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# Effectiveness study of the dual application of new Indigenous entomopathogenic nematode isolate *Heterorhabditis taysearae* and entomopathogenic fungi *Beauveria bassiana* against armyworm (*Spodoptera frugiperda*)

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## Abstract

**Background** The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a polyphagous insect pest species that travel great distances every summer to disperse. They mostly feed on maize and over 350 other crops. In this investigation, two entomopathogens: *Beauveria bassiana* and *Heterorhabditis taysearae*, were utilized to test the efficacy of each against *S. frugiperda* separately and then as a group by inoculating *H. taysearae* two days after fungal infection.

**Results** Evaluations were done on mortality percentage, infective juvenile (IJ) production, and conidia production. The LT<sub>50</sub> continually reduced with increases in nematode and fungus concentrations. For the LC<sub>50</sub> value, the *H. taysearae* isolate was 289 IJs/larva after 96 h of treatment, while for *B. bassiana* isolate, it was 10<sup>6</sup> CFU/ml after 144 h of treatment. Dual infections with *B. bassiana* and *H. taysearae* had a beneficial effect on pest mortality, resulting in 83% mortality, and caused a significant increase in conidia production while utilizing the nematode or fungal separately caused decrease in mortality (63 and 73%, respectively).

**Conclusions** The study's findings indicated a quicker time to death and suggested that combining a moderately pathogenic fungal isolate with nematodes could raise the mortality rate. The mutually beneficial relationship between *B. bassiana* and *H. taysearae* controls *S. frugiperda*.

**Keywords** *Spodoptera frugiperda*, Dual infection, *Beauveria bassiana*, *Heterorhabditis taysearae*

## Background

Heterorhabditidae and Steinernematidae are two families of obligate insect parasites that belong to the entomopathogenic nematodes (EPN). These are attractive possibilities for biological control tactics against economically significant insect pests because of their tremendous reproductive potential, ability to produce large quantities, and low toxicity to non-target animals and the environment (Abd El Azim and Khashaba 2021).

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Regarding Hussain et al. (2009), there is a wealth of data showing that entomopathogenic fungi (EPF), such as *Beauveria bassiana*, are detrimental to a range of pest species. EPF dig tunnels through the insect host's cuticle to reach the hemocoel. The fungus grows its infectious spore stage on the host's cuticle. The insect is then killed by the fungus that forms inside its body (Safavi 2013).

Molecular identification is the most accurate technique for determining phylogenetic relationships because morphological identification is unreliable for classifying novel isolates into specific species. Using genome sequencing of different parts of the EPNs, this technique can identify nuclear genes such as the 18S rRNA gene, internal transcribed spacers (ITS1 and ITS2), the 5.8S and 28S rRNA genes, and ribosomal genes such as the 18S rRNA gene (Stock 2009; Abd El Azim 2022).

Combining biocontrol treatments can significantly improve pest management, according to mounting data. Their greater effectiveness might be explained by additive or synergistic effects. According to a theory on synergistic interactions, one agent may cause stress to the target insect or change its behavior (such as eating or moving), increasing its susceptibility to the other agent (Ansari et al. 2008). Since nematodes primarily target soil-dwelling pests, mutual applications with EPF would seem to be a feasible way to increase levels of pest control (Acevedo et al. 2007).

More than 350 plant species, including cotton, rice, sorghum, millet, maize, sugarcane, and vegetable crops, are consumed by the Fall Armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), an insect pest that inhabits the tropical and subtropical regions of the Americas and recently in Africa and Asia. Larval stage is the harmful and important polyphagous pest's stage (Montezano et al. 2018). If *S. frugiperda* is not controlled properly, it causes large yields' losses. In May 2019, *S. frugiperda* was recorded for the first time in maize field in Aswan Governorate, Upper Egypt (Dahi et al. 2020).

