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BIOCHEMICAL STUDIES ON THE EFFECT OF *Metarhizium anisopliae* VAR *acridum* INFECTION IN THE DESERT LOCUST, *Schistocerca gregaria* FORSKAL

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ABSTRACT

Fifth instar nymphs of the desert locust *Schistocerca gregaria* Forskal were topically treated with 5µl of an oil-based spore suspension of the entomopathogenic fungus *Metarhizium anisopliae* var *acridum.* Haemolymph samples were collected from both control and treated insects every 24 hours starting from day 2 after the treatment.

Estimation of total protein, total carbohydrate, total lipids and cholesterol were carried out. The results showed that the haemolymph content of the treated insects was highly affected and all the studied parameters were lower than in the control insects the decrease in the haemolymph content increased by increasing the post-inoculation period.

Keywords: *Schistocerca gregaria*, Entomopathogenic, *Metarhizium anisopliae* var *acridum,* Haemolymph, Protein, Carbohydrate, Lipid, Cholesterol.

INTRODUCTION

Locusts and grasshoppers (Orthoptera: Acrididae) represent perhaps the most conspicuous of all insect pests and are the most abundant insects of dry grassland and desert. When populations of these insects buildup, certain species exhibit gregarious and migratory behavior, leading to the formation of spectacular swarms (Showler and Potter, 1991). Consideration of Acrididae population dynamics is useful in evaluating the potential for biological control (Lockwood and Ewen, 1997). Indigenous Deuteromycete such as *Beauveria* spp. and *Metarhizium* spp fungi have considerable potential as microbial control agents because they are genetically stable and can be produced cheaply in large quantities. (Prior and Greathead, 1989).The aim of integrated biological control of Acrididae pests is to develop acceptable alternative non-chemical methods for controlling locusts and grasshoppers. The existing control measures still rely heavily on chemical pesticides which are the only reliable method in cases of severe infection (Johnson *et al*. 1988). The purpose of this study is to evaluate the effect of the entomopathogenic fungus *Metarhizium anisopliae* var *acridum* against the desert locust *Schistocerca gregaria* through studying its effect on the haemolymph of survivors.

MATERIALS AND METHODS

1- Rearing of test insects

Nymphal instars of the desert locust *Schistocerca gregaria*, Forskal (Orthoptera: Acrididae) 2 days after ecdysis were used in all experiments. The individuals were taken from the sock culture maintained for several

generations at the locust research section, plant protection research institute (PPRI), A.R.C., Dokki, Giza. . The insects were reared in the laboratory according to (Robert, *et al.,* 2002) in wooden framed cages measuring (60x60x70cm). The cages sides were made of wire gauze with glass top and a small door in the front side, for daily routine feeding, cleaning and handing. A sand layer of 20 cm deep was spread in the bottom of each cage for egg lying, and kept until hatching. Each cage was illuminated and heated by a100 watt electric bulb. Nymphs were transferred to other cages measured (100x100x120cm) without a sand layer for rearing the progeny. All cages were placed on a large table and a suitable container was filled with water and placed under every table leg. The electric wires were painted with grease to protect nymphs from ants attack. The daily routine work includes removing the previous uneaten food, faces and dead nymphs before introducing the fresh food. Both hoppers and adults were fed on brunches of Berseem, *Trifolium alexandrinum* and dry wheat bran fortified with 5% yeast powder as a source of vitamin B_1. Fortified bran was introduced in Petri dishes. The locust's cages were kept at 32 ±2 °C and 30-50% R.H.) . When the cages were empty due to the termination of the life cycle for each generation or when the locusts were removed for experimental purposes, they were disinfected by a diluted solution of an antiseptic agent to avoid contamination with harmful microorganisms.

2- The entomopathogenic fungus

The entomopathogenic fungus used during the study was *Metarhizium anisopliae* var *acridum (=Metarhizium flavoviridae* Driver, *et al*., 2000) isolate (IMI 330189) originally obtained in 1988 from *Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae) from the International Agricultural Center, Nairobi, Kenya. It is specific to locusts and grasshopper conidia of fungus were suspended in cotton seed oil and the resulting spore suspension was used in inoculating insects with dose $5x10^7$ Spores/ml.

