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Influence of *Penicillium aurantiogriseum* and its Mycotoxin Citrinin on Haemolymph of *Schistocerca gregaria* (Forskål)

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ABSTRACT
The influence of entomopathogenic fungi, *Penicillium aurantiogriseum* and its mycotoxin citrinin against the haemolymph of the 5th instar nymphs of *Schistocerca gregaria* (Forskål) were studied under laboratory conditions. This study aims to detect the changes of some biochemical parameters of haemolymph which are valuable in evaluating and predicting the pathogenic effect on *S. gregaria.* Results showed an effect of *P. aurantiogriseum* and its mycotoxin citrinin on haemocytes, phenoloxidase activity and haemolymph protein at 1st, 3rd, 5th, 7th and 9th days post application. Comparison between the two treatments revealed fluctuations in the mean total haemocyte count and detection of plasmatocytes, lymphocytes and granulocytes in different percentages in treated nymphs. Citrinin mycotoxin and *P. aurantiogriseum* disturbed the titre of phenoloxidase enzyme in all intervals after infection. Also, they decreased the total haemolymph protein content significantly during the five days post infection as compared to the control.

***Keywords*:** *P. aurantiogriseum*, mycotoxin, *S. gregaria*, haemolymph, haemocytes, proteins, phenoloxidase.

Introduction

The growing demand for reducing chemical inputs in agriculture and increased resistance to insecticides have provided impetus to the development of alternative forms of insect pest control. Entomopathogenic fungi are potentially the most versatile biological control agents due to their wide host range. These fungi comprise a diverse group of over 90 genera with approximately 750 species, reported from different insects. Entomopathogenic fungi are naturally occurring organisms which are perceived as less damaging to the environment (Rai *et al*., 2014). *Penicillium spp.* was considered as entomopathogenic fungi (Al- Keridis, 2015). Mycotoxins are synthesized by fungi and released as secondary metabolites which are toxic to many insect species. Mycotoxins exhibited insecticidal effects and developmental delays to many insect species (Zeng *et al*., 2006) and caused rapid lethal effects to *Schistocerca gregaria* (Helal *et al*., 2012).

Insect haemolymph is the major extracellular fluid in insects. It makes up from 15% to 75% of the volume of the insect, varying significantly with species and individual physiological state. Haemolymph contains cellular components, proteins, carbohydrates, lipids and salts, which are obtained from food and circulated in haemolymph by dissolving in the aqueous (water) portion of the fluid. The haemolymph additionally bears various immunity-related products that function in protecting the insect against systemic infection. These products are components of the humoral (i.e., non-cellular) part of the insect immune system. To a large extent, the humoral effectors are inducible, i.e., they are undetectable (or nearly so) in uninfected insects, and increase to high titers in response to mechanical wounding and infection by bacteria, fungal pathogens, nematodes, etc. Phenoloxidase is one of the vitally important inducible humoral effectors which are induced very rapidly by a proteolytic cascade and anti-microbial peptides(Chapman, 2013).

In insects, the circulating haemocytes perform primary functions in the body such as phagocytosis, coagulation to prevent loss of blood, encapsulation of foreign bodies, nodule formation, detoxification of metabolites and biological active materials, as well as storage and distribution of nutritive materials to various tissues (Siddiqui and Al-Kalifa, 2012; Chavan *et al*., 2017). They derive from stem cells that differentiate into specific lineages. However, certain haemocyte types are not common in all insects and

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differ among species (Meister and Lagueux, 2003).The desert locust, *Shistocerca gregaria* (Orthoptera: Acrididae) (Forskål) is among the major pests of many tropical and subtropical countries causing extensive damage to the foliar part of many plants (Gesraha, 2007) particularly during years with locust outbreaks. Therefore, the objective of the current study was to assess the effect of *P. aurantiogriseum* and its mycotoxin citrinin on haemocytes, titer of phenoloxidase enzyme and total haemolymph proteins of 5th instar nymphs of *S. greagria*.

