Biochemical Evaluation of *Acremonium* sp. as Biological Control Agent Against the Spiny Bollworm, *Earias insulana* by Scanning Electron Microscopy

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Abstract

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Ultrastructure and physiological effects of the biological control agent *Acremonium* sp. on larvae of the spiny bollworm (SBW) *Earias insulana* (Boisduval) (Lepidoptera: Noctuidae) were investigated. The isolate of *Acremonium* sp., EZ1 (MN25101) was applied on the 4th instar larvae and pupae of the spiny bollworm *E. insulana*, with two ml spore suspension of different concentrations of 6x10^6, 6x10^7 and 6x10^8 spores/ml mixed with four gm of artificial diet. Scanning electron microscopy (SEM) was used to investigate sporulation potential and the extent of damage to the growth rate of *Acremonium* sp. on the exoskeleton of *E. insulana* larvae and pupae five days after inoculation, as compared to the untreated control. Treated larvae showed varied level of cuticle damage. Surface of infected pupae showed varied stages of mycelial growth. The biochemical parameters investigated were: carbohydrate hydrolyzing enzymes activity (trehalas, invertase, and amylase), the total soluble protein, acetyl choline esterase, aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activities. High significant reduction in amylase, trehalase and ALT activities in treated larvae was observed. On the other hand, high significant gradual increase in the amount of total soluble protein and the acetyl choline esterase activity were observed. At the same time AST activity was slightly increased, whereas invertase activity was not significantly different from the control. Scanning electron microscopy clearly showed the ability of *Acremonium* sp. fungus to damage the cuticle of both larvae and pupae of *E. insulana* by spore's germination. In addition, it disturbed the activity of many important enzymes, thus it can play a vital role in the control of the target pest in a safe manner without polluting the environment.

Keywords: *Earias insulana*, *Acremonium* sp., scanning electron microscopy, AST, ALT.

Introduction

E. insulana is one of the major pests that seriously affect cotton plants as well as other vegetable crops. Because chemical pesticides have been used extensively to control this pest, resistance to these chemicals has evolved with time. In addition, extensive use of chemicals increased the residual toxicity and had negative effect on beneficial insects. Many efforts were made to solve these issues by searching for safe, environmentally friendly pesticides (Abdou *et al*., 2017). Entomopathogenic microorganisms proved to be effective in controlling insect pests (Benzina *et al*., 2018). The environmental prevalence of *Acremonium* species as saprobes in soil, pathogens of plants and insects, and opportunistic diseases of humans and other animals is well documented (Abd-ElAzeem *et al*., 2019; Schell & Perfect 1996). The waxy layer of the epicuticle and the other layers of the integument, as well as surface sculpturing of larvae and pupae of *E. insulana* infected with *Acremonium* sp. were investigated using scanning electron microscope. This technique has the potential to reveal unknown characters that could be helpful in studying mechanisms of penetration (Asensio *et al*., 2005; Gabarty *et al*., 2014; Sharma *et al*., 2017). The use of biological insecticides (biocides) including entomopathogens as a component of integrated pest management (IPM) is gaining more attention in recent years.

Materials and Methods

Target pest and entomopathogen used

E. insulana larvae were obtained from the Bollworms Research Department's mass rearing facility at the Agriculture Research Center, Giza, Egypt, and fed an artificial diet free of insecticides contamination (Amer & El-Sayed, 2015).

The EZ1 isolate of A*cremonium* sp. isolated from dead spiny bollworm was used. This isolate was identified by using 18s rRNA (accession number MN25101).

Infection of *E. insulana* larva with *Acremonium* sp.

Four grams of artificial diet were mixed with two milliliters of spore suspension in each dish, and only with water in the case of the control. Each treatment and the control were replicated three times. After mixing and feeding for around 30 minutes, fourth instar larvae were transferred into Petri dishes using a fine brush. Under the glass cover, petri dishes were covered with a thin paper to keep larvae from escaping. All Petri dishes (treatments and control) were incubated at a temperature of 26°C and 70% RH. for 24 hrs. After treatment, all larvae were transferred to a clean artificial diet. Pathogenic fungus development at both the larval and pupal stages were continuously monitored.

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Scanning electron microscopy
Five days after inoculation with *Acremonium* sp., ten infected larvae and pupae were identified. The same number of untreated larvae and pupae were also collected, and all (treated and untreated) were fixed with 2.5% glutaraldehyde. By using a Leica EM TP automatic tissue processor, the samples were dehydrated in a series of alcohol solutions of 10, 15, 30, 50, 70 and 95%, before being dried using a CO_2 critical point dryer (Tousimis Audosamdri-815), followed by gold coating (SPI-Module). The exterior morphology was examined using the high vacuum mode of a scanning electron microscope (JEOL-JSM-5500 LV).

