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Potential Use of *Fusarium* Isolates as Biological Control Agents: *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) Case Study

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Abstract: The cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a notorious agricultural pest with world-wide distribution, extreme polyphagy, high mobility and fecundity, facultative diapause, and significant resistance to chemical insecticides. Isolates from various *Fusarium* species were collected from soil, identified, and tested for their entomopathogenicity against *H. armigera* larvae in field experiments. Fungi of the genus *Fusarium* are ubiquitous and include phytopathogenic as well as entomopathogenic strains. Seven *Fusarium* species were identified and tested, including: *F. algeriense*, *F. chlamydosporum* var. *chlamydosporum*, *F. fujikuroi*, *F. longifundum*, *F. pseudoanthophilum*, *F. solani*, and *F. tonkinense*. All the collected fungi demonstrated a notable insecticidal effect on *H. armigera* larvae in field conditions, while some proved to be significantly lethal. The larval mortality of *H. armigera* ranged from 10 (10^3 conidia/mL) to 91% (10^8 conidia/mL) after 9 days (216 h). Larval survival time in treated plants ranged from 95 h (10^8 conidia/mL) to 208 h (10^3 conidia/mL). According to our results, *F. solani* isolate displayed the highest toxicity against *H. armigera* larvae and could be considered as a promising biocontrol agent of this serious pest.

Keywords: entomopathogenic fungi; *Fusarium* species; *Helicoverpa armigera*; tomato; pest management

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1. Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is one of the most harmful agricultural pests [1,2]. It is a cosmopolitan species with presence in Europe, Asia, Africa, and Oceania [3], and it is known as a polyphagous moth feeding on a plethora of important crops such as cotton, tomato, sorghum, chickpea, and others [4]. Eggs are laid on fruits and flowers and hatched larvae feed on plant tissue, causing significant damage [5,6]. Another parameter that ranks *H. armigera* among the most serious crop pests is its extraordinary resistance to synthetic insecticides [7–10].

Entomopathogenic microorganisms such us fungi, bacteria, viruses, and protozoa are among the most promising alternatives to chemical insect control because of their high efficacy and compatibility with other IPM methods [11–14]. Entomopathogenic fungi (EPF) are natural components of the ecosystem with low mammalian toxicity [15,16]. Additionally, they may grow on cadavers reintroducing more inoculum into the system. Due to this, long term residual persistence, a major disadvantage of conventional insecticides, is desirable for EPF [17].

The focus of research concerning *Fusarium* genus is reasonably limited to the phytopathogenic species such as *Fusarium graminearum* Schwabe (anamorph) (Hypocreales: Nectriaceae) and *F. oxysporum* Schlechtendahl (Hypocreales: Nectriaceae) as they cause diseases of great economic importance [18,19]. Among entomopathogenic fungi, *Fusarium* can

parasitize species of many major insect orders such as Coleoptera, Lepidoptera, Hemiptera, and others [20,21]. It has been well documented that they can be effective against various classes of insect pests [20–23]. There are species of *Fusarium* that can synthesize secondary metabolites with insecticidal action (e.g., Beauvericin), which helps the fungus control its target pest [19,24]. Beauvericin is a cyclic hexadepsipeptide and a common EF metabolite with insect toxicity [25–27].

Although the potential effects of the entomopathogenic *Fusarium* species have not been thoroughly studied yet, the fact that *Fusarium* isolates can cause high insect mortality is undoubtful [28]. Additionally, the advantages of EPF on host specificity and safety towards plants, since the ability to parasitize requires specific adaptations to the host, has been well described in the literature. [21,29–31]. The aim of the investigation was to determine the pathogenicity of several *Fusarium* species, isolated from soil on *H. armigera* larvae under field conditions. We also discuss the key issues regarding the use of *Fusarium* species as biocontrol agents against crop pests.

