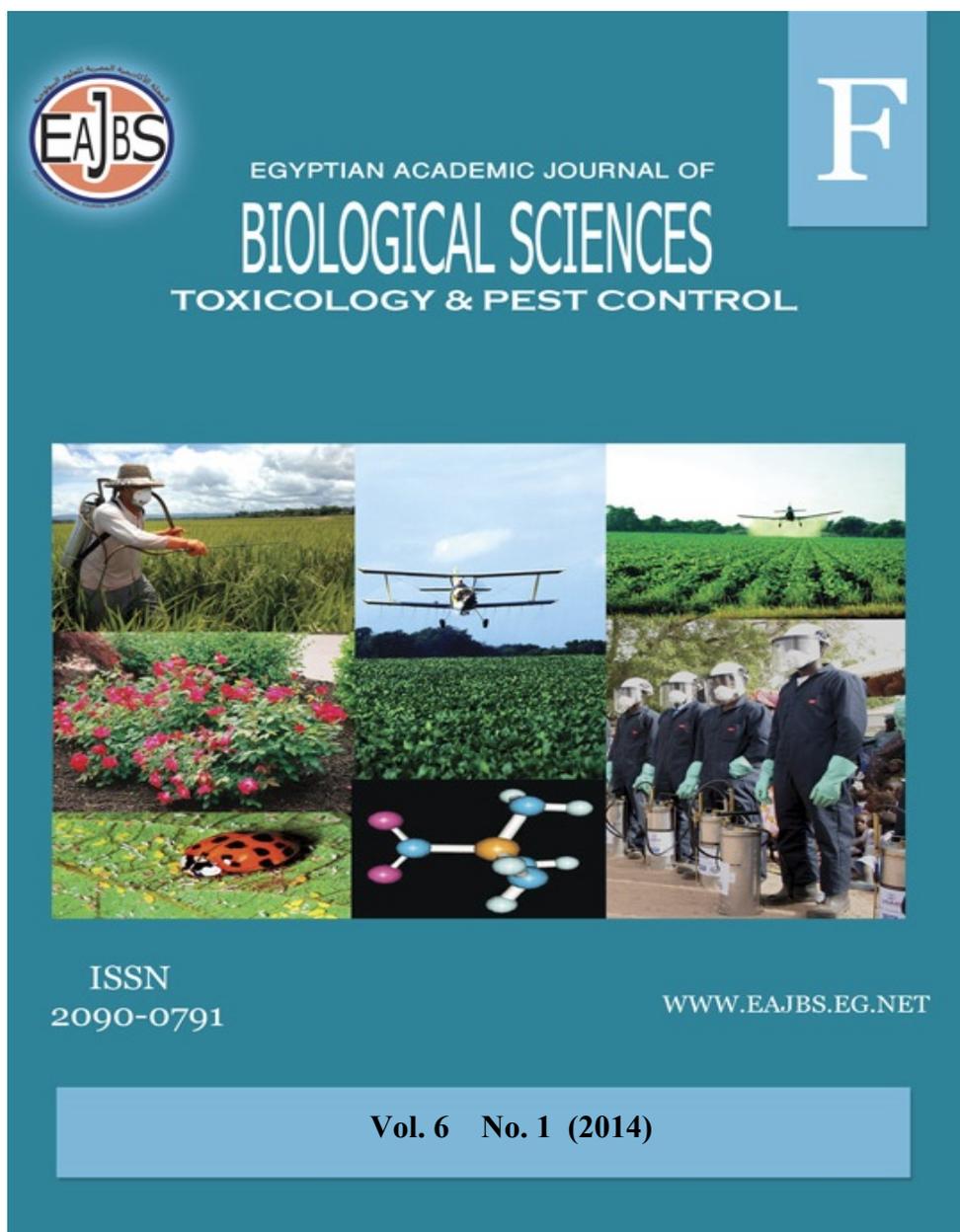


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Biodiversity and enzymatic profile of some entomopathogenic fungi

Sahar S. Ali¹ and Ahmad M. Moharram²

1-Plant Protection Research Institute, Agriculture Research Center, Dokki, Giza, Egypt

2-Department of Botany and Microbiology, Faculty of Science, Assiut University, Egypt.

