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# Parasitizm of Locust by Entomopathogenic Nematode in Relation to Insect Microaggregation Inhibitor

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## ABSTRACT

The present work mainly focused on the susceptibility of *Schistocerca gregaria* (Forskal) 5th instar nymphs to the entomopathogenic nematodes, *Steinernema glaseri* (Rhabditida: Steinernematidae) as a natural exposure experiment. The entomopathogenic nematode, *S. glaseri* turned out to be successful parasitoid of the orthopteran insect *S. gregaria*. The death rate of locusts (sprayed with nematode on clover leafs) was remarkably high. Nematode killed approximately 65% of the locust with in 72 hours post infection at semi-field trial. The impact of parasitation on locusts' immune defense was closely investigated for *S. gregaria* parasitized by *S. glaseri*. Nymphs died within 48-72h after being fed clover leafs contaminated with 1500/ml *S. glaseri* juveniles or previously contaminated with the dual cyclooxygenase/lipoxygenase inhibitor, phenidone. The injection of *S. gregaria* nymphs with, phenidone exhibited significant reduction in microaggegation in response to the nematode injections. At 12h post-injection, insignificant differences were recorded of the individual inhibitor on microaggregation, compared to the ethanol-treated (control) nymphs. Cellular defense components were strongly influenced by parasitation within the first 12h after injection of the nematodes. Nymph's haemolymph was assayed.

**Keywords:** Parasitizm, Locust, Entomopathogenic Nematode

## INTRODUCTION

Entomopathagenic nematodes of the families Steinernematidae and Heterorhabditidae have been used commercially as biocontrol agents of insect pests (Georgis *et al*., 1992) with a clear aim at the avoidance of environmental pollution and health hazards of chemical pesticides. After the infective juvenile nematodes enter their host insect through natural openings (mouth, anus or spiracles), they release bacteria into the insect haemocoel (Poinar and Thomas, 1966; Park and Kim 2000). The relatively rapid death of insect host (24-48 hours) and the wide host range of these nematodes have generated great interest in their use as biological control agents in integrated pest management systems (Woodring and Kaya, 1988; and Burnell and Stock, 2000). In the hamolymph, nematodes can be recognized and activate the defence system of insect host. Insects elaborate two broad categories of defense responses to bacterial infections: humoral and haemocytic (Gupta, 1986 and 1991). Humoral responses require several hours for their full expression and involve induced synthesis of anti-bacterial proteins. The detergent properties of these anti-bacterial proteins disrupt bacterial cell membranes. Insects also synthesize lysozymes enzymes that directly attack bacteria by hydrolyzing their peptidoglycan cell walls (Dunn, 1986).

Haemocytic responses feature direct cellular interactions between circulating haemocytcs and bacteria and typically occur within minutes after infections, rather than hours. Specific cellular defense mechanisms include phagocytosis, nodulation and encapsulation (Miller 1999 and Shairra 2000&2007). The aim of the present work was to elucidate the susceptibility of the locust *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) towards the nematode *Steinernema glaseri* by semi-filed trials. In addition, it was aimed to disrupt the immune reactions of *S. gregaria* in a trail to control this locust species by increasing its vulnerability towards the entomopathogenic nematodes. This disruption was achieved by using the dual cyclooxygenase/lipoxygenase inhibitor, phenidone (immune inhibitors). To ensure the effect of the immune inhibitors on the cellular immune reaction the time course microaggregation was determined.

## MATERIALS AND METHODS

Locust, *S. gregaria* fifth-instar nymphs were established from laboratory colony at the Entomology Department, Faculty of Science, Cairo University. Each weighed about 1.76 gm and was used in all experiments throughout the present work. The used insects were reared according to the method described by Hunter-Jones (1966). For mass culturing of the used nematode species *S. glaseri* was supplied by Dr. El-Sadawy, National Research Centre, Dokki, Giza, Egypt. Last instar larvae of the greater wax moth, *Galleria mellonella* were reared according to Sheble (2002) and used as host according to the methods described by Bedding & Akhurst (1975). The trials were conducted at Agricultural Research Centre, Biological Control Research Department.

