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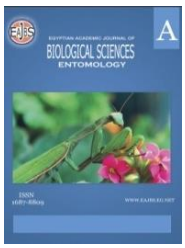

Histological Changes in the Adult Seed Bug, *Graptostethus servus* (Hemiptera: Lygaeidae) Treated with the Entomopathogenic Fungus, *Beauveria bassiana* (Ascomycota: Hypocreales).

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ABSTRACT

The seed bug *Graptostethus servus* (Fabricius) (Hemiptera: Lygaeidae) is a pest, recently recorded in Egyptian oil crops, such as sesame (*Sesamum indicum*), sorghum (*Sorghum bicolor*), cotton (*Gossypium hirsutum*) and sunflower (*Helianthus annuus*). This work is considering the impact of the native isolate of the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales) against *G. servus*. The results revealed that *G. servus* is very vulnerable to *B. bassiana.* LC_{50} value was $2.9x10^6$ spores/ml and the LT_{50} value was 4.16 days. The infection affected various body parts, especially the cuticle and the midgut. The application of *B. bassiana* on the adult's cuticle resulted in an apparent disturbance in the structure of the cuticle and degeneration of its different parts. These changes were observed 48 hours after treatment and increased in severity over time. The fungal infection was not limited to the cuticle, but also affected the epithelial cells of the crop and midgut, as well as the mesenteric caeca. These results highlight the insecticidal potential of the fungus *B. bassiana* against the pest *G. servus*.

INTRODUCTION

Graptostethus servus (Fabricius) (Hemiptera: Lygaeidae) is a pest commonly known as a seed bug (Magsi *et al*., 2018). It is distributed worldwide in Asia, Africa, many countries of the European Union and the South Pacific Island (Péricart, 2001; Kment *et al*., 2005 and Hussain *et al*., 2014).

Graptostethus servus, attacks seeds of many Convolvulaceous and oil crops from other families like sorghum, cotton, sunflower, milkweed and sesame all over the world (Gamberale-Stille and Tullberg ,1999, 2001; Khaing *et al*., 2002 and Kment *et al*., 2005). It was documented as a sap-sucking bug, so it can damage and disrupt these crops, as it was recorded during the summer of 2017, 2018 and 2019 and the winter of 2020 in Giza Governorate, Egypt (Ibrahim and Elshewy,2020).

Also, Chin *et al.* (2018) reported that *G. servus* swarms gathered on native trees,

vegetables, and fruit trees. The bugs attracted to dampness resulted from irrigation and rainwater. They also form big groups in order to mate. It sometimes can cause collateral damage, and appears as needle-thrust spots during feeding on the sap of different parts of the plant. It can also, cause physical damage by smashing stems and scratching leaves, flowers, or fruit as they move in a large swarm on the plants.

There are no concrete investigations on controlling *G. servus* found in the literature. However, conventional insecticides are used excessively, resulting in problems such as resistance, environmental pollution, and the unfavourable impact on non-target organisms (Wang *et al.*, 2011). As a result of these factors, alternative ways of insect pest management, such as biological control using insect pathogenic organisms with several advantages including lower prices, the maintenance of beneficial insects, pollution-free and environmentally safe were used (Carruthers and Hural, 1990; Lacey *et al*., 2001; Freed *et al.*, 2012).

There is great interest around the world in utilizing entomopathogenic fungi as a biological control agent against insects which, unlike other pathogens such as bacteria and viruses that need to be ingested for their action; entomopathogenic fungi require contact with cuticles under favourable temperature and humidity (Islam *et al*., 2021).
The cosmopolitan anamorphic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a very virulent entomopathogenic fungus that is environmentally safe and has been used to control a variety of insect species and some arthropod pests (Ramoutar *et al*., 2010, El-Sheikh 2012 and Perinotto *et al*., 2012, Chu *et al*., 2017) through the mechanism of spore germination, cuticle penetration, and mycelial dissemination mode of action inside the body (Mora *et al*., 2017). This fungus is causing severe diseases leading to reduce pest population and maintenance in the environment for permanent control.