2. Materials and Methods

2.1. Collection, Isolation, and Identification of Fusarium

2.1.1. Sample Collection and Fungal Isolation

Soil samples were collected from Patras Achaia, Greece in 2019. The samples were collected from a depth of 10 cm under the surface soil layer and placed into sealed polyethylene bags after excavation. Seasonally, the samples were collected from 22 points (in total, 176 samples from April 2017 to April 2019) in two suburban green areas in the capital of the prefecture Achaia, Patras. During sampling preparation, the surface litter was removed, and the soil was dug to a depth of 10 cm with a soil core borer. 1000 g from each point were placed in plastic bags and stored at 4 °C, until they were transferred to the laboratory for further processing. After drying the samples with air to avoid possible entomopathogenic nematode (EPN) infestation, as suggested by Quesada-Moraga et al. [32], they were placed on a rough cardboard on the laboratory stalls for 24 h to reduce their humidity. Afterwards, the soil was sieved (Metal, 2 mm × 1 mm, Aggelis Equipment, Athens, Greece) and placed in Sabouraud dextrose agar (SDA) Petri dishes. Each soil sample was tested 10 times; 100 *Sitophilus granarius* L. (Coleoptera: Dryophthoridae) and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) adults were used as insect baits and tested per soil sample. All beetles were transferred to Petri dishes with a layer of SDA. Alternatively, conidia removed from infected beetles were also cultured on the same nutrient agar. To facilitate the incubation and development of the fungi, Petri dishes were maintained at room temperature (25 ± 1 °C) in complete darkness. After fungal development, one more isolation took place to prevent infestation and achieve clear cultivation. The above-mentioned process was carried out inside a laminar flow chamber (Equip Vertical Air Laminar Flow Cabinet Clean Bench, Mechanical Application LTD). The *Fusarium* isolates were then sub-cultured several times on Petri dishes with SDA to ensure purity and monosporic cultures. SDA was amended with streptomycin sulfate and chlor-tetracycline HCl to minimize chances of any bacterial growth. The procedure of DNA sequencing was then used to identify the species.

2.1.2. Morphology of Isolated Fungi

The morphological characteristics of strains were observed by inoculating a fungal mycelial plug (1 cm) on an SDA plate for 10 days. The spore morphology was observed under a phase-contact microscope (100×). Conidial images were taken with an Axio Cam HRC camera. Microscopic features of conidia, conidiophores and chlamydospores were also determined based on Summeral et al. [33].

2.1.3. DNA Extraction, PCR Amplification and Sequence Analysis

All experimental fungal isolates were kept in the laboratory's repository of microorganisms (University of Ioannina). The conidia were scraped from the surface of the plant tissues and from the dead cadavers using a sterile loop, and they were trans-

ferred to potato dextrose agar (in-house technique). The genomic DNA (gDNA) was extracted, adopting the method of Rogers and Bendich [34]. Universal primer sets ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAAC AAGG-3′) were applied and a fragment of the ITS spacer region was expanded. PCR reactions (25 µL) were carried out using Taq 2X Master Mix (M0270) (New England Biolabs GmbH Frankfurt, Germany) and included working concentration of 1X Master Mix, 0.5 µM of each primer and 50 ng of template gDNA. The PCR protocol for amplification of ITS regions included 33 cycles, at 95 °C for 30 s, 55 °C for 40 s, and 68 °C for 1 min, followed by a final elongation at 68 °C for 5 min. PCR products were kept at 4 °C. The quantity and quality of PCR products were determined by gel electrophoresis using 1% agarose gel, which was stained with SYBR Safe DNA Gel Stain (Invitrogen, Waltham, MA, USA) and visualized under UV light (BIO-RAD, Hercules, CA, USA, Molecular Imager Gel Doc XR System).

2.2. Insects

H. armigera larvae were originally collected from biological tomato fields in Kourtesi, Ilia, Greece (37°58′44″ N 21°19′4″ E) and identified stereoscopically. Insect culture was reared in the laboratory on artificial diet (Table 1). In the beginning, the stage 3 vitamins were boiled in distilled water and homogenized using a microwave oven for 1.5 min at 100 °C. Then, the ingredients of stage 1 and stage 2 were mixed separately and blended in a grinder. Ingredients of stage 1 were poured into the ingredients of stage 2 and blended and mixed in the ingredients of stage 3 and then kept at room temperature for 30 min to cool down and stored in the refrigerator (6–8 °C).

Table 1. Artificial substrate of laboratory culture of *H. armigera* (Adapted with permission from Ref. [35]).

Stage 1	Biological yeast powder (60 g), sucrose (60 g), formaldehyde 10% (15 mL), choline chloride 20% (30 mL), distilled water (1200 mL).
Stage 2	Ascorbic acid (12 g), methyl 4 hydroxy benzoate (7.5 g), sorbic acid (4.5 g), streptomycine sulphate (0.1 g), cholesterol (0.6 g), wheat germ oil (0.6 mL) and vitamin mixture (0.6 g). Section 3 includes agar (45 g) and distilled water (1000 mL).
Stage 3 (Vitamin Mixture)	Micotineacitamide (9.30 g), riboflavin (4.64 g), pyridoxine hydrochloride (2.32 g), biotin (0.18 g), vitamin B12 (0.01 g), folic acid (4.64 g) and thiamine hydrochloride (2.32 g).

All biological stages of the insect were kept in room temperature 25 ± 1 °C, 60–70% R.H. and photoperiod 16:8 h L:D. Plastic trays (26 × 51 cm wide, 4 × 4 × 5.5 cm^3), tightly covered with fine muslin cloth for aeration, were used for larval rearing. Pupae were collected daily, transferred to glass vials sealed with cotton and placed in the incubator (24 ± 3 °C, 70 ± 5% RH and L14: D10), until adult emergence [36]. Upon adult eclosion, moths were sexed and transferred to boxes to obtain eggs for future progeny production.

