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Isolation and identification of entomopathogenic fungi associated with the spiny bollworm and evaluation of their metabolites against the insect's biological parameters

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ABSTRACT

Entomopathogenic fungi (EPF), along with their byproducts such as cuticle-degrading enzymes, serve as robust biological agents for controlling agricultural pests. This study focuses on isolating and identifying fungi found in the spiny bollworm (SBW), *Earias insulana* larvae cadavers. The investigation delves into the impact of their metabolites on various stages of *E. insulana*. Among the isolated fungi from the insect larvae cadavers (*Trichoderma koning*, *Fusarium oxysporum*, *Penicillium notatum*, *Trichoderma asperellum*, and *Altarnaria alternata*). *T. asperellum* was the most prevalent, its metabolite displayed substantial larval and pupal mortality rates of 60.73% and 21.82%, respectively. It also disrupted multiple growth stages of the insects, reducing adult emergence, the number of deposited eggs, and hatchability percentage (68.78, 156.89 and 61.90) compared with control (100.00, 273.11 and 93.17), respectively. Consequently, T. *asperellum* underwent scrutiny for producing cuticle-degrading enzymes using modified Czapek's broth medium tailored for each enzyme individually. In each culture filtrate, the activities of proteases and lipases surpassed those of chitinases, potentially explaining the heightened larval mortality rates (70.22 and 68.74%) post-inoculation with proteases and lipases specific culture filtrate, respectively. The verification of *T. asperellum's* identity was accomplished using the 18S rRNA gene of DNA, and the resulting fungal sequence was deposited into NCBI under accession number OQ616502. The crude filtrate derived from *T. asperellum*, contains cuticle-degrading enzymes with notably high proteases and lipases activities. This filtrate exhibits potent effects on *E. insulana* larvae and pupae, significantly impacting their biological processes.

Keywords: *Earias insulana,* Entomopathogenic fungi, *Trichoderma asperellum*, cuticle degrading enzymes.

INTRODUCTION

Cotton, *Gossypium barbadense* holds immense importance as an economic crop. Its versatility and widespread use make cotton a crucial commodity that profoundly impacts various aspects of society and the global economy **(**Haider *et al.,* 2015; Malinga and Laing, 2022). The spiny bollworm (SBW)*, Earias insulana,* (Boisd.) (Lepidoptera: Nolidae) (Zahiri *et al.*, 2013) poses significant challenges to cotton and many other crops. It causes crop damage by feeding on the reproductive structures of cotton plants affecting the quality of cotton fiber produced. Efforts to effectively manage this pest are crucial to mitigate crop losses and support the livelihoods of farmers relying on cotton and other affected crops (Lotfy and Moustafa, 2021).

Microorganisms, particularly Entomopathogenic fungi play a vital role in biological pest control and act as promising alternatives to chemical insecticides. Their ability to infect and kill insects, along with their specificity and environmental safety qualify them to be valuable tools used in sustainable agriculture and pest management practices (Farooq *et al.,* 2020).Entomopathogenic fungi have significant effects on the biology of *E. insulana*; these fungi infect bollworm larvae, leading to their death and subsequently reducing population size in agricultural settings. Reduced fertility by decreased mating success or egg production, contributing to further declines in population size. Even surviving larvae may suffer impaired development, reduced feeding activity, or altered behavior, making them more vulnerable to predation or other stressors. Finally, when applied as biocontrol agents, can spread among bollworm larvae, leading to localized outbreaks and providing sustained control over time (Ullah *et al*., 2019).

EPF produce mixture of cuticle-degrading enzymes which are considered an essential component for pathogenicity, playing an effective role in fungal adhesion and penetration to insect cuticles. Proteases, lipases, and