Therefore, the aim of this work is to study the pathogenicity of the entomopathogenic fungus *B. bassiana*, as well as its histological effect on the cuticle and the gastrointestinal tract of the adult *G. servus*.

MATERIALS AND METHODS

Insect Colony:

G. servus adults and nymphs were obtained from bindweed (*Convolvulus arvensis* L.) at The Experimental Station of the Faculty of Agriculture, Cairo University. Seed bugs were transferred to the lab., housed in polypropylene containers at 25 ± 2°C, 75-85 % RH, with milkweed plant pods for feeding, which were refilled every two days. To avoid cannibalism, pieces of paper were put into the containers, and a saturated piece of cotton was placed in, to provide humidity. Muslin was used to cover all containers to allow for airflow. Adults were depositing their eggs outside the muslin cover, then they were collected and kept in a petri dish containing moistened cotton wool till hatching. As with previous methods, all hatchlings were moved to different containers for maintenance.

Entomopathogenic Fungus Culture:

Isolate of the entomopathogenic fungus *Beauveria bassiana* (Hypocreales: cordycipitaceae) used in these investigations was obtained from soil collected from East Owainat, New Valley Governorate, Egypt. The wax moth larvae, *Galleria mellonella* L., were used to trap the entomopathogenic fungus, (Zimmermann, 1986). *Beauveria bassiana* was cultured on *Sabourad dexstrose* yeast agar media (SDAY), at 25 ± 1 ºC for 14 days (Nada, 2015).

Bioassays Procedure:

Spores were obtained from the culture media by washing them in a sterile aqueous solution containing 0.02 percent Tween 80 and then filtering them through a fine mesh cloth to prevent the mycelium from sticking. The concentrations of spores were quantified using a Haemocytometer (Neubauer improved HBG, Germany 0.100 mm X 0.0025mm^2). The suspensions were produced at concentrations of 10^6, 10^7, $5x10^7$ and 10^8 spores/ml for the bioassay (Nada, 2006). Adults *G. servus* were treated for 10 seconds in each concentration using the dipping method procedure, and the remaining solution was then filtered out with filter paper. Adults in the control group were treated with a sterile aqueous solution containing 0.02 percent tween 80. Each concentration and control were replicated three times, and a total of 25 individuals for each replica were employed. Individually, the treated and controlled insects were kept in transparent plastic containers with ventilation holes in their cover and provided with moistened filter paper. For feeding, all plastic boxes were supplemented with milkweed plant pods, that were replaced every two days. All containers were placed in an incubator at 25°C, 75-90 % RH; mortality was observed on a daily basis for seven days.

Histological Study:

A concentration of $1x10^7$ spores/ml of isolated spores was prepared to use in a histological study using the previously mentioned dipping technique. An aqueous solution of 0.02% Tween 80 was used as a control (Gad and Nada 2020).

After 24,48 and 72 hr of treatment, treated and untreated adults of *G. servus* (n = 5 per each treatment time and control) were immediately fixed in 10% buffered formalin for 24 hr, then dehydrated in an ethanol-xylene series and embedded in paraffin wax, and cut into 4 μm sections. The sections were deparaffinized, rehydrated, and stained with Haematoxylin and Eosin for histopathological examination under an optical microscope to determine the various anomalies that may have appeared in the cuticle and the gastrointestinal tract of the treated *G. servus* adults (Bancroft and Stevens 1996).

Statistical Analysis:

To determine the LC_{50} and LT_{50} values for the toxicity tests, using "Ldp" software (Bakr, 2000), The corrected mortality percentages were statistically calculated according to Finney (1971).

Statistical analysis of obtained data was analysed using SAS procedure, and means were compared by Tukey's HSD (P= 0.05 level) in the same program.