Bioassays

The field experiment was performed during the seasons 2019–2020 and 2020–2021 on a biological tomato field in Kourtesi. All the recommended agronomic practices were adopted on the tomato crop. Conidial suspensions of concentrations of 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 conidia/mL were prepared for *Fusarium* strains. Conidial viability was calculated based on the formula:

$$\text{Viability (\%)} = [G1/(G1 + G2)] \times 100, \quad (1)$$

where G1 refers to the number of germinated conidia, G2 are the number of non-germinated conidia, while the sum of G1 and G2 is equal to 100. Thus, viable conidia percentage was determined by counting a total of 100 conidia per fungal isolate. Fungal isolates presenting $\geq$95% viability was used in the insect bioassays. The range of doses was selected to determine the infectivity of the fungi.

For each replication, 10 3rd-instar larvae of *H. armigera* were placed in a sterile Petri dish and sprayed with 2 mL of conidial suspension of each tested pathogen. Application of pathogen suspensions were applied with airbrush Harder and Steenbeck infinity x cr Plus (0.4 mm nozzle set, 5 mL cup + lid). First, airbrush Harder and Steenbeck was calibrated, and then the water suspension. The spraying sequence was performed in the tomato field and took place from 4:00 to 8:00 a.m. so no additional environmental stress was induced on the tested pathogens.

Following, the sprayed 3rd-instar larvae were transferred onto plants with small green tomatoes. Each plant with treated larvae was covered with a ventilated insect rearing cage (53 $\times$ 53 $\times$ 53 cm) with four sides made of clear Perspex, while two opposite sides were entirely covered with nylon mesh to allow free flow of air through the cages and the bottom side had a hole opening, so that the plant could fit. The hole at the bottom was covered with nylon mesh to allow the plant to grow properly and prevent the larvae from leaving the ventilated insect rearing cages. The experiment's design was the randomized complete block. All standard agronomic practices were followed, and control measures were used against insect pests. The investigation included seven *Fusarium* species that were evaluated on six different conidial concentrations, replicated 10 times and with 10 control replications. Additionally, 10 3rd-instar larvae were placed on each tomato plant and the experiment was performed for two successive years.

The larval survival rate was recorded every three days. Control larvae were sprayed with 10 mL of surfactant solution (H_2O + Tergitol NP9 0.05%). Dead larvae were removed on site, and surface sterilized (2% sodium hypochlorite for a few seconds) to avoid the development of saprophytic fungi. Each dead larva was then examined under stereoscope to determine the cause of death. Visual verification of fungal pathogen was achieved by the appearance of mycelium on the cadavers or with black spots on the dead insect body if the mycelium was not present. The presence of *Fusarium* in infected dead larvae was verified by re-isolation and subsequent pure culture on Petri dish with SDA. Only *Fusarium*-infected dead larvae were included in the results.

2.3. Statistical Analysis

Analysis of variance was performed to compare mean values of larval mortality, with EF conidial dose and time variation as main factors. Experimental data were arcsin-converted to meet the requirements of the parametric analysis for equal variations between treatments, where deemed necessary. Tukey' test, for significance level $\alpha = 0.05$, was used to find statistically significant differences between the factors. The Kaplan–Meier method (non-parametric) was also applied to determine the mean larval survival time. Survival data were compared with the Breslow–Gehan distribution test. Probit analysis was carried out for the estimation of LC_{50} values. All statistical tests were performed with SPSS v. 25.0 (SPSS Inc., Chicago, IL, USA, ver. 25).

3. Results

The present study is the first that evaluates different *Fusarium* isolates against larvae of *H. armigera* on field experiments (Table 2).

Table 2. Isolates of *Fusarium* species that were tested in the present study. All collected fungal isolates were lab cultured and stored at 25 °C in SDA plates.

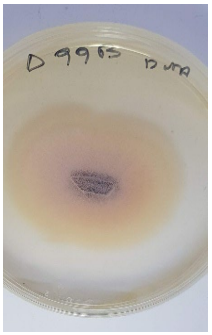
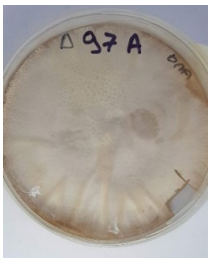
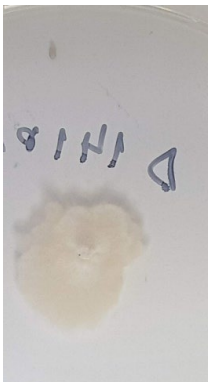
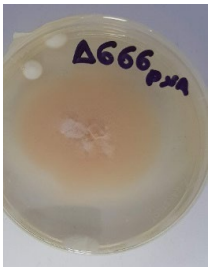
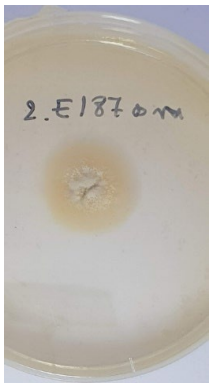
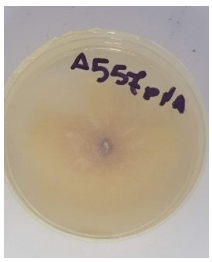
Fungal Species	Isolate	Insect Bait	Collection Site	Request ID	ID Match (%) (NCBI BLAST)	Photo
F. solani	Δ99B	*Sitophilus granarius*	Dassylio Achaia	TYPX96JD016	98.73	
F. chlamydosporum var *chlamydosporum*	E103	*Tribolium confusum*	Elos Agias Patra	TYZV90RD016	98.55	
F. tonkinense	Δ97A	*Sitophilus granarius*	Dassylio Achaia	TYTTA1TV01R	100	
F. longifundum	Δ141	*Sitophilus granarius*	Dassylio Achaia	TYTWYJE2013	99.62	
F. pseudoanthophilum	Δ666	*Tribolium confusum*	Dassylio Achaia	TYW8XYVE013	99.43	