**Susceptibility of *Schistocerca gregaria* 5$^{th}$ instar nymphs to *Steinernema glaseri*.**

To investigate the susceptibility of *S. gregaria* 5$^{th}$ instar nymphs towards the entomopathogenic nematode species, *S. glaseri*, nymphs (n = 30) were allowed to feed for 48 h on 600 gm fresh clover, *Trifolium alexandrinum* leaves that were sprayed with a suspension of IJs in distilled water at rate of 2000, 1500 and 1000 IJs/ml. The infected nymphs were then transferred to clean cages of 1x0.5x1 m containing fresh clover leaves as food devoid from any nematode suspension. Cages were also supplied with about 360 gm of dried autoclaved beach sand moistened with 54 ml distilled water (RH: 65 $\pm$ 5%) and were then kept at 30 $\pm$ 2°C. Equally repeated control treatments were conducted using clover leaves without nematode suspension at each experiment. The number of cadavers was recorded after 24, 48, 72 and 96 h post-infection. The accumulative mortality percentages of the host insect were calculated.

**Effects of both parasitic nematode and the dual cyclooxygenase/lipoxygenase inhibitor, phenidone, against *S. gregaria* nymphs.**

Three wood cages (1x0.5x1m), were supplied with about 10 cm depth dried and autoclaved beach sand moistened with distilled water at photoperiod of 12 h/day. The relative air humidity was 60-70%. Cages were kept at 32 $\pm$ 1°C. About 30 fifth instar nymphs of *S. gregaria* were kept in each cage. First cage, contained *S. gregaria* nymphs fed on clover leaves *T. alexandrinum* which was contaminated with *S. glaseri* using sprayer of nematode suspension about 1500 IJs/ml. Second cage contained *S. gregaria* nymphs fed on clover leaves contaminated with the inhibitor, phenidone at the rate of 0.05 mg/2ml. After 24 h, nematode suspension (1500 IJs/ml) was sprayed on clover leaves. Control cage, contained *S. gregaria* nymphs fed on

clover leaves sprayed with distilled water only. The accumulative mortality was observed after 24, 48, 72 and 96 h.

**Haemolymph volume**

The haemolymph volume of *S. gregaria* 5th instar nymphs was determined for insects exposed to nematode at the rate of 200 IJs/ml and nymphs pre contaminated with (0.05 mg/ml) of the dual cyclooxygenase/lipoxygenase inhibitor, phenidone for 24 h before exposure to nematodes. Determination used the amaranth dye dilution method of Lee (1961) and modified by Gillespie *et al*. (2000). Control nymphs were treated with distilled water only. To measure the haemolymph volume, the tested nymphs were weighed individually and injected with 25 µl of 2% (W/V) amaranth in saline solution (0.140 M NaCl; 0.010 M KCl; 0.004 M $CaCl_2$; 0.004 M $NaHCO_3$; 0.006 M $NaH_2PO_4$; 0.090 M Glucose, pH 6.8). The insects were injected dorsoventrally in an anterior direction between the 3rd and 4th abdominal segments using a microsyringe. A 5 µl sample of haemolymph was collected periodically from each treated and untreated nymph in microcap (Camlab) capillary tubes from punctures in the arthrodial membrane of a hindleg. Samples were diluted to 1 ml saline and the absorbance was determined using a spectrophotometer at 520 nm. A 5µl of haemolymph was taken before injection and diluted to 1ml to minimize the errors due to blood inclusions. Haemolymph volume was determined from at least 10 insects.

The blood volume (V) was calculated according to the following equation:

$$V = (dg_1 / g_2 – a)$$

Where $g_1$: weight of dye injected, $g_2$: weight of dye in the sample, d: volume of sample and a: volume of saline injected with the dye.

