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# Haemolymph Picture and Its Chemical Components in *Schistocerca gregaria* (Forskål) as Affected by Two Entomopathogenic Fungi

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## ABSTRACT

Effects of *Metarhizium anisopliae* var. *acridum* (Metchnikoff) Soroken, *Beauveria bassiana* (Bals.) Vuill. sole and their combination were studied on the haemolymph picture of the 5th nymphal instar of *Schistocerca gregaria* (Forskal), also toxicity effect was evaluated against 3rd nymphal instar of *S. gregaria*. The infection of both fungi (sole or in combination) caused reduction in chemical composition of haemolymph in the 5th nymphal instar of *S. gregaria*, also reduced the activity of Acid phosphatase (except in case of *B. bassiana* infection), prophenyleoxidase, and phenyleoxidase. Five types of haemocytes were recognized. The treatments also reduced the total haemocytes count and resulted in changes of the percentages of the haemocyte types. The combined infection caused potential effect at all observations when 3rd nymphal instar was treated with 1X $10^3$ spores/nymph. While in case of dose 2 X $10^3$ spores/nymph of the mixture, there was an additive effect on day 7 and potential effect on days 10, 14, 18 and 21post infection, the combined infection also accelerated the mortality.

**Key words:** Entomopathogenic fungi, *Metarhizium anisopliae* var. *acridum*, *Beauveria bassiana*, *Schistocerca gregaria*, toxicity effect, haemolymph picture.

## INTRODUCTION

Entomopathogenic fungi have considerable potential for locust and grasshoppers control (Goettel, 1992). *Metarhizium anisopliae* var. *acridum* and *Beauveria bassiana* were efficient against desert locust *Schistocerca gregaria* and clover grasshopper *Euprepocnemis plorans* (Charpentier) nymphs in the laboratory, as well as the common grasshoppers at Sharq Eloinate, Egypt under semi field conditions, while *M. anisopliae* var. *acridum* was a successful biological control agent in the field against *S. gregaria* at Elba Mountains, South east of Egypt, also against common grasshoppers at Baharia oasis, (Abdelatef, 2005 and El-Maghraby *et al.*,2009). Slow speed act of killing is perceived as a potential drawback to the use of fungi in control operations. It seemed possible that combined infection of *M. anisopliae* var. *acridum* and *B. bassiana* might accelerate the speed act of killing and enhance the efficacy of both fungi against *S. gregaria*.

Insect immune responses are one of the potential factors involved the inability of fungus to infect the non-permissive host insect. The insect immune system is divided into two interactive responses, the first is the ability of the insect haemocytes to identify a non-self entity in the haemolymph and the other response is humoral or noncellular response such as; activation of prophenoloxidase (Gillespie *et al.*, 2000). Acid phosphatase (AP) is one of the major lysosomal enzymes in invertebrates (Anderson, 1981), and considered as non specific body response to integument damage or fungal toxins (Serebrove *et al.*, 2006).

The objectives of this study were evaluating the effect of combined infection of *M. anisopliae* var. *acridum* and *B. bassiana* against 3rd nymphal instar of *S. gregaria*, also on the main haemolymph nutrient resources, acid phosphatase, prophynoloxidase and phenoloxidase activity of the 5th nymphal instar haeymolymph of *S. gregaria*. The effect of such combined infection on the haemocytes was also studied.

## MATERIALS AND METHODS

### Insect rearing:

Used insects were the 3rd and 5th nymphal instars of the desert locust, *S. gregaria*. The insects were obtained from the stock culture maintained for several generations at Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt. The culture is usually fortified with insects brought from the field every year. Insects were reared in the laboratory according to (Robert *et al.*, 2002) in framed cages, both hoppers and adults were fed on brunches of Egyptian clover, *Trifolium alexandrinum* and dry wheat bran fortified with 5% yeast powder as a source of vitamin $B_1$. The locust's cages were kept at 30 ± 2 °C and 30-50 % R.H.).

### Entomopathogenic fungi:

Spores of *M. anisopliae* var. *acridum* and *B. bassiana* were mass production as designed method of El-Maghraby *et al.* (2009).

**Toxicity effect of combined infection on 3rd nymphal instar:**

Six treatments were carried out to study the effect of the two fungi on *S. gregaria* 3rd instar nymphs sole and in combination. These treatments were 1- *M. anisopliae* var. *acridum* sole at dose 5 X $10^2$ spores/nymph, 2- *B. bassiana* sole at dose 5 X $10^2$ spores/nymph, 3- combination of *M. anisopliae* var. *acridum* 5 X $10^2$ spores per nymph + *B. bassiana* 5 X $10^2$ spores per nymph, 4- *M. anisopliae* var. *acridum* sole at dose 1 X $10^3$ spores/nymph, 5- *B. bassiana* sole at dose 1 X $10^3$ spores/nymph and 6- combination of *M. anisopliae* var. *acridum* 1 X $10^3$ spores/nymph + *B. bassiana* 1 X $10^3$ spores/nymph. These treatments were calculated according to the results obtained by El-Maghraby *et al.*, 2009. Sixty nymphs (for each treatment) were divided into 4 groups; each group contained 15 nymphs placed in cylinder plastic cage. Plastic cages contain infected insects were kept in an incubator at 31° C and 12:12 hrs light: dark photo period. The infected nymphs were observed daily for mortality, cleaned and fed on *T. alexandrinum*. The combined action of the mixtures was calculated in term of co-toxicity factor according to the equation given by Mansour *et al.*,(1966):

$$\text{Co-toxicity factor} = \frac{\%\ M_O - \%\ M_E}{\%\ M_O} \times 100$$

where:

% $M_O$ (Observed % mortality): the mortality percentage among treated insects with fungi combination.

