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Article

Occurrence and Distribution of Entomopathogenic Fungi in Cultivated Soil and Its Efficacy Against *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) Under Laboratory Conditions

Zainab Saeed [1,2], Usman Khalique [2], Farrah Zaidi [1], Muhammad Zahid [2], Bibi Hajra [1,2], Khush Bakht Latif [1], Syeda Hira Fatima [3,4] and Syed Jawad Ahmad Shah [2,*]

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Abstract

Annually, global crop harvest significantly declines due to various pest attacks. Their effective management is crucial for sustainable agricultural productivity. Native entomopathogenic fungi (EPF) have been recognized as the most promising microbiological control agents against these pests. The present study aimed to isolate locally occurring entomopathogenic fungi and assess their efficacy against the serious noctuid pest, *H. armigera,* under laboratory conditions. EPF was explored in cultivated soil from crops of two distinct agro-ecological zones (plains and foothills) in Khyber Pakhtunkhwa province, Pakistan. Using the Galleria baiting technique, fungal isolates were recovered from collected soil samples. Upon identification, these isolates belonged to 4 different EPF species, viz., *Nomuraea rileyi, Aspergillus parasiticus, A. niger,* and *A. flavus*. Results revealed that soils from the foothills exhibited a comparatively higher percentage distribution of isolates than those from the plains. *Aspergillus niger* was the most abundant fungal species in various localities and crops. The pathogenicity of four isolated species was assessed against *H. armigera* at three concentrations ($1x10^6$, $1x10^7$ and $1x10^8$ conidia/mL). Results revealed that the *H. armigera* larvae were found to be susceptible to all tested EPF species, particularly at high concentration levels. *M. rileyi* was the most effective, causing the highest percent mortality and exhibiting the lowest percentage of pupal recovery and adult emergence, followed by *Aspergillus* species. Probit analysis showed that *M. rileyi* was highly virulent, with the lowest LC_{50} and LT_{50} values. This study reveals the potential of *M. rileyi* to serve as an effective biocontrol agent in integrated pest management strategies against *H. armigera*, and as a promising candidate for bio-pesticide product development. The use of EPF agents will ensure the production of healthier organic crops by eliminating insecticide residue and resistance problems.

Keywords: *Helicoverpa armigera*; microbial control; entomopathogenic fungi; distribution; *Galleria mellonella*

1. Introduction

The tomato fruit worm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), is a highly destructive and cosmopolitan pest (Kriticos et al. 2015, Dinesh et al. 2017, Chen et al. 2018, Fathipour et al. 2020). Its larval stage is highly voracious and infests over 181 host plant species belonging to more than 47 families (Bird 2017, Mishra and Omkar 2021). This pest successfully adapts to various agro-ecosystems due to its polyphagous nature, facultative diapause, high reproductive capacity, and

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migratory potential (Chaitanya et al. 2014, Jones et al. 2019, Katsikis et al. 2020, Riaz et al. 2021). In Pakistan, from the late 20th century to the early 21st century, this pest was identified as a significant factor in yield reduction and threatening major field crops (Karim 2000).

Tomatoes, *Lycopersicon esculentum* (Mill.) are among the valuable crops targeted by *H. armigera*. In Pakistan, tomatoes are the second most produced crop in the horticultural sector (Chohan and Ahmad 2008). The initial instars of *H. armigera* feed on green shoots and foliage, while subsequent stages target buds, inflorescences, and fruiting bodies, resulting in serious damage to post-harvest produce (Wang and Li 1984, Czepak et al. 2013, Pratissoli et al. 2015). This noctuid pest is estimated to cause around 53% fruit loss in tomatoes (Saljoqi et al. 2022). Farmers in Pakistan and around the world mainly rely on chemical applications to control this pest (Shaheen 2008, Fite et al. 2019). However, the excessive use of broad-spectrum insecticides has raised serious concerns about their harmful effects on human health, the environment, non-target species, disturbs ecological balance and leads to pest resurgence and the development of pest resistance (David 2008, Shah et al. 2013, Sun et al. 2019, Riaz et al. 2021). According to the Arthropod Pesticide Resistance Database (APRD) (http://www.pesticideresistance.org/DB/), this pest has become resistant to over 40 active ingredients. This situation has increased interest in finding more eco-friendly alternatives to reduce the harmful impacts of insect pests and support sustainable farming (Sezen and Demirbag 2006).

One such approach is biological control with insect-invading pathogens (Ai et al. 2018). Entomopathogenic fungi (EPF) are eco-friendly microbial agents naturally found in various terrestrial habitats (Sanchez-Peña 2000, Sevim et al. 2010, Mahankuda and Bhatt 2019); widely recognized worldwide in integrated pest management due to their broader host range, ability to cause lethal mycosis in insect pests through cuticle penetration, and capability to control different developmental stages more effectively than other strategies (Vega et al., 2012; Maina et al., 2018). Moreover, EPF strains promote long-term environmental sustainability by germinating on insect cadavers, thereby enhancing their inoculum density and disseminating their network throughout the ecosystem (Moore et al. 2012).

Metarhizium spp. are frequently used or tested as eco-friendly microbiological agents because they are easy to produce on a large scale (Greenfield et al. 2015). *Metarhizium rileyi* (Farl.), formerly known as *Nomuraea rileyi* (Kepler et al. 2014), is a potential EPF that can cause lethal epizootics in various insects, including noctuid pests (Jaronski 2014, Ramos et al. 2024). Various studies have reported the potential of *M. rileyi* for developing a microbial pesticide against lepidopterous pests (Vega-Aquino et al. 2010, Hatting et al. 2012). *M. rileyi* is widely used in control strategies for *H. armigera* (Yuan and Yong 2010, Inglie et al. 2017) due to its primary features, including environmental friendliness and host specificity (Sinha et al. 2016). *Aspergillus* is another important genus within Ascomycota, encompassing more than 250 species (Kotta-Loizou 2021). Some of these species have been identified as entomopathogenic, such as *A. niger* (Kaur et al. 2016), *A. parasiticus* (Nnakumusana 1985, Khan et al. 2025), and *A. flavus* (Askar et al. 2024). *Aspergillus* spp. employ a unique infection method by initiating lipid peroxidation, followed by a series of events that ultimately compromise the host's immunity. They release toxic compounds that cause gut tissue leakage and induce septicemia, leading to the death of the host insect (Karthi et al. 2024).

