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# THE EFFECT OF ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae* VAR *acridum* ON FLIGHT MUSCLES IN THE DESERT LOCUST, *Schistocerca gregaria* (FORSKAL)

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## ABSTRACT

The effect of infecting the flight muscles of adult desert locust *Schistocerca gregaria* Forskal with the entomopathogenic fungus *Metarhizium anisopliae* var *acridum* was studied. The results show that the fungal spores germinated on the cuticle and penetrated the flight muscles 24 hours after the treatment. The histopathological studies of the treated muscles showed that the nucleus, mitochondria, myofibrils and the vacuoles were highly deformed. The activity of trehalase increased during the first two days after the treatment then significantly decreased on the third day. Lactate dehydrogenase activity was always lower in the mycosed insects.

**Keywords:** *Schistocerca gregaria, Metarhizium anisopliae* var *acridum*, Flight muscles, Electron Microscope, Trehalase, Lactate dehydrogenase

## INTRODUCTION

The desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae), is one of the most dangerous economic pest causing severe damages to the crops during its plagues. Fungal pathogens have considerable potential for locust and grasshopper control (Goettel *et al.*, 1995). Deuteromycete fungi such as *Beauveria* spp. and *Metarhizium* spp. are the most promising candidate pathogens for locust control because they are cheap to produce and penetrate directly through the host cuticle, an advantage in harsh climatic conditions. They also have good potential for formulation in oil as ultra-low volume sprays, thus making use of existing application technology, including aerial spraying (Prior and Greathead, 1989). A mycoinsecticide based on the entomopathogenic fungus *M. anisopliae* var *acridum* has been developed and is being commercialized under the tradename "Green Muscle" for the control of locusts and grasshoppers in Africa (Thomas *et al.*, 2000).

Insect fungal pathogens invade their hosts through the cuticle. The fungus secretes proteases and chitinases that hydrolyse protein and chitin which are considered the key components of cuticle (Charnley, 2003) thys facillitatiz the passage of the hyphae through the integument and providing nutrition for the fungus (Charnley and St Leger, 1991; St Leger, 1995).

The fungal conidia germinated within 12 hrs after application to the cuticle, digested and penetrated the epicuticle 12-18 hrs after application, and started to invade the hemocoel by 24 hrs (Gunnarsson, 1988). Biserova and Pflüger (2004) reported that the ultrastructure of locust muscles with different

function was examind, the pleuroaxillary flight steering muscle is compared with a typical flight (power muscles) and a typical leg muscle,in particular with respect to sarcomere length, tracheation, mitochondria, and sarcoplasmic reticulum. The ultrastructure of the pleuroaxillary muscles resembled that of leg muscles.

In many insects, trehalose constitutes the major haemolymph sugar, whereas glucose is often present at much lower concentrations (Wyatt, 1967; Becker *et al.*,1996). Trehalose is syntheized and released into the haemolymph by the fat body, the central organ of intermediary metabolism in insects (Candy and Kilby, 1959, 1961). Wegener *et al.* (2003) found that overt flight muscle trehalase is located in the plasma membrane with the active site accessible to the haemolymph. Trehalase inhibitors are considered valuable tools for studying the molecular physiology of trehalase function and sugar metabolism in insects.

The characteristics of pyruvate kinase, phosphoenolpyruvate carboxykinase, lactate and malate dehydrogenases from *Mermis nigrescens* isolated from locusts (*S. gregaria*) were determined. Lactate dehydrogenase (LDH) activity declined markedly in 21 day parasites and postparasites. Pyruvate kinase activity declined slightly during nematode development and phosphoenolpyruvate carboxykinase activity did not appear before 19 days (Platzer, 1979). The changes in LDH activity in the fat body of the cricket, *Gryllus bimaculatus* after infection with *Nosema grylli* and *Adelina* sp. were recorded by spectro- photometry. *N. grylli* and *Adelina* sp. infections caused 5 and 10 fold increase in LDH activity. Xiahong *et al.* (2005) observed LDH activity at low temperature increased significantly and the ATPase activity decreased with prolonged duration of exposure to low temperatures. Poly acrylamide Gel Electrophoresis (PAGE) of fat body homogenate supernatans of uninfected males, females and larvae revealed the presence of 3 types of isoenzyme patterns; a slow band, a fast band and a band with 5 distinct inner bands.