% $M_E$ (Expected % mortality): the sum of mortality percentage among treated insects with each fungus in sole.

This factor was used to differentiate the results into three categories:

1. Potential (a positive factor of + 20 or more)
2. Antagonism (a negative factor of - 20 or more)
3. Additive (an intermediate values i.e. between +20 and – 20)

**Effect of fungal infection on the haemolymph composition of desert locust:**

To study the effect of the two fungi *M. anisopliae* var. *acridum* and *B. bassiana* on haemolymph of the desert locust, *S. gregaria*, four treatments were applied to 1 day old 5th nymphal instar after the fourth molting. These treatments were: 1- untreated nymphs, 2- treated nymphs with $10^3$ spores/nymph of *M. anisopliae* var. *acridum*, 3- treated nymphs with $10^3$ spores/nymph of *B. bassiana* and 4- treated nymphs with 5X $10^2$ spores/nymph of each fungus (combination of *M. anisopliae* var. *acridum* and *B. bassiana*). Samples of haemolymph were taken daily after treatment till the 8th day. About 10 nymphs of each treatment were used to collect the haemolymph daily, the nymphs were allowed to feed for 2 hrs, and then were chilled on ice for 10 minutes. The arthropodial membrane of the hind leg of each nymph was pierced with sterile needle and the haemolymph was collected using 10 µl capillary pipettes (Gillespie *et al.*, 2000).

For the differential haemocytes count, small haemolymph droplet was placed on clear glass slide, quickly smeared to a thin film on the slide by using an edge of another slide, the smear was then air dried and fixed in 2-3 drops of methanol 95%. The smears were stained with diluted Gemsa stain for 15 minutes then washed with distilled water. The haemocytes were examined and photographed using light microscope under the oil immersion lens. The haemocytes were identified according to (Gupta, 1979). Each heamocyte type was counted and its percentage was calculated.

To perform the total haemocyte count, haemolymph was collected using 100 µl glass capillary tubes, diluted (1:4) with sterile ice cold anticoagulant buffer (0.098 M NaOH; 0.180 M NaCl; 0.017 M EDTA (free acid); 0.041 M citric acid; 440-450 mOs/Kg; pH 4.5) then transferred immediately to an improved Neubauer haemocytometer. The number of cells was counted and the total haemocyte numbers were calculated according to Gillespie *et al.*, 2000,

For protein, carbohydrate, and lipids determination, 0.5 ml of haemolymph were received in small tubes contain traces of phenyl thiouria, the tubes containing the haemolymph were then kept in deep freezing till further determination of total protein, total carbohydrates and total lipids as following: A known volume of the collected haemolymph (0.1ml) was diluted up to 2 ml with saline solution and purified by centrifugation to remove blood cells and pigments. Then the filtrate was collected for haemolymph analysis.

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

**Total carbohydrates** were estimated by the method of Trinder (1969).

**Total lipids** were estimated by modified method of (Knight *et al*, 1972).

For determination of acid phosphates in haemolymph, Powell and Smith (1954) method with slight modification were used, 1 ml citric buffer (pH 4.9), 1 ml of 0.01 M disodium phenyl phosphate, and 0.1 ml nymphal haemolymph were mixed gently and incubated for exactly 30 minutes at 37 °C. At the end of incubation period, 0.8 ml of NaOH was added to stop the reaction. Then, 1.2 ml of 4% sodium carbonate and 4% bicarbonate, followed by 1 ml of

1.5% 4-aminoantipyrine solution and 1 ml of 4% potassium ferricyanide were added. Produced color was measured, immediately, by spectrophoto-meter at 510 nm. The optical density was converted to enzymatic activity units which expressed as mg phenol released/ml haemolymph, using phenol standard curve.

For determination of phenoloxidase in haemolymph, method of Ishaaya, 1972 was used, with some modifications, 200 µl haemolymph, 2ml phosphate buffer (0.2 M, pH 7) and 0.5 µl 2% Catechol were mixed gently, incubated for 5 min at 25 °C. The activity was then recorded at absorbency 470 nm, the optical density was recorded every 1 min for 10 min. The specific activity of phenoloxidase was expressed as units of activity per mg of protein. One unit of activity was defined as the amount of enzyme that increases the absorbance by 0.001 units per min.

To determine prophenoloxidase activity, 100 ml of diluted haemolymph serum was incubated with 100 ml of chymotrypsin (1 mg/ml in phosphate buffer) for 30 min prior to the addition of Catechol. Then, 100 ml of the pre-incubated mixture was assayed as above. The value for phenoloxidase activity was subtracted from the value obtained for phenoloxidase activity after chymotrypsin activation to give the quantity of prophenoloxidase present in samples (Gillespie *et al.,* 2000).

**Statistical analysis**

Data were subjected to analysis of variance using GLM procedure in SAS software, SAS (1999).

