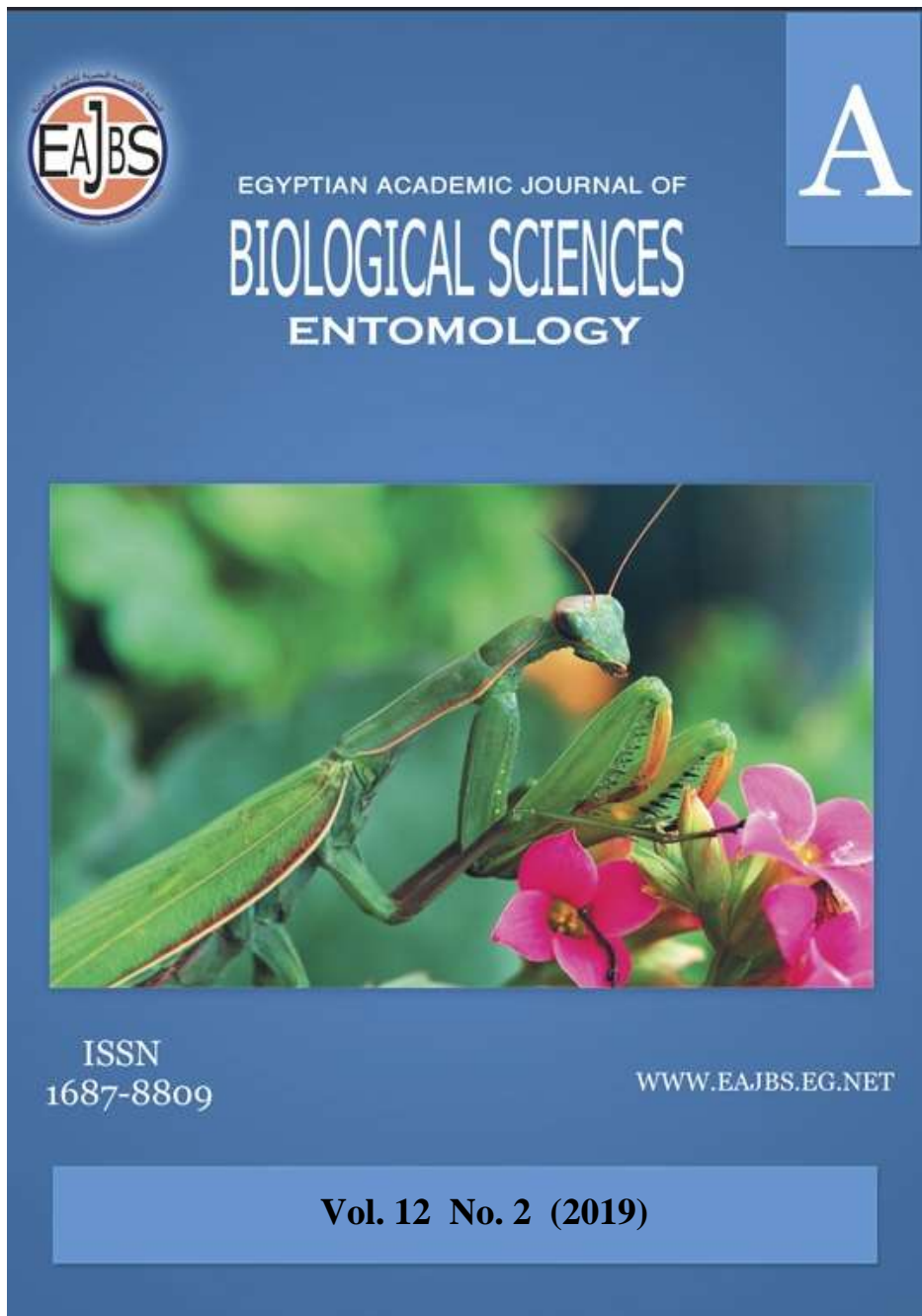


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Fungal Infection Causes Serious Effects on Cuticle and Midgut of the Greater Wax Moth, *Galleria mellonella*