E-mail: sahar_s_ali@yahoo.com

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ABSTRACT

Five fungal isolates belonging to the entomopathogenic fungi were isolated in Bio-insecticide Production Unit, Plant Protection Research Institute, two isolates of *Beauveria bassiana*, two isolates of *Lecanicillium antillanum* and one of *Paecilomyces lilacinus*, in addition to one isolate of *Rhizopus stolonifer* that grows with *Lecanicillium antillanum* in Mycoparasitism relationship. The isolated fungi were recovered from Egyptian soil and from larvae of *Spodoptera littoralis* which were found infected in a sugar beet field. These isolates were identified by conventional techniques in Mycological Center, Faculty of Science, Assiut University. Among these fungal species *L. antillanum* is a new record in Egypt. In addition, the activities of proteases, lipases, amylases and L-asparaginase of isolates fungi were recorded. The results showed that, all isolates were active producers of extracellular hydrolytic enzymes.

INTRODUCTION

Entomopathogenic fungi are natural enemies of insects and arachnids and contribute to the regulation of their host populations. In agriculture, these fungi have been observed to cause mortality in pest populations and several fungal species have been investigated for their potential as biological control agents. Entomopathogenic fungi display different strategies in their attachment to insect cuticle which forms the first formidable barrier to pathogens and fungal spores have to pass through certain discrete stages before breaching the insect cuticle. Conidial contact with an arthropod surface is the first step of the fungal penetration and infection process. Through the combined action of hydrolytic enzymes such as chitinase, protease and lipase, the fungal mycelia are able to penetrate through these barriers (Bidochka and Khachatourians 1990; St Leger *et al.* 1986).

Entomopathogenic fungi produce many enzymes that also are toxic components found in bacteria and animal venoms and thus may be considered as potential virulence factors (Charnley, 2003; Mustafa and Kaur 2010).

Some of the main characteristics of entomopathogenic fungi are their pathogenicity and virulence. Pathogenicity can be defined as the microorganism ability to cause disease while virulence is defined as the degree of pathogenicity that an organism uses in order to kill its host in controlled conditions. These characteristics are related to the proteases production; enzymes that are considered as the most important within the infective process (Mustafa and Kaur 2009). When the epicuticle breaks down by the lipases, the fungus produces great quantities of Pr 1 protease, which degrades the proteinaceous material. The solubilized proteins are degraded by amino peptidases and exopeptidases until amino acids, serving as nutrients for entomopathogenic fungi (Wang *et al.* 2002).

MATERIALS AND METHODS

1- Isolation of entomopathogenic fungi:

a- From soil:

The dilution plate method was used for this purpose. Soil samples were collected from vegetable field in Giza Governorate. From each sample 20g were mixed with 100 ml of 0.185 % tetra sodium pyrophosphate was incubate on a shaker at 28 °C for 3 h, and allowed to settle for 15 sec. The suspension (0.1 ml) was spread on semi-selective medium (glucose, 2 %; peptone, 1 %; agar, 1.8 %) with the antibiotics tetracycline (0.005 %), streptomycin (0.06 %), cycloheximide (0.005 %) and dodine (0.01% v/v) added after autoclaving. Cultures were then incubated at 28 °C for 10-15 days in the dark after which the developing colonies were aseptically subcultured on potato dextrose agar (PDA) medium in plates and slopes for further investigations.

b- From infected larvae:

Larvae of *Spodoptera littoralis* showing symptoms of fungal infection were collected from a vegetable field in Giza Governorate, placed on PDA medium to and incubated at 28°C for 7-10 days to allow growth of infecting fungi. Pure slant cultures of fungi were preserved for further studies.

2. Identification of fungal strains:

By conventional methods: Fungi were cultured on Czapek's yeast extract agar (CYA) or potato sucrose agar (PSA). Fungal cultures were incubated at 28 °C for 7 to 10 days after which the macroscopic and microscopic morphological characteristics were observed. Identification was done according to mycological refereces including, Domsch *et al.* (2007) and Zare & Gams (2004).

