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Biological activities of spores and metabolites of some fungal isolates on certain aspects of the spiny bollworms *Earias insulana* (Boisd.) (Lepidoptera: Noctuidae)

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Abstract

Biological activities of spores and metabolites of some fungi isolated from dead larva of the spiny bollworms (SBW), *Earias insulana* (Boisd.) (Lepidoptera: Noctuidae), against the newly hatched larvae of the pest were carried out. Results showed that the fungi *Metarhizium anisopliae*, *Acremonium* sp., and *Paecilomyces variotii* had affected the newly hatched larvae of (SBW). *Acremonium* sp. was the most potent one as it had the highest newly hatched larval mortality percentage (65 and 58.33%) for its spore suspension and metabolites, respectively, while the lowest one (41%) was for *P. variotii* metabolites. Also, spore suspensions of the all fungal isolates had the highest larval mortality than fungal metabolites. Studying the enzymatic activity showed that *Acremonium* sp. produced protease enzyme on media containing gelatin, which caused the highest larval mortality (72.22%).
These isolates showed different effects on all stages of the pest and decreased pupal weight, adult emergence percentages, deposited eggs, and hatchability percentages than the control. Identification of *Acremonium* sp. EZ1 was confirmed using 18 s rRNA and its accession number MN25101.

Keywords: Spiny bollworms, *Earias insulana*, Entomopathogenic fungi, *Acremonium* sp., *Metarhizium anisopliae*, *Paecilomyces variotii*, Biological activity

Background

The spiny bollworm (SBW), *Earias insulana* (Boisd.) (Lepidoptera: Noctuidae), is an essential lepidoptera pest located in many countries of the Mediterranean basin, as well as in Africa and Asia (Mansour 2004). The SBW is one of the main cotton pests. Its larvae usually attack cotton flower buds, flowers, and bolls causing damage to seeds and fiber, especially at the late growing stage of the cotton plants leading to a decrease in the quality and quantity of the lint and the obtained oil yield (Salem 2008).

Entomopathogenic fungi (EPF) are biological control agents against insect pests. Fungi invade insects through penetrating the body cuticle by a combination of mechanical force and enzymatic degradation depending on the structure and composition of the insect cuticle (Reda et al. 2013). Microbial degradation of insect lipid, protein, and chitin as well as production of lipase, protease, and chitinase has captured the worldwide attention for insect control and has become the object of extensive research (Barra et al. 2015).

Reda et al. (2013) studied the effects of several microorganisms as biological control agents on economic pest showing cuticle degradation against the pink bollworm, *Pectinophora gossypiella* (Saund.), causing a high larval mortality and affected pupation and hatchability. Duarte et al. (2016) investigated the effect of *Beauveria bassiana* and *Metarhizium rileyi* on all biological aspects of diamondback moth (*Plutella xylostella* L.). Ibrahim et al. (2016) showed that *B. bassiana* and *Paecilomyces lilacinus* were virulent against the greater wax moth *Galleria mellonella* L. causing 98.0 and 87.5% larval mortality

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with a lethal time (LT_{50}) of 1.7 and 2.2 days, respectively. Latent effects were markedly obvious on pupation and rates of adult emergence. Also El-Massry et al. (2016) reported the efficacy of *Trichoderma harzianum* on the cotton bollworms *E. insulana* and *P. gossypiella.*

Proteases from a variety of sources (viruses, bacteria, fungi, plants, and insects) have toxicity towards insects. Other proteases play roles in insect development or digestion, but exert an insecticidal effect when overexpressed from genetically engineered plants or microbial pathogens. The sites of protease toxic activity range from the insect midgut to the hemocoel (body cavity) to the cuticle (Robert and Bryony 2010).

The main objectives of this study were to isolate fungi from dead larvae of SBW and evaluate the efficacy of these isolates on the pest and its ability to produce cuticle degrading enzymes.