chitinases are major enzymes produced by EPF (Hajek and St. Leger, 1994). Lipases may exist on the conidial outer layer to enhance the bonding of the conidia to the host by boosting the hydrophobic connections between the conidial surface and the host cuticle by releasing free fatty acids resulting from lipolytic activity. Proteases especially subtilisin protease (Pr1) serves as a crucial factor influencing virulence, are tasked with the enabling the solubility of proteins in the pro-cuticle to aid hyphal entry and supplied nourishment for fungal development. Chitinases with different isoforms produced and collaborate in tandem with proteases to break down host cuticles (St. Leger *et al*., 1987; St. Leger *et al*.,1991; Butt *et al*., 2013; Akyıl and Cihangir, 2018). The combination of these enzymes allows the fungi to penetrate the insect cuticle and ramify throughout the host hemocoel forming yeast-like blastospores, that structure facilitates rapid dispersal in the hemolymph offering a large surface area for nutrient uptake and multiplying in this form until nutrients are exhausted (Akyıl and Cihangir, 2018). *T. asperellum* belongs to a type of filamentous fungus relating to the *Trichoderma* genus. It is recognized for its ability to produce various enzymes with important roles in biological aspects and captured the worldwide attention for insects ' control (Dou *et al.,* 2015).

Understanding the mechanisms of enzyme production and evaluating of their efficacy to be used as biological control agents could lead to better use of EPF in IPM strategies. The main objectives of this study were to identify the most pathogenic fungi isolated from *E. insulana* larvae cadavers, investigate its capacity for generating enzymes that degrade cuticles and evaluate the efficacy of its crude metabolite on various developmental stages of *E. insulana.*

MATERIALS AND METHODS

1. Rearing of the spiny bollworm:

Our study utilized recently emerged larvae of *E. insulana* reared in the Bollworm Research Department, Plant Protection Research Institute, Sharkia Branch, Agriculture Research Centre, Giza, Egypt. These larvae were bred for numerous generations on a specialized diet outlined by (Amer, 2015) ensuring away from insecticidescontamination.

2. Technique for isolation of fungi:

To unveil EPF linked to the deceased *E. insulana* larvae, each cadaver underwent a triple rinse with 70% alcohol, followed by resination using sterile distilled water then, positioned within a Petri dish lined with damp, sterile filter paper. The dishes were then kept at ambient room temperature (28±2 ºC), for a duration of 10 days until the insect's body became coated with fungal. They were placed on an inclined surface for purification and identification. The cultivated fungi were then isolated from Czapeck's Dox's agar medium using the direct plating technique (Inglis *et al.,* 2012), and incubated at room temperature 28±2 °C, for a period ranging between 5 to 7 days. Plates placed in the incubator were examined on a daily basis to monitor the development of fungi, as fungal emergence start to appear; they were placed on slants for purification and identification. The identical procedures were applied to robust larvae to obtain the expected dormant and excluding isolates shared between healthy and dead larvae (Rosa *et al*., 2021).

3. Utilizing a light microscope for the identification of isolated fungi at the species level:

He progressing fungal colonies were inspected on a daily basis, and the purified fungi were identified at the level of species whenever feasible. Macroscopic recognition was carried out through the identification of the colony morphology and is depended on the form of the colony, the elevation, and pigmentation of the projecting hyphae, alongside the foundational hue, rate of expansion, edge attributes, external texture, and penetration depth into the substrate. Microscopic identification relies on discerning the structural features of hyphae, the morphology and positioning of spores, and the existence of distinctive anatomical elements. The process of identifying fungal genera and species was conducted with assistance from the universally acknowledged for identifying tested isolates (Humber, 2005;). Examinations were compared against a structured reference guide for the *T. asperellum* (White *et al*., 1990).

4. Screening of the isolated fungal metabolites against *E. insulana* larvae:

4.1. Evaluation of fungal metabolites:

100 ml of sterilized Czapeck's Dox's medium inoculated with spore suspensions of seven days-old slant, for all five tested fungal isolates then incubated at 28±2 °C for 7 days. Crude metabolites from these isolated fungi were

obtained through growth culture filtrate using filter paper (Whattman No.1.) and assessed for their impact on newly hatched larvae of *E. insulana* and their latent effect on various developmental stages (White *et al*., 1990).