Enzymatic activities of entomopathogenic fungi:

In vitro laboratory studies have been conducted to investigate the enzyme secretion by the isolated fungal strains. These fungi were tested for their abilities to produce protease, lipase, amylase and L-asparaginase. Ten day old colonies were used for preparation of spore suspension inoculums. Fungal spores were suspended in sterile distilled water to which tween 80 (0.02%) was added. The final concentration of spore suspension was around 1×10^6 cells/ml.

a- Proteolytic activities of fungal strains were tested using the method of Paterson and Bridge (1994) based on the hydrolysis of casein incorporated in the medium and distributed in test tubes (10 ml / each tube). Fungal isolates were individually inoculated and the cultures were incubated at 28°C for 14 days. Data were recorded as the depth in mm of the clear zone resulting from casein digestion.

b- Lipolytic activities were similarly estimated using the medium of Ulman and Blasins (1974) in which tween 20

and CaCl_2 were incorporated. The depth in mm of visible precipitate (due to formation of crystals of calcium salt of the oleic acid liberated by the lipase enzyme) was measured.

c- Amylase production: Starch agar medium containing 20 gm soluble starch /liter was used. After autoclaving the medium was dispensed in 9cm sterile Petri plates (20 ml /plate) and left to solidify. Using cork borer a cavities were made in each plate to which 20 ul of fungal spore suspension were added. Cultures were incubated at 28 C for 10 days followed by flooding with Iodine solution (1gm Iodine and 2gm Potassium Iodide in 300 ml distilled water). Appearance of clear zone around and beneath fungal growth indicates hydrolysis of starch. Activity zone was measured in mm.

d- L-asparaginase production by fungal strains was done according to Gulati *et al.* (1997). The test medium contained (g/l): glucose 2.0, L-asparagine 10, K_2PO_4 1.5, KCl 0.52, Mg $\text{SO}_4 \cdot 7\text{H}_2\text{O}$ 0.52, and traces of $\text{Cu NO}_3 \cdot 3\text{H}_2\text{O}$, Zn SO_4 , FeSO_4 . For solidification 20g agar

were added to the medium. After autoclaving the medium was poured in Petri plates (20 ml/plate). Test fungi were inoculated centrally in plates and cultures were then incubated for 7 days. Appearance of pink colour around and beneath the fungal growth (alkaline pH) indicates production of L-asparaginase which acts on the amino-acid L-asparagine yielding aspartic acid and ammonia.

RESULTS AND DISCUSSION

Fungal species isolated in the present study:

Four different fungal species were identified during this work using conventional methods as recorded in Table 1. Two isolates of *Beauveria bassiana* were recovered; one of which from soil and the second from a diseased larva of *Spodoptera littoralis* collected from a sugar beet field, two isolates of *Lecanicillium antillanum* and *Paecilomyces lilacinus* in addition to *Rhizopus stolonifer* were also obtained from the tested soil samples.

Table 1: Fungi isolated and identified by using the conventional methods.

Fungal isolates	Source	Strains (AUMC NO.)
<i>Beauveria bassiana</i> (1)	Soil	(9896)
<i>Beauveria bassiana</i> (2)	<i>S. littoralis</i> larvae	(9908)
<i>Lecanicillium antillanum</i> (1)	Soil	(9905)
<i>Lecanicillium antillanum</i> (2)	Soil	(9907)
<i>Paecilomyces lilacinus</i>	Soil	(9897)
<i>Rhizopus stolonifer</i>	Soil	(9906)

These fungi identified by using the conventional methods and photographed as shown in Figs (1, 2, 3 and 4).

To the best of our knowledge *L. antillanum* (Synonym= *Verticillium antillanum*) is a new record to Egypt. According to Zare and Gams (2004) this species is characterized by white mycelium with cream- colored reverse. Phialides developing on prostrate hyphae, singly or up to 6 in verticils. Conidia of two strains, were classified

into primary (fusiform, sigmoidally curved with narrow pointed ends) and secondary (ellipsoidal and shorter). This fungus was observed parasitizing hyphae of *Rhizopus stolonifer* (Fig. 4) causing reduction in its mycelia growth and delay in the production of sporangia which are also formed in lower number than in normal cultures. Tae-Young Shin *et al.* (2010) determined the optimal isolation conditions of the entomopathogenic fungi and compared