Materials and methods

Rearing of the spiny

Full-grown SBW larvae of field strain were collected from infested cotton bolls in Sharkia Province, Egypt, and reared in the laboratory at the Bollworms Research Department, Plant Protection Research Institute, Agriculture Research Center, Giza, Egypt, for 6 generations. The neonate larvae were transferred into glass tubes (2.5 × 7cm) containing about 4 g semi artificial diet Shorey and Hale (1965). The experiments were performed at constant temperature of 26 ± 1 °C and 75 ± 5% RH. The diet for maintaining laboratory colony preparing by adding boiled water to 250 g kidney beans and 125 g wheat grated then add over heat for 70 min, lifted and left for 20 min to be cooled and clarifying water from them. The diet was blended by 100 ml milk in an electric blender and placed in the refrigerator for 24 h. After that 49 g dry active yeast, 3 g ascorbic acid, 1.75 g sorbic acid, 1.75 g methyl parahydroxy benzoate, 8 ml mixture of vitamins, and 2.5 ml formaldehyde 34–38% were added, all thoroughly blended and kept in the refrigerator for 24 h before being used (Amer and El-Sayed 2015).

The culture was away from any contamination with any microorganisms or pesticides. The dead larvae in culture were obtained at full grown and stored slowly in sterilized tightly closed vials at 4 °C in a refrigerator until needed (Mahfouz and Abou El-Ela 2011).

Microbiological analysis

Fungi isolation technique

In order to reveal any microorganisms associated with the dead spiny bollworm SBW larvae (4th instar), each of the refrigerated individuals was examined through 24–72 h from the time of storage under aseptic conditions. The larvae were surface sterilized by dipping in 2% sodium hypochlorite for 3–5 min to isolate fungi on insect's surface, then passed through 5 separated washings with sterile distilled water (Crecchio and Stotzky 2001). For insuring the appropriate surface sterilization, checks were made by spreading the last washing solution on Czapek-Dox agar medium. Sterilized larvae were dried up between 2 filter papers (Whattman No. 1), then transferred aseptically into a sterile mortar and macerated with a sterile pestle, diluted and plated on Czapek- Dox agar medium for growth incubating at 30 °C for 5–7 days. Incubated plates were inspected daily to observe the colonies growth that were then purified and stored on slants of the desired artificial media at 4 °C. The isolates were cultured periodically until they had been used in the subsequent experiments. Healthy larvae were subjected to the same procedures of isolation for obtaining the expected dormant pathogens.

Screening of fungal isolates for their mortality effect on E. insulana

Spore suspensions were obtained by washing the 7-day-old slant of tested fungal isolates (Dulmage et al. 1971; Mohd-Salleh and Lewis 1983), then inoculated a 100 ml of Czapek- Dox agar medium (Oxoid 1982) composed of (g/l) 20 sucrose, 2.0 $NaNO_3$, 1.0 KH_2PO_4, 0.5 $MgSO_4{\cdot}7H_2O$, 0.5 KCl, and 20.0 agar-agar and dissolved in 1 l tap water, pH 5.0 in a 250-ml Erlenmeyer flask with each suspension. The inoculated broth was incubated at 30 °C for 7 days, while metabolites were obtained by filtration using the filter paper (Whattman No.1.). Spore suspension and filtrate of all isolates were tested for their mortality effect and on biological aspects of *E. insulana* as described in the bioassay method.

Bioassay

Two milliliters from each spore suspension and metabolites was mixed with the artificial diet in each dish, while the diet of control was mixed with water only. Each treatment was replicated 3 times. Batches of 20 1st instar larvae were transferred immediately after hatching using a fine brush to each treated Petri dish after about 30 min from mixing in the diet. Treated Petri dishes were covered by a fine and soft paper below the glass cover to prevent larvae to escape. All treatments were incubated at the constant conditions of 26 ± 1 °C and 70 ± 5% RH. After 24 h of exposure and feeding, dead and alive larvae were counted. The mortality percentages were calculated.

$$Larval\ mortality\% = dead\ larvae/total\ larvae \times 100$$

Mortality data were corrected according to Abbott (1925).