Studies on the effect of entomopathogenic fungi against swarming form and flight process is rare and need more investigation. This research work was extended to study the germination and penetration of the entomopathogenic fungus on cuticle and flight muscles by Scanning Electron Microscope and histological effect on muscles by Transmission Electron Microscope and the activity of trehalase and lactate dehydrogenase.

## MATERIALS AND METHODS

### Test insects

The stock colony of the desert locust, *Schistocerca gregaria*, Forskal was maintained for several years at the Locust Research Division, Plant Protection Research Institute, Agricultural Research Center, Dokki, Cairo. The insects were reared and handled according to Robert *et al.* (2002). The cages were incubated at 32 ± 2°C and 30-50% R.H. and photoperiod of 12:12 dark-light. Fresh clover leaves were used for feeding the insects in winter and the leaves of leguminous plant *Sesbania aegyptiaca* Webster, were introduced during summer. The experimental insects were segregated from the

gregarious stock colony at the beginning of the first nymphal instar and held up in cages (30x30x30 cm) in diameter. The cages had a wooden frame and equipped with zinc bottom covered with thin layer of sand, glass- covered sides and a wire- gauze top provided with a little door. Unconsumed food, dead locusts and faeces were removed daily. The cages were thoroughly washed and sterilized with an antiseptic agent every (4-6 weeks) or whenever they become empty and at the end of every experiment.

**Fungal inoculum:**

The isolate used was *M. anisopliae* var *acridum* (IMI 330189) from commercialized formulation "Green Muscle".

**Scanning Electron Microscope examination**

Insects were topically inoculated on the thorax with 5 µl of an oil-based fungal spore suspension at concentration of $5x10^7$ spores/ml using a microapplicator. Control insects were treated with cotton seed oil only (Prior *et al.*, 1995).

Immature adults of *S. gregaria* were prepared for electron microscopy. The insects were anesthetized with $CO_2$ then killed by twisting the head to break the "neck" membrane. The posterior tip of the abdomen was cut off and the head; with the gut attached was removed. The carcass was cut open ventrally and the fat bodies overlying the flight muscles were removed with tissue paper. Excised thoraces from immature adults were fixed in 1% osmic acid, then 2% glutaraldehyde, dehydrated in ethanol and critical point dried in liquid $CO_2$ . Specimens were sputter- coated with gold and examined with scanning electron microscope JEOL (JSM 5200) at on accelerating voltages of 10 to 15 V. (Inglis *et al.*, 1995).

**Transimission Electron Microscope examination**

Before dissection, the locusts were cooled in the refrigerator and then the flight muscles were exposed, prefixed in situ with 2.5% glutaraldehyde (Sigma) in 0.1M cacodylate buffer (pH 7.2) for about 10min, dissected free and put into fresh fixative for 1-2 h. After washing in 0.1 M cacodylate buffer, specimens were postfixed in 1% $OsO_4$ in the same buffer for 1 h, then washed, dehydrated in an ethanol series, and embedded in Araldite epoxy resin. Semi-thin sections for light microscopy, and ultr-athin sections 80 nm for electron microscopy (EM) were prepared using a Leica EM KMR2 ultramicrotome. Semi-thin sections were stained with toluidine blue, while ultra-thin sections were stained with 4% uranyl acetate and 0.4% lead citrate in distilled water, and examined with a JEOL 1200 EX II transmission electron microscope at the central laboratory, Faculty of Science, Ain Shams University. Pictures were further pocessed by using Adobe Photoshop 6 (Biserova and Pflüge, 2004).

**Estimation of trehalase activity**

Trehalase activity was estimated according to Ishaaya and Swiriski (1976). The free aldhydic group of glucose formed after trehalose digestion was estimated using 3.5 dinitrosalicylic acid reagent.