RESULTS AND DISCUSSION

Pathogenicity of *B. bassiana* against *G. servus*:

Our findings confirmed that *G. servus* adults were very vulnerable to infection with the entomopathogenic fungus *B. bassiana*, and proved the pathogenicity of *B. bassiana* to *G. servus*. LC_{25}, LC_{50} and LC_{90} values were $3x10^5$, $2.9x10^6$ and $2.08x10^8$ spores/ml, respectively (chi2= 0.407, slope= 0.689±018, P<0.816) (Fig. 1). After 4 days of treatment, mortality was noticed. Mean lethal time LT_{25}, LT_{50} and LT_{90} values were 3.12, 4.16 and 7.2 days, respectively (chi2= 0.79, slope= 5.38±1.008, P<0.851) (Fig. 2).

Our results are in harmony with a study performed by Krueger *et al.*, (1991) who reported the virulence of *B. bassiana* against nymphs and adults of hairy chinch bug (*Blissus leucopterus* hirtus). Also, adult mortality of chinch bug increased with increased *B. bassiana* dose, reaching greater than 90% at 10^8 conidia /ml (Boyle and Cutler 2012). In addition, they noticed that chinch bugs mortality increased with exposure time. According to Samuels *et al*., (2002) *B. bassiana* isolates were pathogenic to chinch bug eggs. Bidochka *et al*., (1993) reported that three predominant *Lygus* species were

susceptible to *B. bassiana*, that LT_{50} was 4.97 days and 90 % mortality was achieved in 7 days. *Beauveria bassiana* ARSEF 792 was the most virulent isolate against both nymphs and adults of *Blissus antillus* (Hemiptera: Lygaeidae), immersion of fungi caused 53 and 78% infection, respectively, with LT_{50} values of 7.8 and 5.0 days, respectively (Samuels and Coracini, 2004).

In context with our results, Nada (2021) proved the harmful effect of *B. bassiana* on the immune defenses mechanisms of *G. servus*, which was evident in total protein, total carbohydrates, Esterase and alkaline phosphatase activities.

In agreement with our results, Ahmed *et al*. (2020) indicated that *B. bassiana* caused higher mortality and provide better control with insecticides to be used in integrated pest management for reducing the population density of *Oxycarenus hyalinipennis* (Hemiptera: Lygaeidae).

Moreover, El-Sheikh and EL-Shami (2021) reported that a considerable number of adults and the last nymphal instar of *O. hyalinipennis* inhabiting okra dry pods were infected and killed by *B. bassiana*. Slimier results were observed by Khan *et al.* (2014) who reported that *B. bassiana* caused high mortality with an LC_{50} value of 2.5×10^7 spores/ml on *O. hyalinipennis*, with an LT_{50} value of 4.32 days at a concentration of 3×10^8 spores/ml of *B. bassiana*.

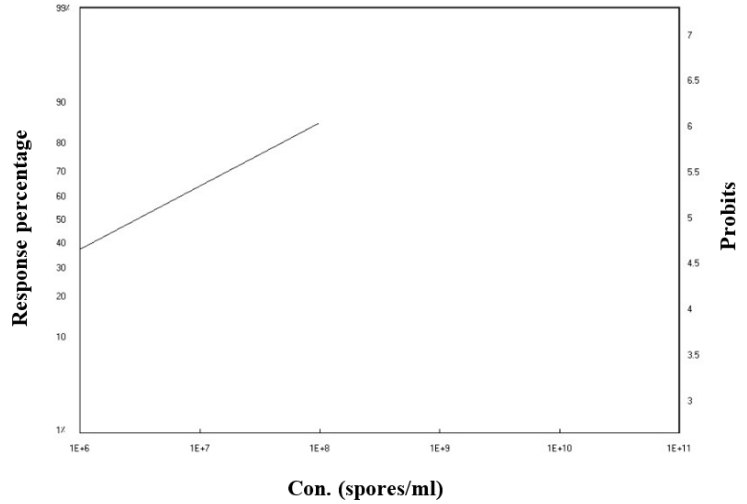

Fig. 1: Insecticidal potency of *B. bassiana* against *G. servus* (expressed as % mortality).