Mensah et al. (2015) reported the effective control of H. armigera through the use of local Aspergillus sp. (BC 639), which also had a minimal impact on beneficial organisms. Similarly, Sebayang et al. (2021) explored native EPF species as microbial agents against *H. armigera* and isolated *A. flavus* and other EPF species from infected *H. armigera* larvae. These species have been utilized as microbial agents against other agricultural pests, including *Oligonychus coffeae* (Mazid et al. 2015), *Dysdercus koenigii* (Kumari et al. 2019), *Spodoptera frugiperda* (Idrees et al. 2021), and *Spodoptera litura* (Kaur et al. 2016, Kaur et al. 2025). However, in Pakistan, the potential of these entomopathogens viz., *M. rileyi*, *A. niger*, *A. parasiticus*, and *A. flavus* has never been investigated against the noctuid pest, *H. armigera*.

Moreover, understanding the composition and distribution of indigenous species is vital for preserving fungal strains in soil, which is necessary for effectively managing local pest populations

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in agro-ecosystems (Meyling and Eilenberg 2006, Hussein et al. 2010). Additionally, laboratory evaluations are crucial to determine effective concentration levels, ensuring the success and safety of future field applications against this pest. The study's objectives were to explore indigenous EPF strains in cultivated soils of KP province and evaluate their potential against the local pest, *H. armigera*, using the topical inoculation method under laboratory conditions. The main parameters examined included the comparative distribution of fungal isolates in plain areas versus foothills and between agronomic versus horticultural crops, along with first mortality, total mortality (%), pupal recovery (%), adult emergence (%), and the determination of LC_{50} and LT_{50}.

2. Materials and Methods

2.1. Study Area

Isolation of entomopathogenic fungi and pathogenicity bioassays were performed in the Biocontrol and Plant Pathology laboratories of Nuclear Institute for Food Agriculture (NIFA), Peshawar. The morphological identification of isolated EPF species was done in the Vector Biology Laboratory of the Institute of Zoological Sciences, University of Peshawar.

2.2. Collection of Soil Samples

Soil samples were collected from four agronomic (rice, maize, sugarcane, mustard) and four horticultural crops (tomato, potato, guava, peach) from distinct geographical terrains in Khyber Pakhtunkhwa province (Plains: Peshawar and Charsadda and Foothills: Abbottabad and Haripur) during 2023-24. The details regarding the geographic coordinates of surveyed regions are given in Figure 1.

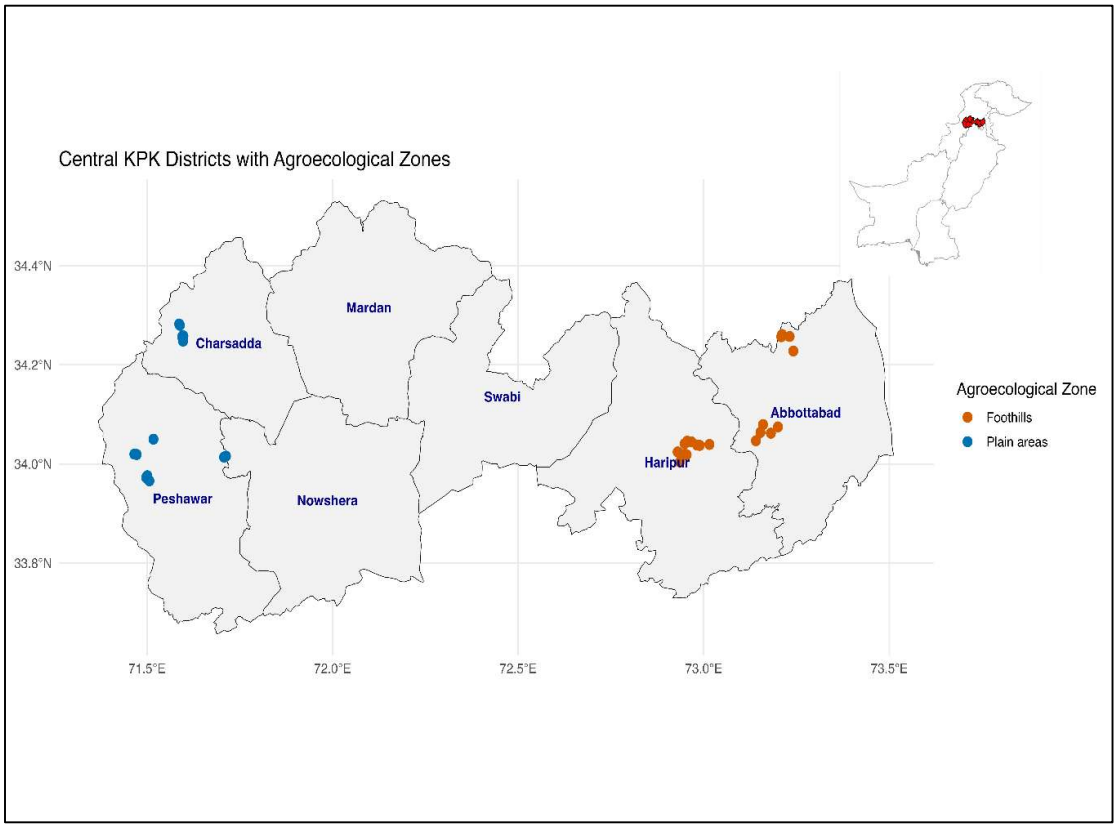

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Figure 1. Map of different agro-ecological zones of Khyber Pakhtunkhwa (KP) province, Pakistan showing soil sampling sites for entomopathogenic fungi isolation in 4 districts. The values on the X-axis present longitudinal coordinates and values on the Y-axis present latitudinal coordinates.

From each locality, five soil samples (~200 g each) were collected from a depth of 5-10 cm at five random points by using a hand shovel with three replicates per point and stored in sterile zip-lock bags (28×20 cm) at 4 °C in a refrigerator until used (Mantzoukas et al. 2022).. The samples from each site were mixed to get a unit sample and sieved through a 16 mm mesh to remove roots, stones, litter, etc. Soil samples were then spread on rectangular trays and kept open to remove excessive moisture to avoid nematode infestation as suggested by Quesada-Moraga et al. (2007).

2.3. Isolation of Entomopathogenic Fungi

Galleria bait method was used for isolating the entomopathogenic fungi from collected soil samples (Zimmerman 1986). The wax moth larvae, *Galleria mellonella* (Lepidoptera: Pyralidae), were originally collected from infested bee combs acquired from local beekeepers. The *G. mellonella* culture was sustained on a suitable diet, specifically bee wax, and kept in rectangular trays within insect rearing cages (60 x 60 x 60 cm) under controlled conditions i.e., 26 ± 2℃ temp., 65±5% R.H. and continuous darkness (Sevim et al. 2010). The dry soil samples were first moistened to field capacity (Quesada-Moraga et al. 2007), and then, after thorough mixing, from each unit sample, five sub-samples were taken into autoclaved glass vials. At the top of the soil, about 1 cm space was left and vial lids were provided with needle holes for proper aeration. Five healthy 3rd instar larvae were added to each vial using sterile forceps and incubated at 26 ± 2℃ for ten days. The vials were inverted daily to ensure regular larval movement through the soil. After incubation, the soil was examined, and the bait larvae were removed. The larval cadavers were first surface sterilized in 3% sodium hypochlorite (disinfectant) for 3 min, then washed with sterile distilled water for 3 min as suggested by Sookar et al. (2008) and individually incubated at 26 ± 2℃ on wet filter paper in sterile glass petri plates for three days to enhance pathogen sporulation.