Materials and Methods

1. Insect culture

Females and males of the desert locust, *Schistocerca gregaria* (Forskål) were obtained from Plant Protection Research Institute, Zagazig city, Sharkia, Egypt. Adults were breed in the laboratory of Entomology Section, Zoology Department, Faculty of Science, Zagazig University, Zagazig city, Sharkia, Egypt, under crowded conditions at 30 ± 2°C, 70 – 80 % R. H. and photoperiod of 8 D: 16 L for several generations. Adults were placed in wooden – framed cages measuring 40×40 × 60 cm and provided with cups of moist sieved sand to serve as ovipositional sites as described by Hassanein (1965).

2. Conidial production

The conidia of the fungus previously isolated from *S.gregaria* cadavers by Hashem (2013) and identified as *P. aurantiogriseum* (Dierekx) 800 (AUMC) were harvested and prepared according to Hicks *et al*. (2001); Helal *et al.* (2019).

3. Extraction of mycotoxin from *P. aurantiogriseum*

Cultivation of fungi, *P. aurantiogriseum* for mycotoxin screening was conducting by: Fungal conidia on Capek's medium fortified by 2 g yeast extract and 10 g peptone of the following composition: Glucose, 10 g; $NaNO_3$, 1 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; Peptone, 10 g and yeast extract 2 g, per liter of distilled water. The cultivation of *P. aurantiogriseum* was made according to Helal *et al.* (2019).

4. Preparation of the crude mycotoxin of *P. aurantiogriseum*

The content of flask (medium + mycelium) was homogenized for 5 minutes in a high speed blender (16000 r.p.m.) with 100 ml chloroform. The extraction procedure was repeated three times. The combined chloroform extract was washed with equal volume of distilled water, dried over anhydrous sodium sulphate, filtered then concentrated under vacuum or a stream of nitrogen to near dryness, and diluted to 1 ml with chloroform.The preparation and identification of the crude mycotoxin citrinin of *P. aurantiogriseum* were accomplished according to Helal *et al.* (2019).

5. Insect infection

The experimental design was arranged with three groups of treatments and three replicates, each experiment consisted of ten 5th instar nymphs of *S. gregaria*. The first group was inoculated with 50µl sterile water, the second group was inoculated with 50 µl of conidial suspension of 8×10^3conidia / ml and the third group was inoculated with 50µl of 15 µl /ml citrinin mycotoxin by the topical application technique beneath the dorsal pronotal shield of nymphal instars.

6. Haemolymph collection

Haemolymph of 5th instar nymphs was drawn from coxal joint by sterile micro syringe. Control and treated haemolymph samples were taken at five successive intervals of 1, 3, 5, 7 and 9 days post treatments. The collected samples (10 µl) were transferred into clean dry centrifuge tubes containing a few crystals of phenylthiourea (PTU) to prevent melanization before analysis according to Al- Mokhlef (2008). The haemolymph was centrifuged at 4000 r.p.m. for 5 min. at 4º C. After centrifugation, the supernatant fluid was stored at – 20 ºC until analysis.

7. Total haemocyte counts (THC)

For haemocyte counting, 3 µl of haemolymph of 5th instar nymphs of *S. gregaria* were placed on a glass slide and smeared to a thin film. The smears were first stained with diluted May- Grundwald

stain for 3 min., then washed with distilled water and stained for a second time with diluted Giemsa for 10 min. then washed again in distilled water according to Guzo and Stolz (1987). The haemocytes were observed under light microscope with × 100 oil immersion objectives and identified according to Gupta (1979). The total haemocyte counts was calculated by diluting haemolymph (1: 4) with sterile ice cold anticoagulant buffer then placed in an improved Neubauer haemocytometer. Total haemocyte counts were counted in a standard haemocytometer according to the formula of Jones (1962):

$$\frac{\textit{Number of haemocyte counted per chamber x dilution x depth factor}}{\textit{Number of 1 mm squares counted}}$$

8. Differential haemocyte count (DHC)

Stained haemolymph preparations were carried out, according to Arnold and Hinks (1979). The haemolymph was smeared on clean glass slides, allowed to dry for 1 minute, and fixed for 2 minutes with drops of absolute methyl alcohol. Fixed cells were stained with Giemsa's solution (diluted 1: 20 in distilled water) for 20 minutes, washed several times with tap water, and dipped in distilled water. The stained smears were air -dried and mounted in DPX with slip cover. The haemocytes were viewed under light microscope at a magnification 10 X 40 =400. The cell shape, cytoplasmic ratio, cytoplasmic inclusions and shape of nucleus were used for classification of haemocytes using the classification scheme of Bréhelin and Zachary (1986). The percentages of haemocyte types were calculated by the formula:

$$\frac{\textit{Number of each haemocyte type}}{\textit{Total number of haemocytes examined}} \textit{ x 100}$$

