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Laboratory evaluation of the effects of neem (*Azadirachta indica)* oil and *Metarhizium anisopliae* against some immature stages of the desert locust *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae)

Ebtisam M. Bashir[1] and Hamadttu A.F. El Shafie[2]

1. Assistant Professor and Head, Department of Plant Protection and Environmental Studies. Faculty of Agriculture, Al Zaiem Al Azhari University, Khartoum North, Sudan.

2. Department of Crop Protection. Faculty of Agriculture. University of Khartoum, Khartoum North, Sudan.
* Corresponding Author e-mail: ebtisammb@hotmail.com
Tel. No: +249 912185581
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Abstract

The biological activity of neem (*Azadirachta indica A. Juss*) oil and the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes), and their combination, were evaluated under the conditions of the Insectary of the Department of Crop Protection, Faculty of Agriculture, University of Khartoum against the 3rd, 4th and 5th nymphal instars of the desert locust (*Schistocerca gregaria*). The test products were used at concentration of 8 ml/L water (for neem oil) and 25 g powder/L diesel (for the fungus), and for combination treatment (8 ml neem oil plus 25 g of *Metarhizium anisopliae*). All products were applied topically on the test insects using Ultra low volume (ULVA+) spraying. Mortality, body weight, developmental time and morphological anomalies were used as parameters to evaluate the efficacy of each product. The suspension of *M. anisopliae* caused 90, 58.9, and 98.8% mortality on the 3rd, 4th and 5th nymphal instars, respectively, during the period from the 7th to the 11th day post-treatment. The neem oil had shown strong antifeedant action against the various instars, and a mortality rate of 32.9, 39.9 and 80% on the 3rd, 4th and 5th instars, respectively. Also, there was a delay in the development period, in addition to deformation and moulting failures.

Keywords: Desert locust, biocontrol, Metarhizium, Plant extract, body weight, mortality

Introduction

The desert locust *Schistocerca gregaria* (Forskal) is regarded as a major pest in Africa that causes serious damages to agricultural crops and vegetation. In the years of major plagues, swarms of the desert locust may attack crops grown in areas extending from West Africa to India (Steedman, 1988). The desert locust is considered as the most important species of all locusts because it forms huge swarms in the gregarious phase that cover about 29 million km^2. Such swarms affect 57 countries in Africa and Asia with an area equivalent to 20% of the land surface of the world (Steedman, 1988; Meinzingen, 1993). Sudan is an important breeding area where breeding takes place in summer and winter.

The Desert locust is a polyphagous pest, although it prefers plants belonging to the Family Poaceae which includes all the major staple food crops in locust affected countries. The actual losses caused by desert locust in various countries are not available. It is estimated that during 1988 the total grain losses caused by locust in Mauritania, Niger, Chad, Sudan, Ethiopia, Senegal, Gambia and Mali were 24,000 tones, i.e. almost 1.3% of the total grain production in these countries (Stein *et al*., 1989).

The control of the desert locust depends on application of chemical pesticides which proved to be highly effective; however, these compounds are more expensive and affect a wide range of non-target beneficial organisms as well as being hazardous to the operators and contribute to environmental pollution. To help reduce these drawbacks of chemical pesticides, entomologist search for other safer and efficient alternative methods for locust control. These alternative control agents may include entomopathogenic fungi such as *Metarhizium anisopliae* var. *acridum* (Merschnikoff), which is named the green muscle and a widely distributed soil-inhabiting fungus works only against the locusts and grasshoppers (Lomer *et al*, 2001). It has been reported to infect approximately 200 species of insects and other arthropods and it is not infectious or toxic to mammals and non-target organisms (Anon, 1999).

Botanical insecticides from the neem tree (*Azadirachta indica* A. Juss) is also effective against locust and act best as insect growth regulators and potent repellent agents. Azadirachtin, which was identified as the primary active pesticidal ingredient in neem oil, affects insects in different ways. Compared to other insect species, *S. gregaria* seems to be extremely sensitive to azadirachtin (Schmutterer, 1990). However, the development and implementation of effective preventive control strategies for desert locust remain a major challenge.