The cadavers with symptoms of fungal infection were transferred to Sabouraud Dextrose Yeast Agar (SDAY) plates comprising 10 g dextrose, 2.5 g peptone, 2.5 g yeast extract and 20 g agar in 1 litre of distilled water (supplemented with antibiotics 100 mg/l to inhibit bacterial growth) (Sevim et al. 2010) inside a laminar flow cabinet in Plant Pathology Laboratory, NIFA. They were kept at 26 ± 2℃ temperature, 60±5% relative humidity in complete darkness till the growth of fungal mycelium. The culture obtained from an infected bait larvae was considered an isolate. The name code for isolates were based according to crop: Maize (M), Sugarcane (S), Mustard (Mu), Rice (R), Potato (P), Tomato (T), Guava (G) and Peach (P); replication (R1-R3) and sample number within crop (S1-S5) and thus samples were labeled as MR1S1, RR2S5, etc.

2.4. Identification of EPF Isolates

The fungal isolates were identified based on morphological traits, such as colony appearance, spore size and shape, etc., using a taxonomic key in Vector Biology Laboratory of the Institute of Zoological Sciences, University of Peshawar (Humber 2005). Pure culture plates of each fungal isolate were then submitted to the First Fungal Culture Bank of Lahore, Pakistan (FCBP) for confirmation. The identified fungal cultures were deposited in the Biocontrol laboratory of Nuclear Institute for Food Agriculture (NIFA), Peshawar, and Vector Biology Laboratory of the Institute of Zoological Sciences, University of Peshawar.

2.5. Insect Rearing

H. armigera larvae were collected from the NIFA field area and initially reared on a natural diet (peas) in plastic cups. Pupae were collected and transferred to glass vials for adult emergence. The emerged adults were coupled, kept in 2.5 L plastic jars covered with muslin cloth as a substrate for oviposition, and provided with a 10% sugar solution. The muslin cloth with eggs was changed daily

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and shifted to plastic boxes, with an artificial diet for hatchlings, comprising of chickpea powder (79.6%), scorbic acid (1%), methyl para hydroxyl benzoate (0.8%), ascorbic acid (0.3%), streptomycin (0.3%), yeast (12.7%), agar (0.1%) and vitamin B12 (0.0001%) by weight in distilled water (1000 ml). After hatching, the third instars of the F1 generation were used in bioassays. All developmental stages of *H. armigera* were kept under controlled conditions of 26 ± 2℃ temperature, 65±5% relative humidity, and L14: D10 hr photoperiod.

2.6. Pathogenicity of Entomopathogenic Fungi on H. armigera

Bioassays were performed in the Biocontrol laboratory of NIFA to evaluate the pathogenicity of isolated EPF species against *H. armigera*. Conidial suspensions were prepared from 2-3 weeks old pure EPF cultures in 10 ml of distilled water with Tween-80 (0.1%) in sterile 15 ml tubes and quantified using a Hemocytometer (Sorathiya et al. 2023). The desired concentrations of each fungal strain, i.e., 1×10^8, 1×10^7, and1×10^6 conidia/ml, were adjusted through serial dilution. Three replicates were used per treatment and each replicate contained nine 3rd instar of *H. armigera*, which were topically inoculated using a micropipette with a volume of 10 microliter of conidial suspensions. In a control treatment, the larvae were treated similarly, using only distilled water. The bioassay was repeated twice and during the experimental period, the larvae were provided with artificial diet and adults were fed with 10% sugar solution.

2.7. Parameters to Be Studied

2.7.1. Percentage Diversity of Soil-Dwelling EPF

To calculate the percentage diversity of a particular identified EPF strain (x), the following formula was used:

$$\%\ \text{Diversity of 'x'} = \frac{\text{Total count of Identified EPF}}{\text{No.of Identified EPF species (x)}} \times 100$$

2.7.2. Concentration-Response Bioassay

Concentration-response tests were conducted for different parameters. In each treatment, as noted above, nine healthy third instars were placed individually in petri plates and topically inoculated with conidial suspension and in the control group, treated with distilled water. The first larval mortality data for each treatment's replication were recorded for 5 days following the inoculation of EPFs. The larvae showing no indication of movement were considered dead. The total mortality and median lethal concentration (LC_{50}) for each fungal treatment were determined at different post-inoculation intervals: total mortality after 7 and 14 days, LC_{50} after 3, 7, and 14 days. The effect of different concentrations of EPFs on the pupal period and adult emergence of *H. armigera* was also assessed. The median lethal time (LT_{50}) was also estimated for each fungal concentration.

2.8. Research Design

A Completely Randomized Design (CRD) was followed, with 3x replicates. The aspect used was the conidial concentration treatments (T) with the following levels for each fungal species: $1\times10^{6,}$ 1×10^7, and 1×10^8 conidia/ml. The bioassay was conducted twice, comprising a total of thirteen treatments, including a control group.

2.9. Statistical Analysis

The bioassay data (percent mortality, pupal recovery and adult emergence) were analyzed using one-way ANOVA with Statistix version 8.1 software to determine means and different parameter values for tested fungal treatments. The means of significant treatments were compared with Tukey's HSD (Honestly Significant Difference) test at a significance level of $\alpha = 0.05$. To estimate LC_{50} and LT_{50} and their associated P values, degrees of freedom, chi-square and slopes of tested EPF species, probit analysis was performed on bioassay data using SPSS v16.

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3. Results

3.1. Isolation and Identification of EPF Isolates

A total of 209 fungal isolates were recovered from baited wax moth larvae. Based on morphological traits identified using a taxonomic key (Humber 2005) and confirmed by the Fungal Culture Bank of Pakistan (FCBP), fungal isolates were classified into four different EPF species: *Nomuraea rileyi*, *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus niger*. Their morphological details are presented in Figure 2.

