

**Effect of entomopathogenic nematode and fungi on mortality and development of *Spodoptera frugiperda* (J.E. Smith) larvae**

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**Running title:** Biological control on fall armyworm

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**ABSTRACT**

The present study was conducted to evaluate the efficacy of entomopathogenic nematode, *Steinernema carpocapsae* Weiser and three entomopathogenic fungi (*Metarhizium anisopliae* (Metschn.) Sorokin, *Trichoderma harzianum* Rifai, and *T. viride* Pers.) against *Spodoptera frugiperda* (J.E. Smith) second and fourth larval instars. The results showed that *S. carpocapsae* caused a pronounced mortality to second and fourth larval instars of *S. frugiperda* using a leaf dipping method at 4 days post inoculation (dpi) with LC<sub>50</sub> values 52.03 and 4.11 infective juveniles (IJs)/ml, respectively. On the other hand, the three tested entomopathogenic fungi caused a strong toxicity on larval instars of *S. frugiperda*. The fungus, *T. harzianum*, displayed the highest insecticidal activity on second larval instar (LC<sub>50</sub>=1.1×10<sup>7</sup> spores/ml) and *M. anisopliae* on fourth larval instar (LC<sub>50</sub>=1.5×10<sup>7</sup> spores/ml) after 10 dpi. Our results showed that *S. carpocapsae* completely inhibited pupation and adult emergence from treated larvae at 250 IJs/ml. The lethal effect of entomopathogenic nematode and fungi against *S. frugiperda* larval instars indicates that these biological control agents could be useful candidates in integrated pest management programs for this invasive insect.

**Keywords:** Fall armyworm; *Steinernema carpocapsae*; *Metarhizium anisopliae*; *Trichoderma harzianum*.

## INTRODUCTION

36  
37 The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is the main  
38 insect pest of many field crops (80 host plants) such as maize, sugarcane, rice, cotton, and other  
39 crops (Murúa *et al.*, 2006; FAO, 2019). The larvae induce huge damages on epidermal leaf  
40 tissue and cause holes in plant leaves, which is the typical damage of this insect pest. However,  
41 large larvae of *S. frugiperda* consume foliage. Larvae may cause death to young plants after  
42 feeding on maize crops (Prasanna *et al.*, 2018; CABI, 2020). This insect pest is classified as an  
43 invasive pest on maize fields in several regions of the world particularly the tropical and  
44 subtropical regions of the Americas and most African countries (Rwomushana *et al.*, 2018). In  
45 Egypt, *S. frugiperda* was recorded infesting maize crop in 2019 and 2020 in several  
46 governorates (Dahi *et al.*, 2020; Gamil, 2020; Mohamed *et al.*, 2022). This invasive pest has a  
47 high dispersal ability, and higher fecundity and fertility (Abrahams *et al.*, 2017; Capinera, 2017;  
48 Mohamed, 2022; Al-Ayat *et al.*, 2022). Due to the wide distribution of *S. frugiperda* in Africa,  
49 chemical insecticides have been commonly applied for the control of this insect pest on infested  
50 crops, particularly maize (Tepa-Yotto *et al.*, 2022). However, the frequent use of high  
51 application rates of these substances is associated with serious problems, such as increased  
52 resistance of insects and detrimental effects on environments, animals, and humans (Yu, 1991;  
53 Prasanna *et al.*, 2018). Thus, alternative strategies have been examined and used for  
54 management of *S. frugiperda*, such as entomopathogenic nematodes and fungi, pheromone  
55 traps, and parasitoids (Mendez *et al.*, 2002; Gutierrez-Martinez *et al.*, 2012; Varshney *et al.*,  
56 2021; Mohamed and Shairra, 2023). Many studies reported the efficacy of biological control  
57 agents such as entomopathogenic bacteria, fungi, viruses, and microbial-derived insecticides on  
58 larvae of *S. frugiperda* (Polanczyk *et al.*, 2000; Molina-Ochoa *et al.*, 2003; Ríos-Velasco *et al.*,  
59 2010; Deshmukh *et al.*, 2020; Kulye *et al.*, 2021).

60 Entomopathogenic nematodes and fungi are important tools in the integrated pest  
61 management (IPM) systems of many insect pests (Brower *et al.*, 1996; Ramanujam *et al.*, 2020).  
62 These methods could be favorable alternatives to synthetic insecticides for the control of this  
63 insect pest owing to their several advantages, such as less risk to the environment and relative  
64 safety for humans as well as an absence of toxic residues in the field crops (Uma Devi *et al.*,  
65 2008). Recently, there has been a growing interest in the application of biological control agents  
66 in the management strategies of *S. frugiperda* (Herlinda *et al.*, 2021; Chen *et al.*, 2022; Idrees  
67 *et al.*, 2023; Mohamed and Shairra, 2023). However, little information is available on the

68 effectiveness of entomopathogenic **nematode and fungi** against *S. frugiperda* in Egypt and no  
69 reports on efficacy of *Trichoderma* spp. on this insect pest. Therefore, we focus our present  
70 study on examining the susceptibility of second and fourth larval instars of *S. frugiperda* to  
71 **entomopathogenic nematode, *Steinernema carpocapsae* Weiser, and three entomopathogenic**  
72 **fungi (*Metarhizium anisopliae* (Metschn.) Sorokin, *Trichoderma harzianum* Rifai, and *T. viride***  
73 **Pers.** Also, the latent effects of entomopathogenic **nematode and fungi** on pupation, adult  
74 emergence and survival were assessed.