The present study adopted some of the available alternative (safer) forms of control that may reduce the reliance on broad spectrum chemicals. It was carried out under laboratory conditions at the Department of Crop Protection, Faculty of Agriculture, University of Khartoum, during the period from May to December, 2004. The main objective was to study the biological effects of the oil suspension of entomopthogenic fungus *Metarhizium anisopliae*, the neem seed oil extract, and their combination against some

Materials and Methods

Mass rearing of desert locust

Immature gregarious adults of the desert locust *Schistocerca gregaria* (Forskal) were obtained from the field of the International Centre of Insect Physiology and Ecology (ICIPE) in Port-Sudan. Two different types of cages were used for mass rearing and egg laying. The rearing cage measured 75 cm x 45 cm x 45 cm and was used for the purpose of mass production of desert locust. The cage was made of iron rod frames covered with light cloth. The second type of cage, which was used for egg laying, measured 120 cm (length) by 100 cm (width) by 100 cm (height) and was constructed from wooden frames having three faces covered with wire mesh and two faces covered with fine cloth, while the bottom board was made of plywood and divided into three divisions. The first and third divisions, each 40 cm wide, were covered with plywood. The second middle division, 20 cm wide, was covered with wire mesh. Twenty holes were made in the first and third divisions to accommodate the plastic oviposition cups (7.5 cm diameter and 11 cm deep) which were filled with sandy soil for egg-laying. The sand used for egg-laying was sieved through a wire mesh (2

mm^2) and moisted daily with tap water to maintain an optimal moisture level for egg-laying. The cups containing egg-pods were removed and replaced by fresh ones for further egg-laying. The locusts were fed ad libitum on fresh leaves of millet seedlings supplemented with wheat bran. The cages were daily cleaned from the dead locusts, feces and unconsumed dried seedlings. They were also checked for damaged legs, wings…etc. and provisioned with fresh food. Approximately equal numbers of male and female adults were put in each cage (30 for each sex).

The extraction process

Collection of neem seeds

Ripened fruits of neem which had dropped to the ground, were collected at Shambat area. The seeds were washed with tap water and shade-dried for 3-4 days on a jute sack. . Seeds obtained after decortications were ground to a fine powder in an electric blender. The neem seed powder thus obtained was kept in a glass vial and used for extraction as described below:

Neem seed oil extract

Neem oil was extracted from the powder which was prepared as mentioned above by using cold pressing method (by hand). The preparation was used at rate of 8ml/litre water, with liquid soap used as emulsifying agent.

Preparation of fungal spores suspension

The commercial powder of *Metarhizium anisopliae* var. *acridum* (green muscle (GM) biopesticide), Strain F_1-985 was obtained from the International Centre of Insect Physiology and Ecology (ICIPE) in Port-Sudan. It was used at a rate of 25 g powder mixed with one liter of diesel oil.

Equipment and spraying technique

The micron sprayer with the trade mark ULVA + (Ultra-low-volume with 6 batteries) was used for spraying. This sprayer is a hand-held with spinning disk. It consists of a plastic tank connected to a feed nozzle. The capacity of the sprayer is one litre of the liquid. The tank of the sprayer was washed each time with tap water and soap thoroughly rinsed before filling any test product.

The experiments

The set of experiments were carried out in the Insectary of the Department of Crop Protection, Faculty of Agriculture, University of Khartoum, during the period extending from May to December, 2004. All the tests were conducted under the prevailing temperature of the insectary. The third, fourth and fifth nymphal instars of *Schistocerca gregaria* (Forskal) were used as test insect at different growth stages to evaluate the efficacy of the prepared treatments of the experiments. All experimental insects were kept in cage designed for the purpose. The experimental cage (25 cm x 25 cm x 25 cm) had 4 faces covered with a wire mesh. One face was covered with cloth sleeve made in the form of a tube which facilitated cleaning, feeding and handling of treated insects. The bottom of the cage was covered with plywood.