Table 2. *Cont.*

Fungal Species	Isolate	Insect Bait	Collection Site	Request ID	ID Match (%) (NCBI BLAST)	Photo
F. fujikuroi	2E187	*Sitophilus granarius*	Elos Agias Patra	TYVNJ10A013	99.83	
F. algeriense	Δ557	*Tribolium confusum*	Dassylio Achaia	TYV2VNSW013	99.88	

Control mortality (H_2O + Tergitol NP9 0.05%) was 2.7% at the end of the experiment. By contrast, fungus *F. solani* resulted in varying degrees of larval mortality, which was proportional to the concentration used (Table 3). Larval mortality ranged on the 9th day of the experiment between 30 to 91%. All applied doses differed significantly from the control, with the lower doses of 10^3, 10^4 and 10^5 inducing 30 to 67% mortality by 9th day. In the doses of 10^6, 10^7 and 10^8 the recorded mortalities were significantly higher compared with the control, in all cases. The main effects and interactions for all factors proved to be significant (Concentration: F = 2.107, df = 6, 1002, $p < 0.001$; Exposure Time: F = 4.031, df = 2, 1002, $p < 0.001$; Concentration × Exposure Time: F = 1.716, df = 15, 1002, $p < 0.001$).

Table 3. Mean mortality (±sd) and mean Median Lethal Time of *H. armigera* larvae exposed to *F. solani* for 216 h of two season experiment. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 3.220, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. solani	10^3	0.0 ± 0.0 c	17.0 ± 5.8 c	30.0 ± 10.0 c	203.3 ± 1.2 a
	10^4	0.0 ± 0.0 c	23.0 ± 5.8 c	43.0 ± 5.8 c	198.2 ± 2.3 b
	10^5	3.4 ± 5.8 c	30.0 ± 15.3 bc	67.0 ± 5.8 b	169.5 ± 1.8 c
	10^6	13.4 ± 5.8 b	33.0 ± 10.0 b	80.0 ± 10.0 a	137.7 ± 2.1 d
	10^7	20.0 ± 10.0 a	43.0 ± 11.5 a	89.0 ± 11.5 a	100.0 ± 0.8 e
	10^8	33.0 ± 15.3 a	53.0 ± 15.3 a	91.0 ± 5.8 a	95.0 ± 1.4 f
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	2.7 ± 1.3 d	2.7 ± 1.3 d	215.0 ± 0.2 g

Fusarium chlamydosporum var. *chlamydosporum* (Hypocreales: Nectriaceae) induced larval mortality that ranged between 23 to 77% on the 9th day of the experiment (Table 4). A significant difference with the control was observed, with the lower doses of 10^3, 10^4, 10^5 and 10^6 recorded mortality rates ranging from 23 to 60% by the 9th day. In the case of the higher doses of 10^7 and 10^8, significant mortality was observed ranging from 73 to 77%. The main effects and interactions for all factors proved to be significant (Doses: F = 3.194, df = 6,

1002, $p < 0.001$; Exposure Time: F = 5.017, df = 2, 1002, $p < 0.001$; Doses × Exposure Time: F = 1.506, df = 15, 1002, $p < 0.001$). *Fusarium tonkinense* (Bugnic.) O'Donnell, Geiser and T. Aoki, (Hypocreales: Nectriaceae) also, showed different degrees of larval mortality (Table 5). On the 9th day of the experiment larval mortality ranged between 20 to 71%. All applied doses differed significantly from the control, with the lower doses of 10^3, 10^4, 10^5, 10^6 and 10^7 resulting in a mortality rate of 23 to 67% by the 9th day. The highest dose of 10^8 induced 71%, a mortality proved to be significantly lethal. The main effects and interactions for all factors proved to be significant (Concentration: F = 2.386, df = 6, 1002, $p < 0.001$; Exposure Time: F = 4.457, df = 2, 1002, $p < 0.001$; Concentration × Exposure Time: F = 2.113, df = 15, 1002, $p < 0.001$).