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76

## MATERIALS AND METHODS

### **Insect rearing**

78 *S. frugiperda* larvae collected from infested plants of maize fields in Ash Sharqia Governorate,  
79 Egypt. The insect samples were transferred to Plant Protection Research Institute, Agricultural  
80 Research Center (ARC), Giza, Egypt for confirming the pest identification based on the  
81 distinctive *S. frugiperda* morphological characteristics such as the inverted “Y” shape in the  
82 head capsule of larvae, a-four black spot forming a square in the 8<sup>th</sup> abdominal segment, and a  
83 trapezoidal pattern of four spots in the 1–7<sup>th</sup> and the 9<sup>th</sup> abdominal segments of *S. frugiperda*  
84 larvae (Passoa, 1991; CABI, 2019; Mohamed *et al.*, 2022). Healthy male and female adults  
85 were selected and allowed to mate and lay eggs in plastic containers. The neonate larvae were  
86 fed on fresh castor bean leaves, *Ricinus communis* L., at insect rearing laboratory, plant  
87 protection Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt under  
88 conditions (28±1°C, 65±5% relative humidity (RH) and 12:12 h of light and dark). The use of  
89 plant materials in the current study complies with international, national and/or institutional  
90 guidelines (FAO, 2018; Al-Ayat *et al.*, 2022).

### **Entomopathogenic nematode**

92 The entomopathogenic nematode, *Steinernema carpocapsae* (All) (Rhabditida:  
93 Steinernematidae) obtained from Biosys Palo Alto, CA (USA) by Dr. Ahmed Azazy. Who  
94 maintained and reared this strain for several years in Plant Protection Research Institute,  
95 Agricultural Research Center (ARC), Giza, Egypt (Azazy *et al.*, 2018). We supplied this strain  
96 from him and we were reared *S. carpocapsae* through larvae of *Galleria mellonella* under  
97 conditions according to Hussein and El-Mahdi (2020). The infective juveniles were transferred  
98 into Erlenmeyer flasks (500 ml) with 150 ml distilled water and stored at 14°C till needed.  
99 Flasks were shaken weekly to improve aeration and survival of infective juveniles (IJs). These

100 IJs were used within the first three weeks after emerging and harvested from White's traps  
101 (Kaya and Stock, 1997). Freshly emerged IJs were kept at least 5 h at room temperature before  
102 usage in the experiments (Mohamed and Shairra, 2023). Water suspensions of *S. carpocapsae*  
103 were prepared at four concentrations (25, 125, 250, and 500 IJs /ml).

#### 104 **Cultures of fungal strains**

##### 105 ***Isolation of Trichoderma spp.***

106 Isolation of two strains of *Trichoderma* spp. (*T. harzianum* and *T. viride*) from Egyptian soil  
107 was done by serial dilution technique (Naher *et al.*, 2019). Ten grams of soil samples were  
108 mixed with 100 ml of sterile distilled water and then mixture was shaken at 100 rpm for 10 min.  
109 using a rotary shaker. Consequently, soil suspension was subjected to serial dilution to isolate  
110 the colonies of *Trichoderma* spp. From each of dilution, 1 ml of the suspension was taken using  
111 a micropipette and transferred into sterilized Petri plates containing Rose Bengal Agar (RBA)  
112 medium (Khang *et al.*, 2013) and incubated at 25±2°C for 5-7 days. The incubated plates were  
113 checked daily, and the fungal colonies were marked and purified on potato dextrose agar (PDA)  
114 medium. Pure cultures were stored on PDA slants at 4 °C in a refrigerator for further use. The  
115 two strains of *Trichoderma* spp. were identified based on their morphological properties  
116 (conidiophore branching patterns, phialide arrangement, and conidia shape and size) (Gams and  
117 Bissett, 1998; Kumar and Sharma, 2011) and molecularly by using ITS-PCR amplification of  
118 the DNA extracted from fungal isolates. The PCR amplification was performed in a total  
119 volume of 50 µl, containing 25 µl Master Mix (sigma), 3 µl of each primer (10 pmol/µl), ITS-  
120 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'),  
121 and 3 µl template DNA (10 ng/µl) and 16 µl dH<sub>2</sub>O. PCR amplification was performed in a  
122 Perkin-Elmer/ GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill  
123 40 cycles after an initial denaturation cycle for 5min at 94 °C. Each cycle consisted of a  
124 denaturation step at 94 °C for 30 s, an annealing step at 45 °C for 30 s, and an elongation step  
125 at 72 °C for 1min. The primer extension segment was extended to 7min at 72 °C in the final  
126 cycle (Abdelgaleil *et al.*, 2023). DNA sequences of *T. harzianum* (OR366537.1) and *T. viride*  
127 (OR366542.1) were submitted in the National Center for Biotechnology Information (NCBI).