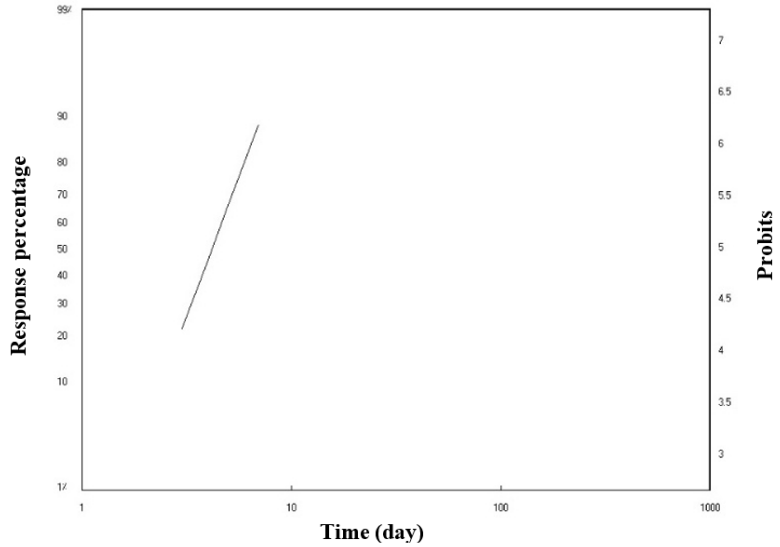

Fig.2: Time–mortality regression line of *B. bassiana* against *G. servus.*

Histological Studies in the Adult *G. servus:*

Many studies used light and electron microscopy to identify many histological alterations in most insects caused by infection with various entomopathogenic fungi (Gabarty *et al*., 2014; Khaleil *et al*., 2016; Ragavendran *et al*., 2017). The fungal treated and untreated adults of *G. servus* were histologically examined after 24, 48 and 72 hr of treatment using a light microscope.

The thin sections of the adult of *G. servus* in this study revealed that the *B. bassiana* spores' suspension brought about intense degeneration and distortion of the adult of *G. servus* cuticle, foregut, midgut and the mesenteric caeca (Figs., 3-5). The untreated adults (Fig., 3 a) showed the normal structure of the bug's cuticle with a normal intact cuticle that clearly differentiated into epicuticle and procuticle layers. On the other hand, the treated individuals' semi-thin section showed many abnormalities in the insect's cuticle after 48 hr of treatment (Fig., 3 b). There was a disorder of the cuticle layers, which has become black-spotted and had not differentiated into its epicuticle and procuticle layers resulting from a direct fungal attack. Also, the treatment caused the destruction of the cuticle -the insect's primary defense line-, specifically the adult epicuticle which is degraded into small pieces after 72 hr of treatment (Fig., 3 c).

Similar results were obtained by Bidochka *et al*., (1993) who revealed that microscopic examination of the thin sections of *B. bassiana*-infected *lygus* bug cadavers revealed massive disintegration of internal muscle tissues while breaching of the cuticle was generally localized to the intersegmental membranes. The same observation was recorded by Amer *et al*., (2008), who noticed many malformations in the cuticle of *Spodoptera littoralis* larvae after being treated with five strains of entomopathogenic fungi.

Our results were in agreement with Haitham and Alleddin (2012), who demonstrated that fungal infection had a significant impact on all areas of the insect's body including the cuticle, intestine and adipose tissue.

By comparing with the control which has an intact wall (Fig., 3 a), this destruction has engendered, as an indicator of disorders and histopathological changes in the cuticle resulting from fungal invasion.

The fungal infection had a clear effect on the gastrointestinal tract, which was clearly evident in the cross-sections of intestinal cells. The crop, was the most affected part of the foregut, as its epithelial cells degenerated and adjacent cell adhesion was lost slowly after 48 hr of infection (Fig., 4 b) compared with those of the control (Fig., 4 a).