$$Corrected\ mortality\% = \frac{mortality\ in\ treated - mortality\ in\ control}{mortality\ in\ control} \times 100$$

The remained alive larvae of each treatment were transferred singly to glass tubes (2 × 7.5 cm) containing about 4 g of untreated control diet and covered with a

piece of absorbent cotton and held under the same conditions as mentioned above. Larvae were examined daily to record the biological parameter, larval duration and pupation percentage; then, pupae were transferred individually to other clean tubes and incubated until moth emergence. Pupal duration, adult emergence percentage, sex ratio (as females), and deformed adults were calculated. Emerged moths from each treatment were sexed and caged in 2 pairs, and eggs deposited on strips of muslin cloth hanged in the chimney cages.

Forty pairs were used from each treatment (male and female) under the previously mentioned rearing conditions. A piece of cotton wool previously soaked in 10% sugar solution was hung inside the jars near its upper opening for moth feeding and changed by new one every 2 days. The upper openings of cages were covered by muslin cloth followed by a tightly secured paper with rubber bands. Each cage was examined daily to record data of several biological aspects such as preovipositional, ovipositional periods, number of deposited eggs, postovipositional period, and longevity of males and females. The deposited eggs were collected daily from strips of muslin cloth then transferred to a convenient glass jar and incubated at the same conditions to record hatchability percentages.

Characterization of most potent fungal isolate

Identification of isolated fungi by light microscope

The developed fungal colonies were examined daily, and the purified fungi were identified to the species level whenever possible. The identification of fungal genera and species was carried out by the help of the following universally accepted keys for identification of the different isolates. Morphology based on colony shape, height, and color of the aerial hyphae as well as the base color, growth rate, margin characteristics, surface texture, and depth of growth into the medium. Tests were contrasted with an ordered key for the genus *Acremonium* sp. (Rifai 1969).

Molecular characterization (sequence of 18S rRNA gene of DNA)

Sequence of 18S rRNA gene of DNA of fungal isolates was done at Sigma Scientific Services Co, Cairo, Egypt, also kindly confirmed by Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt (Figs. 1 and 2). Molecular characterization involved the following steps according to the protocol adopted by Woese and Fox (1977) and Abdel-Salam (2003).

Screening of lipase, protease and chitinase produced by *Acremonium* sp.

In vitro

Seven-day-old fungal culture was used as a standard inoculant. At the end of incubation period for each enzyme (protease, lipase and chitinase) respectively, the fungal cultures were filtered and the clear supernatants were considered the source of crude enzyme (Reda et al. 2013).

The most active isolate of *Acremonium* sp. was screened for lipase, protease, and chitinase production

Description	Max score	Total score	Query cover	E value	Ident	Accession
Eutypa sp. isolate LWU_43 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence;	902	902	99%	0.0	99%	MK299142.1
Sarocladium implicatum isolate 6398 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete se	902	902	99%	0.0	99%	MG551553.1
Hypocreales sp. B1a1037PD2CC996 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete s	902	902	99%	0.0	99%	KP307006.1
Hypocreales sp. B1a1037P30CC481 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete se	902	902	99%	0.0	99%	KP307004.1
Hypocreales sp. B1A0288P15CC1048 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	902	902	99%	0.0	99%	KP306966.1
Hypocreales sp. B1A0316EM2CC923 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete s	902	902	99%	0.0	99%	KP306906.1
Sarocladium implicatum strain C40251PD1CC634 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	902	902	99%	0.0	99%	KP143714.1
Sarocladium implicatum strain C10391P15CC676 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	902	902	99%	0.0	99%	KP143689.1
Eutypa sp. BAB-4035 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 2	902	902	99%	0.0	99%	KM051400.2
Eutypa sp. BAB-3890 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 2	902	902	99%	0.0	99%	KM051384.2
Sarocladium sp. isolate AX- ZMC-2-25 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inte	898	898	99%	0.0	99%	MG783402.1
Sarocladium implicatum isolate CLB67 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tran	898	898	99%	0.0	99%	KU645986.1
Sarocladium sp. BAB-5555 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	898	898	99%	0.0	99%	KU504326.1
Sarocladium implicatum strain MSEF31 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	898	898	99%	0.0	99%	KT310954.1
Sarocladium implicatum strain MSEF2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	898	898	99%	0.0	99%	KT310923.1
Acremonium sp. CT720 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	896	896	97%	0.0	100%	KX056237.1
Sarocladium implicatum strain 04035 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete se	896	896	99%	0.0	99%	KT878353.1
Hypocreales sp. B1B0763P30CC929 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete s	896	896	99%	0.0	99%	KP307012.1
Sarocladium implicatum strain B1a0096EM2CC933 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer	896	896	99%	0.0	99%	KP263106.1
Sarocladium implicatum strain B1a0097P30CC625 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer	896	896	99%	0.0	99%	KP263103.1
Sarocladium implicatum strain C40252EM1CC589 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	896	896	99%	0.0	99%	KP143712.1
Sarocladium implicatum strain B1aA5P15CC632 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2,	896	896	99%	0.0	99%	KP143706.1