##### 128 ***Isolation of M. anisopliae***

129 A strain of the *M. anisopliae* fungus was originally isolated from a naturally infected white  
130 grubs, *Pentodon bispinosus* Kuster, larvae collected from golf playground, Katameya, Cairo,  
131 Egypt. The dead larva was surface-sterilized using a sodium hypochlorite (2% v/v). Then, the

132 larvae were rinsed twice with sterile distilled water and dried between folds of sterilized filter  
133 paper. Surface sterilized larvae were placed on Petri plates of PDA supplemented with  
134 streptomycin sulfate at 100 µg ml<sup>-1</sup> and incubated at 25±2°C (Ayala-Zermeño *et al.*, 2015).  
135 After emergence of fungal hyphae and sporulation, they were sub-cultured by transferring onto  
136 a new PDA plate and incubated at 25±2°C for 15 days. Pure cultures were stored on PDA slants  
137 at 4 °C in a refrigerator for further use. This fungus was identified using molecular techniques  
138 (Abdelgaleil *et al.*, 2023) with accession number OR366543.1 submitted in NCBI.

### 139 **Preparation of spore suspension**

140 The fungal spores were collected from the surface of growing cultures on PDA medium after 7  
141 and 15 days for *Trichoderma* spp. and *M. anisopliae*, respectively. Ten ml of 0.01% (v/v)  
142 Tween-80 solution in sterile distilled water was added to the surface of a Petri plate. The surface  
143 of the medium was then rubbed with a glass rod and the spore suspension was transferred to a  
144 sterile glass vial (50 ml). The spore suspension was vortexed for 5 min and passed through a  
145 layer of sterilized cheese-cloth. The concentrations of spore suspension were calculated using  
146 a haemocytometer and adjusted to 1.0×10<sup>5</sup>, 1.0×10<sup>6</sup>, 1.0×10<sup>7</sup>, and 1.0×10<sup>8</sup> spores/ml for  
147 bioassay experiments.

148

### 149 **Bioassays**

150 The leaf dipping method was conducted according to IRAC method (IRAC, 2018). The stocks  
151 of IJs of *S. carpocapsae* and three strains of fungi were prepared in distilled water. Toxicity of  
152 *S. carpocapsae* was assessed at 25, 125, 250, and 500 IJs /ml. The concentrations of three fungi  
153 were tested at 1.0×10<sup>5</sup>, 1.0×10<sup>6</sup>, 1.0×10<sup>7</sup>, and 1.0×10<sup>8</sup> spores/ml. The castor bean leaves were  
154 cut into small pieces (4 × 4 cm). The leaf sections were dipped for five seconds in each  
155 concentration and then left to complete water evaporation. Three treated pieces were transferred  
156 to each plastic cup (8 cm diameter × 5 cm high). Five newly molted second or fourth instar  
157 larvae were introduced to each cup. The cups were covered with cheese cloth and kept under  
158 the same insect rearing conditions. Four replicates were used in each tested concentration. An  
159 additional series of castor bean leaves were treated with distilled water alone served as control.  
160 Mortality percentages were recorded after 2, 3, and 4 days of treatment with *S. carpocapsae*  
161 and 5, 7 and 10 days for three fungal strains because no mortality was recorded in the first four  
162 days after treatment. On the other hand, the treated larvae with the entomopathogenic nematode

163 and fungi were examined daily until complete pupation and adult emergence. **Percent** pupation  
164 and adult emergence were calculated as following **formula** (Korrat *et al.*, 2019):

165 Pupation (%) = (Number of pupae /Total number of larvae) ×100

166 Adult emergence (%) = (Number of moths /Total number of pupae) ×100

167 Survival (%) = (Number of moths /Total number of larvae) ×100

### 168 **Statistical analysis**

169 Mortality percentages were corrected using Abbott's formula (1925). To estimate the LC<sub>50</sub>  
170 value, the corrected mortality percentages were subjected to probit analysis **using LdP-Line®**  
171 **software** according to Finney (1971). **Pupation** and adult emergence were **analyzed using** one-  
172 way analysis of variance (ANOVA). Mean separations were performed by Tukey's HSD test at  
173 a significance level < 0.05. Statistical analysis was conducted using the software SPSS 21.0  
174 (SPSS, Chicago, IL, USA).