The cross-section in the midgut showed the effect of the fungus on its epithelial cells, that it caused a change in the thickness of these tissue after 48 hr of treatment (Fig., 5 b**)** compared with those of the control (Fig., 5 a), Furthermore, the disappearance of the midguts' epithelial cells which completely dissolved and separated from the basement membrane in some sections, especially after 72 hr of treatment (Fig., 5 c**).**

Fungal infection does not stop at the level of the cuticle or gastrointestinal tract, rather, it went beyond that to infect the accessory organs of the gastrointestinal tract, the mesenteric caeca, the epithelial cells of which were degenerated and separated from its basement membrane after 48 hr of treatment (Fig., 4 b), subsequently lost their functional specialization in increasing the secretion and absorption surface of the stomach.

Histopathological changes in the midgut of *G. servus* are similar to those observed by Soliman *et al*. (2022) on *Nezara viridula* treated with *B. bassiana*, including cell degeneration of the stomach and vacuolation, which invades cells all time after treatment with fungus.

Based on these findings, the action of the fungus on the muscle layers and intestinal cells is probably considered a good control of the fungal colonization of the

digestive canal as was recorded by Benzina *et al*., (2018).

The changes that were observed in the digestive canal, especially the midgut cells, are explained by the fact that this portion of the digestive tract, which is responsible for digestion in insects, is in direct contact with the toxic fungus and so causing death. These results show similarities with those observed by Al-Mehmadi and Al-Khalaf (2010) who found that the earliest changes occur in the midgut and posterior stomach of *Culex pipiens* larvae infected by *Bacillus sphaericus*.

