1997). The mean number of re-isolated juveniles per dead pupa was recorded for each application dose of EPN. Since Nostalgist is a commercial product based on EPF (*Beauveria bassiana* strain Bb-1), it contains additional ingredients. Therefore, only local isolates were used in combined studies that included EPF and EPN to eliminate interference that may be caused by the ingredients of the commercial product.

Besides, adult emergence was recorded after applications and the number of adult individuals emerged from the pupa was recorded in both experiments. Adult emergences are given as percentage (%) for each application.

### Statistical analysis

The assumption of normality of obtained data was checked with the test of Kolmogorov–Smirnov. In addition, square root transformation was applied to the numbers of the re-isolated nematodes in the 3<sup>rd</sup> juvenile stage

that was nonparametric. Then, these data were subjected to one-way ANOVA, followed by Tukey's HSD (honestly significant difference) test ( $P < 0.05$ ). All data were analyzed using SPSS 20.0 software.

## Results

### Experiment 1

Mortality rates in all EPF and EPN applications were found higher and significant than the control ( $P < 0.05$ ), and the mortality percentage on Nostalgist (74%) reached a higher mortality rate than all applications. Additionally, non-significant difference was observed between the recommended highest concentrations (200 and 250 million IJs/100 l water) of *H. bacteriophora* (40 and 44%, respectively) and *B. bassiana* BIM-001 (50%). It was found that there was non-statistical difference between the recommended concentration of *H. bacteriophora* (150 million IJs/100 l water) and the all application concentrations of *S. feltiae* (Table 3). While both EPN caused higher mortality than *B. bassiana* BY2, *B. bassiana* BIM-001 had the highest mortality rates.

On the other hand, it was found that the mean number of 3<sup>rd</sup> juveniles that were re-isolated from dead pupae in experiment 1 was increased linearly with the application concentrations of both nematode species. While the re-isolated juvenile numbers in 250 million concentration were higher and more significant than other doses of *H. bacteriophora*, this was not the same for the *S. feltiae*. In *H. bacteriophora*, the number of juveniles obtained at 200 million concentration ( $256.70 \pm 10.73$ ) was significantly higher than 150 million concentration ( $192.87 \pm 6.09$ ) ( $P < 0.05$ ). In addition, the difference between the number of 3<sup>rd</sup> juveniles obtained at 200 million ( $234.55 \pm 4.80$ ) and 250 million concentrations ( $247.34 \pm 8.80$ ) of *S. feltiae* was significant ( $P < 0.05$ ) than 150 million concentration, but was not differed from each other ( $P > 0.05$ ) (Table 4).

When EPF and EPN were applied separately, adult emergence was determined for Nostalgist, *B. bassiana*

**Table 2** Application concentrations of entomopathogenic fungi and nematodes in experiment 2

Treatments	Concentrations (per pupa/ 5 ml)
<i>Steinernema feltiae</i> + <i>Beauveria bassiana</i> BIM-001	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml
<i>S. feltiae</i> + <i>B. bassiana</i> BY2	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml
<i>Heterorhabditis bacteriophora</i> + <i>Beauveria bassiana</i> BIM-001	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml
<i>H. bacteriophora</i> + <i>B. bassiana</i> BY2	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml
<i>B. bassiana</i> BIM-001	1x10 <sup>8</sup> spores/ml
<i>B. bassiana</i> BY2	1x10 <sup>8</sup> spores/ml
<i>H. bacteriophora</i>	150 million IJs/100 l water
<i>S. feltiae</i>	150 million IJs/100 l water
Control	Sterile distilled water

**Table 3** Mortality rates (%) determined on pupae of *Leptinotarsa decemlineata* (Say) in entomopathogenic fungi and nematodes applications

Treatments	Application concentrations	Mortality Rates
Nostalgist	1x10 <sup>8</sup> CFU/ml	74 a
<i>Beauveria bassiana</i> BIM-001	1x10 <sup>8</sup> spores/ml	50 ab
<i>B. bassiana</i> BY2	1x10 <sup>8</sup> spores/ml	10 c
<i>Heterorhabditis bacteriophora</i>	150 million IJs/100 l water	26 bc
	200 million IJs/100 l water	40 ab
	250 million IJs/100 l water	44 ab
<i>Steinernema feltiae</i>	150 million IJs/100 l water	22 bc
	200 million IJs/100 l water	22 bc
	250 million IJs/100 l water	30 bc

a, b, c, d: Values indicated by different letters in the same column are statistically different from each other ( $P < 0.05$ )

**Table 4** Mean numbers of re-isolated 3<sup>rd</sup> juveniles from *Leptinotarsa decemlineata* (Say) pupae

Application Concentrations	Mean ± S.E.	
	<i>Heterorhabditis bacteriophora</i>	<i>Steinernema feltiae</i>
150 million IJs/100 l water	192.87 ± 6.09 c	194.81 ± 3.58 b
200 million IJs/100 l water	256.70 ± 10.73 b	234.55 ± 4.80 a
250 million IJs/100 l water	315.85 ± 6.63 a	247.34 ± 8.80 a

a, b: Values indicated by different letters in the same column are statistically different from each other ( $P < 0.05$ )

BIM-001, *B. bassiana* BY2, and control as 26, 50, 90, and 100%, respectively. These values determined as 74, 60, 56 and 78, 78, 70 for 150, 200, and 250 million (IJs/100 l water) concentrations of *H. bacteriophora* and *S. feltiae*, respectively.