The trehalase reaction mixture consisted of 0.2 ml of 3% trehalose (substrate), 0.180 ml acetate buffer (pH 5.4) and 20 µl of muscles homogenate.

The dinitrosalicylic acid reagent was prepared by dissolving one gram of 3.5- dinitrosalicylic acid in 20 ml of 2N NaOH and 50 ml of distilled water with the aid of a magnetic stirrer. Potassium sodium tartarate (30 g) was added, and magnetic stirring was continued until a clear solution was obtained. Distilled water was then added to bring the final volume to 100 ml and incubated at 37 °C for exactly 60 min, then 0.8 ml of 3.5- dinitrosalicylic acid reagent was added. The reaction mixture was heated for 5 minutes at 100 °C in a water bath followed by immediate cooling in an ice bath. The optical density (OD) of the produced colour was measured at 550 nm. The enzymatic activity was expressed as µg glucose released/ min/ gm muscle.

**Preparation of standard curve of glucose**

Serial concentrations of glucose solution containing 50, 100, 200, 300 and 400 µg glucose. Avolume of 0.4 ml distilled water were pippeted into test tubes and 0.8 ml of dinitrosalicylic acid solution was then added to each tube. The mixture was heated for 5 min in a water bath at 100° C in boiling water bath and then cooled immediately on ice. The resulting colour was measured spectrophotometrically at 550 nm.

**Estimation of lactate dehydrogenase activity**

Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate; NADH is oxidized to NAD in the process. The rate of decrease in NADH is directly proportional to the LDH activity is determined at 340 nm.

The reaction mixture consisted of phosphate buffer (68 m mol/L, pH 7.5) pyruvate (0.37 m mol/L) and 1.1 m mol/L of NADH.

Hundered microlitres of the sample were mixed with 2.5 ml of the reaction mixture pre-incubated at 37°C. The initial absorbance was read 340 nm. (Kirkton *et al.*, 2005).

LDH activity was calculated according the follwing equation:

LDH activity = factor x Δ A 340 nm/min

Where: Factor = 4468 (as recommed by the used kit; Randox kit, United Kingdom).

Δ A = change in absorbance/min.

**Statistical analysis**

All experiments were in 3-5 replicates. Data were presented as means ± SD. Data were subjected to analysis of variance (ANOVA), and Duncon's multiple range test to differentiate between the means at $P< 0.05$, using Costat Software.

## RESULTS

The present results showed the effect of the entomopathogenic fungus *M. anisopliae* var *acridum* on flight activity of *S. gregaria* by SEM, TEM examination and measuring the trehalase and lactate dehydrogenase activities.

**1. Scanning Electron Microscope (SEM) examination**

Examination of adult stage *S. gregaria* treated with entomopathogenic fungus *M. anisopliae* var *acridum* showed germinated spores on cuticle surface (Fig. 1), and the fungus succeeded in penetrating the insect cuticle and the flight muscles 24 h after treatment. The growth of the hyphae on the inner surface of the flight muscles 24 h after treatment are

shown in (Fig. 2, b). The invaded flight muscles showed clear damages as a result of fungal infection compared with untreated muscles (Fig. 2).

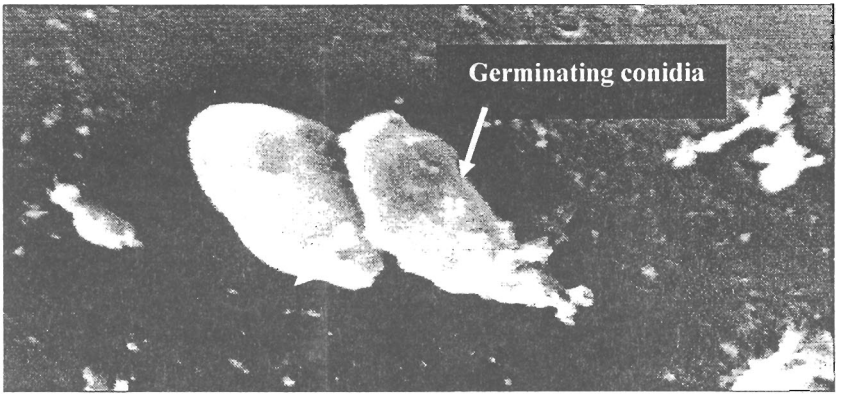


Fig. (1): Scanning electron micrographs showing germinated *M. anisopliae* var *acridum* fungal conidia on adult desert locust cuticle, 24 hr after infection (X7, 500).