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ABSTRACT

The pathogenicity efficiency among two of the entomopathogenic fungi (EPF), *Beauveria bassiana* and *Metarhizium anisopliae* against the last larval instar of the greater wax moth (GWM), *Galleria mellonella* L., under laboratory conditions was compared. The histopathological changes in cuticle and midgut at 48hrs post fungal infection was investigated. The results revealed that, the concentration–mortality relationship showed that larval mortality increase in a linear relationship with conidia concentration and *B. bassiana* causing higher mortality percentage with LC₅₀ 3.1x10²conidia/ml while LC₅₀ of *M. anisopliae* reached 4.6x10³conidia/ml. The histological analysis of *B. bassiana* infection at 48 hrs. post-infection showed extensive histopathological changes in cuticle and midgut that induced nodule formation beneath the cuticle layer and destroying midgut epithelium by separation of columnar layer towards gut lumen with increasing the number of regenerative cells beneath it.

INTRODUCTION

G. mellonella (Lepidoptera: Pyralidae) a serious pest of beehives feeding on wax and pollen (Chang and Hsieh 1992 and Iwona Wojda 2017), it creates tunnels lined with silk which entangles and starves emerging bees, a phenomenon is known as galleriasis. Tunnels also result in massive destruction of the combs and honey is wasted as it leaks out when cell caps are eaten (Haewoon *et al.*, 1995 and Kwadha *et al.*, 2017). In addition, both *G. mellonella* adults and larvae can be vectors for pathogens that can infect honeybees, therefore, most studies have focused on the pest as a model for in vivo studies of toxicology and pathogenicity (Johnson, 2015 and Kwadha *et al.*, 2017).

Entomopathogenic fungi are among the most promising group of biological control against insect pest (Reithinger *et al.*, 1997). Over 500 species of fungi are known to have insect pathogenic properties. Interestingly, *Beauveria bassiana* and *Metarhizium anisopliae* (*Deuteromycotina*, *Hyphomycetes*) represent the most frequently used isolates (Burges and Hussey, 1971) and are known to infect a broader range of insect pests of crops belonging to Lepidoptera, Homoptera,

Hymenoptera, Coleoptera and Diptera. Entomopathogenic fungi (EPF), compared to other entomopathogenic microbial organisms can infect their host via attachment of conidia to the insect cuticle, germination, appressorium formation, penetration into the hemocoel, mycosis (disease development inside insects) and finally external conidiogenesis (Guerra-Agullo *et al.*, 2010 and Toledo *et al.*, 2010). The death of the host occurs as a result of massive tissue destruction, depletion of nutrients or the production of toxins by the fungus (Murrin 1996). These fungal toxins induced alternations of the cuticle and midgut epithelial cells (Bogus and Maria 2000).

The present investigation aims to evaluate the pathogenicity of both *B. bassiana* and *M. anisopliae* against *G. mellonella* larvae as well as the histopathological changes in cuticle and midgut at 48 hrs post fungal infection.

MATERIALS AND METHODS

Entomopathogenic Fungi:

Entomopathogenic fungi isolates used in this study were *Beauveria bassiana* (3873AUMC) and *Metarhizium anisopliae* (5130 AUMC) obtained from Assuit University, Mycological Center.

Conidiospores Production:

The entomopathogenic fungi *M. anisopliae* and *B. bassiana* were grown on sterilized Sabourad Dextrose Agar Yeast media (SDAY) consisted of peptone 10 g/L, glucose 40 g/L, 2g/L yeast extract, agar- agar 20 g/L and 1L. distilled water in standard Petri- dishes (90 mm diameter) then the culture was incubated at 25 ± 2 °C for 10 days. Developed conidiospores were harvested by scraping into sterile 0.01% Tween 80. The suspension was vortexed for five min before filtering through four layers of sterilized muslin. The resulting conidial stock suspension was estimated using an improved Neubauer bright line hemocytometer (Neubauer improved HBG, Germany) under a Leitz Dialu X 20 EB microscope (400 x magnifications). A series of dilutions were made to give range concentrations 10 , 10^2 , 10^3 , 10^4 and 10^5 conidia/ml (Hassan, 2008).

Bioassay:

Thirty individuals of *Galleria mellonella* last larval instar were divided into six replicates each contains five larvae for each fungal infection. For inoculation, each group of larvae was placed in a plastic cup (9 cm diam., 5 cm deep) filled with 200-g of sterile soil that moistened with distilled water (10% w/w) then uniformly pipetted on the soil surface with 10 , 10^2 , 10^3 , 10^4 and 10^5 conidia/ ml from the fungal suspension. After inoculation, cups were incubated at 28 C°. Larval mortality was monitored every 24 hrs over a period of 10 days.