their growth characteristics with non-entomopathogenic fungi on agar media containing various concentrations of copper (II) chloride (CuCl₂) or dodine. Their results showed that dodine medium is more selective, and the optimal concentration of dodine is determined with 50µg/ml. In Canada, Bidochka *et al.* (1998) studied the occurrence of deuteromycetous entomopathogenic fungi in 266 soil samples representing 86 locations across temperate and near northern habitats in Ontario. They succeeded to isolate entomopathogenic fungi by baiting the soil with wax worm larvae, *Galleria mellonella* L., and incubating at 8, 15, or 25°C. Fungi were isolated from 91% of the locations sampled across Ontario and the most abundant species were *Metarhizium anisopliae* (Metschn.) Sorok., *Beauveria bassiana* (Bals.) Vuill., and *Paecilomyces* spp. They also observed that *Beauveria bassiana* was isolated more frequently in soils from near northern locations particularly from larvae baited in soils incubated at 8 and 15°C with no relation to soil type or pH. In Korea, Nguyen *et al.* (2007) isolated *Lecanicillium antillanum* from soil samples collected from Gwangju district.

The fungus was found to be a chitinolytic-nematophagous microorganism showing a high rate of parasitism on *Meloidogyne incognita* eggs with more than 90% infection rate on the third day after treatment. In Egypt, Hussein *et al.* (2010) carried out an extensive work to isolate soil borne entomopathogenic fungi using the wax moth larvae (*Galleria mellionella*) as baits. *B. bassiana* was recovered more frequently than *M. anisopliae*. As an alternative to chemical pesticides, *B. bassiana*, is becoming increasingly popular to protect crop yields from losses due to disease and pest infestation. *B. bassiana* can parasitize more than 700 different kinds of insects (Meyling and Eilenberg 2007). Isolates of *B. bassiana* can antagonize a variety of soil and foliar plants pathogens (Vega *et al.* 2010) while they are in symbiosis with many plant species.

Enzymatic activities of fungal isolates:

The different fungal isolates tested proved to be active producers of extracellular enzymes hydrolyzing proteins (casein), lipids (Tween 80) and starch in addition to their ability to utilize the amino-acid L-asparagine (Table 2 and Figs 5, 6 and 7).

Table 2: Enzymatic activities of isolated fungi.

Fungal strains	Zone of activity in mm			
	Proteolytic	Lipolytic	Amylolytic	L-asparaginase
<i>B. bassiana</i> (1)	35	22	35	45
<i>B. bassiana</i> (2)	30	21	33	42
<i>L. antillanum</i> (1)	30	22	40	40
<i>L. antillanum</i> (2)	25	20	42	42
<i>P. lilacinus</i>	28	21	18	20
<i>R. stolonifer</i>	38	25	35	28

The tested fungi showed higher proteolytic than lipolytic activities. Variations in the enzymatic activities among fungal isolates appeared to be correlated with the fungal species as well as with the substrates attacked by the individual isolates. Isolates belonging to *Beauveria* and *Lecanicillium* exhibited generally higher enzymatic activities than

those of *Paecilomyces* and *Rhizopus*. A recent report by Sánchez-Pérez *et al.* (2014) revealed that, the entomopathogenic fungi pathogen activity depends on the ability of its enzymatic equipment, consisting of lipases, proteases and chitinases, which are in charge of breaking down the insect's integument. Lipases are the first

enzymes synthesized by the entomopathogenic fungi. These break down long chain alkenes and fatty acids to become initial nutrients. Ali *et al* (2009) studied that Lipases are important cuticle degrading enzymes involved in the infection process of entomopathogens by hydrolysing the ester bonds of lipoproteins, fats and waxes present in the insect integument. The importance of lipases in the tegument penetration and breaking down process has also been demonstrated by Da Silva *et al.* (2010). Proteases are released. These enzymes are considered as virulence indicators and they are regulated by certain mechanisms activated by the protein kinase A. Once the epicuticle breaks down, the fungus produces great quantities of protease which degrade the proteinaceous material that is found in the procuticle. Wang *et al.* (2002) reported when the epicuticle breaks down by the lipases, the fungus produces great quantities of Pr1 protease, which degrades the proteinaceous material. The solubilized proteins are degraded by amino peptidases and exopeptidases until amino acids, serving as nutrients for entomopathogenic fungi. Hasan *et al* (2013) studied the amylase activity at different pH by *Verticillium lecanii*. Extracellular enzymes capable of carbohydrate degradation in plant tissues are produced by pathogenic fungi. Plant tissues are analogous in structure to insect cuticle (containing fibrous chitin or cellulose within a matrix of protein, pectic substances or hemicelluloses) Amylase is secreted in a pH dependent manner by isolate. Nageswara *et al* (2014) evaluated ability of *Beauveria bassiana* to produce L-asparaginase by rapid plate assay.