**Inhibition of haemocyte microaggregation.**

**Effect of nematode injection in relation to inhibitor presence.**

Individuals in three groups of test nymphs were injected with one of the the dual cyclooxygenase/lipoxygenase inhibitor, phenidone as 4 µg dissolved in 2 µl of 95% ethanol. Control nymphs were injected with 2 µl of ethanol (95%). Three to ten min after injection of the inhibitor, the nymphs were injected with five individuals of infective juvenile nematodes (Shairra 2007). At 12 h post injection, the nymphs were anesthetized on ice and haemocyte microaggregations were assessed.

**Assay for microaggregation**

Haemocyte microaggregation in the nematode-infected nymphs was assessed at a standard time of 12 h post-infection. Nymphs were anesthetized by chilling on ice and haemolymph was collected by pericardial puncture using Teflon-lined needles (Horohov & Dunn, 1983 and Gunnarsson & Lackie, 1985). Ten µl of haemolymph was collected, mixed with 80 µl of diluting solution (NaCl, 4.65 g ; KCl, 0.15 g; $CaCl_2$, 0.11 g ; crystal violet, 0.05g and acetic acid, 1.25 ml/liter distilled water). A 20 µl of diluted haemolymph was applied to a haemocytometer (AO instrument Co., Buffalo, NY). The haemocytes were allowed to settle for 3 min then a cover slip was applied. The number of cellular microaggregates in each sample was determined by counting four large fields in the haemocytometer using a phase-contrast optics. Numbers of microaggregates were normalized to microaggregates/ml haemolymph. Only haemocyte clusters containing ten cells (about 100 µm) or more were considered microaggregation *in vivo* (Jurenka *et al*., 1997).

**Study of haemocytes**

The haemolymph was smeared on clean glass slide after 12 h post infection. A drop of haemolymph was collected directly onto clean glass slides. The smear was allowed to dry for 1 min then fixed for 2 min with drops of absolute methyl alcohol. Fixed cells were then stained with Giemsa solution (Sigma) and mounted in Canada balsam (Sigma) then the slides were covered with slips and examined microscopically, with Eitz Wetzlar microscope.

**Statistical analysis**

The data presented in percentage values in the present study were used to estimate of $LD_{50}$ and $LD_{90}$ values using a software package "Ldp-line" (Copyright by Ehab, M. Bakr, Plant Protection Research Institute, ARC, Giza, Egypt). The significance of various treatments was evaluated by Duncan's multiple range test ($P \leq 0.05$). All analyses were made using "COSTAT", software package a product of Cohort Software Inc., Berkeley, California.

## RESULTS AND DISCUSSION

**Susceptibility of *S. gregaria* nymphs to the nematodes, *S. glaseri*.**

The efficacy of the entomopathogenic nematode *S. glaseri* on *S. gregaria* 5$^{th}$ instar nymphs is presented in Table 1. The nymphs were fed on clover leaves for 48 h to nematode species at different doses of 2000, 1500 & 1000 IJs/ml separately. Statistical analysis of the accumulative percentage mortalities of *S. gregaria* nymphs revealed a significant increase by increasing the IJs doses in a comparable manner for the tested nematode species. The data in Table1. revealed that, $LD_{50}$ recorded 1505.13, 393.16 and 812.63 over the exposure periods of 48, 72 and 96 h, respectively. On the other hand, $LD_{90}$ recorded 9478.5, 5047.0 and 1274.2 over the exposure periods of 48, 72 and 96 h, respectively.

Table 1: Susceptability of *Schistocerca gregaria* 5$^{th}$ instar nymphs to *Steinernema glaseri* over time.

| (Time) | $LD_{50}$ | $LD_{90}$ |
|---|---|---|
| 48 | 1505.13 | 9478.5 |
| 72 | 393.16 | 5047.0 |
| 96 | 812.63 | 1274.2 |