Histological Preparations:

LC₅₀ value of *B. bassiana* has been used for histopathological investigation. There were 20 larvae inoculated by fungal suspension in Eppendorf tubes, the same with control but treated with distilled water only. At 48 hrs. post-infection, all the treated larvae were fixed in 10% buffered formalin for 24 hrs, then dehydrated in an ethanol-xylene series and embedded in paraffin wax and cut on 4µm, then sections were deparaffinized, rehydrated, and stained by Haematoxylin and Eosin for histopathological examination (Bancroft and Stevens 1996).

RESULTS

Data are shown in Table (1) indicated that both isolates *B. bassiana* and *M. anisopliae* were pathogenic against the last larval instar of *G. mellonella*. The larval

mortality percentage increased in linear relationship with conidia concentration. The highest mortality percentage occurred by 10^5 conidia/ml while the lowest mortality percentage occurred by 10 conidia/ml for both fungi isolates. *B. bassiana* infection was more effective as compared to *M. anisopliae* by recording 26.67, 35.29, 66.67, 73.33 and 86.67 for 10, 10^2 , 10^3 , 10^4 and 10^5 conidia/ml respectively as compared to 13.33 in the control. While *M. anisopliae* was less effective recorded 6.67, 13.33, 46.7, 60.33 and 73.33 for 10, 10^2 , 10^3 , 10^4 and 10^5 conidia/ml respectively as compared to 6.67 in the control. Symptoms could be detected of the infected larvae (Fig. 1).

The calculated LC_{50} and slope values of the tested fungal isolates are presented in Table (2). The LC_{50} for *B. bassiana* reached 1.3×10^2 conidia/ml (slope = 0.452 ± 0.117) while LC_{50} for *M. anisopliae* recorded 4.6×10^3 conidia/ml (Slope = 0.589 ± 0.131). Data indicate that *B. bassiana* was more pathogenic than *M. anisopliae*.

Table (1): Virulence of *B. bassiana* and *M. anisopliae* against *G. mellonella* last larval instar.

Concentrations (conidia/ml)	Mortality % \pm SE	
	<i>B. bassiana</i>	<i>M. anisopliae</i>
10	26.67 ^a	6.67 ^a
10^2	35.29 ^{ab}	13.33 ^a
10^3	66.67 ^{bc}	46.7 ^b
10^4	73.33 ^{cd}	60.33 ^b
10^5	86.67 ^d	73.33 ^{cb}
Control	13.33 ^a	6.67 ^a

Mortality values within the column followed by the same letter are not significantly different (Duncan's test: $P > 0.05$).

Table (2): Calculated LC_{50} for *Beauveria bassiana* and *Metarhizium anisopliae* against last larval instar of *Galleria mellonella*

Nematodes species	LC_{50}	Slope
<i>B. bassiana</i>	3.1×10^2	0.452 ± 0.117
<i>M. anisopliae</i>	4.6×10^3	0.589 ± 0.131

Histopathology:

The figures 2&3 illustrated the histopathological changes in cuticle and midgut tissue of *G. mellonella* treated with LC_{50} of *B. bassiana* at 48 hrs post infection. The changes may be arranged as follows:

The Cuticle:

The histological section at the cuticle of untreated *G. mellonella* (Fig.2A) showing intact cuticle with its epidermal layer. While cross-section in the cuticle of the fungal infected larva at 48 hrs. post-infection (Fig.2B) showing nodule formation with aggregation of hemocytes below cuticle.

The Midgut Tissue:

On the other hand, Histological section at midgut of untreated *G. mellonella* (Fig.3A) showing normal midgut tissue with its epithelial layer, peritrophic membrane, basement membrane and regenerative cells. While cross-section in the midgut of the treated larva at 48 hrs. post fungal infection showed extensive damage to the epithelium layer due to fungal toxins by separation of columnar layer towards gut lumen with increasing the number of regenerative cells beneath it.