The cages were cleaned daily and fresh food was provided for the insects. The stands of the cage were put on container filled with water to keep away ants from invading the cages. From each instar, 150 nymphs were sprayed and allowed to dry for 2 minutes before being transferred to the experimental cage in groups of 10 nymphs with replicates for each treatment. Each treatment was replicated three times and the experimental units were arranged in a completely randomized design.

The different treatments tested in these experiments were as follows:

1. 8 ml neem oil in 1 litre water.

2. 25 g powder of the spores of the fungal *M. anisopliae* formulated in one litre of diesel oil.
3. 8 ml neem oil mixed with 25 g *Metarhizium anisopliae* spores powder in one litre of diesel oil.
4. Control treated with diesel oil only.
5. Untreated control.

The parameters such as mortality, body weight and developmental period were used to measure the performance profile of each test product.

The effect of test products on the nymphal instars

Three sets of experiments were carried out to study the biological activities of the neem preparations and the *Metarhizium anisopliae* on the 3rd, 4th and 5th inst ars of the pest, respectively. The products were applied topically according to the method used by Gillet (1973). The application of all treatments was made in the morning.

The outgrowth of the *M. anisopliae* after treatment

Ten nymphs from each instar (3rd, 4th, 5th), after death, were maintained in sterilized Petri-dishes to observe the time period of the appearance of the green outgrowth of the *Metarhizium* spores.

Statistical analysis

The data were subjected to analysis of variance (ANOVA). Transformation of mortality data were done, using arcsine table from Gomez and Gomez (1984). The least significant difference (L.S.D.) was used to compare the means.

Results

Mortality effects of treatments

The effect on 3rd instar

Table 1 shows the mortality effects of neem oil and *M. anisopliae* applied separately and in combination on the 3rd instar of the pest. On the 3rd and 4th days after treatment, there were no significant mortality differences between all treatments. On the other hand, highly significant **post treatment** mortality differences between the neem oil and neem oil + *Metarhizium* were obtained on the 5th, 6th and 7th days. There was also a significant mortality difference between neem oil + *Metarhizium* and the control. On the 6th and 7th days there were no significant differences between the *Metarhizium* and *the Metarhizium* + neem oil combination. The highest mortality of the 3rd instar nymphs of the desert locust (90.07%) gave by *Metarhizium* while the lowest mortality (32.9%) acheaved with Neem oil (Table 1).

Table 1: Mean percentage mortality of the 3rd instar nymphs of the desert locust *Schistocerca gregaria* treated topically with neem oil and *Metarhizium anisopliae.*

Treatment		Days after treatment				
		3rd	4th	5th	6th	7th
Control (O)	P	0.00 a	0.00 a	0.00 c	1.3 c	2.6 c
	T	0.57	0.57	0.57	6.53	9.23
Control oil	P	0.00 a	0.00 a	0.00 c	4.7 bc	6.9 bc
	T	0.57	0.57	0.57	12.48	15.19
Metarhizium	P	1.3 a	3.9 a	29.7 ab	68.7 a	90.0 a
	T	6.53	11.45	33.00	56.00	71.56
Neem oil	P	0.00 a	0.00 a	9.5 bc	26.5 b	32.9 b
	T	0.57	0.57	17.90	30.99	35.01
Mixture	P	1.3 a	9.5 a	39.9 a	73.8 a	83.7 a
(M+No)	T	6.53	17.90	39.15	59.22	66.15
L.S.D.		NS	NS	**	**	**
		11.87	19.64	20.05	18.51	25.28
C.V.%		221.19	173.48	42.47	21.65	24.78

Means followed by the same letters are not significantly different at P< 0.05, according to LSD test.
P = The percentage of dead insects noticed, T = The data transformed by arcsine transformation.
L.S.D. = Least significant difference, C.V. = Coefficient of variations, ** = Difference significant at the 0.01 level (P< 0.01).