**Table 5** Mortality rates (%) after entomopathogenic fungi and nematodes simultaneously applications of pupae of *Leptinotarsa decemlineata*

Treatments	Application Concentration	Mortality Rates (%)
Control	Distilled sterile water	00 e
<i>Beauveria bassiana</i> BIM-001	1x10 <sup>8</sup> spores/ml	50 bc
<i>B. bassiana</i> BY2	1x10 <sup>8</sup> spores/ml	10 d
<i>Heterorhabditis bacteriophora</i>	150 million IJs/100 l water	26 cd
<i>Steinernema feltiae</i>	150 million IJs/100 l water	22 d
<i>S. feltiae</i> + <i>B. bassiana</i> BIM-001	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml	80 a
<i>S. feltiae</i> + <i>B. bassiana</i> BY2	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml	64 ab
<i>H. bacteriophora</i> + <i>B. bassiana</i> BIM-001	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml	76 ab
<i>H. bacteriophora</i> + <i>B. bassiana</i> BY2	150 million IJs/100 l water+1x10 <sup>8</sup> spores/ml	56 ab

a, b, c, d, e: Values indicated by different letters in the same column are statistically different from each other ( $P < 0.05$ )

## Experiment 2

Lethal effects on pupae of the Colorado potato beetle were determined when EPF and EPN were applied simultaneously in the experiment 2. In the present experiment, the mortality rate was significantly higher (80%) than other EPF-EPN combinations (combination of *S. feltiae* and *B. bassiana* BIM-001). Moreover, there were non-statistical differences between the other EPF and EPN combinations. According to the present results, simultaneous applications of EPF and EPN caused a higher mortality on the pest than the applications of these organisms alone. In the combination of *S. feltiae* and *B. bassiana* BIM-001, the mortality was 80% on the pupae, and these rates were 50 and 22%, respectively, when EPF and EPN were applied separately. *Beauveria bassiana* BY2 was found to cause low mortality rates when applied simultaneously with EPN relative to other isolate ( $P < 0.05$ ) (Table 5).

When EPF and EPN were applied simultaneously, adult emergence was determined for *S. feltiae* + *B. bassiana* BY2, *S. feltiae* + *B. bassiana* BIM-001, *H. bacteriophora* + *B. bassiana* BY2, *H. bacteriophora* + *B. bassiana* BIM-001, and control as 26, 20, 44, 24, and 100%, respectively.

## Discussion

Many studies have been conducted to determine the effects of different strains of *B. bassiana* on the adult, larval, pupal stages, and hatching rates of the Colorado potato beetle (Atanasova and Vasilev 2020). Among these researches, the results of the applications made against the pupa stage under the soil are remarkable. It has been reported that the pupal stage of the pest is sensitive to soil applications and these applications can reduce the next adults' emergence up to 74% (Klinger 2003). Cantwell et al. (1986) recorded the adult emergence 38.8–28–62.6–43.6%, respectively, when the rates of 7.5 and 75 g/m<sup>2</sup> of *B. bassiana* were applied to the pupal

stage of the Colorado potato beetle. In the present study, adult emergence from pupae treated with Nostalgist, *B. bassiana* BIM-001, and *B. bassiana* BY2 was observed as 26, 50, and 90%, respectively. Few studies have been conducted on the effects of EPN on the pupal stage of the Colorado potato beetle. It has been reported that the mortality rate varies between 80 and 90% when the rates of 79-158 nematodes/cm<sup>2</sup> of *S. feltiae* (Mexican strain) were applied to the soil which included the pupae of the Colorado potato beetle under laboratory conditions. In the present study, *S. feltiae* caused 22-30% mortality and *H. bacteriophora* caused 26-44% mortality in the pupal stage of the Colorado potato beetle. In another study, the adults' emergence decreased by 88.4-100% when the rate of 93-155/cm<sup>2</sup> of *S. feltiae* (Mexican strain) was applied in field conditions (Wright et al. 1987). In the present study, adult emergence was determined as 70-78% and 56-74% for *S. feltiae* and *H. bacteriophora*, respectively. Additionally, EPNs were also re-isolated from dead pupae and it was proved that used EPNs caused death in the pupal stage. Although EPNs cause low mortality rates, the presence of EPNs in pupal cadavers suggests the probability of being the source of inoculum.