## RESULTS AND DISCUSSION

**1-Toxicity effect of fungal combination on 3rd nymphal instar:**

Data in table (1) show the toxicity factor of the mixtures of *M. anisopliae* var. *acridum* and *B. bassiana,* at dose of 1X $10^3$ and 2X $10^3$ spores/ nymph of the mixture against *S. gregaria,* on days 7, 10, 14, 18 and 21 post treatments. It is clear that the dose of 1 X $10^3$ spores/ nymph of the mixture caused potential effect at all observations. While in case of dose 2 X $10^3$ spores/nymph of the mixture, there was an additive effect on day 7 and potential effect on days 10, 14, 18 and 21post infection. In general, toxicity factors of the dose $10^3$ were higher than those of dose $10^4$ spores/nymph this may be due to competition between each fungal spore. More over, obtained results of daily corrected mortalities of the treatments: combination of *M. anisopliae* var. *acridum* 5 X $10^2$ spores per nymph + *B. bassiana* 5 X $10^2$ spores per nymph, *M. anisopliae* var. *acridum* alone at dose 1 X $10^3$ spores/nymph and *B. bassiana* alone at dose 1 X $10^3$ spores/nymph were subjected to propit analysis to calculate the time mortality response, the data are shown in table (2). It is clear that the mixtures caused significant decrease in the time required to kill 50 % of the treated nymphs, as well the treatment with *M. anisopliae* var. *acridum* caused significant reduction in the $LT_{50}$ , comparing with *B. bassiana* treatment.

The joint infection of two or more pathogens to same host is very important phenomenon. This phenomenon may occur under field application, when applying one pathogen to infected population with another pathogen. Many authors studied the co-infection of two or more pathogens to many insect species. The results of these works indicate that there were variations in the co-infection effect, where some of them showed antagonism action as the finding of Cossentine and Lewis (1984) who reported that *Vairimorpha necatrix* antagonized the effect of the *Vairimorpha* sp. in infected *Agrotis ipsilon* larvae and Pilarska *et al.,* (2006) who found that there were antagonism between *Nosema* and *Vairimorpha* infection to *Lymantria dispar* larvae, due to the competition between the two pathogens. Other authors reported synergistic action, for example Gothama *et al.,* (1995) stated that there was an additive effect due to co-infection of *Spodoptera exigua* larvae with entomopathogenic nematode, *Steinernema carpocapsae* and *S. exigua* multinucleocapsid nuclear polyhedrosis virus. Also, they suggested that the two pathogens may work independently in there infection to the host larvae. Also, Inglis *et al.,* (1997 and 1999) found out that mortality of *Melanoplus sanguinipes* nymphs inoculated with *B. bassiana* and *M. flavoviride* in combination, significantly increased than of those infected with each pathogen alone. More over Wraight and Ramos (2005) reported that there was a low level synergistic interaction between *B. bassiana* and *Bacillus thuringiensis tenebrionis,* when applied against field populations of Colorado potato beetle larvae. In the present study, the synergetic effect of *M. anisopliae* and *B. bassiana* infection to 3rd nymphal instar of *S. gregaria* may be due to some unknown effects of both pathogens on the physiology of the locust such as; starvation stress, intoxication and/ or slow development.

**2-Effect of fungal infection on total proteins, carbohydrates and lipids in infected 5th nymphal instar:**

Data illustrated in Table (3) show the effect of *M. anisopliae* var. *acridum* and *B. bassiana* on the crude amount of total protein contents of the 5th nymphal instar of desert locust *S. gregaria.* It is clear that the crude protein content in the infected

Table (1): Effect of mixture of *Metarhizium anisopliae* (Ma) and *Beauveria bassiana* (Bb) on mortality percentages in *Schistocerca gregaria* 3rd instar nymphs.

| Days post treatment | *Metarhizium anisopliae* + *Beauveria bassiana* $10^3$ spores/nymph | | | | | *Metarhizium anisopliae* + *Beauveria bassiana* $10^4$ spores/nymph | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ma Sole | Bb Sole | Expected (Ma + Bb) | Observed (combination) | Co toxicity factor | Ma Sole | Bb Sole | Expected (Ma + Bb) | Observed (combination) | Co toxicity factor |
| 7 | 6.78 | 6.78 | 13.56 | 16.67 | +22.92 | 10.17 | 8.33 | 18.50 | 20.00 | +8.09 |
| 10 | 12.50 | 12.50 | 25.00 | 30.36 | +21.43 | 14.27 | 16.07 | 30.36 | 39.26 | +29.41 |
| 14 | 15.39 | 11.54 | 26.93 | 44.44 | +65.08 | 25.00 | 27.78 | 52.78 | 66.67 | +26.32 |
| 18 | 17.65 | 17.65 | 35.30 | 57.69 | +63.46 | 31.37 | 25.00 | 56.37 | 75.00 | +37.84 |
| 21 | 20.83 | 16.67 | 37.50 | 61.22 | +63.27 | 27.08 | 20.40 | 47.49 | 73.47 | +54.70 |

Table (2): Effect of mixtures of *Metarhizium anisopliae* and *Beauveria bassiana* on mortality acceleration of *S. gregaria* 3rd nymphal instar.

| Treatments | $LT_{25}$ | $LT_{50}$ [a] | $LT_{90}$ | Slope |
|---|---|---|---|---|
| *M. anisopliae* $10^3$ (Sole) | 11.34 | 24.66 b | 107.97 | 2.00 |
| *B. bassiana* $10^3$ (Sole) | 14.11 | 36.39 a | 220.36 | 1.64 |
| *M. anisopliae* + *B. bassiana* $10^3$ (combination) | 9.07 | 15.56 c | 43.35 | 2.88 |