Fig. 1 18S ribosomal RNA gene of *Acremonium* sp.

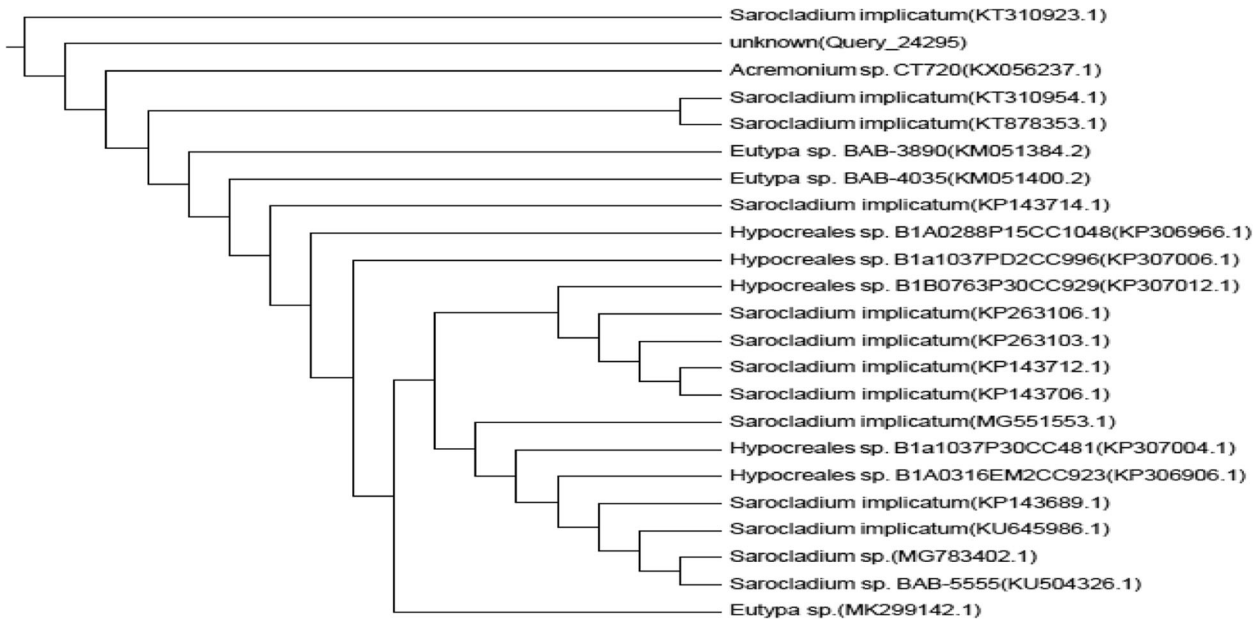

Fig. 2 Phylogenetic dendogram of different fungal isolates accessions revealed by average linkage cluster analysis based on 18S rRNA partial sequence

according to clearing zone technique using Dox-yeast extract-tributyrin agar (Elwan et al. 1977); Dox agar with replacing of $NaNO_3$ by 0.2% gelatin (Ammar et al. 1991) and chitin media which consists of (g/l): colloidal chitin, 0.5; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4$. $7H_2O$, 0.3; K_2HPO_4, 1.36; agar-agar, 20 (CM) (Rajamanickam et al. 2012), respectively.