Biochemical analysis
24 hours following exposure to *Acremonium* sp. spore suspension concentrations of 6×10^6, 6×10^7, and 6×10^8 spores/ml, treated larvae were used for biochemical analysis. Five larvae per treatment plus the control were transferred separately to glass tubes and maintained in a refrigerator (1-7°C) for biochemical analysis. The larvae homogenates were then centrifuged at 3500 rpm for 10 min at 5°C to remove the hemocytes from the homogenates. Supernatants were then chemically analyzed.

Carbohydrate enzymes - Invertase, amylase, and trehalase activities were measured using Ishaaya and Swirski's method, which depended on the digestion of sucrose, starch, and trehalose, respectively (Ishaaya & Swirski, 1976).

Total soluble proteins - A colorimetric measurement method was used to determine the total soluble proteins in the supernatants (Gornall *et al*., 1949). In this approach, alkaline cupric sulphate was used to produce a violet-purple color, whose optical density is directly proportional to the protein concentration present.

Determination of enzymes activity - Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities were colorimetrically measured (Reitman & Frankel, 1957). Acetyl choline-esterase activity (AChE) was measured by acetylcholine bromide as substrate according to Simpson *et al*. (1964).

Statistical analysis
Data obtained was subjected to analysis of variance (ANOVA) using Tukey-HSD software version 6.311 (Costate, 2005).

Results

Scanning electron microscopy
In contrast to insect stages treated with the tested entomopathogenic fungi *Acremonium* sp., neither untreated larvae nor untreated pupae displayed any abnormalities in the cuticle structure. The normal exoskeleton of a larva is characterized by the presence of arranged spines and hairs covering the cuticle of body surface as well as rolls around the spiracles (Figure 1a). Numerous anomalies and malformations on both the exoskeleton of *E. insulana* larvae and pupae was observed when a fungus prevents the spiracles from opening (Figure 1b). The hairs surrounding the exoskeleton apertures were damaged (Figure 1c), or they may have been entirely gone (Figure 1d). Untreated larvae had typical hair surrounding their spines (Figure 2a), whereas infected larvae had huge masses of conidial spore aggregations that descended into the cuticle of the larva (Figure 2b). It was common to observe sheath-like structures that completely covered conidia groups (Figures 2c and 2d).

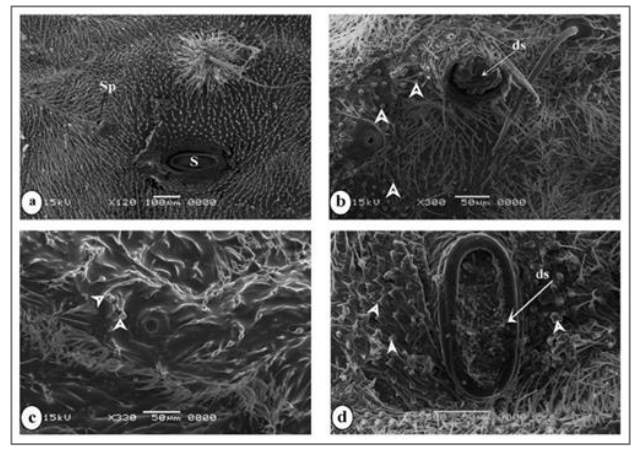

Figure 1. Scanning electron microscope micrographs of the development of *Acremonium* sp. on *E. insulana* larva. (a) untreated 4th instar larvae showing normal spiracle opening, normal hairs and spines that cover all body surface, (b-d) treated 4th instar larvae five days post inoculation with *Acremonium* sp. at concentration of 6×10^8 spores/ml. ds= deformed spiracles appeared blocked with aggregations of conidia, s= spiracle opening, arrows head indicated broken hairs.