Table (3): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on total proteins contents (g/100 ml) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X} \pm SE$ [b] | $\bar{X} \pm SE$ [b] | % Reduction | $\bar{X} \pm SE$ [b] | % Reduction | $\bar{X} \pm SE$ [b] | % Reduction |
| 1st day | 2.10 ± 0.04 a | 2.09 ± 0.03 a | 0.19 | 2.10 ± 0.02 a | 0.13 | 2.10 ± 0.02 a | 0.13 |
| 2nd day | 2.13 ± 0.04 a | 2.18 ± 0.02 a | -2.38 | 2.18 ± 0.03 a | -2.51 | 2.16 ± 0.02 a | -1.38 |
| 3rd day | 2.23 ± 0.04 a | 2.11 ± 0.02 b | 5.21 | 2.12 ± 0.01 b | 5.03 | 2.10 ± 0.02 b | 5.92 |
| 4th day | 2.36 ± 0.02 a | 2.06 ± 0.03 b | 12.92 | 2.08 ± 0.03 b | 12.07 | 2.05 ± 0.01 b | 13.25 |
| 5th day | 2.37 ± 0.04 a | 2.03 ± 0.01 b | 14.71 | 2.03 ± 0.01 b | 14.49 | 1.99 ± 0.01 b | 16.00 |
| 6th day | 2.38 ± 0.01 a | 2.00 ± 0.02 b | 15.93 | 2.02 ± 0.01 b | 14.92 | 1.97 ± 0.01 b | 16.94 |
| 7th day | 2.38 ± 0.02 a | 1.96 ± 0.02 b | 17.56 | 1.99 ± 0.01 b | 16.11 | 1.95 ± 0.02 b | 17.90 |
| 8th day | 2.37 ± 0.01 a | 1.95 ± 0.02 b | 18.02 | 1.96 ± 0.01 b | 17.46 | 1.94 ± 0.01 b | 18.30 |
| Average | 2.29 ± 0.03 | 2.05 ± 0.02 | 10.63 | 2.06 ± 0.02 | 10.06 | 2.03 ± 0.02 | 11.25 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letter in same row mean no significant differences.

Table (4): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on total carbohydrates contents (g/100 ml) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X} \pm SE$ [b] | $\bar{X} \pm SE$ [b] | % Reduction | $\bar{X} \pm SE$ [b] | % Reduction | $\bar{X} \pm SE$ [b] | % Reduction |
| 1st day | 4.17 ± 0.05 a | 4.18 ± 0.04 a | -0.08 | 4.18 ± 0.03 a | -0.08 | 4.19 ± 0.05 a | -0.32 |
| 2nd day | 4.26 ± 0.05 a | 4.30 ± 0.03 a | -1.02 | 4.29 ± 0.02 a | -0.70 | 4.28 ± 0.03 a | -0.47 |
| 3rd day | 4.32 ± 0.03 a | 3.88 ± 0.03 b | 10.18 | 3.92 ± 0.03 b | 9.41 | 3.86 ± 0.01 b | 10.72 |
| 4th day | 4.41 ± 0.02 a | 3.44 ± 0.12 c | 21.94 | 3.68 ± 0.04 b | 16.41 | 3.29 ± 0.04 d | 25.34 |
| 5th day | 4.49 ± 0.03 a | 3.13 ± 0.06 c | 30.31 | 3.47 ± 0.05 b | 22.59 | 2.93 ± 0.04 d | 34.70 |
| 6th day | 4.47 ± 0.06 a | 2.97 ± 0.08 c | 33.51 | 3.19 ± 0.04 b | 28.66 | 2.78 ± 0.04 d | 37.76 |
| 7th day | 4.47 ± 0.08 a | 2.77 ± 0.05 c | 38.15 | 3.01 ± 0.03 b | 32.64 | 2.57 ± 0.04 d | 42.55 |
| 8th day | 4.48 ± 0.04 a | 2.46 ± 0.06 c | 45.06 | 2.92 ± 0,04 b | 34.80 | 2.02 ± 0.06 d | 55.02 |
| Average | 4.38 ± 0.04 | 3.39 ± 0.06 | 22.65 | 3.58 ± 0.03 | 18.28 | 3.24 ± 0.04 | 26.12 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letters in same row mean no significant differences.

insects, slightly increased on the 2nd day post treatment, then the total protein amount decreased significantly on the 3rd day post infection till the end of the experiment. The infection with *B. bassiana* showed the lowest reduction in the amount of total proteins content. While *M. anisopliae* var. *acridum* and *B. bassiana* combination showed the highest reduction in the amount of total proteins content. There were no differences between the treatments in their effect on the total protein contents. The average amounts of total proteins were 2.29, 2.05, 2.06 and 2.03 g/100 ml haemolymph in control, *M. anisopliae* var. *acridum*, *B. bassiana* and *M. anisopliae* var. *acridum* + *B. bassiana* combination treatments, respectively (Table 3).

Data presented in Table (4) show the effect of the same treatments on the total carbohydrates contents of 5th nymphal instar nymphs. Obviously, the infection in the three treatments caused significant reductions in total carbohydrates contents on the 2nd day post treatment. On the fourth day, the infection with *M. anisopliae* var. *acridum* and *B. bassiana* combined caused highest significant reduction, followed by *M. anisopliae* var. *acridum* which was significantly higher than *B. bassiana* infection. The average amounts of total carbohydrates was 4.38, 3.39, 3.58 and 3.24 g/100 ml haemolymph in control, *M. anisopliae* var. *acridum*, *B. bassiana* and *M. anisopliae* var. *acridum* + *B. bassiana* combination treatments, respectively (Table 4).

Data in Table (5) demonstrate that the infection in the 3 treatments caused significant reductions in the amount of total lipids on the 3rd day post treatment. There were no significant differences between the treatments in the reduction of the total lipids amount, except on the 5th day; there were significant differences between *B. bassiana* and *M. anisopliae* var. *acridum* + *B. bassiana* combination treatments. The average amounts of total lipids were 3.65, 2.13, 2.27 and 1.98 g/100 ml haemolymph in control, *M. anisopliae* var. *acridum*, *B. bassiana* and *M. anisopliae* var. *acridum* + *B. bassiana* combination treatments, respectively (Table 5).