From the present work, it was evident that *S. gregaria* nymphs were comparably susceptible to the tested nematode *S. glaseri* with significant different levels of potencies depending upon IJs dosage. Coincided results were also observed by Shairra (2007) she working on *S. gregaria* nymphs was comparably susceptible to the two tested nematodes *Heterorhabditis bacteriophora* (RM1) and *S. glaseri* with significant different levels of potencies depending upon IJs dosage. Generally, the host mortality percentage of was positively correlated with the nematode IJs dose. El-Bishry *et al*. (2002) found that the lethality of *Heterorhabditis* spp. towards *Corcyra cephalonica* larvae was dose dependant. According to Shairra (2000) the mortality percentages of some lepidopterous larvae increased with the increase of IJs dose of each of *H. indicus* and *H. bacteriophora* nematodes. The dose-dependant mortality rate may be due to the fact that, the success of initial penetration of some infective juvenile individuals opens pathways for other IJs. The present results revealed an obvious low percent mortality of *S. gregaria* nymphs at 48h of the natural infection of the nematode *S. glaseri.* This may be due to the low penetration rate

and/or the high immune reactions of *S. gregaria* nymphs towards infection with *S. glaseri.* To eliminate the factor of penetration difficulty of *nematode S. glaseri* into nymphs of *S. gregaria*, the subsequent experiments concerning the immune responses were carried out.

**Effects of both parasitic nematode and an inhibitor against *S. gregaria* nymphs.**

The present results revealed increased mortality percent with time increased of *S. gregaria* nymphs at 48, 72 and 96 h post infection with the nematode *S. glaseri.* On the other hand, this mortality percent increased in parable results when nematodes were combined with the dual cyclooxygenase/lipoxygenase inhibitor, phenidone 71.04% mortality compared to nematode infection 45.21% mortality at 48h (Fig.1) may be due to the inhibition of some immune reactions of *S. gregaria* nymphs. So, to eliminate the factors which affected of *S. gregaria* nymphs to *nematode S. glaseri* and its stimulated bacteria *Xenorhabdus nematophilus*, the subsequent experiments concerning the immune responses were carried out. The high efficacy of the entomopathogenic nematodes against locusts and the indications for phagocytosis-inhibiting factors released from the nematode-associated bacteria are the main results of this study. Ghally (1988) applied *Steinernema feltiae* nematodes to *S. gregaria* fifth instar nymphs by intrahemocoelic injection of infective juveniles, suggesting that the natural route of infection could not be established.

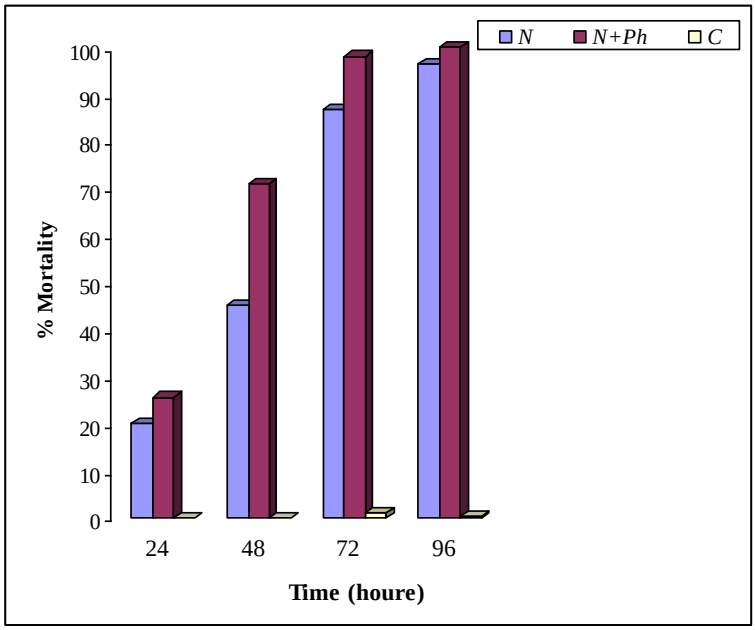


Fig.1: Effect of nematode and nematode-inhibitor against *S. gregaria* nymphs.
Test nymphs were infected with nematodes (*N*), phenidone-treated nymphs pre-infected with nematode (*N+Ph*) and control nymphs(*C*).