9. Phenoloxidase (PO) activity:

Phenoloxidase activity was determined according to a modification of Ishaaya (1971), in a reaction mixture consisting of 0.5 ml phosphate buffer (0.1M, pH 7), 200µl enzyme solution and 200 µl catechol solution (2%). Prior to the initiation of the reaction, the substrate and other ingredients of the reaction mixture were separately incubated at the optimum temperature of the reaction (25º C). Enzyme reaction was initiated by adding catechol solution. Then after exactly 1 min., the optical density was determined, zero adjustment was against a sample blank at 405nm on APEL-PD 303 Spectrophotometer.

10. Determination of total haemolymph protein.

Colorimetric determination of total haemolymph protein of 5th instar nymphs of desert locust inoculated with 50 µl of sterile water, 50 µl of 8× 10^3 conidia / ml conidial suspension of *P. aurantiogriseum* and 50µl of 15µl/ml citrinin mycotoxin were carried out as described by Bradford (1976). Protein reagent was prepared by dissolving 100 mg of Coomassie Brilliant blue G-250 in 50 ml 95% ethanol. To this solution 100ml 85% (W/V) phosphoric acid were added. The resulting solution was diluted to a final of 1 liter. Sample solution (50µl) or for preparation of standard curve 50µl of serial concentrations containing 10 to 100µg bovine serum albumin were pipette into test tubes. The absorbance at 595nm was measured after 2 min. and before 1 hr against blank prepared from 1ml of phosphate buffer and 5 ml protein reagent.

Statistical analysis

Means and Standard errors of total haemolymph protein and phenoloxidase activity of 5th instar nymphs were calculated and compared by using a one- way ANOVA in SPSS Version 14.0.

Results

1. Total haemocyte counts (THC)

The data in table (1) showed the effect of inoculation of 50µl of sterile water, 50 µl of 8×10^3 conidia /ml *P. aurantiogriseum* and 50µl of 15µg/ml citrinin mycotoxin on the mean THC of 5th instar nymphs of *S. gregaria* at 1st, 3rd, 5th, 7th and 9th days after treatment. On the 1st and 3rd days after treatment, the nymphs showed significant decrement in the THC in insects inoculated with conidial suspension and citrinin mycotoxin, respectively (0.44 ± 0.06, 0.68 ± 0.12; 0.68 ± 0.005, 0.61 ± 0.09 ×

10^3 haemocytes/ µl) as compared to control (1.4 ± 0.5, 0.8 ± 0.09 × 10^3 haemocytes/ µl)(p= 0.01, 0.009 and 0.6, 0.4, respectively). Comparison between nymphs inoculated with conidial suspension and citrinin mycotoxin revealed a non-significant changes in the mean of THC at the two intervals, respectively (p=0.35and 0.7). The mean THC in the 5th day after treatment revealed a non -significant increment in insects inoculated with *P. aurantiogriseum* and citrinin mycotoxin to reach 0.81 ± 0.02 and 0.9 ± 0.19 × 10^3 haemocytes/ µl, respectively as compared to control (0.59 ± 0.18 × 10^3 haemocytes/ µl) (p= 0.4 and 0.23). Also, insects inoculated with citrinin mycotoxin demonstrated a non-significant increase as compared to insects inoculated with conidial suspension (p= 0.7). Moreover, significant increase was recorded on the 7th and 9th days after treatment with *P. aurantiogriseum* (0.98 ± 0.07 and 0.75 ± 0.15 ×10^3 haemocytes/ µl, respectively) and after treatment with citrinin mycotoxin (1 ± 0.14 and 0.3 ± 0.03 × 10^3 haemocytes/ µl), respectivelyas compared to control (p= 0.049, 0.041 and 0.029, 0.5). The mean THC decreased in control insects to 0.44 ± 0.16 and 0.18 ± 0.01 ×10^3 haemocytes/ µl, respectively. The comparison between inoculated insects with conidial suspension and inoculated insects with citrinin mycotoxin revealed a non-significant changes on the 7th and 9th days post treatment in the mean THC (p= 0.7 and 0.1, respectively).