Successful penetration of the integument following treatment with conidia resulted in subsequent formation of hyphal bodies within the heamolymph (Vilcinskas and Matha 1997). Fungal reproduction occurred mainly in heamolymph of its host. Moreover, infection may diminish the uptake of nutrients by the host, where the depletion of the nutritive resources could be suggested as reason for total haemolymph proteins, carbohydrates and lipids contents reduction. There is no doubt that there was a competition between the entomopathogens and their hosts on the nutrient resources transported by the haemolymph, thus may be the growth of the fungi causes exhaustion of these resources.

The results go in line with the findings of Gillespie *et al.*, 2000 who reported a reduction of haemolymph proteins content of *S. gregaria,* as result of infection with *M. anisopliae* var. *acridum*. Also, Metaweh *et al.*, 2001, reported a reduction of proteins content in the haemolymph of *Euprocnemis plorans* (Charp.) after infection with *M. anisopla, M. anisopliae* var. *acridum* (formally, *M. flavoviridae*) and *B. bassiana*. Seyoum *et al.*, 2002 stated a reduction of carbohydrates and lipids levels in the haemolymph of *S. gregaria* infected with *M. anisopliae* var. *acridum*, as well were Metaweh *et al.*, 2001 who reported the same reduction in case of *E. plorans* infected with *M. anisopla, M. anisopliae* var. *acridum* and *B. bassiana*.

In the present study, there was slight increase in the protein amounts in the haemolymph of the treated 5th nymphal instar of *S. gregaria*. This increase may be due to the secretion of antimicrobial proteinaceous compounds in response to the cuticular invasion by the fungal spores, Vilcinskas and Matha 1997 and Gillespie *et al.*, 2000.

**3-Activity of Acid phosphatase, prophynoloxidase and phynoloxidase**

Obviously from data illustrated in Table (6), *B. bassiana* infection caused increase in the Acid phosphatase (AP) activity during the course of the experiment, except on the 2nd day, it was equal to control. Such increase was not significant comparing with control treatment. The activity of AP was fluctuated up and down during the first 4 days post infection in *M. anisopliae* var. *acridum* treatment, then declined significantly till the end of the experiment, while such fluctuation in combined infection was during the first 3 days, then declined significantly till the end of experiment. The reduction in those treatments reached 64.29 and 71.43 % by the end of the experiment, respectively (Table 6).

Vincent *et al.*, 1993 suggested that acid phosphatase may play defensive role in the haemolymph of the migratory grasshopper, *Melanoplus sanguinipes* during the infection with *B. bassiana*. In the present study, *B. bassiana* infection may be failed to overcome (AP) activity during the process of infection, while in *M. anisopliae* var. *acridum* and combined infection treatments the infection succeeded to reduce (AP) activity. This may be due to that the fungal metabolites caused toxicity to (AP) production.

Data recorded in Table (7) demonstrate the activity of prophenoloxidase (proPO) in the haemolymph of the 5th nymphal instar of *S. gregaria*. It is clear that on the 5th day post

Table (5): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on total lipids contents (g/100 ml) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X}$ ± SE [b] | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction |
| 1st day | 2.92 ± 0.23 a | 2.92 ± 0.23 a | 0.00 | 2.93 ± 0.26 a | -0.17 | 2.93 ± 0.33 a | -0.14 |
| 2nd day | 3.34 ± 0.31 a | 3.02 ± 0.11 a | 9.52 | 3.08 ± 0.28 a | 8.00 | 2.96 ± 0.06 a | 11.58 |
| 3rd day | 3.88 ± 0.26 a | 2.99 ± 0.15 b | 23.15 | 3.01 ± 0.25 b | 22.50 | 2.85 ± 0.10 b | 26.52 |
| 4th day | 3.92 ± 0.37 a | 2.70 ± 0.17 b | 30.94 | 2.86 ± 0.19 b | 26.94 | 2.60 ± 0.13 b | 33.73 |
| 5th day | 3.74 ± 0.27 a | 2.19 ± 0.19 bc | 41.39 | 2.42 ± 0.13 b | 35.27 | 1.84 ± 0.10 c | 50.89 |
| 6th day | 3.71 ± 0.22 a | 1.26 ± 0.19 b | 66.09 | 1.49 ± 0.31 b | 59.97 | 1.09 ± 0.12 b | 70.62 |
| 7th day | 3.87 ± 0.28 a | 0.98 ± 0.08 b | 74.66 | 1.23 ± 0.15 b | 68.28 | 0.87 ± 0.08 b | 77.66 |
| 8th day | 3.82 ± 0.46 a | 0.95 ± 0.03 b | 75.11 | 1.14 ± 0.20 b | 70.10 | 0.69 ± 0.14 b | 81.82 |
| Average | 3.65 ± 0.30 | 2.13 ± 0.14 | 41.73 | 2.27 ± 0.22 | 37.86 | 1.98 ± 0.13 | 45.84 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letter in same row mean no significant differences.