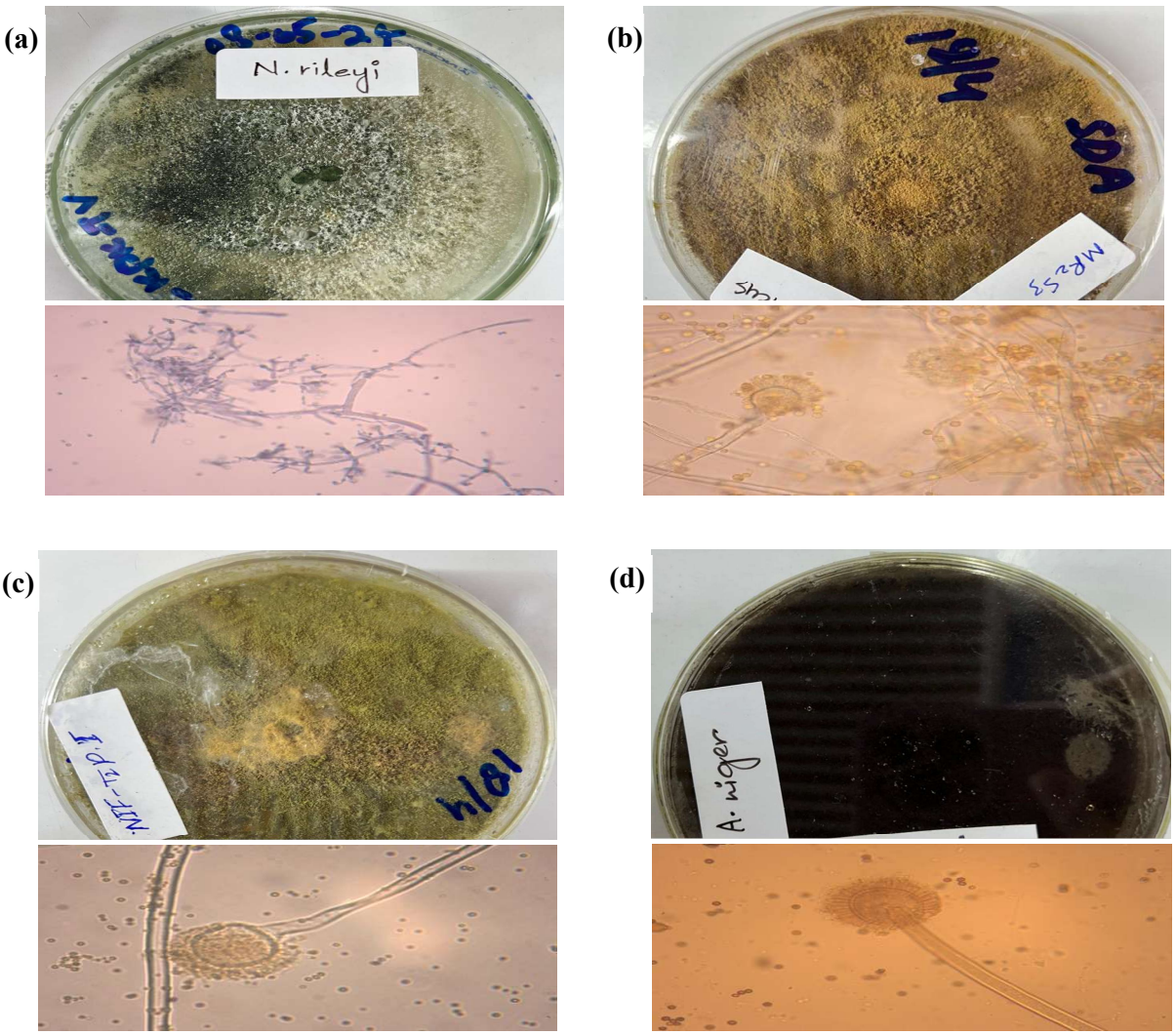

Figure 2. Colony visuals and microscopic view of identified EPF species (a) *Nomuraea rileyi* (b) *Aspergillus parasiticus* (c) *Aspergillus flavus* (d) *Aspergillus niger.*

3.2. Comparative Distribution of Fungal Isolates in Different Agro-Ecological Zones of KP Province

The comparative distribution of EPF isolates was determined in cultivated soil from diverse agro-ecological zones, including plains and foothills of KP Province. Of the total 209 isolates, 133 were obtained from cultivated soils in the plain area districts, i.e., Peshawar and Charsadda, while the remaining 76 isolates came from the foothills districts, i.e., Haripur and Abbottabad. Our results revealed that the comparative distribution of all isolated EPF species was higher in the soil of the foothills than in the plain areas (Figure 3). Overall, fungal species composition was uniform throughout, with the highest percentage distribution of *Aspergillus niger* i.e., 48% in the foothills and

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37% in the plain regions, while *Nomuraea rileyi* had the lowest i.e., 15% in the foothills and 11% in the plain areas.

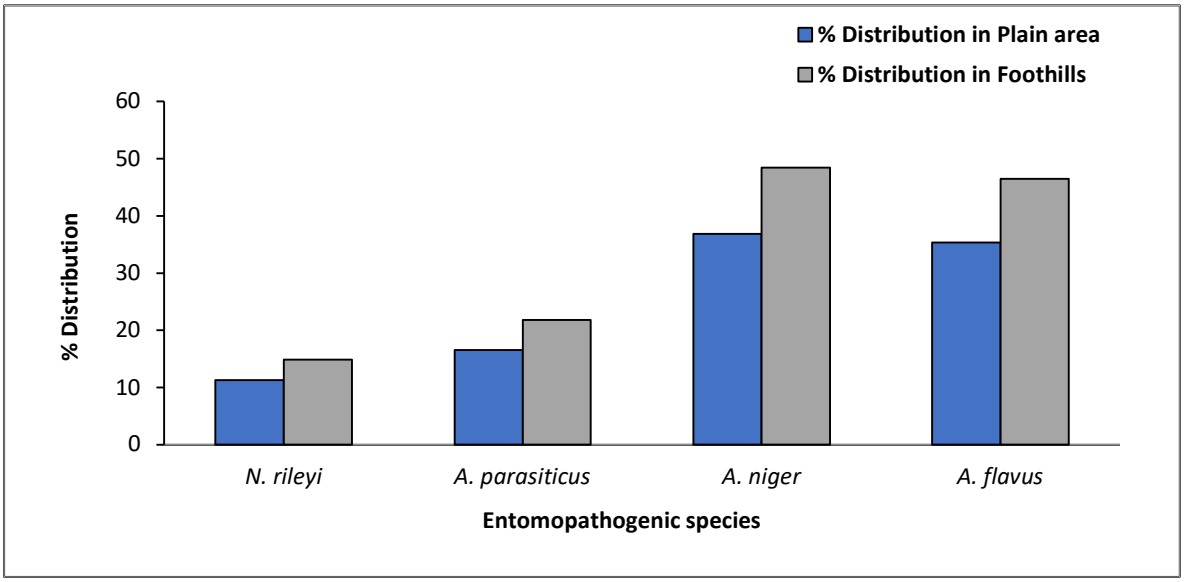

Figure 3. illustrates the percent distribution of entomopathogenic fungi (EPF) isolates in plains vs foothills soil of Khyber Pakhtunkhwa province. The findings suggest that the comparative distribution of all EPF isolates was higher in the soil of the foothills than in the plain areas. Overall, fungal species composition was uniform throughout, with the highest percentage distribution of *Aspergillus niger*, followed by *Aspergillus flavus*, *Aspergillus parasiticus* and *Nomuraea rileyi*.