Table 4. Mean mortality (±sd) and Median Lethal Time of *H. armigera* adults exposed to *F. chlamydosporum* var. *chlamydosporum* for 216 h of two season experiments. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 4.115, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. chlamydosporum var. *chlamydosporum*	10^3	0.0 ± 0.0 c	7.0 ± 5.8 c	23.0 ± 10.0 c	205.0 ± 2.6 a
	10^4	0.0 ± 0.0 c	13.0 ± 5.8 c	33.0 ± 5.8 c	201.0 ± 1.4 a
	10^5	0.0 ± 0.0 c	13.0 ± 15.3 bc	57.0 ± 5.8 b	172.0 ± 2.1 b
	10^6	3.4 ± 5.8 b	20.0 ± 10.0 b	60.0 ± 10.0 a	170.0 ± 1.2 b
	10^7	10.0 ± 10.0 a	33.0 ± 11.5 a	73.0 ± 11.5 a	132.0 ± 1.8 c
	10^8	23.0 ± 15.3 a	43.0 ± 15.3 a	77.0 ± 5.8 a	124.0 ± 0.9 d
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	0.0 ± 0.0 d	2.7 ± 1.3 d	215.0 ± 0.2 e

Table 5. Mean mortality (±sd) and Median Lethal Time of *H. armigera* adults exposed to *F. tonkinense* for 216 h of two season experiments. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 3.893, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. tonkinense	10^3	0.0 ± 0.0 c	10.0 ± 5.8 c	20.0 ± 10.0 c	205.0 ± 0.6 a
	10^4	0.0 ± 0.0 c	13.0 ± 3.8 c	23.0 ± 5.8 c	203.0 ± 1.7 a
	10^5	0.0 ± 0.0 c	13.0 ± 12.3 bc	37.0 ± 5.8 b	195.0 ± 2.1 b
	10^6	3.4 ± 5.8 b	20.0 ± 10.0 b	50.0 ± 10 a	177.0 ± 0.2 c
	10^7	10.0 ± 10.0 a	23.0 ± 11.5 a	67.0 ± 11.5 a	162.0 ± 2.8 d
	10^8	13.0 ± 15.3 a	33.0 ± 15.3 a	71.0 ± 5.8 a	158.0 ± 0.9 e
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	0.0 ± 0.0 d	2.7 ± 1.3 d	215.0 ± 0.2 f

Different degrees of larval mortality were also observed with *Fusarium longifundum* J.W. Xia, L. Lombard, Sand.-Den., X.G. Zhang and Crous (Hypocreales: Nectriaceae), ranging between 13 to 67% on the 9th day of the experiment (Table 6). All applied doses differed significantly from the control, with the lower doses of 10^3, 10^4, 10^5, 10^6 and 10^7 resulting in a mortality rate of 13 to 46% by the 9th day. The main effects and interactions for all factors proved to be significant (Concentration: F = 1.286, df = 6, 1002, $p < 0.001$; Exposure Time: F = 3.411, df = 2, 1002, $p < 0.001$; Concentration × Exposure Time: F = 2.313, df = 15, 1002, $p < 0.001$). The highest dose of 10^8 proved to be significantly lethal. *Fusarium pseudoanthophilum* Nirenberg, O'Donnell and Mubat (Hypocreales: Nectriaceae) also resulted in diverse effects on larval mortality (Table 7). Mortality ranged between 20 to 71%. All applied doses differed significantly from the control, and in the lower doses of 10^3, 10^4, 10^5, 10^6 and 10^7 mortalities were calculated from 13 to 67% by the 9th day. Regarding the highest dose (10^8), 81% mortality rate was recorded, demonstrating its effectiveness. The main effects and interactions for all factors proved to be significant (Concentration:

F = 2.111, df = 6, 1002, $p < 0.001$; Exposure Time: F = 3.216, df = 2, 1002, $p < 0.001$; Concentration × Exposure Time: F = 1.913, df = 15, 1002, $p < 0.001$).