According to Thakur et al. (2022), in a bioassay greenhouse experiment, the mixture of native EPNs, EPF, and EPB demonstrated a strong virulence toward *Spodoptera litura*. According to the study, the most effective biological control strategy, dealing with local pest issues, may involve combining three entomopathogens (*H. bacteriophora* + *B. bassiana* and *H. bacteriophora* + *Bacillus thuringiensis*) in a synergistic blend.

In the present study, the nematode *Heterorhabditis taysearae* alone and in combination with EPF isolate *B. bassiana* (MN337282) was used against the FAW to assess influences on mortality, time to death, and host colonization.

## Methods

### Sampling

Fifteen soil samples (from 20 cm deep) from an area cultivated with peanuts in the Ismailia Egyptian Governorate (N 30 27" 11.2", E 32 16' 20.1") were collected in 2021. Samples from different locations in the field were brought into the laboratory and placed in a plastic container holding 250 g of the subsample stored at 16 °C and were homogenized before the extraction of EPNs.

The *Galleria mellonella* (Galleria trap) method of insect-baiting was used to process the soil samples (Bedding and Akhurst 1975). Six instar *G. mellonella* larvae were placed inside 250 g perforated plastic boxes and then embedded in the soil sample. Samples of soil were kept at 25 °C. Dead *G. mellonella* exhibiting signs of an EPN's infection were removed and replaced every five days. This process lasted five days to complete. Infectious juveniles (IJs) were gathered throughout the course of the next few days and stored at 16 °C in distilled water after the dead *G. mellonella* larvae were moved to White traps (White 1927).

### Nematode identification

#### Molecular characterization of the isolate

The ITS rDNA sequences were used to molecularly characterize the isolate. DNA extraction from EPN was described by Kary et al. (2009). After being ground up in 20 µl of 1 × PCR solution, the nematodes were placed into a 1.5 ml tube, which was sterilized, frozen, and then filled with 20 µl of the same solution. 5 µl of 60 µg/ml Proteinase K was added to the tube after a 15 min incubation period at 70 °C and a 60 °C thawing period. The tube was incubated at 65 °C for two hours and then heated to 95 °C for fifteen min. Following a 15 min centrifugation at 16,000 rpm, the nematode DNA-containing supernatant was gathered and kept at 70 °C until required. To determine the quantity and quality of DNA, a spectrophotometer and a 1% agarose gel electrophoresis were utilized.

Using a 25 µl reaction mix containing 2 µl of the DNA suspension, 2.5 µl of 10X PCR buffer, 2.5 µl of MgCl<sub>2</sub> (25 mM), 2 µl of the dNTP combination (10 mM of each dNTP), 0.6 U of Taq DNA polymerase, 13.4 µl of dd H<sub>2</sub>O, and 1 µl of the forward primer, the ITS region of the nematode rDNA was amplified by PCR. AB28:5'-ATA TGCTTAAGTTCAGCGGGT3 and 1 µl of the reverse primer TW81:5-GTTTCCGTAGGTGAACCTGC-3' (Joyce et al. 1994). Following a 5-min initial denaturation at 94 °C, the ITS rDNA region was subjected to 30 cycles of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min. One final extension took place at 72 °C for 5 min. Next, using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega) and the manufacturer's instructions, the product was placed onto a 2% agarose gel and purified.

Sequence-specific primers and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA) were used by the Macrogen Inc. service located in South Korea to sequence PCR data on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, USA) in both directions. An NCBI BLAST (Basic Local Alignment Search Tool) search yielded around 700 base pairs of sequences (National Centre for Biotechnology Information). The isolate's *Heterorhabditis* sequences were found in the NCBI database, and they were compared to the *Heterorhabditis* sequences found.

#### Phylogenetic analysis

The phylogeny.fr platform (<http://www.phylogeny.fr/phylogeny.cgi>) was used to create a phylogenetic tree (Dereeper et al. 2008). The program Muscle was used to align the ITS sequences. The PhyML program's maximum likelihood approach was utilized to reconstruct the phylogeny, and the bootstrapping technique was employed to assess the internal branch reliability (100). The application TreeDyn was used to render the trees.