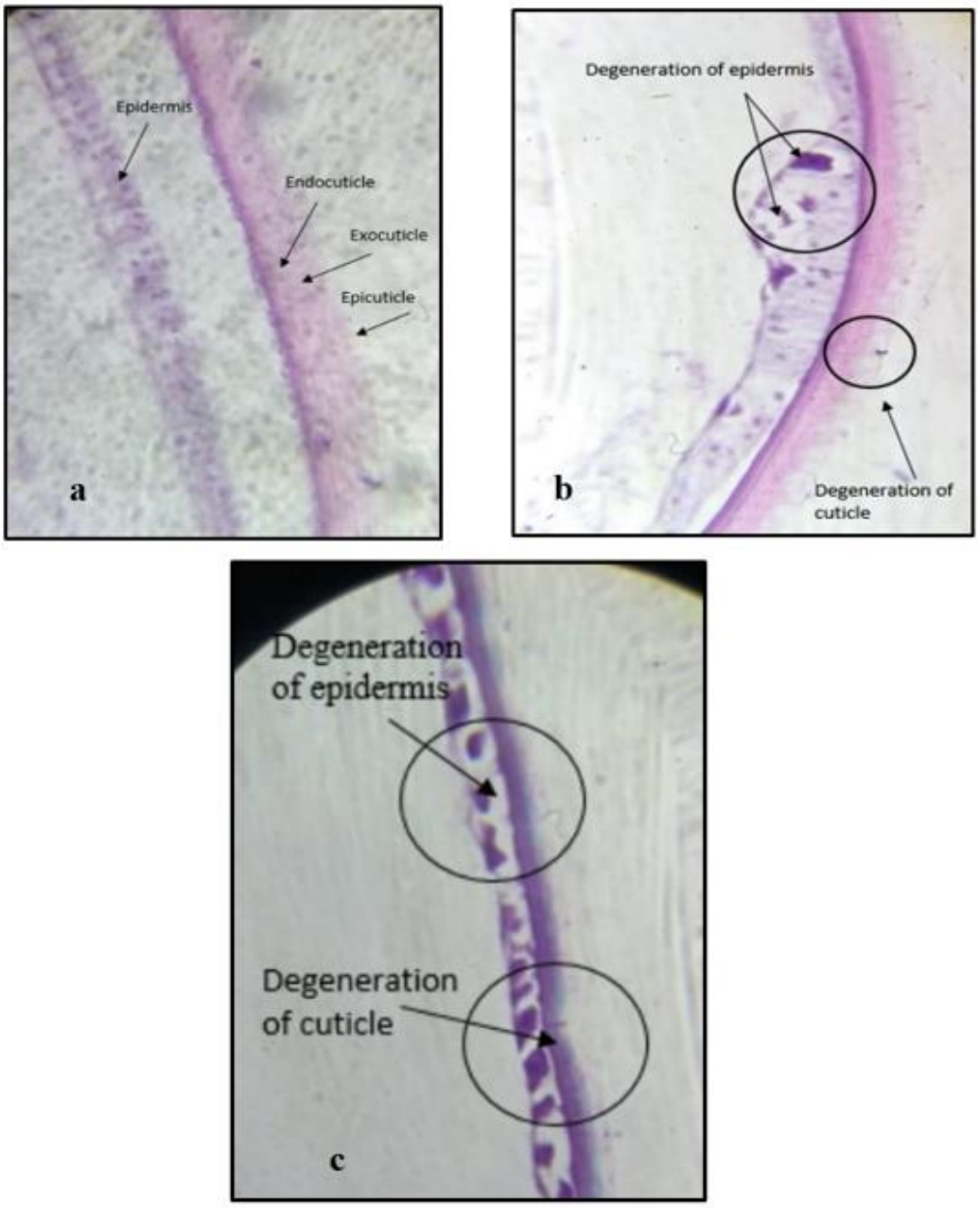

Fig.3: Histoarchitecture of *G. servus* adults' cuticle showing the untreated adult (a), compared with adult after 48 hr of treatment with *B. bassiana* (b), and adult after 72 hr of treatment with *B. bassiana* (c) (G10x40).

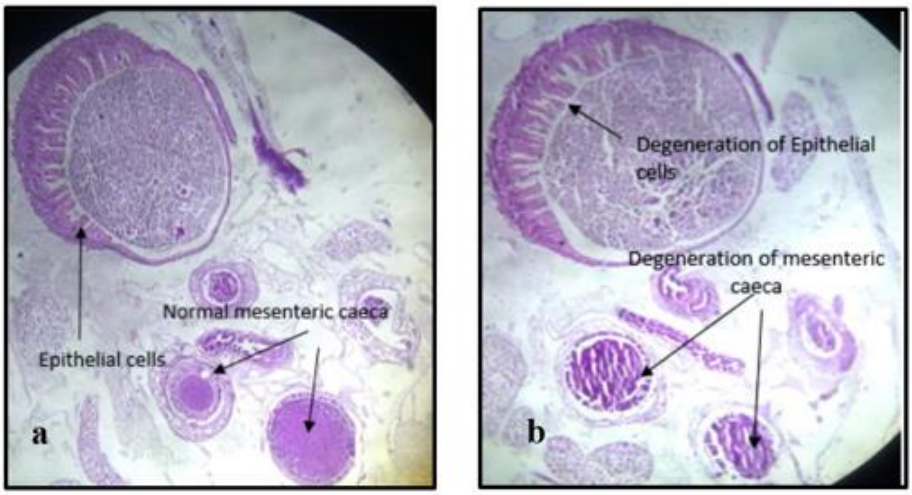

Fig. 4: Degeneration of epithelial cells in the crop and the mesenteric caeca of *G. servus* adult; control (a), and after 48 hr of adult treatment with *B. bassiana* (b) (G 10×40).