3- Characterization of the haemolymph of the 5th nymphal instar after infection with an entomopathogenic fungus

3-1- Inoculation of insects

Fifth instar nymphs 2-3 days old after molting were taken for experiments under laboratory conditions. Five hundred nymphs (250 treatments + 250 controls) were divided into five replications for every experiment. Locusts were chilled at 4°C for 1h prior to topical application beneath the dorsal pronotal shield with 5µl of fungus formulation for the treated nymphs and 5 µl cotton seed oil for control nymphs using a micropipette applicator (Bateman, *et al*., 1993). Nymphs were kept in cages (25x25x60 cm) at 30±2°Cand 30-50% R.H., with a fluorescent lamp as a light source. The control insects were placed in other cages under the same conditions in 16h light and 8h dark (Robert *et al.* 2002). Samples of the haemolymph were taken at different intervals of 2, 3, 4, 5, and 6 days after inoculation. (Metaweh, *et al.* 2001a).

3-2- Samples collection and purification

The haemolymph was collected through a fine puncture in the hind leg membrane and transferred into clean dry centrifuge tubes. Few crystals of phenylthiourea were added to prevent melanization before analysis. A known

volume of the collected haemolymph (0.1ml) was diluted up to 2ml with saline solution and purified by centrifugation to remove blood cells and pigments. Then the filtrate was collected for blood analysis (El Gawhary, 1997).

3-3- Determination of total carbohydrate

Total carbohydrates were estimated according to Trinder, (1969).

3-3-1- Reagents

Solution (1): phosphate buffer (170 m mol/L.ph 7.0). G.O.D. (glucose oxidase) (>5 µl/ml). P.O.D. (peroxidase) (>3 µl/ml) amino phenazone (0.28m.mol/L) and phenol (16m mol/L).

Standard solution has a concentration of 100mg glucose/100ml.

A known volume of solution (1) (1-2.5 ml) was added alone into (0.02 ml) the standard solution and mixed together to prepare the standard. For sample preparation, 2.5ml of solution (1) were mixed with 0.02ml of haemolymph filtrate. The tubes were shaked well and incubated at 37°C for 15 min. The absorbance of the sample (A sample) and that of the standard (A standard) were read 505nm using 1 cm cuvette.

3-3-2- Calculation

mg glucose /100 ml filtrate haemolymph = (A sample/A standard) x 100

3-4- Determination of total lipids

Total lipids were estimated according to the modified methods of Knight, *et al*., (1972).

3-4-1- Phospho-vaniline reagent

Pure vaniline (0.6gm) was dissolved in 10ml ethyl alcohol and the volume was completed to 100ml with distilled water. Phosphoric acid (400ml) was then added. The solution was stored in a dark glass bottle at room temperature.

3-4-2- Standard lipid solution

A known weight of lipid (5mg/ml) consists of , Oleic acid and Palmitic acid in a ratio of 7 : 3 was prepared by dissolving Oleic acid (350mg) and Palmitic acid (15mg) in absolute ethanol (100ml) .

A known volume of haemolymph filtrate (0.1ml) was added to conc. Sulphuric acid (5ml) in a test tube and heated in a boiling water bath for 10 min. After cooling to room temperature. The digest (0.1ml) was added to Phospho-vaniline reagent (6.0ml) after 45 min. The developed color was measured in Bush and Lamp spectrophotometer at 525 nm. Distilled water (0.1ml) was used for blank. The standard lipids solution (0.2ml) was used and treated in the same manner as the unknown.

3-4-3- Calculation

mg lipid/100 ml filtrate haemolymph = (A sample/A standard) x 100

3-5- Determination of total protein

Protein content was determined by Biuret reagent according to the method described by Gornall, *et al*., (1949) as follows:

3-5-1- Reagent

The Biuret reagent was prepared by dissolving 1.5gm copper sulphate ($CuSo_4$.5H2O) and 6 mg Sodium potassium tartarate ($NaKC_4H_4O_6$. $4H_{20}$) in 500 ml distilled water and mixed thoroughly with 300ml of 10% (W/V) Sodium hydroxide (Carbonate free) and the volume was completed

to 1 liter. Potassium iodide (1gm) was added and the reagent was stored in a waxed or plastic bottle.

3-5-2- Standard solution of protein

The standard solution was prepared by dissolving 6gm Albumin protein in 100ml distilled water

A volume of 0.1ml of the haemolymph filtrate was mixed with 5ml of the Biuret reagent in a test tube and incubated for 30min at 20-25 C°. The absorbance (A) was measured at a wavelength 546 nm using 1 cm light path cuvette.