#### The utilization of insects, nematode, and fungi isolates

FAW larvae were grown in Egypt at the Faculty of Agriculture, Cairo University. They were raised in a laboratory and kept in the dark at  $26 \pm 2$  °C. Corn leaves (*Zea mays*) were used to raise the larvae in glass jars.

Dutky et al. (1964) reported that *G. mellonella* larvae in their last instar were infected using the recently isolated EPN. Two weeks following their harvest, the infected juveniles (IJs) that came out of White traps were kept to use. After that, they were kept at 16 °C in distilled water. At  $28 \pm 1$  °C for 14 days, in complete darkness, 90 mm Petri plates containing 50 µg/ml chloramphenicol and 50 µg/ml streptomycin (Meyling and Eilenberg 2007) were cultured for the fungal isolate *B. bassiana* (MN337282), which had previously been isolated by Khashaba (2021). The 0.01% Triton X-100 aqueous solution was used to collect and suspend the conidia. The overall spore count was determined microscopically with a hemocytometer. Dilutions in series were made for different concentrations.

#### Analyzing of biological activity of isolated nematodes and fungi on larvae of Spodoptera frugiperda

##### Nematode isolate

After being collected, the 4th instar larvae of *S. frugiperda* were placed into plastic cups with 50 g of moist sand and 10% distilled water w/w. One larva was contained in each cup, and the median lethal concentration ( $LC_{50}$ ) for EPN and EPF was determined, using 0,100,200,300, and 400 IJs/larvae in nematode concentration–response

experiments. Twenty larvae were used as the control group in two separate tests on each concentration of nematodes. Following that, they were maintained in a dark growth environment at  $26 \pm 2$  °C. The larval death rate was determined after 48, 72, 96, and 120 h by calculating the color shift, which in cases of *Heterorhabditis* is typically reddish-purple. Larval mortality persisted for 5 days.

##### Fungus isolate

Conidia from a 14-day-old culture were cultured on SDAY, collected and suspended in 0.01% Triton X 100, after the agar surface was scraped. The resultant suspension was filtered to remove any last bits of hyphal debris. The primary conidial concentrations were found using a light microscope and hemocytometer ( $10^8$ ). After that, 20 larvae were evaluated with two duplicates of each of the three fungal dilutions, which were adjusted to  $10^5$ ,  $10^6$ , and  $10^7$  spores/ml, using sterile 0.01% Triton X 100 solution from the main concentration. All that was given to the control cups was sterile distilled water. Cups were incubated at  $25 \pm 2$  °C, following inoculation. In the study, the rates of larval mortality were recorded at 72, 96, 120, 144, and 168 h after the hardening of larvae caused by the fungus or the formation of conidiophores. After that, to promote sporulation, the larvae were placed in sterile Petri plates with damp filter paper.

##### Nematode–fungus interactions

To ascertain the interactions between the calculated  $LC_{50}$  of entomopathogenic nematode and fungal isolate, the EPNs were started two days after the EPF infection. After 144 h of infection, the mortality rate was calculated. Every 24 h after infection, larvae were checked, and symptoms and fatalities were recorded. To promote sporulation, cadavers exhibiting signs of fungal infection, such as stiffening or the appearance of conidiophores, were placed in sterile Petri plates on moist filter paper. Larvae exhibiting changes in pigmentation suggestive of an EPN infection were cultured in Petri dishes on white traps to harvest EPNs.

##### Data analysis

Probit analysis was used to determine the lethal time, and concentration values for  $LT_{50}$  and  $LC_{50}$  were calculated according to Finney (1971) using LDPline software.

##### Results

Fifteen soil samples were taken from the governorate of Ismailia's peanut field. A single *Heterorhabditis* isolate (7%), identified by the morphological characteristics of deceased larvae with a brown/black color, was obtained.