## 175 **RESULTS**

### 176 **Toxicity of entomopathogenic nematode, *S. carpocapsae* against *S. frugiperda***

177 The LC<sub>50</sub> values of *S. carpocapsae* against *S. frugiperda* second and fourth instars larvae after  
178 2, 3, and 4 days post-inoculation (dpi) are presented in Table 1. The entomopathogenic  
179 nematode, *S. carpocapsae* showed variable insecticidal activity **with higher toxicity at**  
180 **increasing concentration** and exposure time. *S. carpocapsae* revealed obvious toxicity after 2  
181 dpi as their LC<sub>50</sub> values were 175.26 and 24.60 IJs/ml, for second and fourth instars larvae,  
182 respectively. The toxicity of *S. carpocapsae* increased significantly after 3 and 4 days of  
183 exposure. The LC<sub>50</sub> values were (84.54 and 19.47 IJs/ml) for second and fourth instars larvae,  
184 respectively, after 3 days, while after 4 days, the LC<sub>50</sub> values decreased 52.03 and 4.11 IJs/ml  
185 for second and fourth instars larvae, respectively.

### 186 **Toxicity of the three fungal strains against *S. frugiperda***

187 Toxicity of the three fungal strains against second and fourth larval instars of *S. frugiperda* after  
188 5, 7 and 10 days of exposure expressed as LC<sub>50</sub> values are summarized in Tables 2 and 3. It was  
189 clear that the three fungal strains possessed strong toxicity against *S. frugiperda* larvae. The  
190 fungus, *T. harzianum*, displayed the highest insecticidal activity on second larval instar with  
191 LC<sub>50</sub> values of 5.1×10<sup>7</sup> and 1.1×10<sup>7</sup> spores/ml after 7 and 10 dpi, **respectively. On the other**  
192 **hand, *M. anisopliae* had** LC<sub>50</sub> values of 4.6×10<sup>8</sup> and 6.1×10<sup>7</sup> spores/ml after 7 and 10 dpi,  
193 **respectively.** While the fungus, *M. anisopliae* was highly effective on fourth larval instar after  
194 7 and 10 **dpi** as their LC<sub>50</sub> values 2.5×10<sup>7</sup> and 1.5×10<sup>7</sup> spores/ml, followed by *T. viride* with

195 LC<sub>50</sub> values of  $3.3 \times 10^8$  and  $1.8 \times 10^8$  spores/ml after 7 and 10 dpi, respectively (Table 3). The  
196 highest mortality was achieved by the highest concentration ( $1.0 \times 10^8$  spores/ml) of *T.*  
197 *harzianum*, *T. viride* and *M. anisopliae* was 81.25, 62.50, and 43.75% for second instar larvae,  
198 respectively. **The mortality decreased in fourth instar larvae (50.0, 37.50, and 31.25%) with the**  
199 **same concentration ( $1.0 \times 10^8$  spores/ml) of *M. anisopliae*, *T. viride*, and *T. harzianum*,**  
200 **respectively.**

#### 201 **Latent effects of biological control agents on *S. frugiperda***

202 The **entomopathogenic** nematode and fungi influenced **the pupation**, adult emergence, and  
203 survival **of *S. frugiperda*** (Tables 4 to 6). The **growth and development** of treated larvae  
204 decreased significantly with increasing concentrations of tested biological control agents  
205 compared to untreated second instar larvae (91.3, 93.2, and 85.0%) and fourth instar larvae  
206 (88.7, 94.9, and 84.1%), respectively. **All second and fourth instars larvae treated with *S.***  
207 ***carpocapsae* succumbed to nematode infection particularly at high concentrations and the full**  
208 **mortality (100%) was achieved at 250 and 500 IJs/ml for two tested larval instars and these**  
209 **concentrations were enough to induce complete suppression of pupation, adult emergence, and**  
210 **survival.** Also, **the three tested** fungi significantly decreased the pupation, adult emergence, and  
211 survival percentages with increasing **fungal** concentrations. The highest suppression of  
212 pupation of *S. frugiperda* was achieved by the highest concentration of  $1.0 \times 10^8$  spores/ml of *T.*  
213 *harzianum* (20.0 and 60.0%), *T. viride* (37.5 and 50.0%) and *M. anisopliae* (45.0 and 40.0%)  
214 from treated second and fourth instars larvae, respectively. **Adult emergence was not affected**  
215 **by *M. anisopliae* and *T. harzianum*.** The highest inhibition of adult emergence was obtained by  
216  $1.0 \times 10^8$  spores/ml of *T. viride* (34.2 and 50.8%) from treated second and fourth instars larvae,  
217 respectively. Also, the highest suppression larval survival percentage achieved by the highest  
218 concentration of  $1.0 \times 10^8$  spores/ml of *T. harzianum* (10.0 and 50.0%), *T. viride* (12.5 and  
219 30.0%), and *M. anisopliae* (35.0 and 37.5%) from treated second and fourth instars larvae,  
220 **respectively.**