Table 1: Effect of conidial suspension of *P. aurantiogriseum* and its mycotoxin citrinin on mean total haemocyte counts of 5th instar nymphs of *S. gregaria*

Days	Control (Mean± SE) ×10^3	Conidial suspension (Mean± SE) ×10^3	Citrinin mycotoxin (Mean ± SE) ×10^3
1	1.4 ± 0.5^{ab}	0.44 ± 0.06^{aA}	0.68 ± 0.12^{bA}
3	0.8 ± 0.09^{BC}	0.68 ± 0.005^{BD}	0.61 ± 0.09^{CD}
5	0.59 ± 0.18^{EF}	0.81 ± 0.02^{EG}	0.9 ± 0.19^{FG}
7	0.44 ± 0.16^{dc}	0.98 ± 0.07^{dD}	1.0 ± 0.14^{cD}
9	0.18 ± 0.01^{He}	0.75 ± 0.15^{eI}	0.3 ± 0.03^{HI}

Means with the same small letters are significantly differences p≤ 0.05 Capital letters are non- significantly differences, df1= 2, df2 =6

2. Differential haemocytes count (DHC)

Haemolymph of the 5th instar nymphs of *S. gregaria* revealed the presence of three types of blood cells in control and in infected insects. Table (2) illustrated types and percentages of different haemocytes / 3µl of haemolymph of the 5th instar nymphs inoculated with sterile water (control), conidial suspension of *P. aurantiogriseum* and citrinin mycotoxin after 1, 3, 5, 7and 9 days post treatment.

Table 2: Types and percentage of haemocytes of 5th instar nymphs of *S. gregaria* inoculated with conidial suspension of *P. aurantiogriseum* and its mycotoxin citrinin

Days		Plasmatocytes Percentage	Lymphocytes Percentage	Granulocytes Percentage
Control	**1**	57 ± 2.3^{A}	31.8 ± 3.2^{I}	10.8 ± 0.8^{b}
	3	60 ± 5^{B}	30 ± 5^{J}	10 ± 0^{E}
	5	50 ± 0.5^{ae}	36 ± 1^{K}	13 ± 0.5^{Ff}
	7	53 ± 4^{C}	35 ± 2^{L}	12 ± 2^{G}
	9	54 ± 1^{D}	35 ± 0.0^{M}	12.5 ± 2.5^{H}
Conidial suspension	**1**	58 ± 7.5^{A}	27.5 ± 5.5^{I}	13 ± 1^{bc}
	3	57 ± 7.5^{B}	31.5 ± 8.5^{J}	11 ± 1^{E}
	5	52 ± 2.5^{aQ}	32.5 ± 2.5^{K}	15 ± 0^{Fd}
	7	57 ± 1^{C}	32.5 ± 2.5^{L}	10 ± 1.5^{G}
	9	53 ± 1.9^{D}	35.6 ± 0.6^{M}	11 ± 1.2^{H}
Citrinin mycotoxin	**1**	55 ± 3^{A}	36 ± 2^{I}	9 ± 1^{c}
	3	57 ± 0.5^{B}	34 ± 2^{J}	8 ± 1.5^{E}
	5	63 ± 2.5^{Qe}	27 ± 3^{K}	9 ± 0.5^{df}
	7	57 ± 1.5^{C}	33 ± 2^{L}	9 ± 0.5^{G}
	9	52 ± 2.5^{D}	34 ± 1^{M}	13 ± 1.5^{H}

Means with the same small letters are significantly differences p≤ 0.05, Capital letters are non- significantly differences, df1= 14, df2 =15.