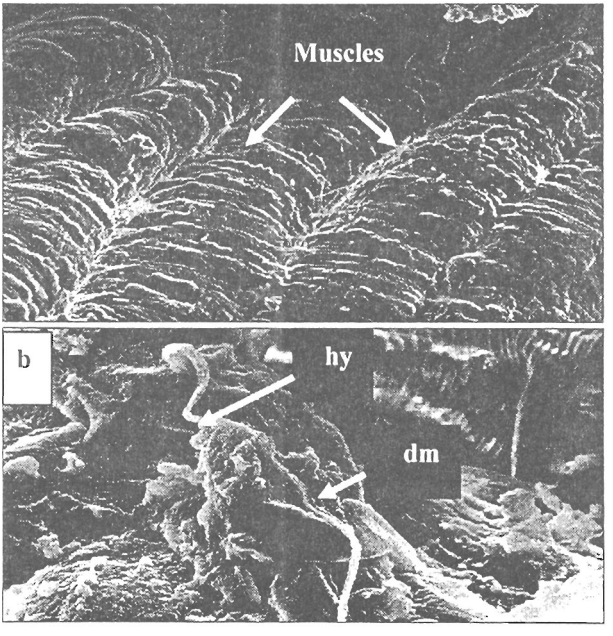


Fig. (2): Scanning electron micrographs showing the penetration of *M. anisopliae* to desert locust flight muscles. (a) The inner surface of flight muscles of control (X7, 500). (b) Penetrated hyphae and damaged flight muscles 24 hr after infection (X7, 500). hy, hyphae; dm, damaged muscles.

**2. Histopathological effects of entomopathogenic fungus *M. anisopliae* var *acridum* on flight muscles of adult *S. gregaria***

Ultrastructure of untreated muscles of adult *S. gregaria* is shown in (Fig. 3 a &b).