## 221 **DISCUSSION**

222 The insecticidal effects of entomopathogenic nematodes and fungi have been reported against  
223 *S. frugiperda* strains present in some countries around the world (Idrees *et al.*, 2023; Mohamed  
224 and Shairra, 2023). Our results showed that entomopathogenic nematode, *S. carpocapsae*,  
225 caused remarkable mortality on second and fourth larval instars of *S. frugiperda* at 4 dpi. The  
226 higher toxicity of *S. carpocapsae* observed in this study is matched with previous reports

227 indicated that *S. carpocapsae* was very toxic against larval instars of *S. frugiperda* (Acharya *et*  
228 *al.*, 2020; Fallet *et al.*, 2022; Sayed *et al.*, 2022). Guo *et al.* (2023) reported that *S. carpocapsae*  
229 at concentrations ranging between  $31.67 \pm 1.97$  and  $59.25 \pm 6.06$  IJs/mg caused complete  
230 mortality (100%) of *S. frugiperda* larvae. Mohamed and Shairra (2023) showed that *S.*  
231 *carpocapsae* was more virulent than the other nematode, *Heterorhabditis indica* (EGAZ2) and  
232 effective against all larval instars and complete mortality was obtained after 48–72 h of  
233 exposure at concentrations of 150–2400 IJs/larva. Generally, *S. carpocapsae* infection was  
234 faster and has higher efficacy on larval instars of *S. frugiperda* than the tested fungi. The  
235 enhanced effectiveness of the nematode could be attributed to its mutualistic relationship with  
236 *Xenorhabdus nematophila*, a species of enteric bacteria (Stilwell *et al.*, 2018). The bacterial  
237 symbiont is carried in a bacterial pouch by the non-feeding resistant stage known as IJs. When  
238 the IJs locate a host that is susceptible to them, they enter the insect through one of its natural  
239 openings (the mouth, spiracles, or anus) and hemocoel, and subsequently release the symbiotic  
240 bacteria. Septicemia is caused by the bacterial cells growing in the hemocoel and killing the  
241 host in less than 48 h. The nematodes consume the host tissues that the symbiotic bacteria have  
242 broken down (Hazir *et al.*, 2003; Hussein, 2022).

243 The three tested entomopathogenic fungi caused strong toxicity on larval instars of *S.*  
244 *frugiperda*. The fungus, *T. harzianum* displayed the highest insecticidal activity on second  
245 larval instar and *M. anisopliae* on fourth larval instar after 10 dpi. However, this is the first  
246 study on the toxicity of entomopathogenic fungi, *T. harzianum* and *T. viride* against *S.*  
247 *frugiperda* in Egypt. Similar results were obtained by Ramanujam *et al.* (2020) on the toxicity  
248 of fungi, *M. anisopliae* and *Beauveria bassiana* (Balsamo) Vuillemin against second instar  
249 larvae of *S. frugiperda* ( $LC_{50} = 1.1 \times 10^7$  and  $1.9 \times 10^7$  spores/ml), respectively. Also, our results  
250 are supported by Garcia *et al.* (2011) who found that the concentration ( $1 \times 10^9$  conidia/ml) of *B.*  
251 *bassiana* induced 96.6% mortality of the second instar larvae of *S. frugiperda*. Morales-Reyes  
252 *et al.* (2013) showed that *M. anisopliae* and *B. bassiana* caused mortality ranging between 10  
253 to 65% in second instar larvae of *S. frugiperda* at two concentrations ( $1 \times 10^6$  and  $1 \times 10^7$   
254 conidia/ml) (Ramanujam *et al.*, 2020). Our results showed a potential toxicity of *S. frugiperda*  
255 by *T. harzianum* and *T. virens* and no previous reports described efficacy of these fungi on  
256 larval instars of *S. frugiperda*. Our findings showed that the tested fungi may be beneficial in  
257 the biological control of *S. frugiperda* due to their capacity to infiltrate insect tissues by  
258 penetrating the cuticle directly, parasitize the insect bodies, and use the host insects as a source

259 of nourishment for the development of new conidia (Skinner *et al.*, 2014). Insecticidal  
260 secondary metabolites produced by fungi, such as in *T. harzianum*, which may produce  
261 peptaibols and induce significant insect mortality rates, are another possible product of fungal  
262 strains (Charnley and Collins, 2007; Rahim and Iqbal, 2019). Furthermore, *T. viride* has a  
263 potential for producing compounds that may have antifeeding qualities against several kinds of  
264 insect pests (Vijayakumar and Alagar, 2017).