The data presented showed a strong consideration of entomopathogenic nematodes as potentially successful parasite in locust control. Voss and Dreiser (1992) suggested that with improved methods for mapping locust breeding areas researchers will be allowed to concentrate future control programs on suitably moist habitats. For most locust species the development of ‘‘outbreaks’’ is linked to preceding rainfall or floods resulting in favorable conditions not only for locust breeding (Abraham *et al.*, 1991; Barrass, 1974) and crops but also for nematode survival. Additionally, modified nematode formulations like sodium alginate capsules and alfalfa wheat pellets or the use of rehydratable, desiccated infective juveniles

(Wojcik and Georgis, 1988) may help to reduce the required amounts of water. Lower application rates could be reached by combining biotechnical and biological control methods. For instance, Parkman and Frank (1993) inoculated *Steinernema scapterisci* into female crickets by using nematode-equipped sound traps. Also, the present study may be help of application methods using combining biotechnical and biological control methods to control locusts or grasshoppers.

**Haemolymph volume**

Haemolymph was collected from the 5$^{th}$ instar nymphs of *S. gregaria*, which were infected with dose of 200 IJs/nymph of the nematode suspension, *S. glaseri* as described. The haemolymph volume of the nematode infected nymphs and nematode treated nymph pre infected with standard dose of the dual cyclooxygenase/lipoxygenase inhibitor, phenidone (0.05mg/ml) was declined from about 232 µl haemolymph/nymph in the control insects to approximately 150 µl haemolymph/nymph for the nematode infected ones. Also, the combination of nematode with the dual cyclooxygenase/lipoxygenase inhibitor, phenidone recorded approximately 178 µl haemolymph/nymph which also decreased from control insects at a standard time of 12 h post-infection. A decrease in the haemolymph volume of *S. gregaria* nymphs was observed in all treatment as a response to the nematode *S. glaseri* infection. This was accompanied with the high number of nodules observed in different body segments. Similarly Lee (1961) detected an insignificant change in the blood volume of *S. gregaria* and *Locusta migratoria* nymphs, after injection of *Bacillus thuringiensis.* Whereas, more or less significant decrease was observed in the adult stage. Also, Gillespie *et al*. (2000) detected a significant decrease in haemolymph volume of mycosed *S. gregaria* challenged with the fungus, *Metarhizium flavoviride*.

**Influence of inhibitor and nematode on microaggregation over time.**

The ethanol-treated (control) nymphs produced a moderate change in haemocyte microaggregation at each time point. It was 54.83x $10^2$m/ml haemolymph at 2 h post-injection, and increased to 244.8x$10^2$m/ml haemolymph at 12 h post-injection. At 18 and 24 h post-injection, it decreased to 149.5x$10^2$ and 140.8 x $10^2$ m/ml haemolymph, respectively (Table 2). Comparable results to the ethanol-treated (control) nymphs were obtained with the nematode-injected nymphs. The haemocytic microaggregation increased significantly by increasing the post-injection period reaching a maximum value of 656.33x$10^2$m/ml haemolymph at 12 h post- injection. They declined being 448.6 x $10^2$ and 316.0 x $10^2$m/ml haemolymph at 18 and 24 h, respectively post-injection, respectively. In case of the dual cyclooxygenase/ lipoxygenase inhibitor, phenidone-nematode-treated nymphs, the number of haemocyte microaggregates insignificantly changed throughout the next 24h post-injection in comparison with the ethanol-treated nymphs. Whereas haemocytic microaggregates decreased significantly in comparison with that of the nematode injected nymphs at each time point, being 210.2x102m/ml haemolymph at 2h post-injection, 301.2x102m/ml haemolymph at 6h post-injection, and 360.0x 102m/ml haemolymph at 12 h post-injection. They then decreased reaching 155.75x 102m/ml haemolymph and 180.23 x102m/ml haemolymph at 18 and 24 h, respectively post-injection (Table 2). The dual cyclooxygenase/lipoxygenase inhibitor, phenidone reduced the formation of microaggregates in locust nymphs (Table 2). Similar results were obtained by Miller *et al*., 1996 & 1999 and Shairra, 2007. They observed that, the inhibitory effects of the phospholipase A2 inhibitor, dexamethasone, on nodulation were apparent 1 h after infection in larvae of the tenebrionid beetle, Zophobas atratus and in adults of the cricket, Gryllus assimilis; also, in locust, S. gregaria nymphs within 2h of injection, and nodulation was significantly reduced, relative to control tenebrionid beetle, over 24h. and over 22 h to control crickets.