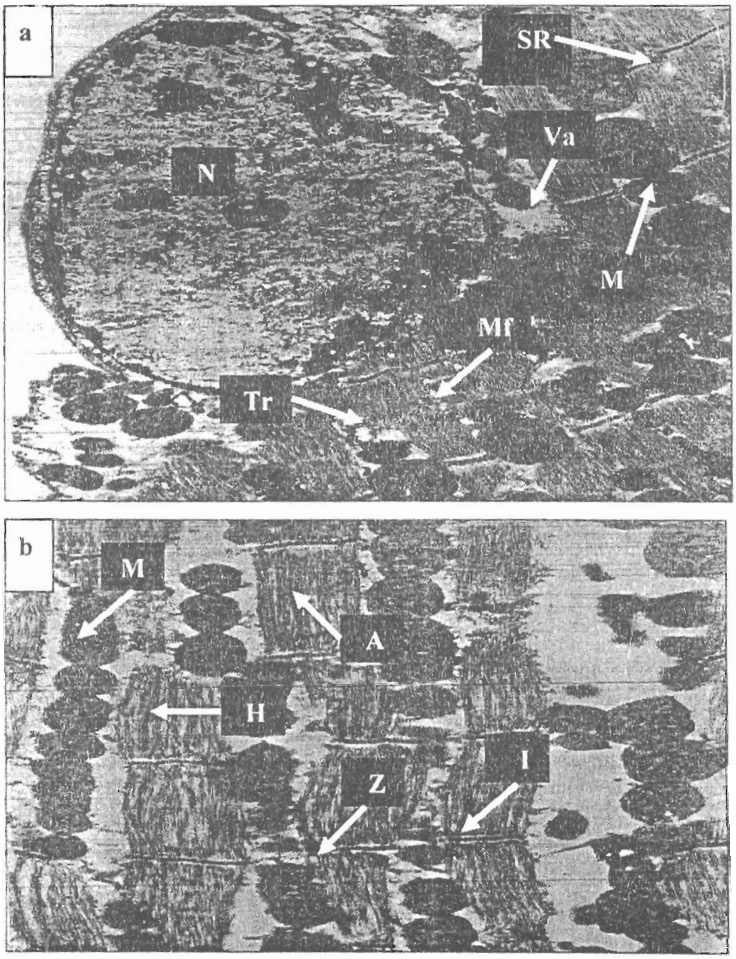


**Fig. (3): Electron micrographs of longitudinal sections of untreated muscle fibres of adult of *S. gregaria* (a) showing Myofibrils (Mf), Nucleus (N), oval-shaped mitochondria (M), Sarcoplasmic reticulum (SR), Vacuole (Va) and Tracheae (Tr). (b) showing Z-line (Z), I,H and A-bands of sarcomere, mitochondria (M) and Extracellular space (Ex) (RX, 5000).**

The muscles are close-packed and each muscle is made up of a number of elongated fibers, and a fiber into myofibrils. Myofibrils (Mf) are composed of sarcomeres. In addition, muscle contains sarcoplasmic reticulum (SR) which is an extensive network of internal membranes broken into vesicles that run longitudinally on the surface of the muscle fibers. Muscles contain abundant and often large, irregulary shaped mitochondria (Mt), nuclei (N) and intracellular tracheoles (Tr). Intracellular tracheoles are not really inside the plasma membrane of the muscle, but are merely pushed into the muscle interior. The contractile fibrils that filled the cytoplasm of each large fiber demonstrate their patterned organization. The fine structure of myofibrils reveals the presence of at least two kinds of filaments in the fibrils (Nation, 2002).

The fibrils are clearly constructed of filaments and the distribution of those filamints is related to the alternating light and dark bands. From the several bands in the striation pattern, the Z-line is commonly selected as marking for the limits of the sarcomere. This line is comparatively denser, especially in contracted fibrils, and may be correctly regarded as a kind of septum that is continuous transversely across the fibril. Other bands are: isotropic band I which is bisected by the Z-line and the anisotropic A which is the denser and is bisected by the narrow light band (H band).

The histopathological examination of the muscles of insects showed different deteriorations after the treatment with the entomopathogenic fungus *M. anisopliae* var *acridum*. Fungal infected muscles 48 and 72 h after infection showed great differences in the nucleus compared with control. Nuclei of treated insects appeared to be greatly damaged and irregular in shape containing small areas of heterochromatin and large vacuoles (Fig. 4 and 5). Nucleus appeared smaller in size, deformated in shape and is surrounded by less numbers of mitochondria and SR.

Muscle fibers from infected insects treated showed degeneration and disorganization of muscle fibers. The mitochondria were clustered and their size was somewhat smaller than those of control muscle fibres. Some mitochondria were elongated while others were swollen with irregular shapes. In electron micrograph of longitudinal section, myofibrils were more indistinct, I and A-bands were less defined, Z-line was diffused and indistinct. H zone is completely unclear and undefined.