The effect on 4th instar

There were no significant differences between all treatments except for the 8th day after treatment. There was a highly significant mortality difference between the treatments and control. The highest and lowest percentage mortality 76.4 and 39.9 achieved with mixture and neem oil respectively (Table 2).

Table 2: The percentage mortality among the 4th instar of *S. gregaria* sprayed with neem oil and *M. anisopliae*.

Treatment		Days after treatment					
		3rd	4th	5th	6th	7th	8th
Control (O)	P	0.00 a	4.7 a	6.9 a	6.9 a	6.9 a	6.9 c
	T	0.57	12.48	15.19	15.19	15.19	15.19
Control oil	P	0.00 a	9.0 a	14.2 a	28.4 a	28.4 a	28.4 bc
	T	0.57	17.41	22.12	32.22	32.22	32.22
Metarhizium	P	4.7 a	9.0 a	25.0 a	29.2 a	36.0 a	58.9 ab
	T	12.48	17.41	30.00	32.71	36.85	50.15
Neem oil	P	0.00 a	4.7 a	20.00 a	20.00 a	26.2 a	39.9abc
	T	0.57	12.48	26.56	26.56	30.78	39.15
Mixture	P	11.8 a	28.4 a	38.0 a	46.0 a	56.8 a	76.4 a
(M+No)	T	20.11	32.22	38.07	42.70	48.93	60.92
L.S.D.	P	NS	NS	NS	NS	NS	**
	T	16.35	24.73	26.33	20.94	20.64	28.57
C.V.%		131.35	73.89	49.26	38.54	34.60	27.94

Means followed by the same letters are not significantly different at P< 0.05, according to LSD test.
P = the percentage of dead insects noticed, T = The data transformed by arcsine transformation.
L.S.D. = Least significant difference, C.V. = Coefficient of variations, ** = Difference significant at the 0.01 level (P< 0.01).

The effect on 5th instar

Table 3 summarizes the effect of topical application of the test products on the 5th nymphal instar. On the 3rd, 4th and 5th days, there were no significant mortality differences between all treatments. However, on the 6th day there was significant difference between the mixture of *Metarhizium* and neem oil (which caused 26.2% mortality) and the untreated control (where there was zero mortality). On the 7th day, no significant difference was observed between treatments, but a significantly different effect was recorded between the mixture and the untreated control. On 8th and 9th day, there were significant difference between the treatments, and highly range of percentage mortality amongenymphs (71.6% -92.4%) achieved with mixture. However on 10th and 11th days there were no significant difference between treatments were observed (Table 3).

Table 3: The percentage mortality among the 5th instar of *S. gregaria* sprayed with neem oil and *M. anisopliae*.

Treatment		Days after treatment								
		3rd	4th	5th	6th	7th	8th	9th	10th	11th
Control (o)	P	0.00a	0.00a	10.0a	0.00b	13.0b	15.7bc	19.3bc	9.3b	9.3b
	T	0.57	0.57	18.44	0.57	21.15	32.36	26.07	26.07	26.07
Control oil	P	4.7a	9.5a	9.5a	9.5ab	9.5b	9.5c	11.8c	17.0b	11.8b
	T	12.48	17.90	17.90	17.90	17.90	17.90	20.11	24.34	20.11
Metarhizium	P	4.7a	4.7a	19.3a	6.9ab	43.2a	60.1a	63.8a	92.4a	98.8a
	T	12.48	12.48	26.07	26.07	41.07	50.84	59.22	74.00	83.85
Neem oil	P	4.7a	10.0a	36.6a	19.3a	43.2a	53.4ab	63.6ab	80.0a	80.8a
	T	12.48	18.44	37.22	26.07	41.07	46.92	52.86	60.44	83.85
Mixture (M+No)	p	10.00a	10.0a	32.8a33	26.2a	43.2a	71.6a	92.4a	98.8a	98.8a
	T	18.44	18.44	4.92	30.78	41.07	57.78	74.00	83.85	83.85
LSD	P	SN	SN	SN	*	*	**	**	**	**
	T	14.54	14.81	15.84	18.38	17.31	26.41	28.95	33.21	27.84
C.V%		70.79	60.01	32.36	55.81	29.32	25.93	24.88	23.88	19.61