4.2. Bioassay:

Mixing four grams of artificial diet manually with two ml of respective fungal crude metabolites and distilled water for treated and untreated dishes, respectively then left about 30 min before experimentation to dryness. Newly hatched *E. insulana* larvae (25 larvae) were individually placed in Petri dishes covered with fine tissue paper below a glass cover to prevent high humidity and larval escape. Each treatment was repeated four times under constant conditions (26±1ºC and 70±5% RH). Larval mortality was recorded after one day post-feeding. Surviving larvae from treated and untreated dishes were singly transferred to glass tubes (2 x 7.5 cm) comprising approximately four grams of diet without any processing shielded by a segment of absorbent cotton and maintained under the conditions till pupal stage. Each pupa was separately relocated to sterilized glass tubes and kept under incubation until the emergence of the moth. The biological parameters, encompassing larval and pupal mortality, deformities, duration, pupation percentage, and sex ratio, were meticulously noted.

Emerging moths from each treatment were segregated by sex then coupled in glass cages (two pairs/cage). Four replicates were used for each treatment and control. A segment of cotton batting saturated with a solution comprising 10% sugar suspended at upper opening of jar for moth nutrition and replace every day. A layer of muslin fabric covered the top apertures of the cages, then firmly fastening a sheet of paper using rubber bands. Daily recordings of periods before egg-laying, during egg-laying, laid eggs, after egg-laying period and male/female longevity were made. Collected eggs were transferred daily to another glass jar for hatchability assessment under identical conditions.

5. Screening of the most potent isolate crude enzymes against *E. insulana* larvae:

5.1. Assessment of *T. asperellum* enzymatic activity: protease, lipase, and chitinase production:

The most active isolate underwent screening for lipase, protease, and chitinase production using specific broth mediums for each enzyme. Lipase activity was assessed using Dox-yeast extract tributyrin broth medium (Abd-ElAzeem *et al.*, 2019), while protease activity was determined by substituting $NaNO_3$ in Dox media with 0.2% casein (Ammar *et al*., 1991) Chitinolytic activity was determined on chitin broth media using colloidal chitin, 0.5; as a carbon source and yeast extract, 0.5; as a nitrogen source (Rajamanickam *et al*., 2012). 100 ml of sterilized modified Czapek's broth medium was inoculated with 1 ml of ($1x10^7$) fungal spores and then incubated under static conditions at 28±2ºC. After 7 days of incubation, the flasks contents underwent centrifugation, and the resulting supernatant (cell-free cells) was utilized as a crude enzyme source (Sahab *et al.,* 2019). The activity of each enzyme was tested by the clear zone technique, 20 ml of specific modified Czapek's agar media was poured into a Petri dish. After solidification 1 cm well was made on each agar plate and 0.1 ml (100 µl) of the above (CFF) was added to each well. Following a 24-hour incubation period at 28±2°C. The transparent (Clear) area surrounding the well was quantified in millimeters, correlating the zone's diameter increase with enzyme activity augmentation.

5.2. Effectiveness of *T. asperellum* crude enzymes against *E. insulana* larvae:

The crude culture filtrate obtained from the most active fungal species, and tested for cuticle degrading enzyme production was screened against *E. insulana* larvae as mentioned previously in section (4.2.).

6. Molecular characterization of the most potent fungal isolate (sequence of 18S rRNA gene of DNA):

The identification of the most potent isolate was confirmed using 18S rRNA gene of DNA of fungal isolates according to (Tarini *et al.*, 2010) and the retrieved sequence was BLAST searched with non-redundant sequences on the NCBI database. For the multiple sequence alignments, FASTA sequences were imported into MEGA 6.0 software, and aligned with ClustalW muscle algorithm (Edgar, 2004). The phylogenetic tree of the target sequence was constructed with neighbor-joining method of MEGA 6.0 (Tamura *et al.*, 2011).

Statistical analysis:

The obtained results were converted from percentages to Arcsine before statistical analysis was performed and analyzed according to Little and Hills, (1975) using CoStat computer program Cohort Software. P. O. Box 1149, Berkeley CA 9471 (CoStat, 2005).