### Molecular characterization

A 700 bp PCR product containing the ITS1-5.8S-ITS2 sequences was used to do a BLAST against the nucleotide collection (nr/nt) database to compare the EPN isolate to other noteworthy species that were kept in Gen-Bank. The discovered isolate exhibited 99% identity with *H. taysearae* based on sequence homology, and it has been deposited under the accession number *Heterorhabditis taysearae* EAG2 isolate (MW544011).

### Phylogenetic analysis

The NCBI-acquired EPN sequences of the “*H. taysearae*” group were compared to the ITS sequences of the nematodes isolated from the positive sample using BLAST as a match. The novel isolate was categorized alongside other *H. taysearae* isolates in a clade. Using the Phylogeny.fr program, they were added together and placed in a clade alongside additional isolates of *H. taysearae* that were found in the GenBank database (Fig. 1). *H. taysearae* (EF043443) from Ireland and *H. taysearae* (KC633186) from the Gaza Strip belong to the same clade, according to the phylogenetic tree.

### EPFs and EPNs bioassay

There were variations in the mortality rates of the larvae for both the single and combined entomopathogens. Within five days, *H. taysearae* caused mortality in larvae, where after 120 h, the concentration of 400 IJs/ larvae caused the greatest larval mortality (63%) and 100 IJs/ larvae caused the lowest one (16%) after 96 h. Within 7 days, the fungal isolate *B. bassiana* caused death of larvae. At 168 h, the concentration of  $1 \times 10^7$  CFU/ml had the highest mortality percentage (73%), while the concentration of  $1 \times 10^5$  CFU/ml had the lowest mortality percentage (28%) at 168 h.  $LT_{50}$  and  $LC_{50}$  values were calculated (Tables 1 and 2), which showed the effects of pathogens individually. It was shown that  $LT_{50}$  steadily reduced with increases in nematode and fungus. *B. bassiana* isolate demonstrated virulence, with  $LT_{50}$  values of 95 h at a concentration of  $10^7$  CFU/ml, while *H. taysearae*

**Table 1** Mortality percentage of the 4th instar larvae of *Spodoptera frugiperda* exposed to the different concentrations of *Heterorhabditis taysearae* and the values for  $LT_{50}$  and  $LC_{50}$

Concentrations (IJs/Larvae)	48 h	72 h	96 h	120 h	$LT_{50}$ (hrs)
100	11.6	11.6	16.6	16.6	–
200	11.6	28.3	33.3	33.3	–
300	16.6	41.6	51.6	51.6	90
400	28.3	63.6	63.3	63.3	66
$LC_{50}$	2080	314	289	289	

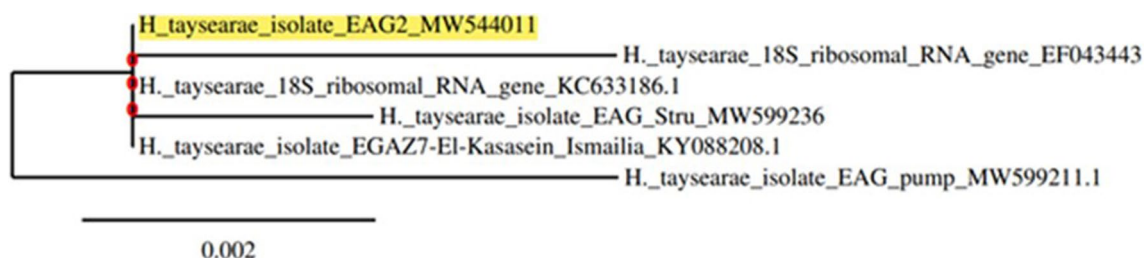
was virulent with  $LT_{50}$  values of 66 h at 400IJs/larvae. For the  $LC_{50}$  value, the *H. taysearae* isolate was 289 IJs/larva after 96 h of treatment, while for *B. bassiana* isolate, it was  $10^6$  CFU/ml after 144 h of treatment.