Means followed by the same letters are not significantly different at P< 0.05, according to LSD test.
P = The percentage of dead insects noticed, T = The data transformed by arcsine transformation.
L.S.D. = Least significant difference, C.V. = Coefficient of variations, ** = Difference significant at the 0.01 level (P< 0.01).

Effect of treatments on body weight of the tested insects

There was no significant difference in the body weight of the 4th and 5th nymphal instars treated with the different formulations. The treatments showed an effect which was not significantly different from the control (Figure1).

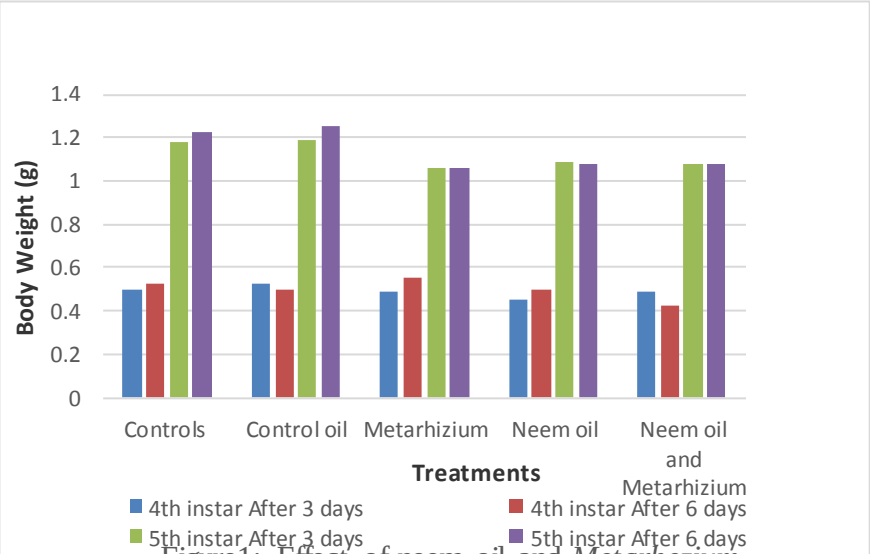

Figure1: Effect of neem oil and *Metarhezium anisoplae* on the body weight of the 4th and 5th instars of *S. gregaria*

Hormonal effect of neem oil on the tested insects

The effects of neem oil on the developmental period of the 3rd, 4th and 5th instars are depicted in Figure 2. It is clear that the neem oil treatment significantly increased the developmental period of various nymphal instars compared with the untreated control. Deformation in the wings and reduction of flight ability of the adults locusts that developed from treated nymphs were also observed.

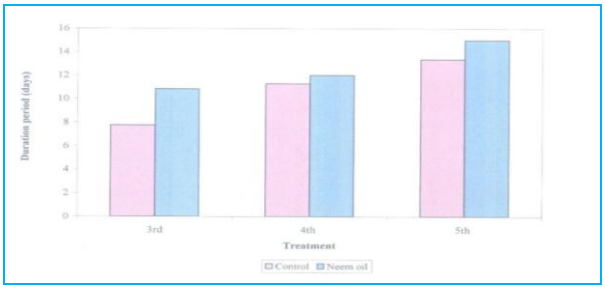

Figure2: The duration period 0f of 3rd, 4th and 5th instar after treatment with neem oil

Characteristics of Metarhizium infected nymphs

Before death, the nymphal instars of *S. gregaria* sprayed with *M. anisopliae* formulated in diesel oil showed characteristic bright red color (Plate 1). They were slowly incapacitated during this period and feeding and mobility were drastically reduced, compared to the healthy nymphs, after the 3rd or 4th day following treatment. The infected insects were observed clinging to or perching on the wire mesh of the experimental cage.