Plasmatocytes predominated in the haemolymph of the 5th instar nymphs followed by lymphocytes and granulocytes in control and in infected insects. Plasmatocytes recorded the highest percentage on the 3rd day and the lowest one on the 5th day post inoculation with sterile water. They listed significant changes after treatment with conidial suspension and citrinin mycotoxin on the 5th day post treatment (p= 0.7 and 0.2) and non-significant differences on the other four periods as compared to control (p = 0.82, 0.63, 0.44, 0.86; 0.6, 0.63, 0.39, 0.7, respectively). Haemolymph of the infected insects recorded non-significant changes in the percentage of lymphocytes at all periods after the two inoculations as compared to control (p= 0.39, 0.76, 0.48, 0.61 and 0.89, respectively for conidial suspension and p= 0.40, 0.42, 0.08, 0.68 and 0.84, respectively for citrin mycotoxin). Meanwhile, the percentage of granulocytes increased on the 1st day post treatment with conidial suspension and decreased on the 5th day post treatment with citrinin significantly as compared to control (p= 0.23 and 0.037). Citrinin mycotoxin caused non-significant disturbance in the percentage of plasmatocytes (p =0.5, 1, 0.3, 0.92 and 0.9) and lymphocytes (p = 0.1, 0.6, 0.27, 0.9 and 0.73) after the five periods of inoculation as compared to conidial suspension of *P. aurantiogriseum*, respectively. On the other hand, significant decrement in granulocyte percentages was caused by the mycotoxin on the 1st and 5th days post treatment as compared to conidial suspension (p= 0.04 and 0.007).

Phenoloxidase activity

Phenoloxidase enzyme showed fluctuations in its activity in the haemolymph of the 5th instar nymphs of *S.gregaria* inoculated with conidial suspension of *P. aurantiogriseum* and its mycotoxin citrinin at the five periods of post inoculation (1st, 3rd, 5th, 7th and 9th days) as compared to the control (Figure 1). It elevated significantly at 1st and 9th days (37.3 ± 0.32 and 51.8 ± 0.04 mg/ml, p= 0.29 and 0.0, respectively) and non- significantly at 5th day (37.15 ± 0.12, p= 0.0) post inoculation with *P. aurantiogriseum* as compared to control (19.7 ± 0.27, 17.2 ± 1.4 and 30.3 ± 3.78 mg/ml, respectively). The activity of phenoloxidase suppressed significantly at the 3rd and 7th days post inoculated instars with the conidial suspension (11.43 ± 3.3 and 14.8±0.08 mg/ml, p= 0.66 and 0.0, respectively) as compared to the control (21.05 ± 5.7 and 36.1±1.1 mg/ml, respectively).) On the other hand, the activity of phenoloxidase showed a non-significant increment in insects inoculated with citrinin mycotoxin as compared with the control on the 1st and 3rd days post inoculation (22.5 ± 0.3 and 22.2 ± 0.1 mg/ ml, p= 0.29 and 0.66, respectively). Inversely, there was a significant decrement in the activity on the 5th and 7th days (14.4 ± 0.4 and 22.6 ± 0.4 mg/ ml, respectively, p=0.0) post inoculation and highly significant increase in the 9th days (88.6 ± 0.2 mg/ ml, p=0.0) post inoculation with citrinin mycotoxin as compared to control insects. Meanwhile, citrinin mycotoxin inhibited significantly the activity of phenoloxidase as compared with the effect of conidial suspension inoculation of *P. aurantiogriseum* at 1st and 5th days post inoculation (p= 0.0) and promoted its effect significantly at 3rd, 7th and 9th days post treatment (p= 0.0, 0.01 and 0.0, respectively) as shown in figure (1).