### Entomopathogenic nematode and fungi interactions

*Heterorhabditis taysearae* was inoculated two days after *B. bassiana* treatment at a concentration of 289 IJs/ larva for *H. taysearae* isolate, while for *B. bassiana* isolate was  $10^6$  CFU/ml according to  $LC_{50}$  value, the two entomopathogens worked in concert to suppress the 4th instar larvae of FAW. The combined effect was 83% mortality rate at 144 h, which was higher than what was observed when the two entomopathogens were inoculated separately (Fig. 2).

### Emergence of infections and symptoms after various treatments

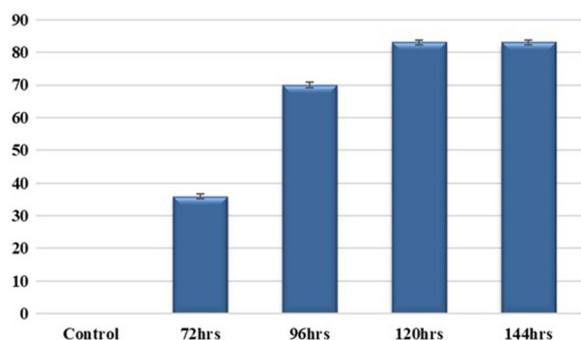
By day 10, 60% of the cadavers for *B. bassiana* produced conidia, and 75% of the infected larvae showed evidence of fungal colonization. Eighty percent of the larvae of the nematode *H. taysearae* showed symptoms of infection, and by day six, IJs had emerged from seventy percent of the cadavers. At the dual infection, 70% of the larvae developed conidia after *B. bassiana* was infected 48 h before *H. taysearae*, of which 50% produced conidia. Thirty percent of the cadavers had IJs and showed signs of nematode infection.



**Fig. 1** Maximum likelihood ITS phylogenetic trees of *Heterorhabditis taysearae* species using ITS sequence. Based on nucleotide sequences of *H. taysearae* (*H. taysearae* isolate EAG2 MW544011) and reference strains (alignment length approximately 700 bp using phylo-tree analysis (Phylogeny.fr program))

**Table 2** Mortality percentage of the fourth larval instar of *Spodoptera frugiperda* exposed to the different concentrations of *Beauveria bassiana* and the values for LT50 and LC50

Concentrations (CFU/ml)	72 h	96 h	120 h	144 h	168 h	LT50 (hrs)
1*10 <sup>5</sup>	11.6	21.6	23.3	28.3	28.3	276
1*10 <sup>6</sup>	13.3	28.3	33.3	43.3	43.3	163
1*10 <sup>7</sup>	31.6	50	68.3	73.3	73.3	95
LC50	4.74X10 <sup>8</sup>	1.37X10 <sup>7</sup>	2.42X10 <sup>6</sup>	1.19X10 <sup>6</sup>	1.19X10 <sup>6</sup>	

**Fig. 2** Efficacy percentage of *B. bassiana* with *Heterorhabditis taysearae* in combination for the control of 4th instar larvae of *Spodoptera frugiperda*. Treatments consisted of (control), and the combination of *B. bassiana* and *H. taysearae* (10<sup>6</sup> CFU/ml and 289 IJs/larva) at different times

## Discussion

Fifteen soil samples were taken from the Governorate of Ismailia's peanut field. Based on the physical characteristics of dead larvae (brown/black color), one isolate of *Heterorhabditis* was found (7%). The soil sample was positive for the occurrence of *Heterorhabditis*. ITS sequences were used to identify the nematodes that were isolated from the positive sample down to the species level. According to the results obtained, the isolate EAG2 (MW544011) indicated a 99% similarity with *Heterorhabditis taysearae*. Because of several characteristics, including the sandy clay loam type of the soil sample and the temperature range (23°–25°), it was anticipated that the recovery rate for EPNs in this region would be high. The results agree with Shamseldean and Abd Elgawad (1994) who first isolated *H. taysearae* from Egyptian sandy soil at Behera Governorate.