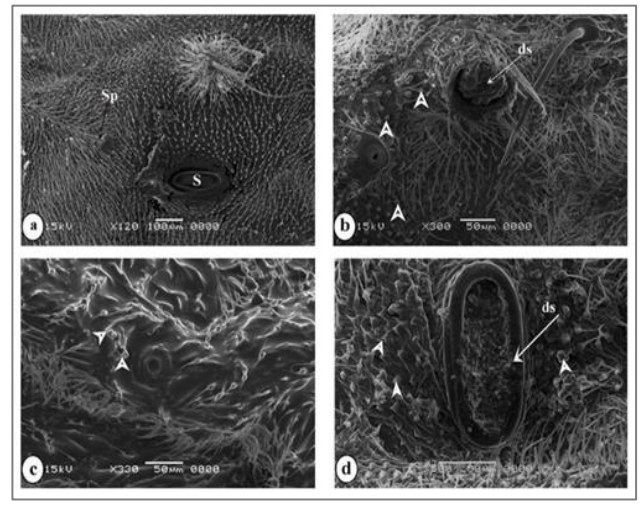

Figure 2. Scanning electron microscope micrographs of the development of *Acremonium* sp. on *E. insulana* larva. (a) untreated 4th instar larvae showing normal cuticle, (b-d) treated 4th instar larvae five days post inoculation with *Acremonium* sp. at concentration of 6×10^8 spores/ml showing deformed cuticle invaded with conidial masses, (c) conidia. Arrowhead indicated broken hairs.

When higher magnification for examining the larval epicuticular layer was used, tiny hair projections or surface sculpturing consisting of a solid exocuticular core covered by a thin layer of epicuticle was observed (Figure 3a); numerous hairs loaded with conidial clusters appeared broken (Figures 3b and 3c); and the appearance of conidia as well as increased mycelial extrusion points were also noted (Figure 3d).

When the external surface of *E. insulana* control pupae were examined by SEM, the pupal surface was found covered with silk-like structures, but a closer look showed very fine filaments (Figure 4a). The outer surface of pupae emerged from treated larvae showed many abnormalities which appeared during conidial germination and formation inside the filaments (Figure 4b). Conidia pustules were gathered near and enclosed within a sheath of aspersoria (Figure 4c and 4d).

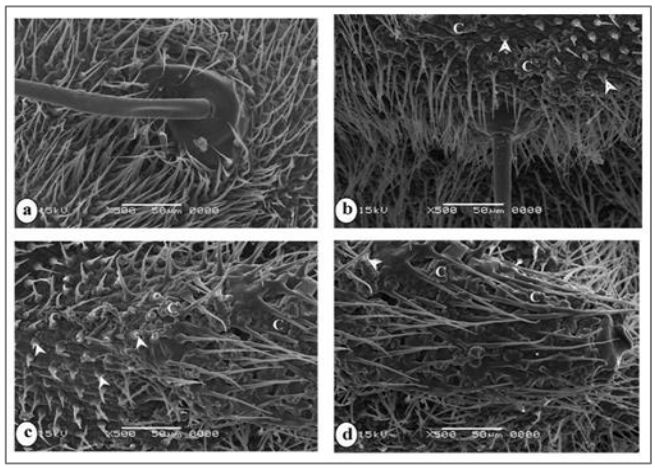

Figure 3. Scanning electron microscope micrographs of the development of *Acremonium* sp. on *E. insulana* larva. (a) untreated 4th instar larvae showing normal cuticle, (b-d) treated 4th instar larvae five days post inoculation with *Acremonium* sp. at concentration 6×10^8 spores/ml showing spines swollen with numerous masses of conidia, and heavy aggregation enveloped in an extensive sheath surrounding the conidia, (c) conidia. Arrowhead indicated broken hairs.

Biochemical studies

The biochemical response of the spiny bollworm larvae was assessed 24 hrs. after treatments with spore suspension of *Acremonium* sp. at concentrations of 6×10^6, 6×10^7 and 6x108 spores/ml. Results of the investigated biochemical parameters are presented in Table 1.

Carbohydrate hydrolyzing enzymes - The statistical analysis recorded high significant reduction in amylase and trehalase activities (1.699 and 26.54 mg glucose/g body weight/min) at concentrations of 6×10^8 and 6×10^6 spores/ml, respectively, as compared to the control (7.003 and 41.085 mg glucose/g body weight/min, respectively), whereas no significant difference in invertase activities was observed.

Total soluble protein (TSP) - The three tested spore concentrations caused significant gradual increase in the amount of total soluble protein. The highest significant increase was 3.34 mg/g at spore concentration of 6×10^8 spore/ml, as compared to the control (1.01 mg/g).

Acetylcholine esterase - High significant increase in AChE activity were noticed in all spore concentrations used and produced 160.16 µg AChE/min/g body weight at the highest concentration compared with 151.17 µg AChE/min/g body weight for the control.