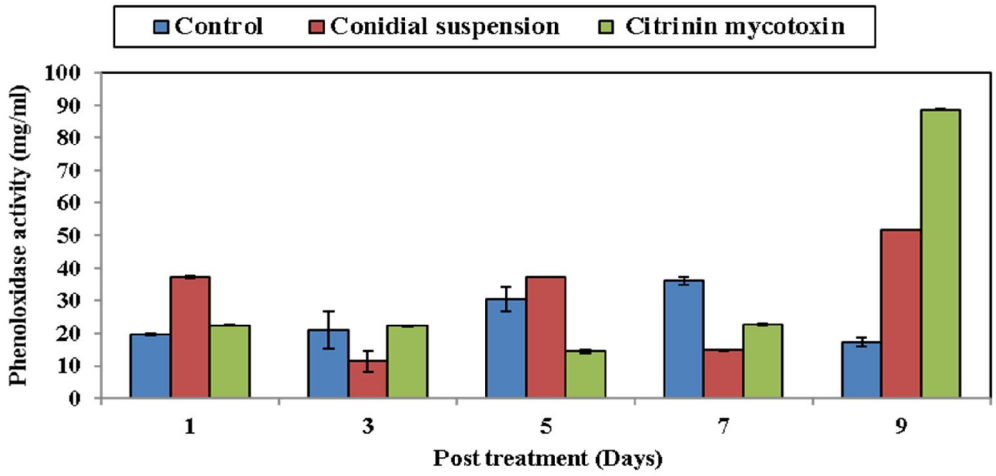

Fig. 1: The effect of conidial suspension *P. aurantiogriseum* and its mycotoxin citrinin on phenoloxidase activity (mg/ ml) in haemolymph of 5th instar nymphs of *S. gregaria.*

3. Total haemolymph protein content

Obtained results in figure (2) revealed that inoculation of 5th instar nymphs with *P. aurantiogriseum* reduced the concentration of total haemolymph protein content as compared to control instars inoculated with sterile water at different days post treatment. The total haemolymph protein content recorded a remarkable significant decrease in the 1st day after inoculation with conidial suspension (7.6 ±0 .3 mg/ ml) as compared to control (55 ± 4.5 mg/ ml, p= 0.006). The same decrease recorded on the 3rd, 5th, 7th and 9th days post treatment (7.57 ± 0.57, 7.38 ± 1, 10.38 ±1 and 6.5±0.46 mg/ ml, respectively) as compared to control (33 ± 4.7, 24± 3.3, 44 ±5.2 and 26±3 mg/ ml, p= 0.0, 0.08, 0.0 and 0.14, respectively). Moreover, insects inoculated with citrinin mycotoxin showed a significant decrease in total haemolymph protein content in the 1st, 5th, 7th and 9th days post inoculation (50.7 ±0.67, 13.5 ± 1.6, 9.5 ± 0.8 and 20.6 ± 2 mg/ ml, respectively, p=0.0) as compared to control (55 ± 4.5, 24± 3.3 , 44 ±5.2 and 26±3 mg/ ml, respectively) and a significant increase in the 3rd day post inoculation (43.5± 0.11 mg/ ml) as compared to control (33 ± 4.7 mg/ ml, p=0.0). Meanwhile,

Total haemolymph protein contents in insects inoculated with citrinin mycotoxin revealed a highly significant increment in the 1st, 3rd and 9th day post treatment (50.7, 43.5, and 20.6 mg/ml, respectively) as compared to insects inoculated with conidial suspension (7.6, 7.57 and 6.5 mg/ ml, respectively) (p= 0.00, 0.00 and 0.002). The 5th day post inoculated insects with citrinin mycotoxin recorded a non-significant increase in total haemolymph protein content (13.5 ± 1.6 mg/ml) as compared to inoculated insects with conidial suspension (7.38 ± 1.0 mg/ml) (p=0.11), while, total haemolymph protein content in 7th day post treatment showed a non-significant increase in insects inoculated with conidial suspension as compared to insects inoculated with citrinin mycotoxin (p= 0.8) (Figure 2).