3.3. Comparative Distribution of EPF Isolates in Different Cropping Systems

The percent distribution of fungal isolates was compared across different cropping systems viz., agronomic and horticultural crops of Plains and Foothills in KP Province. Of 209 isolates, the highest number (119) was found in various horticultural crops. The percent distribution of nearly all fungal isolates in horticultural crops was relatively higher than in different agronomic crops (Table 1). Overall, the highest percent distribution was recorded for *A. niger* i.e., 43% in horticultural crops and 39% in agronomic crops, while *A. parasiticus* had the lowest percent distribution with 6% in horticultural and 5% in agronomic crops. (Table 1).

Table 1. Effect of various cropping categories (agronomic vs horticultural) on % distribution of EPF species. The values presented reflects the number of isolates of each EPF species and its % distribution frequency.

Fungal Species	Isolates in Agronomic Crops	% distribution	Isolates in Horticultural Crops	% distribution
Nomuraea rileyi	18	20	10	8
Aspergillus parasiticus	5	6	25	21
Aspergillus niger	39	43	43	36
Aspergillus flavus	28	31	41	34

3.4. Concentration-Response Bioassays

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1. First larval mortality of *H. armigera* exposed to different EPF concentrations

First mortality is the duration in which different EPF concentrations caused the first larval death to be recorded over 5 days. Results showed that at relatively high concentration levels (1×10^8, 1×10^7 conidia/ml), the first larval mortality in all fungal treatments occurred in a minimum time i.e., within 2 days. *Nomuraea rileyi* was found to be the most effective, causing the first mortality in the minimum time (Table 2).

Table 2. First mortality of *H. armigera* after being exposed to different concentrations of the tested EPF species. The sign (+) represents the first death of *H. armigera* and the sign (-) represents no death over 5 days of fungal exposure.

Treatments[a]	First Mortality of *H. armigera* (in days)				
	1 day	2 days	3 days	4 days	5 days
Nomuraea rileyi					
1×10^6	-	-	-	+	-
1×10^7	-	+	-	-	-
1×10^8	+	-	-	-	-
Aspergillus parasiticus					
1×10^6	-	-	-	-	+
1×10^7	-	+	-	-	-
1×10^8	-	+	-	-	-
Aspergillus niger					
1×10^6	-	-	-	-	+
1×10^7	-	+	-	-	-
1×10^8	-	+	-	-	-
Aspergillus flavus					
1×10^6	-	-	-	-	+
1×10^7	-	-	-	+	-
1×10^8	-	+	-	-	-
Control (water)	-	-	-	-	-

2. Total Percent Mortality of *H. armigera* exposed to different EPF concentrations at three post-inoculation intervals

The total mortality for each treatment was recorded at two post-inoculation intervals (PII): 7, and 14 days against the third instar of *H. armigera*. The recorded data were analyzed using ANOVA to assess the differences among treatments and the group means were compared with Tukey's HSD (Honestly Significant Difference) test at a significance level of $\alpha = 0.05$. Our findings revealed that at 7d PII, *N. rileyi* exhibited the highest mortality percentage in the third instar at 1×10^8 conidia/ml followed by *A. niger* at the same conidial concentration, while the lowest mortality was recorded in control group ($F=13.1$, $P=0.000$). At 14d PII, the highest mortality against the third instar was recorded

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with higher concentrations of tested EPF and no mortality occurred in the control treatment (F=10.1, P=0.000) (Table 3). Among the tested EPF species, *N. rileyi* demonstrated the highest total mortality with 1×10^8 conidia/ml at different post-inoculation intervals.

Table 3. Total Mortality (%) of third instar of *H. armigera* after being treated with different concentrations of the tested EPF species at two post-inoculation intervals. The values presented reflect means (±SE), and different letters indicate statistically significant differences (P≤0.05, Tukey HSD test).

Treatments	Mortality after 7 days	Mortality after 14 days
Nomuraea rileyi		
1×10^6	44.4 BC	66.7 ABC
1×10^7	55.6 BC	100.0 A
1×10^8	100.0 A	100.0 A
Aspergillus parasiticus		
1×10^6	44.4 BC	55.6 BC
1×10^7	66.7 ABC	88.9 AB
1×10^8	55.6 BC	100.0 A
Aspergillus niger		
1×10^6	33.3 CD	44.4 C
1×10^7	44.4 BC	66.7 ABC
1×10^8	77.8 AB	100.0 A
Aspergillus flavus		
1×10^6	66.7 ABC	66.7 ABC
1×10^7	66.7 ABC	77.8 ABC
1×10^8	66.7 ABC	100.0 A
Control		
Water	0.0 D	0.0 D
Sig. Level	P=0.000	P=0.000

3. Effect of different EPF concentrations on Pupal Recovery (%) and Adult Emergence (%) of *H. armigera*

The statistical analysis revealed that different conidial concentrations of *N. rileyi* significantly reduced the percent pupal recovery and adult emergence of previously treated *H. armigera* larvae (Table 4). The highest percent pupal recovery was recorded in the control group, which significantly decreased with increasing concentration levels and reached its lowest at higher concentrations of 1×10^8, 1×10^7 conidia/ml of *N. rileyi*, and 1×10^8 conidia/ml of other tested species (F=13.1, P=0.000). Similarly, the lowest percent emergence was observed in higher concentrations of the tested EPF

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species. While the highest percent adult emergence was recorded in the control group (F=3.01, P=0.00) (Table 4).

Table 4. Effect of different concentrations of tested EPF species on % pupal recovery and % adult emergence of *H. armigera*. The values presented reflect means (±SE), and different letters indicate statistically significant differences (P≤0.05, Tukey HSD test).