*Heterorhabditis taysearae* EF043443 and KC633186 are all members of the same clade, according to the phylogenetic tree, as was the isolate *H. taysearae* EAG2 (MW544011). Given the vast geographic separations between the “taysearae subgroup” nematode occurrence sites (Iran, Gaza Strip, and Egypt), these occurrences may be caused by “latitudinal clades,” as described by Dolgin et al. (2008). *H. taysearae* isolates from all over the world may therefore belong to “latitudinal clades.”

In the present study, the percentage of *S. frugiperda* larvae that died rose with time and in response to an increase in pathogen concentration. Similar findings were also reported by Garcia et al. (2008) as the mortality % of fall armyworm larvae positively correlated with *H. indica* concentration. Also, Ansari et al. (2008) demonstrated that administering EPFs and EPNs simultaneously or at short intervals of two days with other pests can create favorable interactions that are synergistic. The results agree with the outcomes of Degaga and Degaga (2023) showing that FAW larvae could potentially be controlled by 5 fungal isolates found in maize fields and showed mortality rates ranging from the highest (96.67%) and lowest (80.0%). Also, Shinde et al. (2022) evaluated 2 native strains of *H. indica* against larval instars of *S. frugiperda*, and both strains showed great potential in managing *S. frugiperda* under laboratory conditions.

According to Koppenhofer and Grewal (2005), an additive effect occurs when two agents combine to act independently of one another with no interaction, whereas synergistic or antagonistic effects occur when the interaction between the agents makes the combination more or less effective in controlling pests. In the work of Ansari et al. (2008), one of the primary causes of a synergistic interaction was the larvae's initial fungal infection, which made them weaker and prevented them from feeding normally. Additionally, by creating a stressful environment, altering behavior, and releasing more CO<sub>2</sub> in reaction to fungal colonization, the fungus made the host more vulnerable to nematodes.

This work demonstrated that 83% control of *S. frugiperda* 4th instar larvae can be achieved by using *B. bassiana* and *H. taysearae* in combination. Both *B. bassiana* with *H. taysearae* provided a significant control when used alone but efficacy increased in combination. This is in line with the findings of Ansari et al. (2010), who found that combining *M. anisopliae* V275 with *S. kraussei* resulted in 100% control over BVW infesting strawberries in grow bags during the winter, as opposed to a single application. Additionally, in all combined treatments, Ibrahim et al. (2019) demonstrated synergistic interaction between the nematode and fungus species. Shapiro et al. (2004) found that there was antagonism

between the fungi *Paecilomyces fumosus* and *B. bassiana* and the nematodes that caused dual infections of *Curculio caryae* (Coleoptera: Curculionidae). Also, Usman et al. (2020) observed additive interactions when EPNs *S. riobrave* and *S. carpocapsae* were combined with EPFs *M. brunneum* and *I. javanica* when tested on the fruit fly (*Rhagoletis pomonella*).

## Conclusion

The combination of nematodes and fungi applied together in biocontrol provided a promising approach that may outperform ecologically benign means of controlling a variety of insect pests more than single applications. The economic feasibility will always be the deciding factor, although public concern about the environmental effects of conventional insecticides and the emergence of infections resistant to pesticides may encourage it.

## Abbreviations

EPF	Entomopathogenic fungi
FAW	<i>Spodoptera frugiperda</i> : <i>S. frugiperda</i>
<i>B. bassiana</i>	<i>Beauveria bassiana</i>
<i>H. taylorae</i>	<i>Heterorhabditis taylorae</i>

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## Author contributions

E. K. participated in the work and manuscript writing. A. A. participated in the work and manuscript writing. G.K. participated in the work and manuscript writing. All authors read and approved the final manuscript.

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## Availability of data and materials

All datasets are presented in the main manuscript.

## Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing Interests

On behalf of all authors, the corresponding author states that there is no competing interests.

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