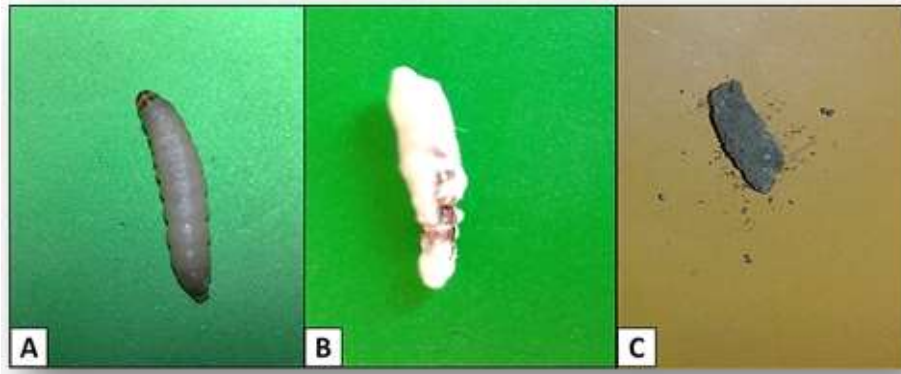


Fig. 1. Symptoms of cadavers of *G. mellonella*: (A) Untreated larva. (B) larva infected by *B. bassiana*. (C) larva infected by *M. anisopliae*



Fig.2: Histopathology of *G. mellonella* cuticle infected by *B. bassiana*. (A) Cross section of untreated larva showing normal cuticle (C) with its epidermis layer (e). (B) Cross section in cuticle at 48 hrs from fungal infection showing aggregation of haemocytes and nodule formation (N F) beneath the cuticle layer. Abbreviations: (F b) fat body. (M) muscle (H&E x 200).

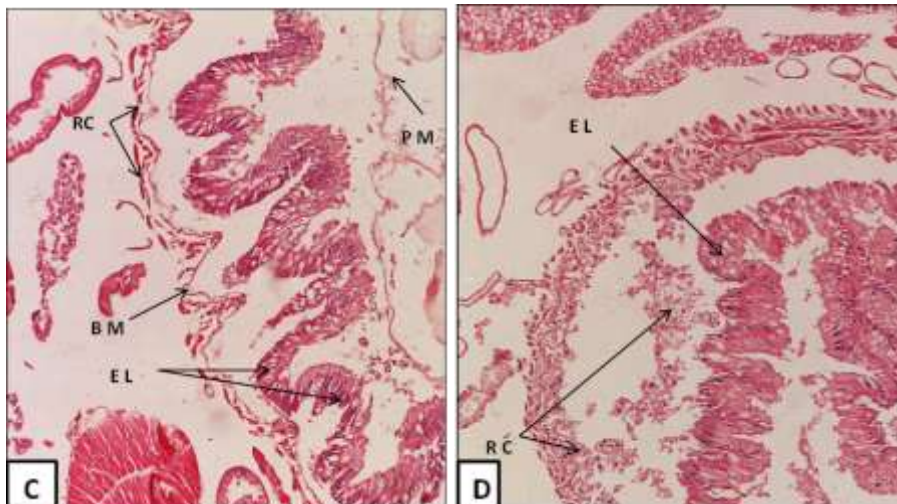


Fig.3: Histopathology of *G. mellonella* midgut infected by *B. bassiana*. (C) Cross section of untreated larva showing normal midgut tissue with its epithelial layer (E L), peritrophic membrane (P M), basement membrane (B M) and regenerative cells (R C). (D) Cross section in midgut at 48 hrs from fungal infection showing extensive damage of epithelial layer by separation toward gut lumen with increment in formation of regenerative cells beneath the columnar epithelium (H&E x 200).

DISCUSSION

Entomopathogenic fungi are important factors regulating insect populations. *M. anisopliae* and *B. bassiana* are important natural control agents and sources of mycopepticides for many Noctuidae pests management worldwide (Asi *et al.*, 2013 and Han *et al.*, 2014). This study showed variability in the pathogenicity of both of the tested fungal isolates towards *G. mellonella* larvae, whereas *B. bassiana* isolated was more virulent than *M. anisopliae* in which concentration of about 3.1×10^2 conidia/ml induced 50% larval mortality while *M. anisopliae* induced the same effect at 4.6×10^3 . This variation among isolates could be related to, the attachment way of spore into insect cuticle, the speed of germination of the conidia of each isolate and/ or the presence of collagenous protective coat that enables fungi to overcome the innate immunity of insects when the fungus comes into contact with hemolymph (Anand *et al.*, 2009 and Momein, 2010).