3-5-3- Calculation

mg protein/100 ml filtrate haemolymph = (A sample/A standard) x 100

3-6- Determination of total cholesterol

Total cholesterol was determined by the enzymatic colourimetric method of Richmond, (1973). All reagents used in this determination were supplied by Ames Division Miles Lab. Inc, England.

3-6-1- Reagents

Solution (1) contains buffer-enzyme-chromogents.

Solution (2) contains Phenol.

Solution (3) one volume of solution (1) + one volume of solution (2). Where one liter of solution (3) contains the following, phosphate buffer 10 mmol PH = 7.7 Cholesterol ester hydrolase ≥ 140µl. cholesterol oxidase ≥ 80µ .peroxides≥ 500µ. Phenol 10mmol, 4 amino-phenazone 0.5 mmol potassium ferrocyanide 2µ mol and Sodium cholate 3 mmol.

Solution (4): is the standard solution

The reagents were kept at 5°C for one month.

Solution (1) and (2) were mixed instantly before use.

3-6-2- Procedure

Volume (2-2.5 ml) of solution was pipetted into a test tube to form the blank. The standard test tube was made by placing the standard solution (3) (2.5 ml) and solution (4) (0.02 ml) into a test tube. The standard solution was added to this tube and 0.2 ml of the unknown sample. After well mixing the test tubes were incubated at 37 C° for 10 minutes or at room temperature for at least 30 minutes and measured at wave length of 410 nm cuvette.

3-6-3- Calculation

mg cholesterol/100 ml filtrate haemolymph = (A sample/A standard) x 100

5- Statistical analysis

Data were analyzed by analysis of variance (ANOVA) means, within row, bearing different subscripts are significantly different ($P<0.05$).

RESULTS

Characterization of the haemolymph of the fifth nymphal instar of desert locust, *S. gregaria* after infection with an entomopathogenic fungus, *M. anisopliae* var *acridum* was done to investigate the effect of fungal infection on total proteins, carbohydrates, lipids and cholesterol.

1. Effect on Protein levels

A dose of 5 x10^7spore/ml of the entomopathogenic fungus, *M. anisopliae* var *acridum* was topically applied on 5th nymphal instar of the desert locust, *S. gregaria* and the chemical analysis of the haemolymph was carried out in period from day 2 until day 6 after treatment.

Table (1): Determination of total protein of the 5th nymphal instar of desert locust, *S. gregaria* after infection with an entomopathogenic fungus, *M. anisopliae* var *acridum*

Days	Controls	Treatments
2nd	4106±45a	4040±36a
3rd	5623±166a	5053±60b
4th	6226±65a	4920±150b
5th	5420±160a	4165±37b
6th	4660±40a	4110±98b

b= significant a= non significant means ± S.D. (P< 0.05).

Table (1) showed the effect of one dose of entomopathogenic fungus (5 x 10^7 spores/ml) on the resulting 5th nymphal instar after administration on 2-day old nymph of the last stadium. The entomopathogenic fungus caused a decrease in the haemolymph protein content compared to that of the control. There was significance difference in the total protein content in both the treated and control insects in 2nd day of application but the difference appeared starting from 3rd day of application and the reduction in haemolymph protein content continued by time even 6th day of application to reach minimum values at the end of the experiment.

2. Effect on total Carbohydrate levels

The effect of the entomopathogenic fungus dose (5 x 10^7 spores/ml) on total carbohydrate content in the inoculated 5th nymphal instar when applied on 2-day old nymphs are summarized in table (2).

Table (2): Determination of total carbohydrate of the 5th nymphal instar of desert locust, *S. gregaria* after infection with an entomopathogenic fungus, *M. anisopliae* var *acridum.*

Days	Control	Treatment
2nd	336±6a	332±12a
3rd	411±7a	396±5b
4th	380±07a	346±10b
5th	392±6a	346±10b
6th	538±9a	318±8b

b= significant a= non significant means ± S.D. (P< 0.05).

It was found that carbohydrate levels were decreased markedly in the treated 5th nymphal instar than that of the untreated at all periods post inoculation. The values of carbohydrate contents during periods of application (from 2nd to 6th day after application) in treated nymphs 396,346,346 and 318

mg/100ml haemolymph but in control were 411,380,392 and 538 mg/100ml haemolymph.