In vivo

The culture filtrates of lipase, protease, and chitinase media of tested strains after incubation for 7 days at 30 °C were obtained and screening against SBW.

Statistical analysis

Obtained results were analyzed according to Little and Hills (1975), using CoStat computer program Cohort Software, P. O. Box 1149, Berkeley CA 9471 (CoStat Statistical Software, 2005).

Result and discussion

Ten fungal isolates from naturally dead larvae of the SBW were preliminary bioassayed for pathogenicity against neonate larvae of the pest. The most effective isolates were identified morphologically and biochemically as *Metarhizium anisopliae*, *Paecilomyces variotii*, and *Acramonium* sp.

Table 1 Effect of some fungal isolates on larval, pupal and adult stages of the spiny bollworm *Earias insulana*

Isolate	% of larval mortality	% of pupal mortality	% of adult emergence	% of deformed adult
Metarhizium anisopliae s.s.	51.67bc	7.87	85.18b	6.94bc
Metarhizium anisopliae f	41.67c	6.81	90.67b	4.56c
Acremonium s.s.	65a	14.48	73.89c	14.48a
Acremonium f	58.33ab	13.40	71.03c	12.71ab
Paecilomyces variotii s.s.	50bc	9.76	86.90b	4.67c
Paecilomyces variotii f	41.00c	9.40	87.01b	6.27bc
Control	3.3	0	100a	0c
P	< 0.0001***	ns	0.0001***	0.0113*
$LSD_{0.05}$	10.29		8.76	7.28

Same letters means non-significant effect while different letters means significant effect
* means significant effect
*** means very highly significant effect
s.s. spore suspension of each isolate, *f* filtrate of each isolate

Table 2 Effect of some fungal isolates on duration in days of some developmental stages of spiny bollworm *Earias insulana*

Isolates	Larvae	Pupa	Female longevity				Male longevity
			Preoviposition	Oviposition	Postoviposition	Total	
Metarhizium anisopliae s.s.	14.41a	10.40 cd	2.15	7.78	5.3	15.23b	14.40bc
Metarhizium anisopliae f	14.57b	10.59bc	2.17	7.82	5.34	15.33b	14.63b
Acremonium s.s.	14.59b	10.10d	2.24	7.39	4.82	14.45c	13.95c
Acremonium f	14.93b	10.2d	2.28	7.62	5.34	15.24b	14.55bc
Paecilomyces variotii s.s.	14.60b	10.63bc	2.15	7.62	5.41	15.18b	14.55bc
Paecilomyces variotii f	14.71b	10.80b	2.28	7.74	5.46	15.48b	14.86b
Control	16.42a	11.52a	2.66	9.46	5.78	16.63a	15.85a
P	0.0004***	< 0.0001***	ns	ns	ns	< 0.0001***	0.0005***
$LSD_{0.05}$	0.7015	0.3157				0.61	0.60

Same letters means non-significant effect while different letters means significant effect
* means significant effect
*** means very highly significant effect

Data in Table 1 show the effect of selected isolates on percentage of larval, pupal mortality rates, adult emergence, and deformed adults. Analysis of variance revealed highly significant effects on larval mortality percentage for all fungal isolates than the control (3.3%). *Acremonium* sp. showed the highest larval mortality (65.00 and 58.33%) for its spore suspension and metabolites, respectively. The pupal mortality indicated that effects of the 3 isolates for their spores and metabolites were insignificant; also *Acremonium* sp. showed the highest pupal mortality (14.48 and 14.40%) for spores and metabolites, respectively. Regarding adult emergence percentage, data indicated that there were highly significant effects between all isolates than the control. *Acremonium* sp. showed the lowest value of adult emergence (73.89 and 71.03%) compared with control (100%). On the other hand, there were significant effects between all isolates concerning deformed adult percentage (14.48 and 12.71%) for spores and metabolites compared with control (0%). From the previous results, it was obvious that *Acremonium* sp. was the most active isolate.