RESULTS

Five different fungal isolates were isolated from naturally dead *E. insulana* larvae. Metabolites of these fungi were preliminarily tested for their pathogenicity against larvae of the *E. insulana* immediately after hatching. Analysis of

variance in table (1) explored significant larval mortality percentage between tested fungal isolates *(Trichoderma koning, Fusarium oxysporum, Penicillium notatum, Trichoderma asperellum* and *Altarnaria alternata*.). Metabolites of *T. asperellum* displayed the highest larval mortality of 60.73% compared to the control (3.31%) Table (1). Also, *T. asperellum* metabolites showed the highest mortality for pupa of 21.82% compared to the control (0.00%). The pupal mortality was insignificant between all tested fungal isolates. Moreover, treatment with metabolites of different tested fungi significantly on adult emergencies as well. *T. asperellum* showed the lowest value of adult emergence 68.78% compared to the control (100.00). On the other hand, no significant effects were recorded between all isolates concerning deformed adults compared with control. *T. asperellum* showed the highest value of deformation (12.94%) compared with control (0.00).

Table 1. Effect of some fungal metabolites on larval, pupal and adult stages of the spiny bollworm *E. insulana*.

Isolates	Larval mortality%	Pupal mortality%	Adult emergence%	Deformed adult%
T. koningi	44.82b	20.56	74.11b	9.39
F. oxysporum	42.73b	17.72	79.95b	6.86
P. notatum	42.34b	17.15	78.32b	5.56
T. asperellum	60.73a	21.82	68.78b	12.95
A. alternata	41.03b	18.07	75.21b	5.12
Control	3.31c	0.00	100.00a	0.00
P.	< 0.0001***	NS	0.0045**	NS
LSD $_{0.05}$	7.66	---	13.34	---

Same letters mean non-significant effect while different letters mean significant effect.

Data in Table (2) illustrated the effect of tested fungal metabolites on duration of some developmental stages. Larval durations showed highly significant effect on tested fungal metabolites while the metabolites of *T. asperellum* shortened the duration of larvae (12.64 days) than other tested metabolites and also than control (15.85 days). Non-significant effect recorded for pupal duration when treated with tested fungal metabolites; the shortest duration recorded for *T. asperellum* (9.23 days) compared with control (10.35 days). Longevity of emerged females fed as larvae on the treated diet with five isolates metabolite proved a significant effect on the pre-ovipositional, and ovipositional periods, while non-significant differences were found between post-ovipositional periods and control. Regarding the duration of males and females' moths for all tested isolates had high significant effects than on the control (P=19.64 & 18.70), respectively.

Table 2. Effect of some fungal metabolites on duration in days of some developmental stages of the spiny bollworm *E. insulana*

Isolates	Larval duration (days)	Pupal duration (days)	Female longevity				Male longevity (days)
			Pre oviposition (days)	Oviposition (days)	Post Oviposition (days)	Total (days)	
T. koningi	15.18abc	9.30	2.34b	10.81ab	5.49	18.64ab	19.44a
F. xysporum	14.42bc	9.39	2.22b	10.05bc	5.50	17.77ab	19.33a
P. notatum	15.40ab	9.27	2.34b	10.82ab	5.54	18.70ab	19.64a
T. sperellum	12.64d	9.23	2.286b	9.45c	5.61	17.34b	18.29b
A. alternata	14.23c	9.53	2.30b	10.90ab	5.47	18.66ab	19.27a
Control	15.85a	10.35	2.58a	11.61a	5.83	20.02a	19.65a
P.	0.0004***	NS	0.0291*	0.0452*	NS	0.0492*	0.0054**
LSD 0.05	1.05	---	0.20	1.29	---	1.61	0.63

Same letters mean non-significant effect while different letters mean significant effect

The data in table (3) showed how the tested fungal metabolites effect on the sex ratio and the fecundity of *E. insulana* moths; that there was no significant effect of the five fungal metabolites on the sex ratio. There was a significant effect was observed on the number of deposited eggs, the lowest egg number recorded with *T. asperellum* metabolites (156.89) compared with control (273.11). Also, a significant effect was recorded with the hatchability percentage; the lowest hatching eggs recorded in treatment with *T. asperellum* (61.90) compared to the control (93.17).

Table 3. Effect of some fungal metabolites on sex ratio, fecundity and fertility of spiny bollworm *E. insulana*.