265 Also, the second instar larvae were more susceptible to biological control agents than  
266 fourth instar larvae. The tested findings conform with Fallet *et al.* (2022) who observed that *S.*  
267 *carpocapsae* caused rapid and complete mortality in second and third larval instars of *S.*  
268 *frugiperda* but the rate decreased in six instar larvae to 75% (Sayed *et al.*, 2022; Acharya *et al.*,  
269 2020). According to Elbrense *et al.* (2021), the differences in the vulnerability and death rates  
270 among larvae in developmental instars may ultimately be connected to their morphological  
271 features, sizes, behaviours, and immunological defense systems. Besides, the reproduction rate  
272 of *S. carpocapsae* has been influenced directly by different developmental stages of the host  
273 insects (Park *et al.*, 2001). Besides their effects on larval mortality, the tested biological control  
274 agents induced significant reduction in pupae and adults as well as reduced adult emergence  
275 particularly *S. carpocapsae* which caused complete inhibition of pupation and adult formation  
276 emergence of *S. frugiperda*. These findings conform with the results of previous studies on the  
277 activity of biological agents against *S. frugiperda* (Park *et al.*, 2001; Acharya *et al.*, 2020; Liu  
278 *et al.*, 2022). The inhibition on the growth and development of *S. frugiperda* by biological  
279 control agents observed is due to their inhibitory effects on insect development (Korrat *et al.*,  
280 2019; Idrees *et al.*, 2023).

## 281 CONCLUSION

282 The biological control agents including entomopathogenic nematode, *S. carpocapsae*, and fungi  
283 (*Trichoderma* spp. and *M. anisopliae*) could be potentially applied for the control of *S.*  
284 *frugiperda* larval instars. Therefore, these biological control agents may be useful for the  
285 management of this invasive insect and should be implemented in IPM programs. The use of  
286 biological control agents with different modes of action is highly important to delay the  
287 development of insect resistance. Also, the uses of ecofriendly products have less impact on  
288 non-target organisms, mammals, and the environment. The efficacy of these biological control  
289 agent and their effects on non-target organisms should be evaluated under field conditions.

290 **Disclosure statement**

291 The authors declare no potential conflict of interest.

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508 **Table 1.** Toxicity of entomopathogenic nematode, *Steinernema carpocapsae* against second  
 509 and fourth larval instars of *Spodoptera frugiperda* at different concentrations (infective  
 510 juveniles, IJs/ml) after 2, 3, and 4 days post inoculation.

Larval instars	Exposure time (days)	LC <sub>50</sub> <sup>a</sup> (IJs/ml)	95% confidence limits (IJs/ml)		Slope <sup>b</sup> ± SE	(χ <sup>2</sup> ) <sup>c</sup>	P <sup>d</sup>
			Lower	Upper			
Second instar	2	175.26	103.52	429.94	1.58± 0.12	24.29	0.000
	3	84.54	46.68	197.88	1.94±0.12	56.27	0.000
	4	52.03	27.97	120.71	2.25±0.13	46.49	0.000
Fourth instar	2	24.60	13.53	36.49	1.15± 0.15	2.57	0.277
	3	19.47	7.88	46.72	1.67± 0.11	16.39	0.000
	4	4.11	0.21	11.37	0.89± 0.23	1.26	0.261

511 <sup>a</sup>The concentration causing 50 % mortality.

512 <sup>b</sup>Slope of the concentration-mortality regression line ± standard error.

513 <sup>c</sup>Chi square value.

514 <sup>d</sup>Probability value.

515

516 **Table 2.** Comparative toxicity of three entomopathogenic fungi against second instar larvae of  
 517 *Spodoptera frugiperda* after 5, 7, and 10 days post inoculation.

Fungal strains	Exposure time (days)	LC <sub>50</sub> <sup>a</sup> (spores/ml)	95% confidence limits (spores/ml)		Slope <sup>b</sup> ± SE	(χ <sup>2</sup> ) <sup>c</sup>	P <sup>d</sup>
			Lower	Upper			
<i>Metarhizium anisopliae</i>	5	2.0x10 <sup>9</sup>	2.4x10 <sup>8</sup>	1.1x10 <sup>11</sup>	0.23±0.04	2.63	0.452
	7	4.6x10 <sup>8</sup>	6.4x10 <sup>7</sup>	1.2x10 <sup>11</sup>	0.22±0.06	0.47	0.789
	10	6.1x10 <sup>7</sup>	1.2x10 <sup>7</sup>	8.6x10 <sup>9</sup>	0.26± 0.03	9.97	0.041
<i>Trichoderma harzianum</i>	5	6.0x10 <sup>9</sup>	5.2x10 <sup>8</sup>	1.1x10 <sup>12</sup>	0.24±0.05	2.12	0.547
	7	5.1x10 <sup>7</sup>	1.1x10 <sup>7</sup>	1.7x10 <sup>9</sup>	0.22±0.06	0.48	0.785
	10	1.1x10 <sup>7</sup>	3.0x10 <sup>6</sup>	1.1x10 <sup>8</sup>	0.22± 0.06	1.04	0.593
<i>Trichoderma viride</i>	5	3.8x10 <sup>10</sup>	2.6x10 <sup>8</sup>	4.9x10 <sup>12</sup>	0.05± 0.02	0.18	0.996
	7	4.6x10 <sup>9</sup>	2.3x10 <sup>8</sup>	8.3x10 <sup>11</sup>	0.13±0.02	0.62	0.891
	10	5.3x10 <sup>8</sup>	1.5x10 <sup>8</sup>	5.7x10 <sup>9</sup>	0.42±0.07	3.47	0.177