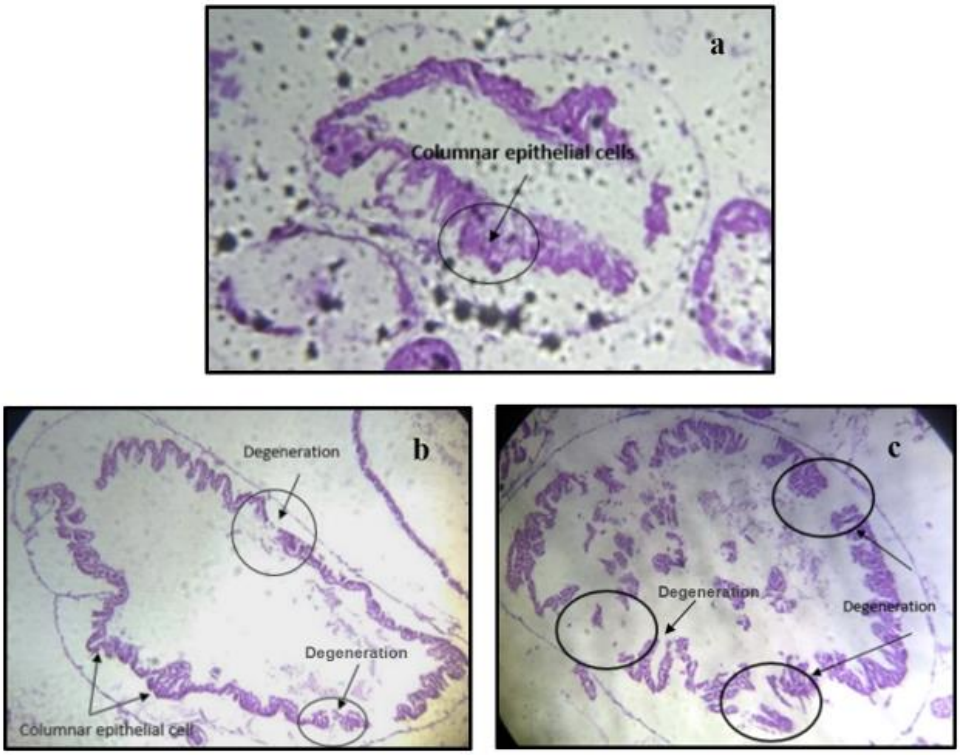

Fig.5: Histoarchitecture of *G. servus* midgut epithelial cells showing the untreated adult (a), compared with adult after 48 hr of treatment with *B. bassiana* (b), and adult after 72 hr of treatment with *B. bassiana* (c) (G10x40).

Conclusion

The application of the entomopathogenic fungus *B. bassiana* on the adult of *G. servus* induces disturbances in all parts of the body, including the structure of the cuticle, stomach and intestine, witnessing the potency of this fungus against the adult of the *G. servus*, which qualifies it as a potential agent through Integrated Pest Management (IPM) programs for controlling *G. servus*.

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ARABIC SUMMARY

التغيرات النسيجية في الطور البالغ لحشرة *Graptostethus servus* المعاملة بالفطرالممرض للحشرات *Beauveria bassiana*

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أجريت الدراسة الحالية لتحديد القدرة الامراضية لفطر *Beauveria bassiana* الممرض للحشرات و تأثيره على بعض التغيرات النسيجية في جدار الجسم والقناة الهضمية للطور البالغ لحشرة *Graptostethus servus* بعد 24و48 و72 ساعة من المعاملة. فقد أوضحت الدراسة أن قيمة الـ LC_{50} كانت 2.9 10X ^{6} جرثومة/مل و أن قيمة الـ LT_{50} كانت 4.16 يوماً و قد أوضحت النتائج ظهور العديد من التشوهات والتراكيب غير الطبيعية في جدار الجسم وأنسجة القناة الهضمية الأمامية والوسطى في الطور البالغ للحشرة بالإضافة لتغيرات في الملحقات المباشرة للقناة الهضمية (الزوائد الأعورية) . فقد بدأ ظهور إضطراب واضح في بنية جدار الجسم وتدهور أجزائه المختلفة بعد 48 ساعة من المعاملة ، وازدادت حدته بمرور الوقت. وقد كان للعدوى بالفطر تأثير واضح على الجهاز الهضمي والذي ظهر في المقاطع العرضية لخلايا القناة الهضمية. وقد كانت الحوصلة هي الجزء الأكثر تضرراً من المعدة الأمامية ، حيث ظهر أثار تحلل في الخلايا الطلائية لها بعد 48 ساعة من المعاملة ، كما سببت العدوى في تغيير سمك الخلايا الطلائية للمعدة الوسطى بعد 48 ساعة من المعاملة و التي إنفصلت عن الغشاء القاعدي لها وتحللت تماماً في بعض المناطق بعد 72 ساعة من المعاملة. كما فقدت الزوائد الأعورية بنيتها الأساسية والوظيفية نتيجة تحلل الخلاية الطلائية لها.