Transaminase enzymes (AST and ALT) - The relative activity of AST recorded a slight increase as compared with control 44.73 and 43.36 µg pyruvate/g body weight/min, respectively. High reduction in the relative activity of ALT was obtained (0.33 µg pyruvate/g body weight/min at spore concentration of 6×10^8/ml), whereas the control produced 1.06 µg pyruvate/g body weight/min.

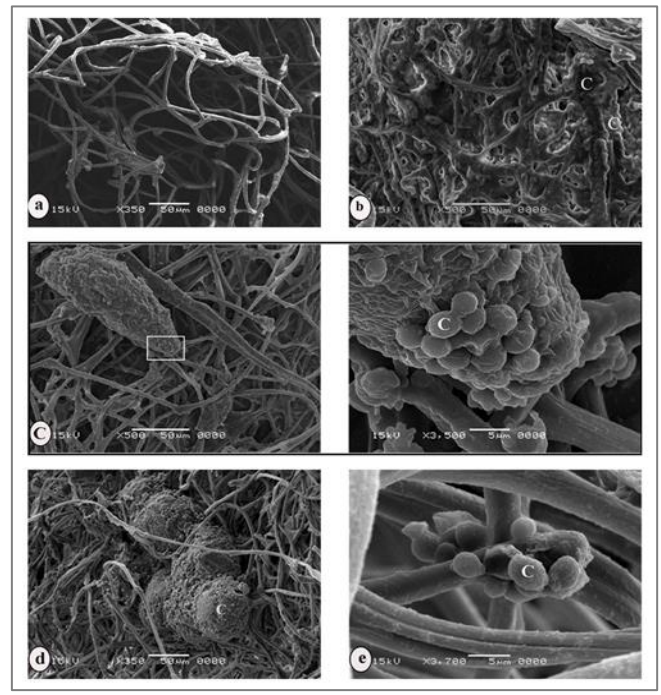

Figure 4. Scanning electron microscope micrographs of the development of *Acremonium* sp. on *E. insulana* pupa. (a) Untreated 4th instar larvae showing normal silk filaments, (b-e) pupa originated from treated 4th instar larvae with *Acremonium* sp. at concentration 6×10^8 spores/ml showing degradation of silk filaments and accumulation of conidial masses in between, (c) conidia.

Table 1. Effect of *Acremonium* sp. fungal spore suspension on some biochemical activities of spiny bollworm *E. insulana*.

Conc. of spore suspension/ml	Carbohydrate enzymes			Total soluble protein	Acetyl cholinesterase	AST	ALT
	amylase	invertase	trehalse				
6×10^6	1.76	57.58	26.39	2.93	155.65	44.73	0.37
6×10^7	1.70	56.79	26.86	3.03	158.82	44.48	0.37
6×10^8	1.69	55.17	26.54	3.34	160.16	43.36	0.33
Control	7.00	51.10	41.08	1.01	151.17	43.36	1.06
$LSD_{0.05}$	0.3472	6.866	0.9977	0.1601	1.2948	1.0405	0.0859

AST= Aspartate aminotransferase, ALT= Alanine aminotransferase. Total soluble protein units (including enzymes) is µg pyruvate/g body weight/min

Discussion

As fungal spores of *Acremonium* sp. germinate on the host's cuticle surface, they tear the insect cuticle (larvae and pupae) through mechanical force and enzymatic degradation. The cuticle and its underlying epidermis make up the majority of the insect's integument, which serves as both an exoskeleton, a water-tight barrier against desiccation, and a sensory interface with the environment (Vincent & Wegst, 2004). *Acremonium* sp. was tested for its potential as an entomopathogen against larvae and pupae of *E. insulana*, and was successful in dissolving the exoskeleton shield. Earlier research employing the clearing zone technique on the same tested fungi for synthesis of cuticle degrading enzymes (lipase, protease, and chitinase) showed that *Acremonium* sp. produced strong protease activity which broke down the cuticle and released nutrients to the fungus (Benserradj & Mihoubi, 2014). Insect suffocation may result from *Acremonium* hyphae and granules generated in and over the tissue that can reach the larvae's integument and obstruct the spiracle, which is responsible for allowing air into the respiratory system (Pekrul & Grula, 1979). *B. bassiana* conidia were able to penetrate directly through the integument, as well as through the respiratory system (Haitham & Alleddin, 2012). Results obtained in this study showed specific destruction of the larval epicuticle, which was degraded into small pieces.