Table 2: Mean microaggregate x $10^2$/ml haemolymph of *S. gregaria* nymphs over time post injection.

| Time(hr) | Control | Nematode | phenidone |
|---|---|---|---|
| 2 | 54.83±1.208[a] | 314±8.151[c] | 210.25±3.350[b] |
| 6 | 165.5±5.777 [a] | 341±18.453 [c] | 301.25±9.068[b] |
| 12 | 244.8±8.261[a] | 656.33±33.377 [c] | 360±6.670 [b] |
| 18 | 149.5±5.038 [a] | 448.66±19.156[b] | 155.75±14.840[a] |
| 24 | 140.8±3.333 [a] | 316.01±12.136 [c] | 180.23±11.331[b] |

# Means followed by the same letter in the same raw are not significantly differents (P > 0.05).

## Studies of haemocytes:

Our study reveled that, Prohemocytes are small, round cells that may be precursors from which some other cell types develop. Granular hemocytes contain conspicuous cytoplasmic granules that can be discharged as part of a defensive response to invading parasites. Plasmatocytes usually contain few granules and are characterized by their ability to change from round or spindle-shaped cells in suspension to extensively flattened, ameboid cells after attaching to a substrate. Spherule cells contain very large cytoplasmic granules, which may contain mucopolysaccharides. Oenocytoids are large cells that synthesize prophenoloxidase (Fig. 2). Using the classification of Gupta (1979) and Subejio. Paijo. (2010). Five different haemocyte types were distinguished in Giemsa stained preparation of blood film of the fifth instar nymphs of *S. gregaria* .These types were identified as Granulocytes (G), Plasmatocytes (P), Prohemocytes (Pr) , Oenocytodis (O) and Spherulocytes (S ). Plasmatocytes and granular hemocytes are usually the two most abundant haemocyte types, although their proportions can vary between species and within a species at different developmental stages. Hemocytes, especially plasmatocytes, also aggregate in a type of coagulation response, sealing wounds to prevent haemolymph loss. Another function of hemocytes is in synthesis of the extracellular matrix that covers tissues exposed to the haemolymph. Granular hemocytes appear to be the primary cell type involved in this aspect of haemocyte function.

Haemolymph serves important roles in the immune system and in transport of hormones, nutrients, and metabolites. Hemolymph of many insects contains lysozyme, an enzyme that degrades bacterial cell walls. In addition, low-molecular-weight antimicrobial peptides are synthesized in response to bacterial. Shairra (2000).