Data in Table 2 represent the effect of the previous selected isolates on duration (in days) of some developmental stages (larvae, pupae, and adult longevity) of SBW. Statistical analysis showed a significant influence in the developmental period of survived larvae for all isolates. The 3 isolates shortened the larval duration than control. There was a highly significant effect on the pupal period for all isolates which showed shorter periods than in the control. Longevity of emerged females treated with the isolates proved significant effect on the preovipositional, ovipositional, and postovipositional periods.

Regarding longevity of males, data indicated that all tested treatments for the 3 isolates had significant effects on the longevity of males than on the control. Number of eggs, hatchability percentage, and sex ratio are presented in Table 3. Data indicated that there was significant effect in percent of sex ratio, while a highly significant effect was noticed in number of eggs and hatchability percentage than the control. Nada and Abdel-Azem (2005) revealed a significant effect between the control and treated larvae of *P. gossypiella* with *P. violacea* for the same previous aspects.

Acremonium sp. was selected for further study as the most active fungus causing the highest larval mortality. Screening of the selected isolates for production of lipase, protease, and chitinase enzymes, using clearing zone technique, was carried out. The results revealed that *Acremonium* sp. exhibited high activities of protease, while no activity of chitinase and lipase. So, further study was completed on protease in vivo.

The results showed that the screening of proteolytic, chitinolytic, and lipolytic activity of *Acremonium* sp. filtrates against SBW (in vivo) were (72.22, 5.50) and (5.20) compared with the control (5%). These results indicated that it had a proteolytic activity and caused the highest larval mortality effect. The same results of protease were produced from *P. violaceae* (Nada and Abdel-

Table 3 Effect of some fungal isolates on sex ratio, fecundity, and fertility of spiny bollworm *Earias insulana*

Isolate	% of sex ratio	Eggs no.	% of hatchability
Metarhizium anisopliae s.s.	51.51	188.21	72.19b
Metarhizium anisopliae f	51.66	193.04	74.19b
Acremonium s.s.	46.66	172.4	60.79d
Acremonium f	50.00	171.01	63.85c
Paecilomyces variotii s.s.	49.98	177.63	72.75b
Paecilomyces variotii f	47.66	187.42	72.98b
Control	49.99	196.41	90.58a
P	ns	ns	< 0.0001***
$LSD_{0.05}$			2.16