In conclusion, entomopathogenic fungi easily isolated from soil and insect larvae using suitable isolation techniques. More work is needed to recover these fungi from different types of Egyptian soil, different species of insects and other

natural sources. Also, enzymatic and toxic compounds that can contribute to virulence of these fungi need more investigations.

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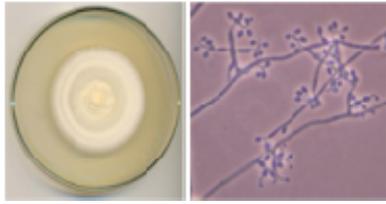


Fig. 1: *Beauveria bassiana*: Colony (left), hyphae, rachis-like conidiogenous cells bearing spherical to oval shaped conidia x1000 (right)



Fig. 2: *Lecanicillium antillanum*: Verticillate arrangement of phialides on prostrate hyphae, primary curved fusiform conidia with pointed ends and secondary smaller ellipsoidal conidia (X1000)

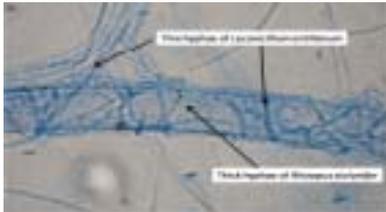


Fig. 3: Mycoparasitism of *Lecanicillium antillanum* (coils of thin hyphae) on *Rhizopus stolonifer* (larger thick hyphae X1000).

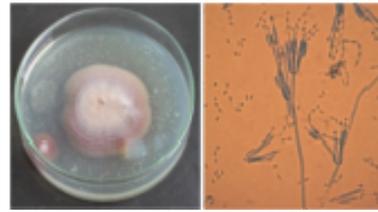


Fig. 4: *Paecilomyces lilacinus*: Colony (left), branched conidiophores, metulae, phialides and conidial chains (right X1000).



Fig. 5: Amylolytic activity of *Beauveria bassiana* (Bb) and *Lecanicillium antillanum* (La).



Fig. 6: L- asparaginase activity of *Beauveria bassiana* (pink colour around and beneath the colony).

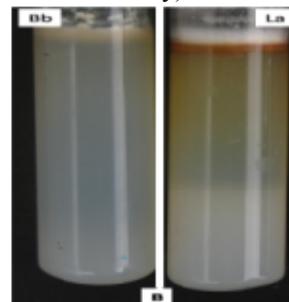
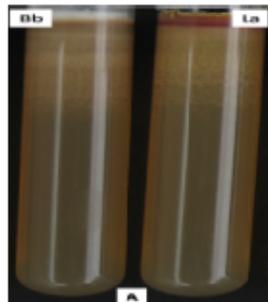


Fig. 7: Lipolytic (turbid zone, A) and proteolytic (clear zone, B) activities of *B. bassiana* (Bb) and *L. antillanum* (La)

ARABIC SUMMERY

التنوع البيولوجي والنشاط الانزيمي لبعض الفطريات الممرضة للحشرات

سحر سيد علي^١ و أحمد محمد محرم^٢

١- معهد بحوث وقاية النباتات بمركز البحوث الزراعية ، الدقي ، جيزة.

٢- قسم النبات والميكروبيولوجي بكلية العلوم جامعة اسيوط.

تم عزل خمس عزلات فطرية ممرضة للحشرات بوحدة انتاج المبيدات الحيوية بمعهد بحوث وقاية النباتات وذلك من التربة المصرية باستخدام البيئات المتخصصة وهم عزلتين من فطر *Beauveria bassiana* وعزلتين من فطر *Lecanicillium antillanum* وعزلة من فطر *Paecilomyces lilacinus* كما تم عزل فطر *Rhizopus stolonifer* وقد تم تعريفهم بواسطة الطرق التقليدية في التعريف بمركز الفطريات بكلية العلوم جامعة اسيوط كما تم التعرف علي النشاط الانزيمي لجميع الفطريات التي تم عزلها من خلال دراسة افرازاتهم لانزيمات البروتيز والليباز والاميليز و الاسبرجينيز.