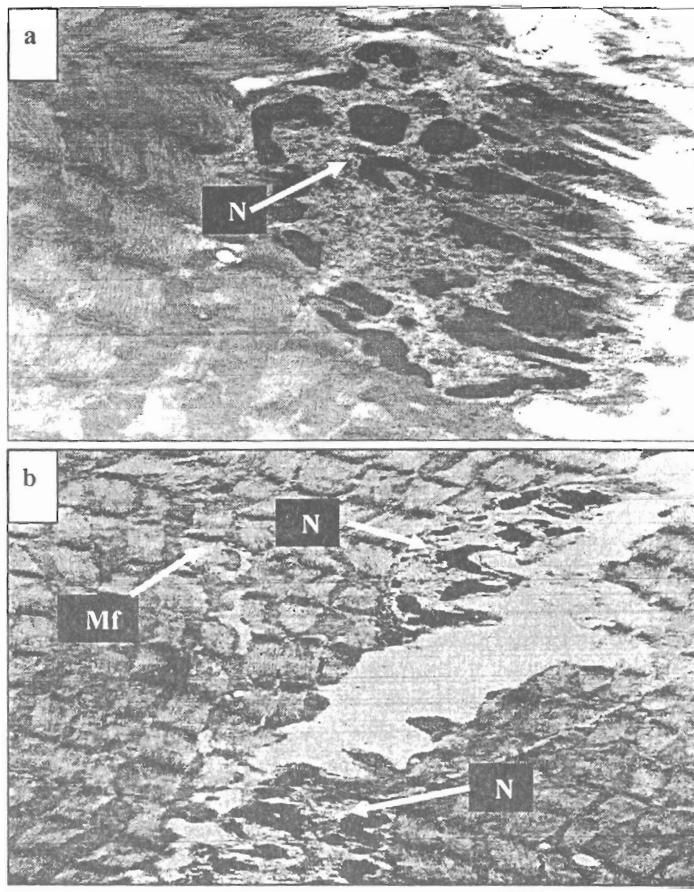


Fig. (4): Electron micrographs of longitudinal sections through flight muscle fibres infected from insects with *M. anisopliae* var *acridum*, 48 h after infection showing the malformation in shape and size of nucleus (resolution,a, RX, 6000); (resolution,b, RX, 3000).the myofibrils (Mf) were indistinct; N, nucleus.