Several studies agree with our finding. Hussein, (2007) reported that *B. bassiana* isolates were highly pathogenic than *M. anisopliae* isolate against *G. mellonella*. Also Oreste, (2012) showed that *B. bassiana* isolates were the most virulent against *G. mellonella*. Similar data were also reported by several authors (Zayed 2003, Amer *et al.*, 2015 and Ibrahim *et al.*, 2016).

The mortality percentage in this study appeared to be concentration dependent (Table 1) and this result is almost similar with the data obtained by Liu *et al.* (2002), Wright *et al.* (2005) and Arici *et al.* (2010) who reported that the mortality in infected pest with *B. bassiana* increased with increase in concentration of conidia suspension.

El-Sinary (2002) and Quesada-Moraga *et al.* (2006) explained that the efficiency of the entomopathogenic fungi began clearly at 48 hrs after inoculation so histopathological changes due to LC₅₀ concentration of *B. bassiana* infection in cuticle and midgut was detected in this study at 48 hrs post fungal infection. Our results showed that the cuticle of the treated larvae induced nodule formation and progressive melanization, indicating direct attack of the fungus on the defense system of the insects (Fig. 2). Bogus and Maria (2000) mentioned that cross-section of *G. mellonella* infected by fungi revealed a progressive melanization at the epidermal cells by the accumulation of hemocytes at the place of infection. The nodule formation at the point of entrance of the fungal hyphae was identified as a cellular encapsulation (Chouvenc *et al.*, 2009). Bogus *et al.*, (2007) indicated that exposure to the fungal infection increased phagocytosis, whereas nodulation or encapsulation responses were increased in *G. mellonella* suggests that such hemocyte-mediated immune responses make some contribution toward protecting the insect from the fungus.

Midgut tissue of infected larva showed progressive destruction of the columnar layer by separation towards gut lumen (Fig. 3). These results are consistent with El-Sinary (2002) who showed that the efficiency of the entomopathogenic fungi began after 48 hrs from infection as a progressive deterioration of the columnar cells. Quesada-Moraga *et al.* (2006) indicated that death of *S. littoralis* larva by fungal species *B. bassiana* was due to toxic protein extracted by fungi leading to a progressive bleeding of the midgut epithelium into the gut lumen with lyses of the epithelium layer. Moreover, increment of regenerative cells production may be tissue tend to repair the occurred destruction as explained by Quesada-Moraga *et al.*, (2006) who found that the progressive increase of the stem cells could allow regrowth of the midgut epithelium.

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ARABIC SUMMERY

العدوى الفطرية وتأثيرها على الجليد والمعى الوسطى لدودة الشمع الكبرى *Galleria mellonella*

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اجريت هذه الدراسة بغرض المقارنة بين كفاءة وتأثير اثنين من الفطريات *Beauveria bassiana* و *Metarhizium anisopliae* ضد دودة الشمع الكبرى *Galleria mellonella* فى ظل الظروف المختبرية. كذلك دراسة التغيرات الهيستولوجية لكلا من الجليد و المعى الوسطى للحشرة بعد 48 ساعه من العدوى بالفطر وقد اوضحت النتائج ان هناك علاقة طرديه بين تركيز جراثيم الفطر ونسب الموت للحشرة حيث تزداد نسب الموت بزيادة تركيز الفطر كما ان فطر *Beauveria bassiana* كان اكثر فاعلية من *Metarhizium anisopliae* حيث سجلت LC_{50} لكلا منهما $10^2 \times 3.1$ و $10^3 \times 4.6$ جرثومه / مللى على التوالي . كما لوحظ تغيرات هيستولوجيه فى الجليد والقناه الهضميه للعائل بعد 48 ساعه من العدوى الفطريه تمثلت فى تكوين تجمعات عقديه تحت طبقة الجليد و تدمير خلايا epithelium للمعى الوسطى بانفصالها عن الغشاء القاعدى و انتاج اعداد كبيره من الخلايا الجذعية اسفلها.