Table 6. Mean mortality (±sd) and Median Lethal Time of *H. armigera* adults exposed to *F. longifundum* for 216 h of two season experiment. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 4.815, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. longifundum	10^3	0.0 ± 0.0 c	13.0 ± 5.8 c	13.0 ± 5.8 c	208.0 ± 0.6 a
	10^4	0.0 ± 0.0 c	17.0 ± 5.8 c	23.0 ± 5.8 c	202.0 ± 3.7 b
	10^5	3.4 ± 5.8 b	27.0 ± 15.3 bc	37.0 ± 5.8 b	193.0 ± 3.3 c
	10^6	3.4 ± 5.8 b	33.0 ± 10 b	40.0 ± 10.0 a	187.0 ± 2.2 d
	10^7	6.7 ± 10 a	37.0 ± 11.5 a	46.0 ± 11.5 a	182.0 ± 1.8 e
	10^8	13.0 ± 15.3 d	40.0 ± 15.3 a	67.0 ± 5.8 a	161.0 ± 2.9 f
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	0.0 ± 0.0 d	2.7 ± 1.3 d	215.0 ± 0.2 g

Table 7. Mean mortality (±sd) and Median Lethal Time of *H. armigera* adults exposed to *F. pseudoanthophilum* for 216 h of two season experiments. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 2.678, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. pseudoanthophilum	10^3	0.0 ± 0.0 c	7.0 ± 5.8 d	13.0 ± 5.8 e	208.0 ± 0.3 a
	10^4	3.4 ± 5.8 a	17.0 ± 11.5 c	27.0 ± 15.3 d	189.0 ± 2.1 b
	10^5	3.4 ± 5.8 a	23.0 ± 15.3 c	47.0 ± 15.3 cd	157.0 ± 2.3 c
	10^6	3.4 ± 5.8 a	33.0 ± 5.8 b	57.0 ± 5.8 c	148.0 ± 1.2 d
	10^7	10.0 ± 10.0 b	37.0 ± 11.5 ab	67.0 ± 5.8 b	132.0 ± 3.8 e
	10^8	20.0 ± 0.0 d	40.0 ± 10.0 a	81.0 ± 5.8 a	106.0 ± 1.9 f
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	0.0 ± 0.0 e	2.7 ± 1.3 f	215.0 ± 0.2 g

Finally, *Fusarium fujikuroi* Nirenberg (Hypocreales: Nectriaceae) also appeared to induce varying degrees of larval mortality (Table 8). Larval mortality ranged on the 9th day of the experiment between 17 to 60%. All applied doses differed significantly from the control, with the lower doses of 10^3, 10^4, 10^5 and 10^6 causing mortalities from 17 to 69% by the 9th day. Concerning the higher doses of 10^7 (73%) and 10^8 (77%), they induced a mortality that proved to be significantly lethal. The main effects and interactions for all factors proved to be significant (Concentration: F = 1.811, df = 6, 1002, $p < 0.001$; Exposure Time: F = 2.647, df = 2, 1002, $p < 0.001$; Concentration × Exposure Time: F = 2.913, df = 15, 1002, $p < 0.001$). *Fusarium algeriense* I. Laraba and O'Donnell (Hypocreales: Nectriaceae) also resulted in differing degrees of larval mortality (Table 9). Larval mortality ranged on the 9th day of the experiment between 10 to 61%. All applied doses differed significantly from the control, and all doses of had produced mortality of 10 to 60% by the 9th day. The main effects and interactions for all factors proved to be significant (Concentration: F = 3.411, df = 6, 1002, $p < 0.001$; Exposure Time: F = 3.992, df = 2, 1002, $p < 0.001$; Concentration × Exposure Time: F = 2.943, df = 15, 1002, $p < 0.001$).

Table 8. Mean mortality (±sd) and Median Lethal Time of *H. armigera* adults exposed to *F. fujikuroi* for 216 h of two season experiments. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 5.330, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. fujikuroi	10^3	0.0 ± 0.0 c	10.0 ± 10.0 b	17.0 ± 5.8 d	205.0 ± 1.3 a
	10^4	10.0 ± 10.0 ab	23.0 ± 11.5 b	40.0 ± 10.0 c	182.0 ± 3.1 b
	10^5	10.0 ± 5.8 b	33.0 ± 0.0 b	50.0 ± 0.0 c	175.0 ± 1.4 c
	10^6	10.0 ± 10.0 ab	40.0 ± 10.0 b	60.0 ± 10.0 b	160.0 ± 1.2 d
	10^7	13.0 ± 0.0 a	53.0 ± 20.8 a	73.0 ± 5.8 a	150.0 ± 1.8 e
	10^8	20.0 ± 5.8 b	56.7 ± 5.8 a	77.0 ± 0.0 a	145.0 ± 2.1 f
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	0.0 ± 0.0 c	2.7 ± 1.3 e	215.0 ± 0.2 g

Table 9. Mean mortality (±sd) and Median Lethal Time of *H. armigera* adults exposed to *Fusarium algeriense* for 216 h of two season experiments. Means of the same column followed by the same letter are not significantly different (Tukey test, a = 0.05), Median Lethal Time (hours ± sd) was estimated by the Kaplan–Meier method (F = 5.232, df = 6, $p < 0.001$), HAT: Hours After Treatment.