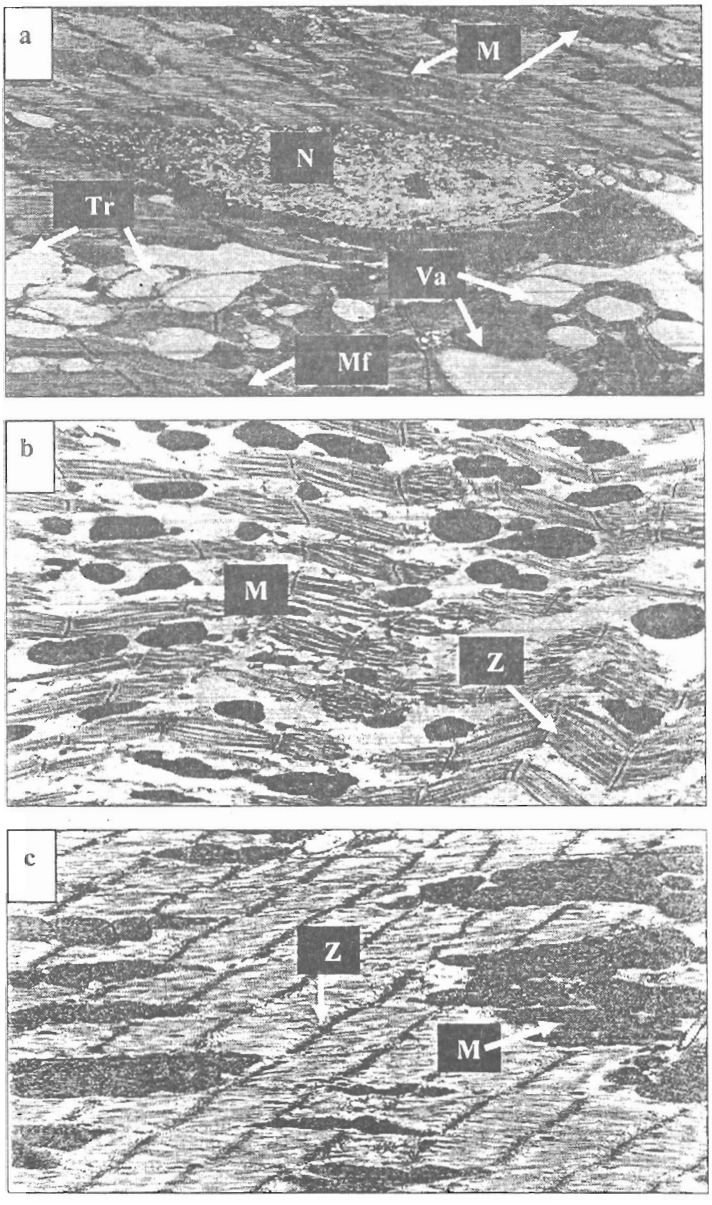


Fig. (5): Electron micrographs of longitudinal sections through flight muscle fibres from infected insects with *M. anisopliae* var *acridum*, 72 h after infection. (a) showing the malformation in nucleus (N) and large vacules (Va); mitochondria (M);Tracheae (Tr) (RX10X). (b) Showing the myofibrils (Mf)were more indistinct. the mitochondria (M) smaller than those of control muscle fibres and irregular in shape (RX, 3000).(c) myofibrils; Z-line and mitochondria (M), (RX, 4000).

### 3. Effect of *M. anisopliae* var *acridum* on trehalase activity

The changes in trehalase activity during the experimental period in the flight muscles of the control insects and the insects mycosed by entomopathogenic fungus *M. anisopliae* var *acridum* were shown in Table (1).

In the treated locusts, flight muscles have asignificantly trehalase activity higher than control in the first and second days but in the third day mycosed locusts have significantly lower activity than control insects ($P< 0.05$).

**Table (1): Effect of *M. anisopliae* var *acridum* on the activity of trehalase.**

| Duration | Control | Treatment |
|---|---|---|
| 24h | 2452±79* | 3983±21* |
| 48h | 2716±15* | 3299±29* |
| 72h | 3260±30* | 3044±39* |

(µg glucose released /min/gm muscle) (*) means ± S.D. ($P< 0.05$).

### Effect of *M. anisopliae* var *acridum* on lactate dehydrogenase

Lactate dehydrogenase activity increased in the flight muscles of control insects than those treated with the fungus through out the whole experiment this increasing was greater at first day where 6.06 U/gm muscle then, became 4.83 U/gm muscle in the second day and smaller value in last day (3.38 U/gm muscle). In the treated muscles the values of enzyme were 3.12

U/gm muscle in first day and increased after 48h to become 4.16 U/gm muscle but turned to be 3.05 U/gm muscle after 72 hrs. as shown in Table (2). ($P< 0.05$).

**Table (2): Effect of *M. anisopliae* var *acridum* on the activity of lactate dehydrogenase.**

| Duration | Control | Treatment |
|---|---|---|
| 24h | 6.06±0.13* | 3.12±0.07* |
| 48h | 4.83±0.08* | 4.16±0.12* |
| 72h | 3.38±0.03* | 3.05±0.06* |

U/gm muscles (*) means ± S.D. ($P< 0.05$).

## DISCUSSION

### Scanning Electron Microscope (SEM) examination

Strain IMI 330189 of *M. anisopliae* var *acridum* germinated on the cuticle and penetrated the cuticle then flight muscles of *S. gregaria* after 24 h from infection. It is likely that the penetration involved the production of cuticle degrading enzymes, as suggested by Gunnarsson (1988). The hyphal bodies penetration of *M. anisopliae* var *acridum* into cuticle then, muscles confirms the potential of its enzymes for consumption nutrients during invasion.

**Histopathological studies**

All the insect muscles are made up of a number of long fibers. Multinucleate cells usually run along the whole length of the muscle. In hemimetabolous insects, such as the desert locust *S. gregaria*, a complete set of muscles is present in the nymphal stage. Flight muscles, however, remain small and functionless until the last nymphal instar and develop rapidly just before and imaginal moult.