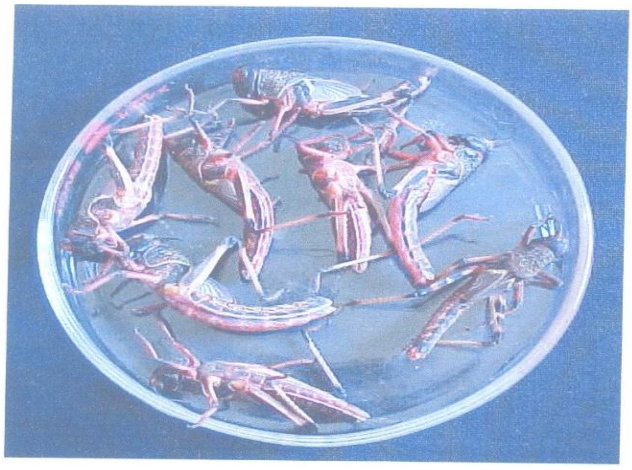

Plate 1: The red color of *Schistocerca gregaria* nymph after treatment with *M. anisopliae*

The outgrowth of *M. anisopliae* on infected nymphs

Plate 2 and Figure 3 show the different periods of time that elapsed from infection by *Metarhizium* and death till the appearance of fungal outgrowth (i.e., green muscle) on the 3rd, 4th and 5th nymphal instars of the desert locust.

Plate2. The outgrowth of green muscle *M. anisopliae* on the nymphs of *Schistocerca gregaria*

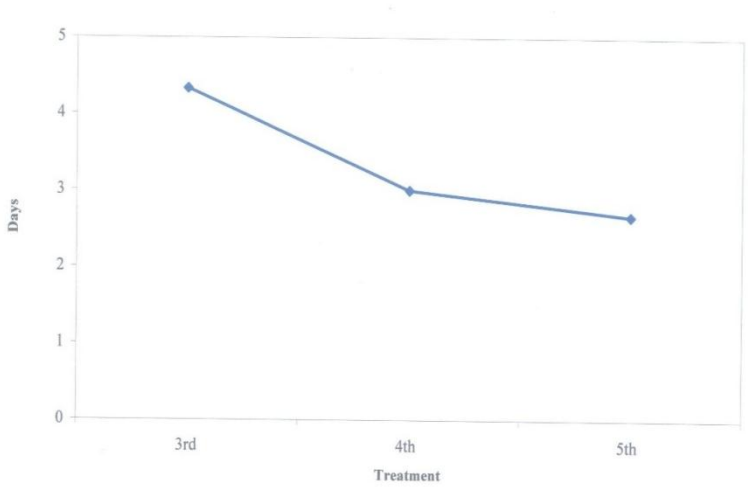

Figure3: The outgrowth of *M. anisopliae* on 3rd, 4th and 5th instar after treatment

Discussion

The recent developments in botanical insecticides indicated that the neem products are the best control agents, (Schmutterer, 1990; Ascher, 1983; Schmutterer *et al*, 1982). All parts of neem are biologically active and can be used in pest control, with seed kernel being the most potent. Extract and pure compounds derived from seed kernel have been found to affect the insects on different steps of host selection. Micro-organisms acting pathogenically on insects have long been known, their introduction in insect pest management has been of minor importance so far.

In this study, the mortality resulting from application of the neem oil was significantly high on the 3rd, 4th and 5th nymphal instars of *S. gregaria* where mortalities of (32.9%, 39.9%, and 80%) were recorded, respectively. These results are comparable with the findings of Schmutterer and Freres (1990) who reported that the neem oil pressed from neem seed and used at a rate of 0.08 to 0.1% were effective and caused 65-100% mortality on *S. gregaria*. Wilps *et al*. (1992) found that neem seed oil (0.5 percent active ingredient) at a rate of 1 L/h caused 8 to 22 percent adult mortality. Also, similar results were reported by Mordue and Blackwell (1993) who found that the desert locust *S. gregaria* is deterred in feeding bioassay at 0.001 ppm quantities of azadirachtin.