518 <sup>a</sup>The concentration causing 50 % mortality.

519 <sup>b</sup>Slope of the concentration-mortality regression line ± standard error.

520 <sup>c</sup>Chi square value.

521 <sup>d</sup>Probability value.

522

523 **Table 3.** Comparative toxicity of three entomopathogenic fungi against fourth instar larvae of  
 524 *Spodoptera frugiperda* after 5, 7, and 10 days post inoculation.

Fungal strains	Exposure time (days)	LC <sub>50</sub> <sup>a</sup> (spores/ml)	95% confidence limits (spores/ml)		Slope <sup>b</sup> ± SE	(χ <sup>2</sup> ) <sup>c</sup>	P <sup>d</sup>
			Lower	Upper			
<i>Metarhizium anisopliae</i>	5	3.6x10 <sup>7</sup>	1.6x10 <sup>7</sup>	1.1x10 <sup>8</sup>	0.43±0.06	3.81	0.149
	7	2.5x10 <sup>7</sup>	1.1x10 <sup>7</sup>	7.5x10 <sup>7</sup>	0.42±0.06	0.95	0.622
	10	1.5x10 <sup>7</sup>	8.4x10 <sup>6</sup>	4.3x10 <sup>7</sup>	0.80±0.12	0.89	0.344
<i>Trichoderma harzianum</i>	5	2.2x10 <sup>13</sup>	2.6x10 <sup>12</sup>	4.1x10 <sup>14</sup>	0.19±0.19	2.84	0.584
	7	1.0x10 <sup>9</sup>	1.5x10 <sup>8</sup>	1.1x10 <sup>11</sup>	0.27±0.06	1.39	0.497
	10	3.4x10 <sup>8</sup>	1.2x10 <sup>8</sup>	2.1x10 <sup>9</sup>	0.50±0.09	0.84	0.358
<i>Trichoderma viride</i>	5	1.3x10 <sup>10</sup>	1.2x10 <sup>9</sup>	4.8x10 <sup>12</sup>	0.39±0.09	0.33	0.847
	7	3.3x10 <sup>8</sup>	8.2x10 <sup>7</sup>	4.9x10 <sup>9</sup>	0.34±0.06	3.13	0.209
	10	1.8x10 <sup>8</sup>	5.5x10 <sup>7</sup>	1.4x10 <sup>9</sup>	0.37±0.06	2.92	0.232

525 <sup>a</sup>The concentration causing 50 % mortality.

526 <sup>b</sup>Slope of the concentration-mortality regression line ± standard error.

527 <sup>c</sup>Chi square value.

528 <sup>d</sup>Probability value

529 **Table 4.** Latent effects of entomopathogenic nematode, *Steinernema carpocapsae* on pupation,  
 530 adult emergence and survival of *Spodoptera frugiperda*.

Larval instar	Concentration (infective juveniles, IJs/ml)	Pupation %	Adult emergence %	Survival %
Second instar larvae	0.0	91.3±2.1a	93.2±2.0a	85.0±1.0a
	25	42.5±6.3b	22.9±7.8b	10.0±4.6b
	125	30.0±5.7b	12.5±7.2b	2.5±1.4b
	250	0.0±0.0c	0.0±0.0b	0.0±0.0b
	500	0.0±0.0c	0.0±0.0b	0.0±0.0b
	F	163.7	16.1	60.4
	P	<0.01	<0.01	<0.01
Fourth instar larvae	0.0	88.7±9.0a	94.9±4.8a	84.1±8.5a
	25	35.0±2.9b	37.5±6.0ab	10.0±4.5b
	125	20.0±2.0c	25.0±6.1ab	5.0±2.5b
	250	0.0±0.0d	0.0±0.0b	0.0±0.0b
	500	0.0±0.0d	0.0±0.0b	0.0±0.0b
	F	540.5	5.2	211.1
	P	<0.01	<0.01	<0.01

531 Values in columns within each compound followed by the different letters are significantly different at Tukey's  
 532 HSD (P < 0.05, df=4,15).