In the present study, the available electron micrographs revealed sever effects of *M. anisopliae* on the flight muscles such as: malformation in nuclei and distortion of Z-bands, disorganisation of A, I and H bands, appearance of gaps and vacuoles in the sarcomere and the mitochondria appeared irregular shapes, clustered and their size was some what smaller than those of control. Similar results were obtained when using plant extracts and synthetic pyrothroids on the muscles of different locust species (Shinga *et al.*, 2002; Biserova and Pflüger, 2004), they found that the remarkable affected muscles treated by different insecticides may be explained the disturbance of proteins in treated nymphs which appeared abnormal in shape and failed to completely shed their exuvia due to hormonal balance changed. So these nymphs metamorphosed to adults with many morphological aberrations.

The above mentioned effects could be due to reprogramming of muscle cell by treatment causing autolysis of the tissue, similar to the programmed cell death of many larval muscles seen during metamorphosis (Lockshin, 1985). In the cricket *Acheta domestica*, the indirect muscles deteriorated early in adult life, stimulated by an increase in the haemolymph titre of juvenile hormones and the fine structure of such degenerating muscles resembles that seen in flight muscles from methoprene treated locusts (Chudakova, 1978).

Hyphae were able to invade various cells and tissues directly such as muscles and caused damage for them by enzymatic activity so that the flight decreased.

**Effect of *M. anisopliae* on activity of trehalase**

Trehalose is the main blood sugar in insects, which is hydrolysed to two glucose units by trehalase. Homogenates of locust flight muscles are rich in trehalase. Flight muscle trehalase is located in the plasma membrane with the active site accessible to the haemolymph (Wegener *et al.*, 2003).

In the present study, flight muscles of the control insects have significantly lower trehalase activities than the treatment during first and second day but in the third day mycosed locusts were significantly lower than control locusts.

When locusts are infected with *M. anisopliae* var *acridum*, a competition between locust and pathogen for energy reserves take place and *Metarhizium* spp. utilizes trehalose as the sole source of carbon and produce both extracellular and intracellular forms of trehalase (Seyoum *et al.*, 2002). The effect on flight is associated with a reduction in food intake. However, these events appear to be merely correlated rather than indicating cause and effect. Mycosis caused a significant reduction in haemolymph lipid and carbohydrate.

These results are showen increased consumption of trehalose by the fungus to obtaine the sole source of carbon and food reduction caused decreased trehalose after 72 h from infection.

**Effect of *M. anisopliae* on Lactate dehydrogenase**

The effect of *M. anisopliae* in LDH activity in the flight muscles decrease in LDH activity and this result contrasted with Chiang (1971) who found that the changes in LDH activity in the fat body of the cricket, *Gryllus bimaculatus* after infection with *Nosema grylli* and *Adelina* sp. caused 5 and 10 fold increase in LDH activity.

Kirkton *et al.* (2005) reported that in *S. americana* the rise in mass-specific during jumping, active aerobic metabolic rates with age indicates that problems with longer tracheae can be overcome; however, the reduced endurance, higher lactate concentrations and increased oxygen sensitivity of locomotory performance in older animals indicate that larger/older grasshoppers have smaller safety margins for oxygen delivery during hopping. Sewify and Hashem (2001) reported that the effect of *M. anisopliae* on oxygen uptake of the wax moth *Galleria mellonella* L. was icreased during the first 3 days after initial infection of larvae.

This results suggest that decrease in LDH activity of mycosed insects may due to increase of oxygen uptake.

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## تأثير الفطر *Metarhizium anisopliae* var *acridum* الممرض للحشرات على عضلات طيران الجراد الصحراوى *Schistocerca gregaria* Forskal

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تم دراسة تأثيراصابة عضلات طيران الحشرات الكاملة للجراد الصحراوى بالممرض الحشرى. أظهرت النتائج إنبات جراثيم الفطر على جدار الجسم وإختراقه داخل عضلات الطيران بعد 24 ساعة من المعاملة. أوضحت الدراسة الهستولوجية وجود تشوه فى النواة والميتوكندريا والميوفايبر ووجود فجوات وفراغات.

نشاط إنزيم التريهاليز زاد أثناء اليومين الأوليين بعد المعاملة ثم انخفض فى اليوم الثالث. انخفض نشاط إنزيم اللاكتات ديهيدروجينز فى الحشرات المعاملة عنه فى الحشرات المقارنة.