3. Effect on Lipids levels

The effect of the entomopathogenic fungus (5 x 10^7 spores/ml) on total lipid contents in the inoculated 5th nymphal instar when applied within the second day of this instar are summarized in table (3) . On 2nd day post inoculation there were no significant differences between the treated and the untreated nymphs but on 3rd, 4th, 5th and 6th day, significant differences were noticed the values of total lipids in infected nymphs were 171 ,145 , 143 and 105 mg/ 100ml haemolymph but in control were 214 ,184 ,210 and 187 mg/ 100ml haemolymph. From these results, it was observed thatthe entomopathogenic fungus application to the 5th instar nymphs caused continuous reduction in the total lipid content in the haemolymph.

Table (3): Determination of total lipid of the 5th nymphal instar of desert locust, *S. gregaria* after infection with an entomopathogenic fungus, *M. anisopliae* var *acridum*

Days	Control	Treatment
2nd	19.8±1.52a	15.66±0.56b
3rd	18.56±0.4a	11.76±1.07b
4th	15.5±0.3a	9±0.3b
5th	19.1±0.6a	8.4±0.5b
6th	11.4±0.52a	7.43±0.49b

b= significant a= non significant means ± S.D. (P< 0.05).

4. Effect on Cholesterol levels

The values of total cholesterol extracted from the haemolymph of 5th nymphal instar of desert locust infected with the entomopathogenic fungus, *M. anisopliae* var *acridum* in table (4) showed significant differences between treated and untreated nymphs during 2nd, 3rd, 4th, 5th and 6th days of application.

Table (4): Determination of total cholesterol of the 5th nymphal instar of desert locust, *S. gregaria* after infection with an

Days	Control	Treatment
2nd	230±9a	235±8a
3rd	214±4.1a	171±3.2b
4th	184±49a	145±11b

entomopathogenic fungus, *M. anisopliae* var *acridum*

5th	210±7a	143±5.5b
6th	187±6.3a	105±6b

S= significant Ns= non significant means ± S.D. (P< 0.05).

DISCUSSION

Infection the 5th nymphal instar of the desert locust, *S. gregaria* with the entomopathogenic fungus, *M. anisopliae* var *acridum* (5 x 10^7 spores/ml) during period from day 2 to day 6 of the treatment caused reduction of total protein, total carbohydrate, total lipid and total cholesterol compared with untreated nymphs. These analyses were carried out after 2 days after inoculation because at this time the fungus has invaded the body cavity and reached to the haemolymph (Bateman *et al*., 1993).

The effect of mycosis on haemolymph protein in the locust could be due either to a direct effect of the fungus through their use and consumption by the germinated spores and hyphal bodies of the fungus for nutrition and the total starvation had no significant effect on the total carbohydrate (Cheeseman and Goldsworthy, 1979). Where efficient mechanisms of carbon assimilation in vivo are likely to be critical for host specificity and virulence. Competition for carbon between the pathogen and host may determine the rate and extent of infection however, the fungal enzymes involved in carbon uptake have not been identified an extracellular trehalase has been purified from *invitro* cultures of *Langenidium* spp., a fungal parasite of mosquitoes (Domnas and Warner, 1991).

A decline in the concentration of the major haemolymph storage carbohydrate, Trehalose, during the infection process suggests that this disaccharide is used the entomopathogenic fungus during the colonization of the host (Gillespie *et al*., 2000b)**.** Although the fungal trehalase was not identified *invivo.* Interestingly mutants of *Metarhizium* hypervirulent toward the mosquito *Culex pipiens* exhibit enhanced starch degrading ability (Al-Aidroos and Seifert, 1980). In light of the substrate preference exhibited by the extracellular-glucosidase purified from *M. anisopliae* (Cobb, 1996). It is possible that the hypervirulent observed towards mosquitoes resulted from increased degradation of glycogen and Trehalose during the invasion of insect tissues the degradation and utilization of Trehalose by the fungus not only depletes a valuable energy reserve of the insect but will also interfere with the osmotic regulation of the insect haemolymph (Gillespie *et al*., 2000b). Or indirectly to a semi-starved condition brought on by the reduced food intake in infected insects (Seyoum *et al*., 1994). The latter seems unlikely as 3 days of total food deprivation had no significant effect on blood lipid and carbohydrate, and, in common with *Locusta migratoria* (Cheeseman and Goldsworthy, 1979) Also The decline observed in infected insects one alternative explanation is that mycosed insects are unable to mobilize lipid from their fat body or that there is so little lipid in their fat bodies that they are unable to maintain plasma levels (Goldsworthy and Mordue, 1973). The