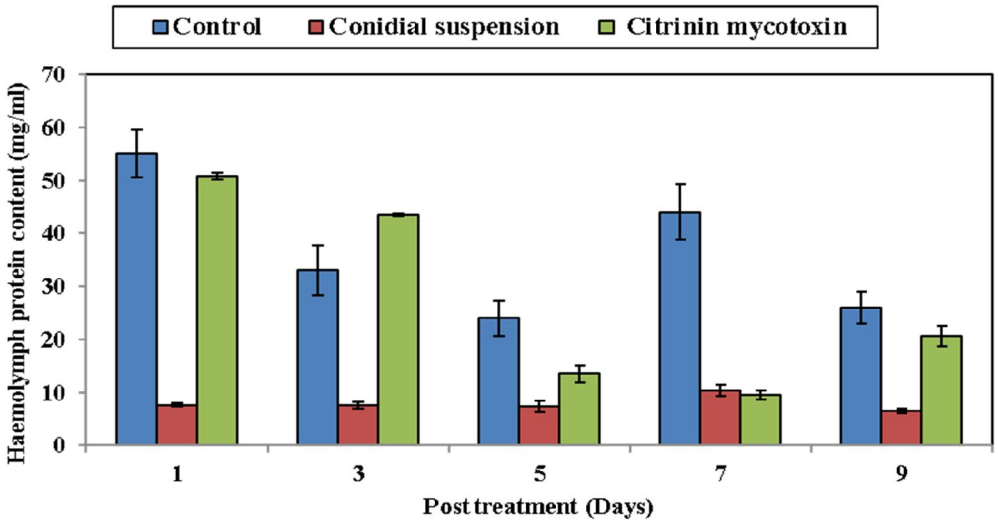

Fig. 2: Effect of conidial suspension of *P. aurantiogriseum* and its mycotoxin citrinin on total haemolymph protein content (mg/ml) of 5th instar nymphs of *S. gregaria*.

Discussion

The current study investigated the effect of inoculation of 5th instar nymphs of *S. gregaria* with *P. aurantiogriseum* and citrinin mycotoxin on the total haemocyte counts, differential haemocytes, the titer of phenoloxidase, which is considered as an insect immune barrier and total hamolymph protein. Haemocytes circulated in insect haemolymph were derived from stem cells that differentiate into specific lineages. However, certain haemocyte types are not common in all insects and differ among species (Meister and Lagueux, 2003). Haemocytes have been studied mostly in different orders (Chiang *et al*., 1988; Lavine and Strand, 2002 and Ribeiro and Brehelin, 2006). Little work has been reported in the available literature for Orthopterans, notably the acridids (Barakat *et al*., 2002; Tanani, 2010; Ghoneim *et al*., 2015). However, our results revealed fluctuations in the mean total haemocyte counts in treated insects. It decreased significantly at 1st and 3rd days and increased significantly at 5th, 7th and

9th days post treatment in inoculated nymphs with *P. aurantiogriseum* and citrinin mycotoxin as compared to control. This increment may be due to the enhanced encapsulation of the toxic molecules through the process of melanization, melanin deposition during encapsulation which is commonly initiated by haemocytes circulation in the plasma (Rolff and Siva- Jothy, 2002; Nappi and Christensen, 2005). Moreover, it could be considered as an immune response against pathogen (Ordas *et al*., 2000). Fluctuation of the total haemocyte counts during fungal infection in the present study corresponded to similar results reported by Hung and Boucias (1992) and Zibaee *et al*. (2011). On the other hand, the decrease in total haemocyte counts in inoculated insects with citrinin mycotoxin may result from formation of nodules induced by fungal metabolites and inhibition of larval hematopoietic function or the cell proliferation (Zibaee *et al*., 2011; 2012). In addition, it may be attributed to the death of pathological cells by degeneration (Sendi and Salehi, 2010).

The insect haemocytes differentiated into 6 types as prohaemocytes, plasmatocytes, granulocytes, adipohemocytes, spherule and enocytoids (Chapman, 2013). The haemocytes of 5th instar nymphs of *S. gregaria* were differentiated into 3 types as plasmatocytes, lymphocytes and granulocytes. This study calculated the percentage of each type according to the total haemocytes count in the three inoculated treatments, so the percentage of these types was convergent in all cases. Our results disagree with a lot of previously mentioned records for various insects; plasmatocytes and granulocytes played a role in phagocytosis of fungal spores (Zibaee *et al*., 2011). Secondary fungal metabolites (mycotoxins) suppressed phagocytosis along with nodule formation (Vey *et al*., 2002).