Table (6): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on acid phsphatase activity (activity units) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X}$ ± SE [b] | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction |
| 1st day | 13.00 ± 1.00 a | 14.33 ± 1.53 a | -10.26 | 13.33 ± 0.58 a | -2.56 | 12.33 ± 1.53 a | 5.13 |
| 2nd day | 13.33 ± 1.53 a | 12.33 ± 1.53 a | 7.50 | 13.67 ± 1.53 a | -2.50 | 15.00 ± 2.00 a | -12.50 |
| 3rd day | 13.67 ± 1.53 a | 15.00 ± 2.00 a | -9.76 | 13.67 ± 1.15 a | 0.00 | 16.00 ± 2.00 a | -17.07 |
| 4th day | 13.33 ± 1.53 ab | 16.67 ± 1.53 a | -25.00 | 16.33 ± 1.53 a | -22.50 | 12.00 ± 1.00 b | 10.00 |
| 5th day | 13.33 ± 1.53 a | 09.00 ± 1.00 b | 32.50 | 15.67 ± 1.53 a | -17.50 | 08.67 ± 1.53 b | 35.00 |
| 6th day | 13.33 ± 0.58 a | 08.00 ± 1.00 b | 40.00 | 14.67 ± 1.53 a | -10.00 | 07.67 ± 2.52 b | 42.50 |
| 7th day | 14.33 ± 2.08 a | 05.67 ± 1.53 b | 60.47 | 15.00 ± 2.65 a | -4.65 | 05.00 ± 1.00 b | 65.12 |
| 8th day | 14.00 ± 1.00 a | 05.00 ± 1.00 b | 64.29 | 14.33 ± 1.53 a | -2.38 | 04.00 ± 1.00 b | 71.43 |
| Average | 13.54 ± 1.35 | 10.75 ± 1.39 | 20.62 | 14.58 ± 1.50 | -7.69 | 10.08 ± 1.57 | 25.54 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letter in same row mean no significant differences.

Table (7): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on prophenoloxidase activity (activity units) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X}$ ± SE [b] | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction |
| 1st day | .0157 ± .0012 a | .0153 ± .0006 a | 2.13 a | .0157 ± .0015 a | 0.00 | .0150 ± .0010 a | 4.26 |
| 2nd day | .0153 ± .0015 a | .0157 ± .0006 a | -2.17 | .0153 ± .0006 a | 0.00 | .0150 ± .0010 a | 2.17 |
| 3rd day | .0157 ± .0021 a | .0153 ± .0021 a | 2.13 | .0153 ± .0015 a | 2.13 | .0147 ± .0021 a | 6.38 |
| 4th day | .0160 ± .0010 a | .0147 ± .0015 a | 8.33 | .0150 ± .0020 a | 6.25 | .0140 ± .0010 a | 12.50 |
| 5th day | .0153 ± .0032 a | .0123 ± .0015 b | 19.57 | .0150 ± .0010 a | 2.17 | .0117 ± .0006 b | 23.91 |
| 6th day | .0153 ± .0025 a | .0107 ± .0015 bc | 30.43 | .0140 ± .0017 ab | 8.70 | .0100 ± .0010 c | 34.78 |
| 7th day | .0150 ± .0026 a | .0090 ± .0010 c | 40.00 | .0130 ± .0010 b | 13.33 | .0083 ± .0006 c | 44.44 |
| 8th day | .0160 ± .0026 a | .0070 ± .0010 c | 56.25 | .0103 ± .0006 b | 35.42 | .0067 ± .0012 c | 58.33 |
| Average | .0155 ± .0021 | .0125 ± .0012 | 19.57 | .0142 ± .0012 | 8.58 | .0119 ± .0010 | 23.32 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letter in same row mean no significant differences.

Table (8): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on phenoloxidase activity (activity units) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X}$ ± SE [b] | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction |
| 1st day | 0.020 ± .001 a | 0.021 ± .002 a | -3.28 | 0.021 ± .002 a | -1.64 | 0.021 ± .002 a | -4.92 |
| 2nd day | 0.020 ± .002 a | 0.019 ± .002 a | 1.69 | 0.020 ± .002 a | -3.39 | 0.021 ± .002 a | -5.08 |
| 3rd day | 0.020 ± .003 a | 0.021 ± .002 a | -3.33 | 0.019 ± .001 a | 5.00 | 0.018 ± .002 a | 11.67 |
| 4th day | 0.020 ± .003 a | 0.018 ± .001 a | 11.48 | 0.019 ± .002 a | 4.92 | 0.016 ± .001 a | 21.31 |
| 5th day | 0.021 ± .002 a | 0.015 ± .001 bc | 27.42 | 0.018 ± .001 ab | 12.90 | 0.014 ± .001 c | 32.26 |
| 6th day | 0.019 ± .002 a | 0.014 ± .001 bc | 27.59 | 0.017 ± .001 ab | 12.07 | 0.012 ± .002 c | 36.21 |
| 7th day | 0.021 ± .002 a | 0.012 ± .002 b | 40.32 | 0.017 ± .001 a | 17.74 | 0.011 ± .002 b | 46.77 |
| 8th day | 0.021 ± .002 a | 0.009 ± .002 c | 57.14 | 0.015 ± .001 b | 30.16 | 0.008 ± .001 c | 61.90 |
| Average | 0.020 ± .002 | 0.016 ± .001 | 20.16 | 0.018 ± .001 | 9.88 | 0.015 ± .001 | 25.31 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letter in same row mean no significant differences.