Treatment	Concentration	Exposure Time			Median Lethal Time
		72 HAT	144 HAT	216 HAT	
F. algeriense	10^3	6.7 ± 5.8 b	6.7 ± 5.8 b	10.0 ± 10.0 d	209.0 ± 0.3 a
	10^4	6.7 ± 5.8 b	13.0 ± 11.5 b	20.0 ± 10.0 c	204.0 ± 2.1 b
	10^5	6.7 ± 5.8 b	20.0 ± 0.0 a	30.0 ± 0.0 c	192.0 ± 1.4 c
	10^6	10.0 ± 10.0 ab	26.0 ± 10.0 a	43.0 ± 10.0 b	185.0 ± 2.2 d
	10^7	10.0 ± 0.0 ab	33.0 ± 20.8 ac	53.0 ± 5.8 a	179.0 ± 2.8 e
	10^8	13.0 ± 5.8 a	36.7 ± 5.8 c	60.0 ± 0.0 a	162.0 ± 1.1 f
Control	H_2O + Tergitol Np9	0.0 ± 0.0 c	0.0 ± 0.0 d	2.7 ± 1.3 e	215.0 ± 0.2 g

Probit analysis results showed that the median lethal concentrations (LC50) of *Fusarium* species were estimated from 3.88×10^3 conidia/mL (for *F. solani*) to 11.85×10^6 conidia/mL (for *F. algeriense*) (Table 10).

Table 10. Estimated lethal concentrations (LC_{50}) for *Fusarium* species on 3rd-instar larvae of *H. armigera*. **LC**: lethal concentrations; **CI**: confidence limit; **SE**: standard error; **χ^2:** Chi-squared goodness of fit test, LC values are considered significantly different when 95% confidence limits fail to overlap.

Fungal Isolate	df	LC_{50} (±95% CI)	Slope ± SE	Intercept	χ^2	*p*
F. solani	5	3.88×10^3 (7.22×10^2–4.47×10^4)	1.54 ± 0.70	0.133	1.53	0.74
F. chlamydosporum var. *chlamydosporum*	5	2.13×10^4 (8.25×10^3–1.07×10^5)	2.74 ± 0.13	0.033	2.74	0.90
F. tonkinense	5	1.19×10^6 (0.9×10^5–1.4×10^7)	3.94 ± 0.21	−1.033	3.09	0.82
F. longifundum	5	2.17×10^6 (1.2×10^5–3.2×10^7)	4.13 ± 0.13	−1.166	3.20	0.96
F. pseudoanthophilum	5	8.96×10^5 (2.2×10^4–5.4×10^5)	3.15 ± 0.13	−0.013	2.81	0.88
F. fujikuroi	5	7.85×10^5 (2.8×10^4–4.6×10^5)	4.43 ± 0.47	−2.266	3.23	0.93
F. algeriense	5	11.85×10^6 (5.2×10^5–9.4×10^7)	4.97 ± 0.38	−1.866	4.20	0.97

4. Discussion

The excessive use of chemical insecticides not only involves risks for human health, but also generates pest resistance and harms the environment by having high persistence and

by affecting non-target organisms. As a result, the implementation of eco-friendly methods of pest management is vital. The value of using natural products as a safe alternative to chemical pesticides for the control of serious agricultural pests is demonstrated by many researchers. These products consist of natural ingredients that are environmentally safe and have low mammalian toxicity [37–39].

The EPF include fungal species that are pathogenic to insects. These fungal pathogens play a vital role in reducing insect population dynamics in comparatively shorter time than other measures [40]. Our investigation once again confirmed the entomopathogenic nature of some species of *Fusarium*. All seven species tested showed significant lethal effects on the survival of *H. armigera* larvae, especially on higher concentrations, but a notable mortality rate was also recorded on lower ones. It is believed that the interaction with the insect host can be more powerful and manageable in cases of absence of previous long-term relationships [41]. The lack of past interaction boosts the efficiency of EPF as the insect hosts have not developed defensive mechanisms on the new pathogen [42]. The selection of locally adapted virulent strains in agroecosystems is the initial step in commercialization and large-scale application [43–45]. Additionally, indigenous strains of EPF have ecological compatibility with insect pests, positive effects on the local environment, lessened pesticide residues in food, reduced risk of significant impact on non-target organisms, and increased biodiversity in managed ecosystems [46–48].

The entomopathogenic effect of the *Fusarium* species has been reported by investigators on certain insect species, such as the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae) [49], the spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae) [50], the wheat stem sawfly, *Cephus cinctus* (Norton) (Hymenoptera: Cephidae) [51] and eggs and larvae of the tobacco cutworm, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) [52]. As reviewed by da Silva Santos et al. [53], the most frequently reported entomopathogenic *Fusarium* species lay between four species complexes of *Fusarium incarnatum-equiseti*, *F. fujikuroi*, *F. solani,* and *F. oxysporum. F. solani* and *F. fujikuroi* (homotypic synonym: *Gibberella fujikuroi)* showed a high mortality rate on the larvae of *H. armigera* and once again confirmed the existing literature claiming the entomopathogenic traits of these species as they have been tested on a variety of insects [51]. Our results include the first ever report on the entomopathogenity of *F. chlamydosporum* var. *chlamydosporum, F. pseudoanthophilum*, *F. tonkinense* (Bugnic.) (basionym: *Cylindrocarpon tonkinense)* and *F. algeriense* as they induced mortality at 77%, 71%, 71%, and 61%, respectively, at high conidial concentrations. Lastly, there is substantial evidence of the *F. incarnatum–equiseti* species complex being effective against insects and our results identified that one member of the complex, *F. longifundum,* induced 67% mortality on *H. armigera* larvae.