It is also known that EPF and EPN generally showed synergistic interactions in pest management and can be used together successfully (Shahid et al. 2012). When the combination of EPF and EPN was applied to the pre-adult stages, this caused lethal effects on different harmful species. In the same way, Ibrahim et al. (2019) recorded 100% mortality in the larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) 2 days after the application of the combination of *H. zealandica* (7 IJ/larva) and *B. bassiana* (3.1 × 10<sup>2</sup> conidia/ml). The highest mortality rate in pre-adult periods was observed when *H. bacteriophora* and *B. bassiana* (WG-11) were combined. In the present study, *H. bacteriophora* + *B. bassiana* BIM-001 that were applied simultaneously caused 76% mortality on pupae of Colorado potato beetle, when applied separately and led to 50 and 26%.

In studies on the Colorado potato beetle, Hussein et al. (2016) found that the mortality rate on the last larval stage of *L. decemlineata* was 98% on the 7th day in Petri dishes when the combination of *S. feltiae* with *Isaria fumosorosea* CCM 8367 were applied. Zemek et al. (2017) also determined that the mortality rate was 44 and 45%, respectively, when *I. fumosorosea* CCM 8367 and *S. feltiae* were applied separately to the last stage larvae of *L. decemlineata* in the potting soil. When *I. fumosorosea* CCM 8367 and *S. feltiae* were applied together, the mortality rate was 84%. In the present study, when *S. feltiae* and *B. bassiana* BIM-001 were applied simultaneously to the pupae of the Colorado potato beetle, the mortality rate was found as 80%. Additionally, the mortality rates were 50 and 22% in

*B. bassiana* BIM-001 and *S. feltiae* applications, respectively. *Heterorhabditis bacteriophora* and *B. bassiana* BIM-001 that applied simultaneously caused 76% mortality in the pupa of the Colorado potato beetle. When these EPF and EPN were applied separately, the rates were 50 and 26%. Obtained results of this study showed that the BY2 isolate of *B. bassiana* caused lower mortality rates than BIM-001 isolated from Colorado potato beetle, both singly and when combined with nematodes.

Kamionek et al. (1974) found that when EPFs and EPNs were applied simultaneously against insects of the order Coleoptera or Lepidoptera in Petri dishes, the lethal infection time was shorter than when the fungus was applied alone. This may be since EPNs release bacteria shortly after entering the insect body. Ansari et al. (2008) reported 100% larval mortality when EPNs were applied 7 to 14 days after *M. anisopliae* application. Dlamini et al. (2020) determined a synergistic effect and the highest mortality in the application of *Steinernema yirgalemense* 1 or 2 weeks after the application with different isolates of *B. bassiana* and *M. anisopliae* on *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) larvae. These studies showed that more successful results were obtained when EPN was applied after EPF application. However, in the present study, it was determined that the mortality rate increased significantly even in simultaneous applications. The mortality rate was lower than *H. bacteriophora* when *S. feltiae* was separately applied to the pupae of the Colorado Potato Beetle. When *B. bassiana* isolates (BIM-001, BY2) were combined with *S. feltiae*, death of pupae reached higher rates than the combination of the EPF isolates with *H. bacteriophora*. Additionally, it was determined that mortality rates of pupae increased in treatments where *B. bassiana* isolates were applied simultaneously with *S. feltiae*. Moreover, BY2 isolate of *B. bassiana* also had a low mortality rate when used alone, while a high mortality rate was observed when combined with *S. feltiae*. Consequently, it can be concluded that there was a synergistic relationship between *S. feltiae* and *B. bassiana* isolates (BIM-001, BY2).

## Conclusions

The combination of the EPF and EPN was more effective than single applications against the pre-adult stages of different agricultural pests. Similarly, in this study, they were tested together, and up to 80% of death obtained under laboratory conditions. It was determined that the use of EPF and EPN combination led to higher mortality rates in the pupal stage of the Colorado potato beetle. The use of entomopathogens is a valuable and an alternative tool in pest control. The results suggest that the combined

effect of EPN and EPFs may be promising for the control of pupae of the Colorado Potato Beetle under field conditions. The obtained results show that similar experiments need to be done in natural conditions as well.

#### Abbreviations

CFU: Colony-forming unit; EPN: Entomopathogenic nematode; EPF: Entomopathogenic fungus; HSD: Honestly significant difference; IJs: Infective juveniles; L: Liter; mL: Milliliter.

#### Acknowledgements

We thank Bioglobal Company (Antalya, Turkey) for providing entomopathogenic nematodes preparations. Additionally, we are grateful to Agrobrest Group (Izmir, Turkey) for Nostalgiist.

#### Author contributions

Study conception and experimental design were performed by AUy and OD, data collection was carried out by AUy and FGÖ, and data analysis was done by TN. All authors read and approved the final manuscript.

#### Funding

Not applicable

#### Availability of data and material

The datasets are available from the corresponding author on reasonable request.

#### Declarations

#### Ethics approval and consent to participate

Not applicable

#### Consent for publication

Not applicable

#### Competing interests

Not applicable

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Received: 11 March 2022 Accepted: 18 June 2022

Published online: 29 June 2022

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