Isolates	Sex ratio%	Eggs no.	Hatchability%
T. koningi	49.39	204.67bc	79.61b
F. oxysporum	51.06	193.16bc	78.29b
P. notatum	50.73	208.82b	79.85b
T. asperellum	50.07	156.89c	61.90c
A. alternata	49.67	198.58bc	78.96b
Control	50.27	273.11a	93.17a
P.	NS	0.0087 **	< 0.0001 ***
LSD $_{0.05}$	---	50.7678	4.7079

Same letters mean non-significant effect while different letters mean significant effect

From the results obtained it is clear that the *T. asperellum* isolate is the most active. The molecular identification confirmation was done using ITs1 and ITS4, and the retrieved sequence was submitted to the gene Bank NCBI under accession No. MK942509 (Figure 1). Consequently, *T. asperellum* was selected for further study as the most effective fungus causing disturbance in spiny bollworm biological activities and high larval mortality. The selected isolate was screened for the production of lipase, protease and chitinase enzymes, using the clearing zone technique. The results revealed that *T. asperellum* exhibited high activities of protease and lipase causing a clear zone with a diameter of 28mm and 21mm, respectively as a result of hydrolysis of specific media containing casein as protein substrate and tributrine as lipid substrate while no chitinase activity. The screening of proteolytic and lipolytic activity of *T. asperellum* filtrates against SBW newly hatched larvae depicted mortality of 70.22 and 68.7%, respectively compared to the control (3.31%).

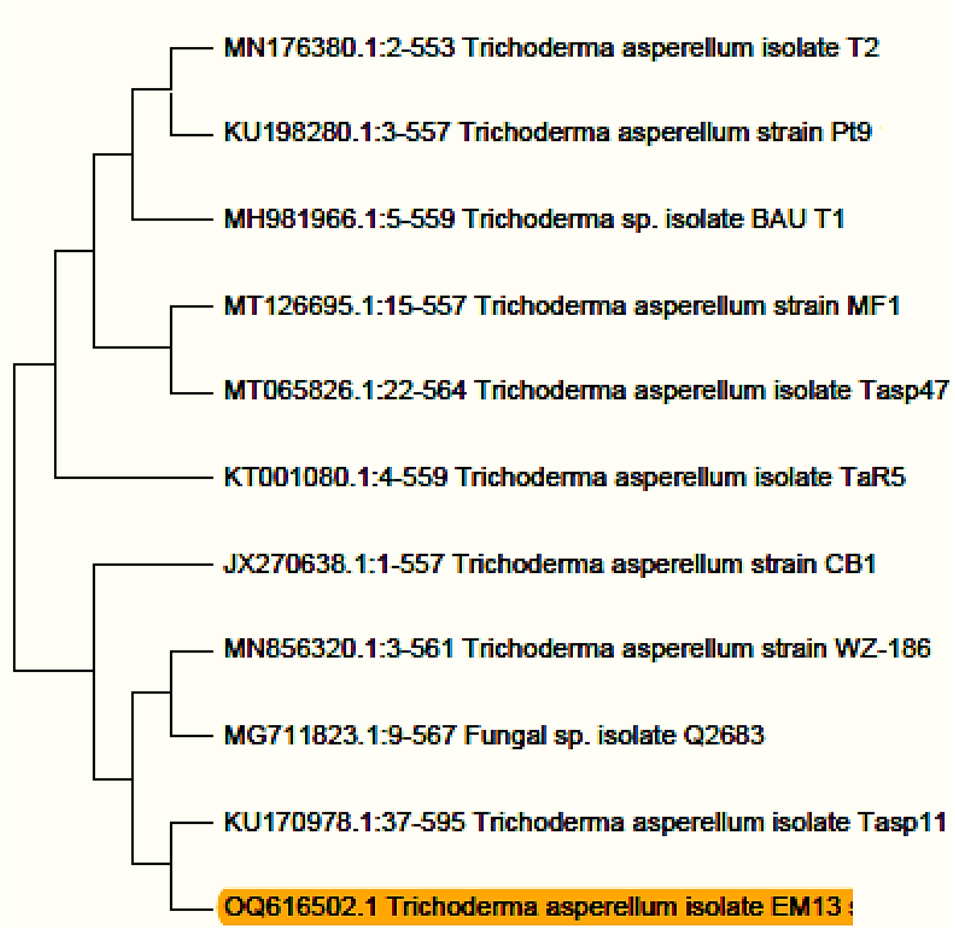

Fig. 1. Phylogenetic dendrogram of different Trichoderma isolates accessions revealed by average linkage cluster analysis based on 18S rRNA partial sequence.