It is well known how this fungus, as a plant pathogen, can attack and kill its host plant. Some explanation of the entomopathogenic effect of the fungus was based on the production of toxins, such as beauvericin [54], fusarin C [55,56], and moniliformin [57,58]. Some strains of *Fusarium avenaceum* (Fr.) Sacc. (Hypocreales: Nectriaceae) can synthesize enniatin cyclic peptides, primarily enniatins A, B and B1 under laboratory conditions [54], but these enniatins may have a role in plant pathogenicity [59].

The effectiveness against *H. armigera* is of an importance as it is characterized by its resistance to many chemical insecticides [60,61]. *F. solani* appeared to be the most lethal against *H. armigera* larvae, even though there have been some reports on the species' mutualistic relationship with insects. The lethal effect observed by Ganassi et al. [62] reported that *Fusarium proliferatum* (Matsush.) Nirenberg (Hypocreales: Nectriaceae) isolates cannot produce significant amounts of toxic metabolites, which could be attributed to extracellular enzymes (e.g., proteases and chitinases). In fact, the capacity to synthesize enzymes involved in the degradation of the cuticle of insects has been demonstrated for some entomopathogenic fungi [63]. Ferron et al. [64] reported the production of chitinase and protease by hyphomycetes. So, treatment with formulations of *Fusarium* induce the appearance of brown-black deposits of melanin on the integument of treated nymphs [62]. The same symptoms mentioned above were observed on the treated *H. armigera* larvae with *Fusarium*

isolates. It is known that melanin and its precursors have fungistatic properties [65] and inhibit microbial proteases and chitinases [64].

As mentioned before, research about the entomopathogenic *Fusarium* species has been neglected, in contrast to the phytopathogenic species. As chemical insecticides are becoming obsolete, alternative methods should be investigated thoroughly; even fungal species that were once considered a threat to cultivations could prove to be more than helpful on pest management. In 2015, Adsure and Mohite [66] tested the effect of *Metarhizium anisopliae* (Metschn.) Sorokīn (Hypocreales: Clavicipitaceae), *B. bassiana* (Bals. -Criv.) Vuill. (Hypocreales: Cordycipitaceae), *Nomuraea rileyi*(Farl.) Samson, (Hypocreales: Clavicipitaceae), against *H. armigera* on chickpea under field conditions. The bio-efficacy of *B. bassiana* studied by Prasad et al. [67] against *H. armigera* with four different doses sprayed topically against fourth-instar larvae recorded 76.7% mortality at the highest dose (0.25 mL $\times$ 10^8 spores/mL). Ebrahimi et al. [68] studied the effect of the entomopathogenic nematode, *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae), on survival and plasma phenoloxidase activity of *H. armigera* in the lab. Another study conducted by Majeed et al. [69] documented the pathogenicity of indigenous soil isolate of *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) on *H. armigera*. Additionally, Mishra and Sobita [70] demonstrated that *B. bassiana* recorded significant larval mortality against *H. armigera* in the field.

As confirmed by our experiments, *Fusarium* species can be used for *H. armigera* management. Due to the epizootics caused by the fungi, after the first 24 h, the larvae showed no changes, feeding normally and responding to external stimuli. After that, the larvae became sluggish, lethargic, and if made to lie on their back, were crippled, showing inability to straightening themselves. Finally, they undergo a moribund stage and death occurs after some time. Mycelial growth (mummification) follows 36–48 h after death.

It is also important to mention that *F. solani* and *F. fujikuroi* can also infect humans, the first one on a greater scale, but mostly immune-compromised patients are affected [19]. However, problems such as pathogenicity to plants and humans may be concerning but easily manageable nowadays, through genetic modification. With the implementation of genetic markers that detect entomopathogenicity and plant pathogenicity and gene silencing, new strains can be developed and applied as biological controls to pest management that will not pose a threat to either the environment or humans.

In conclusion, tested *Fusarium* isolates demonstrated noteworthy larvicidal action against *H. armigera*. Further investigations need to be conducted on other pests to establish the broad effectiveness of these *Fusarium* isolates. The high mortality on *H. armigera* larvae can signify that similar results could be recorded on other economically important moth species. Moreover, our results encourage the inception of studies on practical aspects of *Fusarium*-based insecticides, such as long-term stability, mass production, storage, and formulation issues.

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