Treatments	Pupal recovery (%)	Adult emergence (%)
Nomuraea rileyi		
1×10^6	33.3 BCD	50.0 A
1×10^7	0.0 D	0.0 A
1×10^8	0.0 D	0.0 A
Aspergillus parasiticus		
1×10^6	44.4 BC	50.0 A
1×10^7	11.1 CD	33.3 A
1×10^8	0.0 D	0.0 A
Aspergillus niger		
1×10^6	55.6 B	50.0 A
1×10^7	33.3 BCD	33.3 A
1×10^8	0.0 D	0.0 A
Aspergillus flavus		
1×10^6	33.3 BCD	100.0 A
1×10^7	22.2 BCD	66.7 A
1×10^8	0.0 D	0.0 A
Control		
Water	100.0 A	100.0 A

4. LC_{50} and LT_{50} Values of the Tested EPF Species Against H. armigera

Using SPSS v16 and a probit analysis on mortality data, the LC_{50} and LT_{50} values were calculated for the EPF species, setting P<0.05 as the significance level. We measured LC_{50} values at three post-inoculation intervals: 3, 7, and 14 days, against the third instar of *H. armigera*. Our findings showed that the LC_{50} values of the tested EPF species were time-dependent and decreased significantly as the post-inoculation interval increased. At 3d, the lowest LC_{50} was recorded for *N. rileyi* (1.36×10^8 spores/ml), followed by *A. niger*, *A. flavus* (1.15×10^9 spores/ml) and *Aspergillus parasiticus* (1.66×10^9 spores/ml) (Table 5). At 7d, *N. rileyi* was the most virulent, with the lowest LC_{50} (2.40×10^6 spores/ml), followed by *A. parasiticus* (3.14×10^6 spores/ml) and *A. niger* (8.06×10^6 spores/ml), while *A. flavus* was the least effective, showing the highest LC_{50} (3.18×10^7 spores/ml) (Table 6). At 14d, the lowest LC_{50}

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was recorded for *A. flavus* (4.00×10^5 spores/ml) followed by *N. rileyi* (7.19×10^5 spores/ml), *A. parasiticus* (8.05×10^5 spores/ml) and *A. niger* (1.88×10^6 spores/ml) (Table 7).

Table 5. LC_{50} values (conidia/ml) of entomopathogenic fungi against third instar of *H. armigera* after 3 days.

Fungal Species	N	LC_{50}^* (conidia/mL)	Slope ± SE	Intercept	X^2 (df=1)	P
Nomuraea rileyi	27	1.36×10^8	0.52 ± 0.34	-4.20	0.20	0.65[b]
Aspergillus parasiticus	27	1.66×10^9	0.43 ± 0.36	-3.94	0.36	0.55[b]
Aspergillus niger	27	1.15×10^9	0.39 ± 0.34	-3.53	0.01	0.92[b]
Aspergillus flavus	27	1.15×10^9	0.39 ± 0.34	-3.53	0.01	0.92[b]

N=number of treated insects, SE=Standard error, X^2 =Chi square value, df=degree of freedom, P=Probability value.

Table 6. LC_{50} values (conidia/ml) of entomopathogenic fungi against third instar of *H. armigera* after 7 days.

Fungal Species	N	LC_{50}^* (conidia/mL)	Slope ± SE	Intercept	X^2 (df=1)	P
Nomuraea rileyi	27	2.40×10^6	0.94 ± 0.39	-5.96	2.08	0.15[b]
Aspergillus parasiticus	27	3.14×10^6	0.28 ± 0.30	-1.85	0.00	0.99[b]
Aspergillus niger	27	8.06×10^6	0.59 ± 0.32	-4.08	0.34	0.56[b]
Aspergillus flavus	27	3.18×10^7	0.28 ± 0.30	-2.14	0.00	0.99[b]

N=number of treated insects, SE=Standard error, X^2 =Chi square value, df=degree of freedom, P=Probability value.

Table 7. LC_{50} values (conidia/ml) of entomopathogenic fungi against third instar of *H. armigera* after 14 days.

Fungal Species	N	LC_{50}^* (conidia/mL)	Slope ± SE	Intercept	X^2 (df=1)	P
Nomuraea rileyi	27	7.19×10^5	2.97 ± 5.10	-17.38	0.00	0.95[b]

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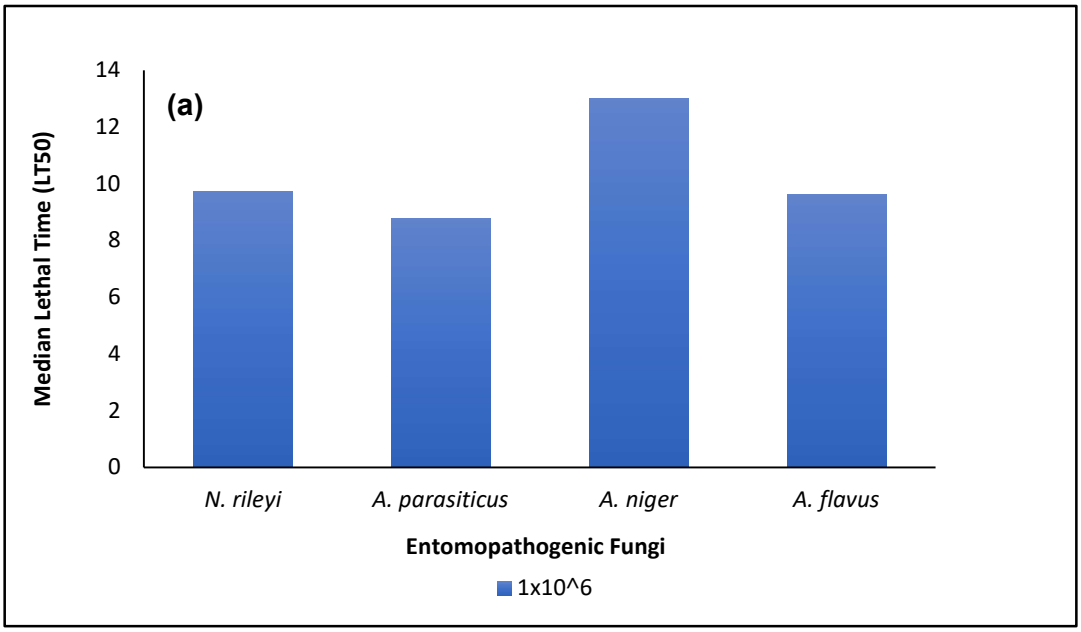
Aspergillus parasiticus	27	8.05×10^{5}	1.20 ± 0.58	-7.10	0.08	0.77^{b}
Aspergillus niger	27	1.88×10^{6}	1.02 ± 0.42	-6.40	0.02	0.31^{b}
Aspergillus flavus	27	4.00×10^{5}	0.77 ± 0.44	-4.31	0.86	0.35^{b}

N=number of treated insects, SE=Standard error, X^2 =Chi square value, df=degree of freedom, P=Probability value.

Median Lethal Time (LT_{50})

Median lethal time (LT_{50}) was also estimated for each fungal concentration, i.e., 1×10^{6}, 1×10^{7}, and 1×10^{8} conidia/ml. At 1×10^{6} conidia/ml, the lowest LT_{50} was observed in *A. parasiticus* (8.77 days), followed by *A. flavus* (9.62 days), *M. rileyi* (9.72 days), and *A. niger* (12.9 days) (Figure 4 a-c). At 1×10^{7} conidia/ml, *M. rileyi* showed the lowest LT_{50}, followed by *A. flavus* and *A. parasiticus*, with *A. niger* having the highest LT_{50} (10.6 days) (Figure 4 b). Similarly, at 1×10^{8} conidia/ml, *M. rileyi* was the most virulent with the lowest LT_{50} (5.45 days), followed by *A. niger* (8.25 days) and *A. parasiticus* (8.49 days), while the highest LT_{50} was recorded for *A. flavus* (8.55 days) (Figure 4 c).