DISCUSSION

EPF are an interesting group of fungi that infect insects and are extensively used in integrated pest management strategies. Most EPF are facultative pathogens that infect insects and consequently colonize insect cadavers. However, it is a challenge to determine whether the fungus isolated from an insect cadaver was responsible for the insect's death (Inglis *et al*., 2012) Several factors may determine or affect the susceptibility of the insect's host to infection by EPF including, the fungal strain, the host's physiological state, nutrition, defense mechanisms, presence of other microorganisms as well as several environmental parameters. Our study revealed that *T. koning, F. oxysporum, P. notatum, T. asperellum* and *A. alternata* were the most common fungus associated *E. insulana* larvae cadavers. All isolates able to produce a wide spectrum of bioactive secondary metabolites that belong to different classes of natural compounds (Petit *et al.,* 2009; Zin and Badaluddin, 2020; Mahmoud *et al.,* 2021; Ibrahim *et al.,* 2021). The study evaluates the efficacy of crude metabolites of different indigenous fungal isolates isolated from *E. insulana* larvae cadavers on different developmental stages of *E. insulana*. The crude metabolites of all tested fungal isolates exhibited significant larval mortality and insignificant pupal mortality of *E. insulana*. Similarly,

The ethyl acetate metabolite extract of *Aspergillus flavipes* AUMC 11390 exhibited insecticidal efficacy against *E. insulana*, resulting in death of larvae and pupae as well as a reduction in adult emergency and deformity of emerging adults. According to Ibrahim *et al.* (2021), the effects of the fungal extract treatment continued throughout adulthood, resulting in a decrease in the number of eggs produced, the hatchability %, and the lifespan of both males and females. Furthermore, when S. litura larvae were fed a diet enriched with ethyl acetate extract of A. flavus, the percentage of adult emergence, lifespan, and fecundity were reduced (Abd-ElAzeem *et al.,* 2023). This may be a result of the secondary metabolites produced by fungi that have poisonous properties that enable them to overcome humoral and cellular defenses in order to infiltrate their hosts (Gillespie *et al*., 2000). Moreover, the relationship between extracellular enzyme activity and fungal virulence (Gupta *et al*., 1994; Montesinos-Matías *et al*., 2011).

Cuticle-degrading enzymes protease, lipase and chitins are compromised important virulence factors used for insects' cuticle penetration and degradation (Butt *et al.* 2016). Several studies have assayed the activity of extracellular enzymes in the presence of insects' cuticles (Ramzi and Zibaee, 2014; Dhawan and Joshi, 2017). Our results indicated that *T. asperellum* crude metabolites have proteolytic and lipolytic activity that caused the highest larval mortality effect. When overexpressed from genetically modified plants or microbial diseases, proteases can have an insecticidal impact in addition to their responsibilities in insect growth and digesting. The insect midgut, hemocoel, and cuticle are among the locations where proteases exhibit harmful action (Robert and Bryony, 2010; Sargin et al., 2013; Cristina and Gheorghe, 2017; Sabry et al., 2023). Conversely, Reda et al. (2013) found that *Streptomyces vinaceusdrappus's* protease and lipase enzymes were harmful to *P. gossypiella*, the pink bollworm. The protease generated by Acremonium sp. was found to be active against *E. insulana* by Abd-ElAzeem et al. (2019), who also observed a significant larval death rate. The capacity of the fungus strain Acremonium sp. EZ1 (MN25101) to destroy the cuticles of both E. larvae and pupae was recently demonstrated using scanning electron microscopy (Sabry *et al*., 2023).

CONCLUSION

Our work underscores the importance of utilizing biological control methods using EPF as *Trichoderma asperellum*, for managing pests like *Earias insulana* in cotton crops. Understanding the mechanisms of action and effectiveness of these fungi can lead to more sustainable and effective pest management practices while minimizing environmental impacts associated with chemical insecticides.

Abbreviation:

EPF Entomopathogenic fungi
SBW Spiny bollworm
IPM Integrated pest management
RH Relative humidity

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