Phenoloxidase (PO) is a vital enzyme responsible for a number of crucial processes, such as defense, wound healing, sclerotization, and pigmentation. Phenoloxidase detected in both cuticle and haemolymph is derived from a pro-enzyme, prophenoloxidase. The PO cascade takes part in the melanization of haemocytes attached to the surface of the parasite (Pech and Strand, 2000). The present data showed fluctuation in the enzyme activity in haemolymph of *S. gregaria* inoculated with conidial suspension and citrinin mycotoxin as compared to at the five periods post treatment. Meanwhile, a comparison between the activity of phenoloxidase in insects inoculated by conidial suspension of *P. aurantiogriseum* and citrinin mycotoxin revealed non- significant decrease at 1st and 5th days and non-significant increase in 3rd, 7th and 9th days post inoculated insects with citrinin mycotoxin. Initial enhancement of enzyme activity may be due to the consequences of the invasion of fungal pathogen into the host haemolymph. In locusts and cockroaches prophenoloxidase stored in haemocytes until a pathogen induces its release (Brehe'lin *et al*., 1986). Phenoloxidase generated quinones which may serve as toxic metabolites that might be harmful to the intruders (Ashida and Yamazaki, 1990). Products of PO activity, i.e. melanin and its oxidized precursors have been shown to have fungistatic activity (St. Leger *et al.,* 1988). Several studies have shown that phenoloxidase levels are elevated in response to natural fungal infection or injection of fungal components (Gillespie *et al.,* 2000). Invaders that are able to penetrate successfully into the insect hemocoel will face a battery of cell defenses, including the phagocytosis of small pathogens and the formation of multicellular layers that encapsulate large intruders by the blood cells. Decreased enzyme activity after infection might be due to suppression of host enzyme activity by releasing inhibitor factors by the invading fungal pathogen (Shelby *et al*., 2000). The decline may be also due to the immunosuppressive effect of fungal proteins or toxic metabolites. Certainly, destruxins prevent PO production by *Locust* haemocytes probably by destroying the cells that produce prophenoloxidase (Cerenius *et al.,* 1990).

Proteins play an important role in the haemolymph of insects not only in specific transport functions, but also in their enzyme action. The high protein concentration is an indication of a greater metabolic activity of the tissue. They are not only responsible for the structure of the cell but also concerned with every function of the cell including respiration, catalysis of enzyme reactions, transport of materials, regulation of metabolism, movement and defense reactions (Rajitha and Savithri, 2013). Inoculation of 5th instar nymphs of *S. gregaria* with *P. aurantiogriseum* and citrinin mycotoxin caused disturbance in the concentration of total haemolymph protein content as compared to the control in the 5th instar nymphs inoculated with sterile water at different days post treatment. Gillespie *et al*. (2000) recorded reduction in total haemolymph protein concentration of adult *S. gregaria* during the course of infection with *M. anisopliae*. The loss of protein from host haemolymph during parasitism may be due to secretion of proteolytic enzymes from the parasite into the haemocoel of the insect and hydrolysis of the host´s proteins. Helal *et al.* (2012) proved that *P. aurantiogriseum* secreted protease enzyme. An increment in mean total haemolymph protein content in the present work on the 9th day post treatment

with citrinin was in accordance with Barakat and Abokersh (2016). They reported that total haemolymph protein of *S. gregaria* infected with *B. bassiana* were significantly higher at the first three days post treatment, but lower at the 4th day post treatment as compared with untreated insects. The observed decrease of total haemolymph protein contents following fungal infection may be attributed to fungal pathogenicity. Pathogens may cause a complete elimination of some haemolymph enzymes, soluble and sticky proteins, which may be involved in anti-fungal immunity. Some native proteins may be converted into lipoproteins or glycoproteins after infection. This could be attributed to intensive consumption of haemolymph proteins during growth and multiplication of the fungi, together with cessation of feeding observed on the infected insects (Sabbour, 2001; Seyoum *et al*., 2002 and Barakat and Abokersh, 2016). In contrary, the late increase of total proteins observed in this study at the 9th day following infection may be caused by citrinin mycotoxin effect within the haemocoel. Also, the decrease in the mean of total haemolymph protein may explain the reason of the decrease in the haemocyte count.

Conclusion

Inoculation of conidial suspension of *P. aurantiogriseum* and its citrinin mycotoxin in 5th instar nymphs of *S. gregaria* caused changes in total and differential haemocyte counts, phenoloxidase activity and disturbance of total haemolymph protein content during the treatment periods. These results suggested that entomopathogenic fungi and their mycotoxins secreted by them caused a disturbing action on the insect's haemolymph and may be interrupted by the hormonal balance or enzymatic hierarchy in nymphs, which can explain their effects on proteins and haemocytes.

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