The neem oil caused significant delay in development and increased the nymphal period besides causing deformity among the test insects. Similar observations were made by Schmutterer (1990) and Nicol and Schmutterer (1991) they found that the delay in development is a typical effect of neem products, especially azadirachtin which regulates insect development. The application of *Metarhizium anisopliae* at 25 g powder/L diesel oil caused satisfactory rates of mortality in nymph of *S. gregaria* and nearly 100% mortality (58-98% mortality) was observed within 6-11 days after treatment. Similar results were obtained in a field test in Niger that was carried out with formulation of neem oil mixtures using ULV application

with rates of 1-2 L/ha and 2-5 x 10^{12} conidia per hectare against the variegated grasshopper. A reduction in the population of approximately 90% was obtained after 15 days from treatment (Prior and Streett, 1997). Also these results are comparable with the findings of Asghar and Port (2013) who reported that topical spray of *M. anisopliae* at concentration of 1.5 10^{8} spores/ml on the third nymph of the grasshoppers *Uvarovistia zebra* caused 43% mortality.

The combined effect of neem oil and *M. anisopliae* showed that they are compatible products. They caused knockdown effect when directly sprayed together on test insects, and the maximum mortality was achieved during the period from 6 to 11 days after application of the mixture. When the two products were applied at rate of 25 g + 8 ml/L, they caused significantly higher mortality (76.4-98.8%) than the control (2.6-19.3%). A synergistic activity against the desert locust was observed. *Metarhizium* could be used against the nymph populations of *S. gregaria*.

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التقييم المعملى لتأثير زيت النيم وفطر الميتاريزيوم على بعض الاطوار غير الناضجة للجراد الصحراوى

ابتسام محمد بشير[1]* و حمدتو عبدالفراج الشفيع[2]

1- استاذ مساعد. قسم وقاية النبات والدراسات البيئية. جامعة الزعيم الازهرى
2- حمدتو عبدالفراج الشفيع- قسم وقاية المحاصيل – جامعة الخرطوم
* المؤلف المراسل ebtisammb@hotmail.com

المستخلص

تمت دراسة تقييم الشاط الحيوي لزيت بذرة النيم وفطر الميتاريزيوم (*Metarhizium anisopliae*) كل على حدا او مجتمعين لمكافحة الطور الثالث والرابع والخامس للجراد الصحراوي *Schistocerca gregaria* تحت ظروف معمل تربية الحشرات بقسم وقاية المحاصيل جامعة الخرطوم. استخدم التركيز 8 مل/لتر ماء لزيت النيم و25 جرام لكل لتر ديزل لفطر الميتاريزيوم وخليط النيم و الميتاريزيوم بتركيز 8 مل + 25 جرام على التوالي. تم تطبيق هذه المستخلصات بالرش المباشر على الحشرات المستهدفة باستخدام الرشاشة ذات الحجم الديق. تم تقييم هذه المركبات من حيث قدرتها على قتل الحشرات وتأثيرها على الوزن وعلى فترة التطور لكل طور وإحداث تشوهات بعد الانسلاخ. المعلق الزيتي لفطر الميتاريزيوم كذلك اعطى نسبة موت 90، 58,9، و98,8% من اليوم السابع إلى الحادي عشر في الأطوار الثالث والرابع والخامس على التوالي. أظهر زيت النيم فعالية أقوي كمانع للتغذية وقتل الحشرات حيث كانت نسبة الموت في كل من الطور الثالث والرابع والخامس 32,9، 39,9 و80% على التوالي بالإضافة لتأثيره في زيادة فترة التطور لكل طور وإحداث التشوهات بعد الانسلاخ وحدوث موت أثناء الانسلاخ.