533

534 **Table 5.** Latent effects of three entomopathogenic fungi on pupation and adult emergence of  
 535 second instar larvae of *Spodoptera frugiperda*.

Fungal strains	Concentration (spores/ml)	Pupation (%)	Adult emergence (%)	Survival (%)
<i>Metarhizium anisopliae</i>	0.0	91.3±2.1a	93.2±2.0a	85.0±1.0a
	1.0×10 <sup>5</sup>	70.0±4.0b	81.8±7.2a	57.5±6.3b
	1.0×10 <sup>6</sup>	67.5±4.8bc	74.1±3.5a	50.0±4.0bc
	1.0×10 <sup>7</sup>	50.0±4.1cd	81.7±6.8a	40.0±4.3c
	1.0×10 <sup>8</sup>	45.0±5.0d	79.2±7.2a	35.0±2.9c
	F	18.5	1.1	28.5
	P	<0.01	0.432	<0.01
<i>Trichoderma harzianum</i>	0.0	91.3±2.1a	93.2±2.0a	85.0±1.0a
	1.0×10 <sup>5</sup>	57.5±8.5b	93.8±6.2a	52.5±4.8b
	1.0×10 <sup>6</sup>	52.5±7.5b	91.7±8.3a	47.5±7.5b
	1.0×10 <sup>7</sup>	45.0±3.2b	55.0±2.8a	25.0±2.9b
	1.0×10 <sup>8</sup>	20.0±2.0c	50.0±6.1a	10.0±4.1b
	F	23.5	2.2	21.4
	P	<0.01	0.126	<0.01
<i>Trichoderma viride</i>	0.0	91.3±2.1a	93.2±2.0a	85.0±1.0a
	1.0×10 <sup>5</sup>	58.0±8.3b	95.8±4.2a	55.0±5.0ab
	1.0×10 <sup>6</sup>	60.0±4.7b	66.7±4.1ab	40.0±2.0bc
	1.0×10 <sup>7</sup>	53.0±2.5b	37.5±6.2b	17.5±1.4cd
	1.0×10 <sup>8</sup>	37.5±3.0b	34.2±8.2b	12.5±4.3d
	F	13.8	7.1	16.6
	P	<0.01	<0.01	<0.01

536 Values in columns within each compound followed by the different letters are significantly different at Tukey's  
 537 HSD (P < 0.05, df=4, 15).

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541 **Table 6.** Latent effects of three entomopathogenic fungi on pupation and adult emergence of  
 542 fourth instar larvae of *Spodoptera frugiperda*.

Fungal strain	Concentration (spores/ml)	Pupation (%)	Adult emergence (%)	Survival (%)
<i>Metarhizium anisopliae</i>	0.0	88.7±9.0a	94.9±4.8a	84.1±8.5a
	1.0×10 <sup>5</sup>	80.0±4.6ab	93.8±6.3a	75.0±5.0ab
	1.0×10 <sup>6</sup>	75.0±2.8b	83.9±5.9a	62.5±2.5b
	1.0×10 <sup>7</sup>	50.0±4.0c	91.6±8.3a	45.0±3.0c
	1.0×10 <sup>8</sup>	40.0±2.0c	93.8±6.0a	37.5±2.5c
	F	60.8	0.54	35.8
	P	<0.01	0.709	<0.01
<i>Trichoderma harzianum</i>	0.0	88.7±9.0a	94.9±4.8a	84.1±8.5a
	1.0×10 <sup>5</sup>	80.0±8.2a	95.0±5.0a	75.0±5.0ab
	1.0×10 <sup>6</sup>	75.0±9.5a	91.7±8.3a	70.0±8.1ab
	1.0×10 <sup>7</sup>	75.0±3.0a	71.7±5.0a	50.0±5.7b
	1.0×10 <sup>8</sup>	60.0±6.1a	83.3±9.6a	50.0±5.8b
	F	0.9	1.20	4.46
	P	0.486	0.358	0.014
<i>Trichoderma viride</i>	0.0	88.7±9.0a	94.9±4.8a	84.1±8.5a
	1.0×10 <sup>5</sup>	80.0±7.1ab	87.5±7.2a	70.0±6.7a
	1.0×10 <sup>6</sup>	75.0±5.0abc	62.5±6.3ab	35.0±5.0b
	1.0×10 <sup>7</sup>	55.0±5.0bc	58.3±4.2b	35.0±2.9b
	1.0×10 <sup>8</sup>	50.0±4.c	50.8±4.8b	30.0±4.0b
	F	6.2	6.9	8.2
	P	<0.01	<0.01	<0.01

543 Values in columns within each compound followed by the different letters are significantly different at Tukey's  
 544 HSD (P < 0.05, df=4, 15).