*** means very highly significant effect

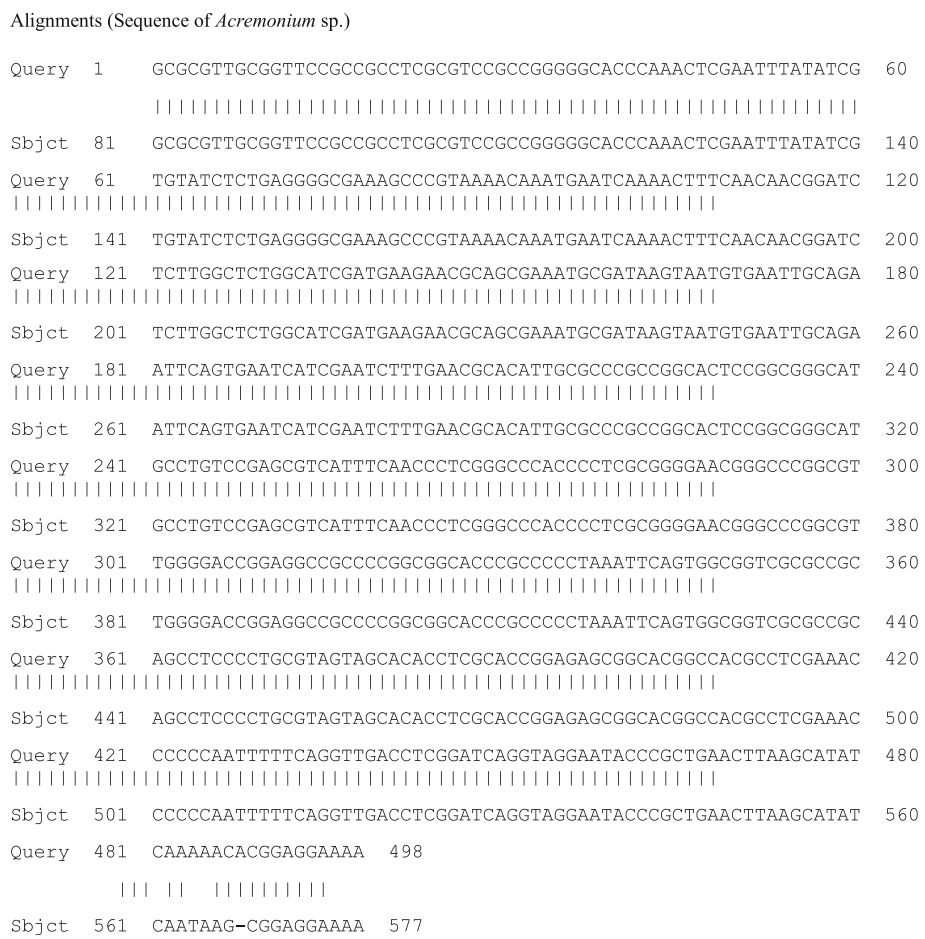

Fig. 3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer1 and 1.5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

Azem 2005). Also, Jain et al. (2012) purified protease from *Acremonium* sp. for commercial purpose. Also, Reda et al. (2013) studied pathogenicity of protease and lipase enzymes produced from *Streptomyces vinaceus-drappus* against the PBW *P. gossypiella*.

Sargin et al. (2013) and Cristina and Gheorghe (2017) investigated the virulence of different EPF, like *M. anisopliae*, *B. bassiana*, and *Paecilomyces* sp. associated with cuticle-degrading enzymes. These enzymes usually hydrolyze the major components of the insect's cuticle (protein, chitin, and lipid) through the infection process.

The results indicated that the protease filtrates gave a high mortality percent in *E. insulana* larvae. Also Nada and Abdel-Azem (2005) reported similar results on *P. gossypiella* by protease secreted by *Paecilomyces* sp.

Molecular identification of the selected fungus

The PCR product of the selected fungus (*Acremonium* sp.) was sequenced using forward primer ITS1 (1). The resulting DNA sequences of the PCR compared with the published sequences were made using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast) and investigated whether homologs to the Gen Bank data.

Figure 3 illustrates the sequence of the eluted PCR products of the selected fungus was homologs (97%) with the sequence of *Acremonium* sp. Phylogenetic tree of the tested isolate showed a position of *Acremonium* sp. isolate also constructed from the evolutionary distance matrix based on the partial 18S rRNA gene sequences (Fig. 1).

Conclusion

The fungus *Acremonium* sp. had the ability to produce cuticle degrading enzyme (protease), so it can play an important role in the control of *E. insulana* in a safe manner and reduce environmental pollution by pesticides.

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Authors' contributions
The conception and design of the study were done by all authors, 1st author EMA isolate entomopathogenic fungi, screening of fungal isolates for their mortality effect and screening of lipase, protease and chitinase produced by *Acremonium* on *E. insulana*. The rearing of the spiny bollworm and detection the effects of the fungal isolates spore suspension and metabolites by WAZM and EMA. The identification of the most potent fungus by HMS and EMA. All authors read and approved the final manuscript.

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Availability of data and materials
All data are available in the manuscript, and the materials used in this work are of high transparency and grade.

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals

Consent for publication
The manuscript has not been published in completely or in part elsewhere

Competing interests
The authors declare that they have no competing interests

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