The biochemical parameters are thought of as sensitive indicators to monitor abnormalities that occur inside the body (Rashwan, 2013) and external damage was accurately evaluated by SEM. Furthermore, fungi have evolved adaptations that allow them to invade the immune system of insects to complete the infection process (Taşkın & Asksoylar, 2011). Total protein content in treated insects may change as an indication that different enzymes have become less active (El-Kordy *et al*., 1995). In addition, carbohydrate hydrolyzing enzymes amylase, trehalase and invertase, which considered as major enzymes in carbohydrates metabolism, play a principal role in the digestion and utilization of carbohydrates. Amylases secreted by larva salivary glands hydrolyze starch to monosaccharides (Riberiro *et al*., 2000), and invertase secreted in the gut larva hydrolyze sucrose to monosaccharides, glucose and fructose (Heil *et al*., 2005). Glucose produced from trehalose by trehalase enzyme for internal supply of chitin synthesis (Sabry, 2018). Reduction in amylase and trehalase digestive enzyme may be related to stress conditions utilized by tested fungus application and this is in agreement with research conducted earlier (Fahmy 2008). El-Lebody *et al*. (2021) found that endophytic fungi *Aspergillus flavus* MRDS 301 and *Curvularia lunata* MRDS 302 caused decrease in proteins and subsequently significant reduction in α-amylase and invertase relative as compared to the control. This study indicated significant elevation in AChE activity in treated insects and this may be related to the production of a secondary metabolite which elevate Ach receptors in insect muscles, which agrees with Bourne *et al*. (2016) who suggested that in peripheral and central synapses of the insects' nervous system, AChE enzyme terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter. In addition, Jia *et al*. (2016) noticed that AChE increased in *Locusta migratoria* under different treatment conditions with the fungus *M. anisopliae* during the early period after treatment but decreased during later period. AST enzyme plays significant role in the formation of non-essential amino acids, in metabolism of nitrogen waste and gluconeogenesis (Mordue & Goldworthy, 1973). In this study, an increase in AST activity may be attributed to a problem with amino acid metabolism, which ultimately lead to an increase in the enzyme metabolic activity. Continuous release of AST by larvae of *E. insulana* treated with *Acremonium* sp. can be caused by the need to increase aspartic acid's dominance during the process of gluconeogenesis, particularly when there is poor carbohydrate metabolism. *Verticillium lecanii*, on the other hand, caused a considerable inhibitory effect on the total activity of ALT and AST in the 4th instar larvae of the pink bollworm *Pectinophora gossypiella* (Rashad *et al*., 2015).

According to the findings of this study, the EZ1 isolate (accession number MN25101) of *Acremonium sp*. can be used as a biological control component in the integrated management of *E. insulana* to reduce the danger of employing chemical pesticides, which have an unintended negative impact on both the environment and the health of mammals.

الملخص

صبري، هند محمد، وردة أحمد زكي المدني، هند سعد الطحاوي وإيمان محمد عبد العظيم. 2023. التقييم الحيوي الكيميائي/البيوكيميائي للفطر *Acremonium* sp. كعامل مكافحة حيوية ضدّ دودة اللوز الشوكية (*Earias insulana*) باستخدام المجهر الالكتروني الماسح. مجلة وقاية النبات العربية، 41(2): 140–145. https://doi.org/10.22268/AJPP-41.2.140145

تمّ تقييم فعالية فطر *Acremonium* sp. المعزول من دودة اللوز الشوكية بالاستعانة بالمجهر الالكتروني الماسح وبعض الدراسات الحيوية الكيميائية/البيوكيميائية. كشف الفحص بالمجهر الالكتروني الماسح جلياً أنّ للفطر المعزول تأثيرٌ واضح على اليرقات من حيث تلف جلدها، وظهور نموات الفطر على سطح العذارى في مراحل متفاوتة ممّا أدى إلى حدوث تشوهات. علاوةً على ذلك، يعزى الاضطراب في نشاط بعض الأنزيمات، مثل الأنزيمات المحلّلة للكربوهيدرات وكلٍّ من أنزيمات الكيتيناز والبروتياز والفينول أوكسيداز، إلى تأثير الفطر المعزول في المختبر. وعليه يمكن اعتبار الفطر *Acremonium* sp. عنصراً واعداً في المكافحة الحيوية لدودة اللوز الشوكية دون اللجوء إلى المبيدات.

كلمات مفتاحية: *Earias insulana*، *Acremonium* sp.، المجهر الالكتروني الماسح، AST، ALT.

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