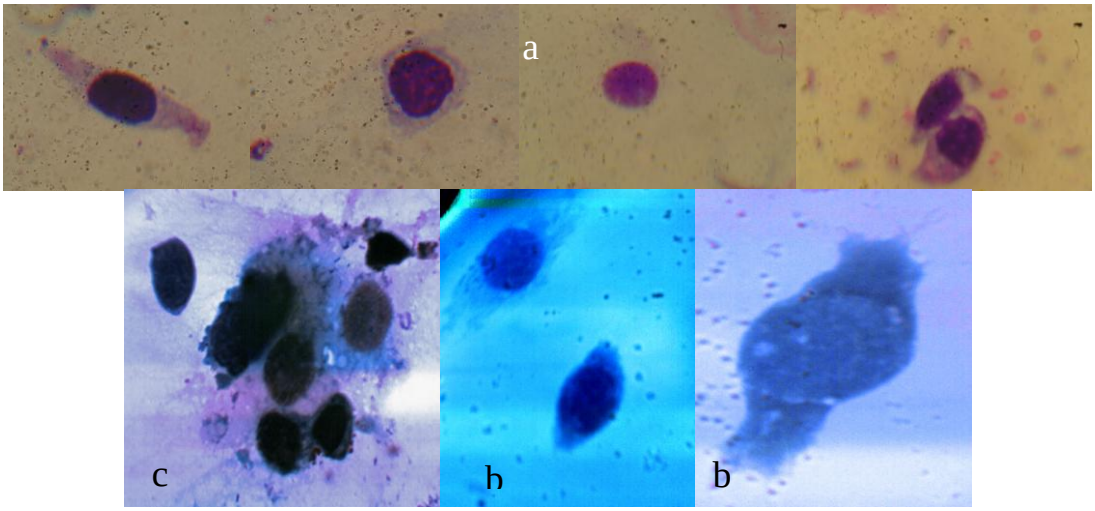


Fig. 2: Different types of *S. gregaria* haemocytes (fifth instar nymph).

a- Control of 5th instars' nymph of *S. gregaria* haemocytes Phase-contrast appearance of main types of hemocytes, prohemocytes, elongated plasmatocyte; granulocyte; and spindle shape prohemocytes. X1000.

b- Plasmatocyte of *S. gregaria* after 12h. Infected with nematodes *S. glaseri*. x1000.

c- Aggregation showed of some types of hemocytes of *S. gregaria* after 12h. post infection with nematodes *S. glaseri* and its released bacteria. x1000.

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## ARABIC SUMMARY

### التطفل على الجراد بواسطة النيماتودا الممرضة مع تثبيط التجمعات الدموية للحشرة

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تستهدف الدراسة تقييم حساسيه العمر الخامس من الجراد الصحراوي شيستوسيركا جريجاريا (فورسكال) للإصابة بسلالة من النيماتودا *Schistocerca gregaria* التابعة لعائلة الاشتينرنيماتدي كمثال لقياس حساسية العمر الخامس للجراد الصحراوي عن طريق التعرض الطبيعى للنيماتودا.

قد وجد خلال التجربة ان نسبة الموت للحشرات قد إزدادت زيادة معنوية كلما إزدادت الجرعات المختبرة ( 1000، 1500، 2000 فرد نيماتودي لكل ميللي من المياة المقطرة).

قد تم اختيار الجرعة المناسبة للحقن وهي 5 فرد من الاطوار الشابة للنيماتودا بعد 12 ساعة من الحقن وذلك لدراسة ردالفعل المناعي لحوريات الجراد شيستوسيركا جريجاريا.

عند دراسه تأثير تغيير الوقت على التجمعات الدموية بعد الحقن بالنيماتودا فقد سجلت النتائج المتحصل عليها ان هناك زيادة معنوية في تكوين تلك التجمعات الدموية اثناء الساعات الأولي للحقن واستمرت الزيادة حتى 12، 18 ساعة بعد الحقن، بينما أظهرت التكوينات للتجمعات الدموية نقصانا معنويا عند مقارنه حقن الحشرات المعاملة بالنيماتودا والفينادون (مركب الفينادون المثبط المزدوج لكل من انزيمي السيكلواوكسي جينييز والليبوكسى جينييز على تكوين التجمعات الدموية) بالحشرات الأخري المعاملة بالنيماتودا فقط.

ومن تلك النتائج يتضح ان مركب الفينادون قد يكون له دور فعال في تثبيط التجمعات الدموية للخلايا التى تقوم حوريات الجراد الصحراوي بتكوينها كرد فعل مناعي للحقن بالنيماتودا *Schistocerca gregaria*.

عند دراسه انواع خلايا الدم لحوريات الجراد الصحراوي، وجد خمس أنواع مختلفه من خلايا الدم هي:
Granulocytes,plasmatocytes,prohemocytes, oenocytodis and spherulocytes.
وجد ان بعض الخلايا مثل plasmatocytes and Granulocytes لهم دور هام جدا في مناعه الحشرة.