Table (9): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on total haemocytes count (X $10^3/\mu l$) of haemolymph of 5th nymphal instar of *S. gregaria*.

| Days Post treatment | control | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | *B. bassiana* $10^3$ spores/nymph (sole) | | Combination [a] | |
|---|---|---|---|---|---|---|---|
| | $\bar{X}$ ± SE [b] | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction | $\bar{X}$ ± SE [b] | % Reduction |
| 1st day | 3.46 ± 0.37 a | 3.86 ± 0.30 a | -11.56 | 3.66 ± 0.27 a | -5.78 | 3.98 ± 0.47 a | -15.03 |
| 2nd day | 3.56 ± 0.50 a | 3.72 ± 0.33 a | -4.49 | 3.56 ± 0.50 a | 0.00 | 3.20 ± 0.63 a | 10.11 |
| 3rd day | 3.62 ± 0.40 a | 3.56 ± 0.31 ab | 1.66 | 3.26 ± 0.33 ab | 9.94 | 2.94 ± 0.60 b | 18.78 |
| 4th day | 3.58 ± 0.29 a | 3.04 ± 0.62 ab | 15.08 | 3.14 ± 0.56 ab | 12.29 | 2.84 ± 0.43 b | 20.67 |
| 5th day | 3.66 ± 0.53 a | 2.96 ± 0.47 bc | 19.13 | 3.24 ± 0.49 ab | 11.48 | 2.64 ± 0.59 c | 27.87 |
| 6th day | 3.70 ± 0.47 a | 2.82 ± 0.45 b | 23.78 | 3.06 ± 0.65 b | 17.30 | 2.52 ± 0.25 b | 31.89 |
| 7th day | 3.52 ± 0.37 a | 2.54 ± 0.43 b | 27.84 | 2.84 ± 0.67 b | 19.32 | 2.30 ± 0.36 b | 34.66 |
| 8th day | 3.36 ± 0.36 a | 2.42 ± 0.60 bc | 27.98 | 2.80 ± 0.48 ab | 16.67 | 2.20 ± 0.41 c | 34.52 |
| Average | 3.56 ± 0.41 | 3.12 ± 0.44 | 12.44 | 3.20 ± 0.49 | 10.19 | 2.83 ± 0.47 | 20.52 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
[b] Same letter in same row mean no significant differences.

Table (10): Effect of *Metarhizium anisopliae* and *Beauveria bassiana* sole and in combined infection on percentages of the haemocytes type of 5th nymphal instar of *S. gregaria*.

| Days Post | control | | | | | *M. anisopliae* var. *acridum* $10^3$ spores/nymph (sole) | | | | | *B. bassiana* $10^3$ spores/nymph (sole) | | | | | Combination [a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| treatment | Pr | Pl | Sp | Gr | Sph | Pr | Pl | Sp | Gr | Sph | Pr | Pl | Sp | Gr | Sph | Pr | Pl | Sp | Gr | Sph |
| 1st day | 26.33 | 55.50 | 2.17 | 9.83 | 6.17 | 26.67 | 55.17 | 2.00 | 9.67 | 6.50 | 26.83 | 54.50 | 2.17 | 10.17 | 6.33 | 26.83 | 53.33 | 2.20 | 11.17 | 6.83 |
| 2nd day | 27.67 | 51.50 | 2.33 | 10.50 | 8.00 | 24.83 | 46.33 | 2.50 | 14.17 | 12.17 | 27.67 | 46.17 | 2.33 | 13.33 | 10.50 | 26.33 | 47.67 | 2.50 | 14.33 | 9.17 |
| 3rd day | 25.17 | 57.50 | 2.33 | 10.17 | 4.83 | 23.83 | 41.33 | 2.83 | 17.50 | 14.50 | 26.33 | 44.83 | 1.83 | 15.17 | 11.83 | 23.17 | 47.33 | 2.00 | 15.33 | 12.17 |
| 4th day | 26.33 | 59.00 | 1.83 | 7.67 | 5.17 | 19.83 | 41.50 | 3.33 | 18.83 | 16.50 | 24.33 | 43.33 | 2.17 | 17.83 | 12.33 | 20.67 | 42.50 | 2.33 | 19.83 | 14.67 |
| 5th day | 22.67 | 65.00 | 2.50 | 6.50 | 3.33 | 19.67 | 37.17 | 4.17 | 21.33 | 17.67 | 22.50 | 42.67 | 2.67 | 17.67 | 14.50 | 19.83 | 38.83 | 3.50 | 21.33 | 16.50 |
| 6th day | 22.83 | 66.17 | 2.67 | 5.17 | 3.17 | 20.17 | 31.00 | 4.17 | 24.83 | 19.83 | 22.33 | 41.83 | 2.83 | 17.67 | 15.33 | 18.67 | 34.00 | 4.50 | 23.17 | 19.67 |
| 7th day | 22.17 | 58.83 | 2.33 | 10.50 | 6.17 | 22.17 | 32.50 | 4.00 | 22.50 | 18.83 | 17.83 | 38.33 | 3.50 | 20.67 | 19.67 | 19.50 | 36.00 | 3.50 | 21.83 | 19.17 |
| 8th day | 23.67 | 57.33 | 2.50 | 9.67 | 6.83 | 20.83 | 33.00 | 3.33 | 22.17 | 20.67 | 22.17 | 29.00 | 3.17 | 24.17 | 21.50 | 20.67 | 36.83 | 3.50 | 21.17 | 17.83 |

[a] *M. anisopliae* var. *acridum* + *B. bassiana* $10^3$ spores/nymph.
(Pr = proheamocyte Pl = plasmatocyte Sp = spindle shaped cell Gr = granulocyte Sph = spherulucyte)