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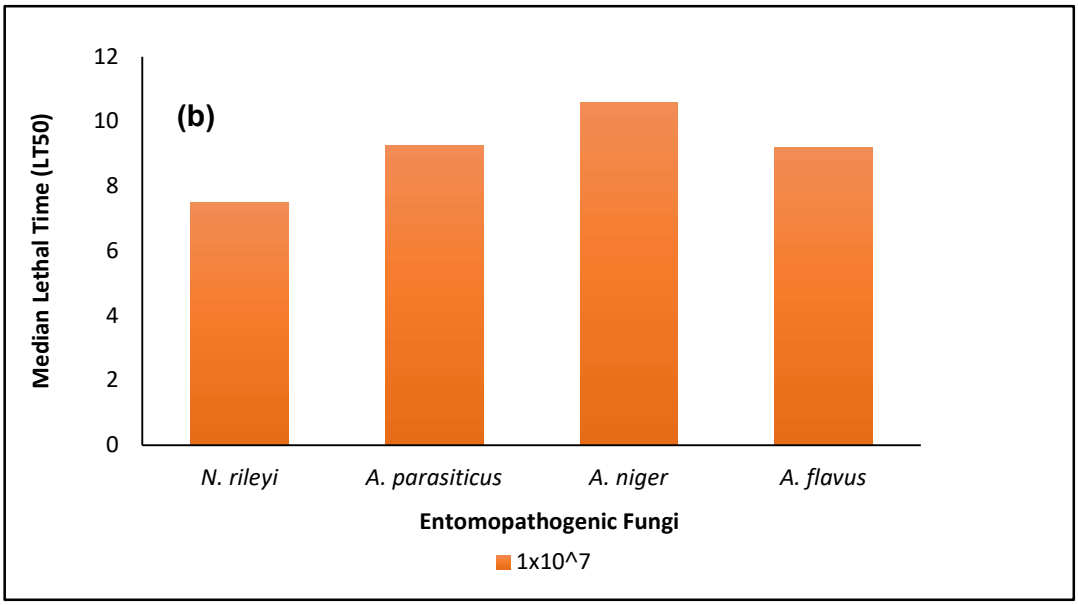

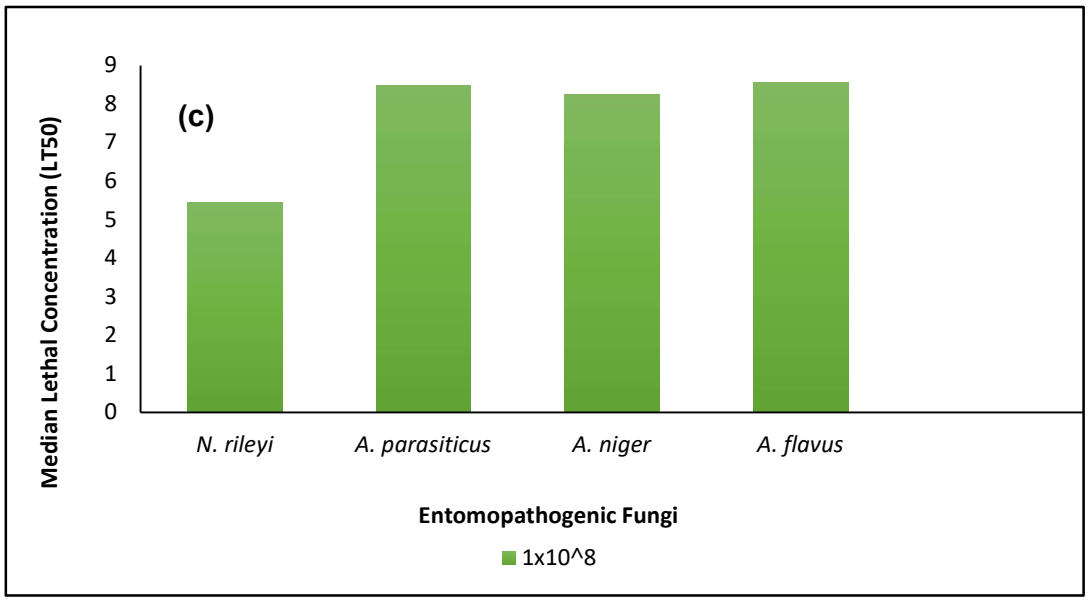

Figure 4. a-c: LT_{50} (days) at three conidial concentrations (a) 1×10^6 conidia/ml (b) 1×10^7 conidia/ml (c) 1×10^8 conidia/ml of entomopathogenic fungi (EPF) to kill 50% population of the exposed *H. armigera* larvae. The results suggest that with increasing conidial concentration of EPF species (except *A. parasiticus*), the LT_{50} values significantly decrease, with the minimum values recorded at 1×10^8 conidia/mL of tested EPF species.

4. Discussion

Many studies have reported the isolation and identification of fungal entomopathogens in different habitats (Sookar et al. 2008, Tkaczuk et al. 2014, Gorczyca et al. 2018). However, in Pakistan, due to limited knowledge about the occurrence and distribution of native fungal species, no microbial pesticide has been registered or made commercially available to farmers for local pest control (Iqbal et al. 2021). This study confirms the presence of potential EPF in cultivated soils. Our results revealed that the distribution percentage of fungal isolates was higher in the soil of the foothills compared to the plains, indicating a significant presence in the soils of the higher altitude districts (Haripur and Abbottabad). These findings align with those of Wakil et al. (2013), who evaluated the occurrence of fungal entomopathogens in different regions of Punjab province and reported the highest

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distribution of fungal isolates in soil from higher altitudes (above 600 m). While Liu et al. (2021) identified ultraviolet exposure as a major factor negatively affecting the occurrence and persistence of EPF species in soil, which contradicts our findings, as foothill soils, being at higher altitudes, are more exposed to ultraviolet radiation than those in plain areas. The difference may be due to variations in the organic content and soil texture that significantly influence the occurrence of EPF in soil habitats (Uzman et al. 2019).