cholesterol is the biosynthesis precursor of ecdysone hormone in insects so, the estimation of the Juvenile hormone mimic role on this metabolite deemed necessary to evaluate its action upon the precursor of moulting hormone (ecdysone) in *S. gregaria* (Rees, 1985).These results are agreement with that of (Metaweh *et al.*, 2001a) found that total lipid, total carbohydrate, total protein and total cholesterol in haemolymph of treated-grasshopper, *Eurpepocnemis plorans plorans* (5^{th} instar nymphs) with the entomopathogenic fungus, *M. flavoviridae* (5 x10^6spores/ml) decreased on untreated samples. Also (Gillespie *et al*., 2000a) who found that the desert locust, *S. gregaria* when inoculated topically with *M. anisopliae* var *acridum* the total protein and total lipid of the haemolymph decreased during the course of infection moreover And adult desert locust *S. gregaria* 3days after inoculation with the entomopathogenic fungus *M anisopliae* var *acridum* (4 x 10^7spores/ml) had significantly less lipid and carbohydrate in the haemolymph than controls this was not due to reduced food intake as 3 days of complete starvation had not effect on haemolymph titers of energy reserves in controls (Seyoum, *et al.*, 2002). And (Sewify and Moursy, 1993) who found that the aphid *Brevicoryne brassicae* when treated with *Verticillium lecanii* (10^7spores/ml) had significantly lower concentration of total crude lipids compared to the concentration of untreated aphids. in the same way (Boucias and Pendland, 1984) found that whole lipids , polar and hydrocarbon fractions of lipids extracted from lepidopteran cuticle were active in stimulating germ tube formation of Deuteromycetes *Nomuraea rileyi* also (Latge *et al.*, 1987) mentioned that the formation of *Ciodioblus obscurus* germ tubes were stimulated by lipidoidal extracts of the aphid cuticle. Steel and hall (1985) stated that the fat body is generally regarded the principal site of trehalase biosynthesis in insects. they added that haemolymph trehalase is an important source of energy and chitin biosynthesis. The Haemolymph trehalose decreased significantly during mycosis of locusts by *M. anisopliae.* All these results suggested that this fungus may take advantage of competing nutrient utilization against the insect (Hua *et al*., 2007). (Polanowski *et al*., 1997) showed that the desert locust, *S. gregaria* when treated with the entomopathogenic fungus, *M. anisopliae* the total protein level of the haemolymph decreased during the course of infection. But (Gurwattan *et al*., 1991) found that Vegetative development of *Beauveria bassiana* in the haemocoel of the beet armyworm, *Spodoptera exigue* did not cause significant alterations in the profile of proteins pulse labeling tissue explants revealed that vegetative growth of *B. bassiana* hyphal bodies did not impact the biosynthetic**.**

Accordingly it could be concluded that the entomopathogenic fungus acted mainly on the haemolymph of the inoculated 5^{th} nymphal instar of the desert locust, *S. gregaria* causing decrease in protein, carbohydrate, lipid and cholesterol levels.

Finally the entomopathogenic fungus, *M. anisopliae* var *acridum* could effect the energy reserves in the haemolymph of the desert locust, *S. gregaria* so could control in the 5^{th} nymphal instar and kill them this gave the biological control importance in the control of the nymphal instar of the desert locust.

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دراسات بيوكيميائية على تاثير الاصابة بالفطر *Metarhizium anisopliae* var *acridum* على الجراد الصحراوى *Schistocerca gregaria* Forskal

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**** قسم بحوث الجراد والنطاط, معهد بحوث وقاية النباتات, مركز البحوث الزراعية**

عوملت حوريات العمر الحورى الخامس للجراد الصحراوى موضعيا بـ 5 ميكروليتر من معلق جراثيم الفطر فى قاعدة زيتية. جمعت عينات الهيموليمف من كلا من الحشرات المعاملة والمقارنة كل 24 ساعة بداية من اليوم الثانى بعد المعاملة.

نفذ تقدير البروتين الكلية, الكربوهيدرات الكلية, الدهون الكلية والكوليسترول.

اظهرت النتائج ان:

مكونات الهيموليمف للحشرات المعاملة تاثرت بشكل كبير وكانت كل مقاييس الدراسة اقل عما فى المقارنة. يزداد الانخفاض فى مكونات الهيموليمف كلما زادت فترة ما بعد المعاملة.