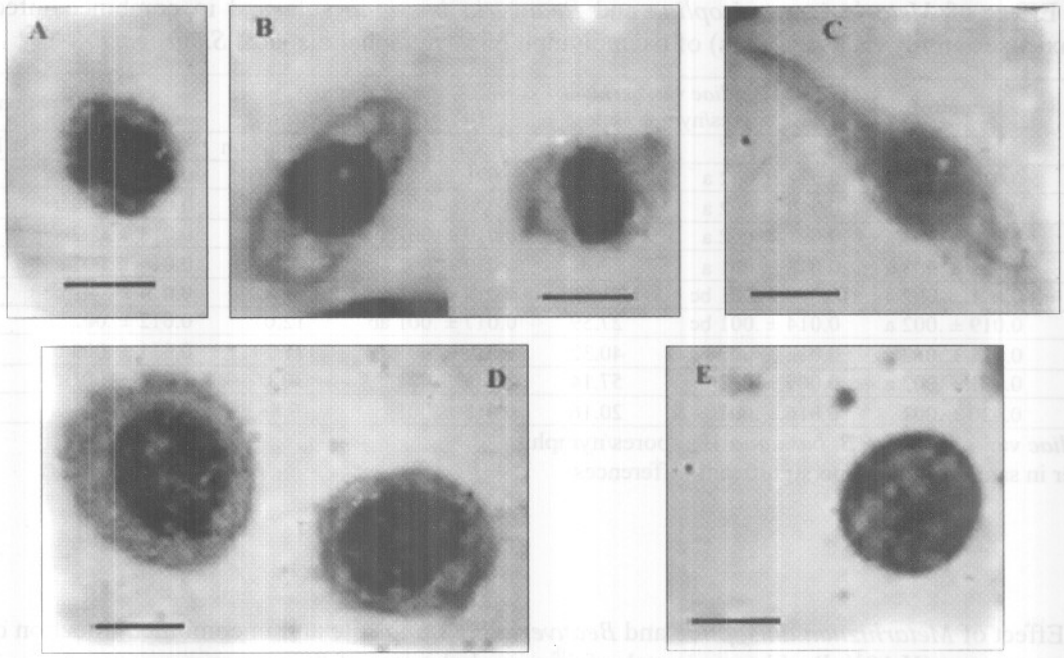


Fig. (1): Haemocytes types of *Schistocerca gregaria*:
(A) Prohaemocyte (B) Plasmatocyte (C) Spindle shaped cell
(D) Granulocyte (E) Spheriloucytes, Bar= 8μ

treatment, the prophenoloxidase activity was significantly reduced in both *M. anisopliae* var. *acridum* and the combined treatments, while in case of *B. bassiana* infection, there was significant reduction only during the last 2 days of the experimental period. The reduction in the activity reached 56.25, 35.42 and 58.33 % by the end of the experiment, in *M. anisopliae* var. *acridum*, *B. bassiana* and the combination treatments, respectively (Table 7).

Data in Table (8) show the phenoloxidase (PO) activity was noticeably at the same trend as prophenol-oxidase activity in the treated nymphs. The reduction in the activity reached 57.14, 30.16 and 61.90 % by the end of the experiment, in *M. anisopliae* var. *acridum*, *B. bassiana* and the combination treatments, respectively (Table 8).

Huxham *et al.*, 1989 cleared that destruxins prevent production of (PO) by the locust haemocytes. This probably is destroyed by the cells that produce (proPO) (Cerenius *et al.*, 1990). The present study suggested that *B. bassiana* metabolites may be also destroyed the same cells but less effectivlly.

**4- Haemolymph heaymocytes in *S. gregaria* 5th nymphal instar:**

In the present work, 5 types of heaymocyte were recognized; these types were identified according to Gupta 1979 as: Prohaemocyte, Plasmatocyte, spindelshiped haemocytes Granulocyte and Spherulocyte (Fig. 1).

**Total heaymocyte count**

In table (9), it is perceptible that, the combined treatment caused significant reduction in the total heaymocyte count (THC) on the 3rd day post treatment. Also, *M. anisopliae* var. *acridum* infection caused significant reduction in the THC on the 5th day post treatment, while in *B. bassiana* infection, it declined significantly on the 6th day post infection. The reduction in the THC reached 27.98, 16.67 and 34.52 % by the end of the experiment, in *M. anisopliae* var. *acridum*, *B. bassiana* and the combination treatments, respectively (Table 9).

Data illustrated in Table (10) show percentage of the haemocyte types of 5tph nymphal instar of *S. gregaria* in the control, *M. anisopliae* var. *acridum*, *B. bassiana* and the combined infection treatments. It is clear that plasmatocytes were the most dominant haemocyte type, followed by prohaemocyte, granulocyte, spherulocyte, then spindelshaped haemocyte. The infection caused increase in the percentage of occurrence of both granulocyte and spherulocyte, also noticeable decrease in the plasmatocyte percentage and slight decrease in the proheamocyte percentage. It was observed also that, *M. anisopliae* var. *acridum* hypha occurred in the examined slides on the 3rd day post treatment and increased in number dally, while in *B. bassiana* infection, the hypha occurred on the 4th day post treatment and in case of combined infection, the hypha occurred on the 2nd day post

infection.

Lackie *et al.,* 1985 observed greater number of plasmatocytes in control desert locust, which is in harmony with the present results. During the infection there was increase in the proportion of spherulucytes and granulocytes, this may be due to their role in production of Humoral defence reaction.

The insect immune system is subdivided into humoral and cellular defense responses. Humoral defenses include the production of antimicrobial peptides and the complex enzymatic cascades that regulate coagulation or melanization of hemolymph. In contrast, cellular defense refers to hemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Lavine and Strand 2001). It is clear that combined infection of both used fungi caused greater reduction to the 5$^{th}$ nymphal instar of *S. gregaria* immune system followed by *M. anisopliae* var. *acridum* then *B. bassiana* infection, such effect may explain superior effect of the combined infection and *M. anisopliae* var. *acridum* treatments.

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