The comparative distribution of fungal isolates was higher in different horticultural crops than in agronomic fields. Our results are in partial agreement with Qayyum et al. (2021), who reported higher fungal distribution frequency in fruits (52.50%) and vegetables (42.50%) than in field crops (37.50%). Similarly, Sookar et al. (2008) also observed that the occurrence and distribution of EPF were significantly influenced by habitat type. They recovered a larger number of *Metarhizium anisopliae* isolates from the vegetable soil than from other plantations. Notably, in our results, *Aspergillus* was the most prevalent genus, with *Aspergillus niger* found as the most abundant EPF species among the fungal isolates, followed by *A. flavus*. Our results are supported by Qayyum et al. (2021), who reported *A. niger* as the most abundant fungal strain with a distribution frequency of 27.50% followed by *A. flavus* with 22.50% occurrence in sampled soil. This is because various ecological and environmental factors influence the distribution and abundance of EPF in the soil habitat (Tkaczuk et al. 2014). Similarly, Wakil et al. (2013) recovered *Aspergillus* as the most common genus from soil samples.

The virulence of isolated EPF species, identified as *N. rileyi*, *A. parasiticus*, *A. niger*, and *A. flavus* was evaluated in bioassays against the third instar of *H. armigera*. The larvae were found susceptible to all tested fungal entomopathogens, resulting in significant mortality, especially at their highest concentration 1×10^8 conidia/ml. Our results showed a dose-dependent mortality response, with *N. rileyi* as the most effective treatment, causing the highest percentage mortality, followed by *Aspergillus* species. These findings are partly consistent with those of Hazarika et al. (2016), who reported concentration-dependent mortality with *N. rileyi* and identified it as a promising biocontrol agent, causing 86% mortality in *H. armigera* at a concentration of 1×10^9 spores/mL. The difference in the maximum mortality recorded in the present study and Hazarika et al. (2016) is attributed to variations in strains and the concentrations assessed in both studies. Our results are highly consistent with those of Dev et al. (2021), who found *M. rileyi* to be highly virulent against *H. armigera*, with the highest mortality observed in the third instar at 1×10^8 conidia/ml. *M. rileyi* causes host-preferential epizootics in susceptible larvae mainly by blocking respiratory structures, leading to host death (Sabbour and Abdel-Rahman 2013). Similarly, Ramos et al. (2024) recorded an effective mortality rate (70 to 98.7%) in *Spodoptera frugiperda* larvae when treated with 24 different isolates of *M. rileyi* in bioassays.

Several studies have reported the entomopathogenic potential of *Aspergillus* species against various insect pests, including lepidopterans (Fitriana et al. 2021), with limited research focused on *H. armigera.* Mensah et al. (2015) assessed the efficacy of *Aspergillus* sp. (BC 639) against *Helicoverpa* spp. and predatory insects both in vitro and in cotton fields. They reported effective pest control using *Aspergillus* spp. with minimal effects on beneficial organisms, maintaining crop yield. Similarly, Kaur et al. (2016) reported a 63% larval mortality in *Spodoptera litura* using ethyl acetate extract of *A. niger* compared to 3% in the control group.

The pupal recovery and adult emergence of treated *H. armigera* larvae were significantly reduced with increasing conidial concentration. The lowest percent pupal recovery and percent emergence were observed at different concentrations of *N. rileyi,* followed by *Aspergillus* species. These findings partially agree with those of Mantzoukas (2019), who reported concentration-dependent pupation and emergence when exposing *H. armigera* larvae to different concentrations of *Metarhizium robertsii,* with the lowest pupation and emergence observed at the highest concentration (1×10^7 conidia/ml). However, Shanthakumar et al. (2010) found no significant effect of EPF concentration on pupation and recorded 90% pupal recovery in *S. litura* at both lower and higher concentrations of *N. rileyi* (10^4 and 10^7 conidia/ml). Similarly, Hatting et al. (2012) observed accelerated pupation in *H. armigera* after

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treating the larvae with *N. rileyi* using both topical and oral inoculation methods. The observed discrepancies in pupal recovery results may be attributed to the genetic makeup of fungal strains isolated from different geographical regions (Bidochka et al. 2000, Couceiro et al. 2022). Additionally, variations in methodology, including inoculation techniques and the susceptibility of targeted pests to fungal infections, also explain the contrasting outcomes.

Probit analysis revealed that the estimated LC_{50} values of the tested EPF species were time-dependent and significantly decreased as the PII increased. *N. rileyi* was found to be most virulent, exhibiting the lowest LC_{50} at 7d PII, followed by *A. parasiticus*, *A. niger* and *A. flavus*. These results are in accordance with those of Liu et al. (2019), who reported an LC_{50} of 6.24×10^{6} conidia/ml for *M. rileyi* against third-instar *S. litura*. Similarly, Ramos et al. (2024) documented the LC_{50} range (2.04×10^{5} to 1.05×10^{6} conidia/mL) of 5 different *N. rileyi* isolates against *S. frugiperda* larvae. However, LC_{50} results for *N. rileyi* are in partial agreement with those of Dev et al. (2021), who determined the LC_{50} of 2.81×10^{5} spores/ml for *M. rileyi* against *H. armigera* third instar. This partial variation in the findings may be attributed to the genotypic variation, related to their adaptation to different native environments (Bidochka et al. 2000, Couceiro et al. 2022).

LT_{50} values of almost all fungal treatments (except *A. parasiticus*) were dose-dependent and significantly decreased with increasing conidial concentrations. These findings are supported by Yang et al. (2024), who also observed concentration-dependent LT_{50} values of *M. rileyi* against *S. frugiperda* larvae. Contrary to these results, Ramos et al. (2024) recorded LT_{50} values for 1×10^{8} conidia/ml concentration of five *M. rileyi* isolates ranging from 7.04 days to 9.46 days against the second instar of *S. frugiperda*. The observed discrepancies in the LT_{50} results may be attributed to experimental conditions and targeted lepidopterous species.

Based on our findings, the distribution of entomopathogenic fungi in the different agro-ecological zones and cropping systems was not uniform throughout the soil habitat. EPF species viz., *N. rileyi*, *A. parasiticus, A. flavus* and *A. niger*, isolated from cultivated soil in Khyber Pakhtunkhwa province of Pakistan, effectively managed the local pest, *H. armigera* in laboratory conditions. Among all the fungal species tested, *N. rileyi* showed the most promising control, followed by *Aspergillus* species. *N. rileyi* caused deadly mycosis in *H. armigera* and showed a detrimental impact on its biological parameters as well. Hence, it is potentially suitable for the eco-friendly management of this pest and would be a sustainable alternative to synthetic insecticides. Naturally occurring EPF causes deadly mycosis in insect pests, with the potential to infect all their life stages. Their incorporation into IPM strategies will ensure the production of healthier organic crops by addressing the insecticide residue and resistance issues. Further, field trials using the extracts of isolated EPF species against *H. armigera* are needed.

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Data Availability Statement: All data supporting the findings of this study are included in the manuscript. The raw data can be made available by the corresponding author upon reasonable request.

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Conflicts of Interest: All authors declare no conflict of interest.

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