COMBINED EFFECT OF ENTOMOPATHOGENIC 
Steinernema SP. AND ENTOMOPATHOGENIC FUNGUS 
Metarhizium anisopliae ON THE DESERT LOCUST 
Schistocerca gregaria (FORSKAL)

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ABSTRACT

Combined effect of Steinernema sp. and entomopathogenic fungus 
Metarhizium anisopliae was investigated against desert locust, Schistocerca gregaria 
under laboratory conditions. Interaction of nematode and fungus isolates in vitro 
revealed that Steinernema sp. isolates SFN.27 and ATs inhibited conidiospores 
germination of isolates C1 and C2 of M. anisopliae while nematode isolate AT4 did 
not inhibit the germination of fungal isolate C3. S. gregaria 3rd instar and adult stage 
were exposed to different concentrations of M. anisopliae (C3) and Steinernema sp. 
(AT4). Insects were applied with nematode simultaneously or 1 and 2 days after 
application of M. anisopliae. The simultaneous and sequential application with both 
pathogens resulted in additive or a synergistic effect on S. gregaria. These 
applications increased the mortality of 3rd instar and adult stage of S. gregaria and 
reduced time until mortality. The results showed that the simultaneous treatment of 
both Steinernema sp. and M. anisopliae (C3) at the same host S. gregaria encourage 
nematode development and inhibit fungal mycoses. However, the sequential 
exposure treatments encourage fungal mycoses and inhibit nematode development.

INTRODUCTION

The desert locust, Schistocerca gregaria (Forskal) has threatened 
ariculture crops and semi-desertic zones of Northern Africa, the Near East 
and South West Asia for thousands of years, because it is a much feared 
pest, has great mobility and a vast invasion area. Despite the development of 
improved monitoring and control technologies, this threat continues to the 
present day. For example, there have been a major desert locust plagues 
since 1360s, some lasting more than ten years, and several upsurges during 
the last 25 years, the most recent being in 2004 (Anon., 2006). Now there are 
deep concerns about environmental and health risks associated with use of 
chemical insecticides. Chemical control of desert locust plagues is expensive 
and environmentally damaging (Brader, 1988; Everts, 1990). In 1988, during 
the plagues of the desert locust, Schistocerca gregaria, 10 million ha in 10 
countries of northern and north – western Africa were sprayed with 
approximately 13 million liters of insecticides at a coast of approximately US$ 
100 million (Anon., 1990), and the hazardous effect on the other 
environmental agents (Anon., 1996). Entomopathogenic nematodes and
fungi are biocontrol agents for use in integrated pest management (IPM). Entomopathogenic nematodes in the family Steinemermatidae is soil inhabiting insect pathogens that possess potential as biological control agents (Gaugler, 1981; Kaya, 1985; Poinar, 1986; Gaugler and Kaya, 1990; Kaya and Gaugler, 1993). The third – stage infective juveniles (IJs) of these nematodes are mutualistically associated with the bacteria Xenorhabdus spp. (Kaya, 1993). Together, nematodes and their associated bacteria possess unusual virulence, killing insects within 24-48 hr. Entomopathogenic fungi Metarhizium anisopliae has been employed against a variety of different insect pests and has demonstrated an excellent capacity suppression of pest population (Inglis et al., 2001). It had proven safety (McCoy, et al., 1988; Gillespie, 1988), and ease of production and contact action, which allows direct penetration of the host cuticle without ingestion (Prior & Greathead, 1989; Payne, 1988; Prior, 1989). Both entomopathogen nematode and fungi exist naturally in soil and cause epizootics in soil borne stage of insect population under favourable conditions (Kaya, 1987; Shamseldean and Abd-Elgawad, 1994). The present work was performed to determine compatibility of fungus and nematode, the type of interaction that occur when both pathogens are combined against 3rd nymphal instar and adult stage of S. gregaria and the effect of dual infections with Steinemermata sp. and M. anisopliae to reduce the period of lethal infection to the desert locust S. gregaria.

MATERIAL AND METHODS

Insect maintenance:

Rearing of the greater wax moth, Galleria mellonella (L):

Larvae of G. mellonella obtained from bee hives were transferred to transparent plastic rearing jars (17 x 17 x 27 cm), containing 250 g of old wax combs pieces that were kept in 1 kg-glass jars covered with a lid of muslin for aeration internally supported with a disc of metal wire-mesh and incubated at 20 ± 2 C° with a photoperiod (L:D) 8 : 16 and relative humidity 65 ± 5 % in the insect rearing laboratory (Alwa, 1999). The healthy emerging moths were then regularly taken and kept in similar jars provided with strips of corrugated paper as oviposition sites. The egg-carrying paper strips were removed daily and transferred to jars containing old wax as a source of food. Jars were held at 28 ± 2°C for both egg incubation and larval development. The wax was renewed when needed. Presence of pollen in some cells of comb is necessary for feeding larvae. Larvae complete their growth in about one month. Mature larvae climbed up the jar’s inner wall and waved their silky cocoon, and then they were collected gently by hand, divided into groups, held in plastic boxes, covered with perforated lids and stored in the refrigerator at 10°C until needed.

Rearing of the desert locust, Schistocerca gregaria:

Insects were reared in wooden framed cages. Three sides of the cage were made from wood and the fourth side was made from glass, with a wire gauze top. The front side of the cage was provided with a small door to
facilitate daily routine work and maintenance of the insects. The bottom was furnished with a sandy layer of 20-cm. depth 10-15 % humidity for egg laying. An electric bulb (150-watt) adjusted to a photo phase of 12 hours was placed in each cage in order to maintain an ambient temperature of 32 ± 2 °C. The insects were reared and handled under the crowded condition as described by Hunter – Jones (1961). Fresh clover in winter and the leaves of leguminous plant Sesbania aegyptiaca, in summer were used in feeding insects.

Pathogens:
The pathogens used in the present work were the entomopathogenic nematode Steinernema sp. isolates ATS and SFN. 27 were collected from soil sample, while AT4 isolate collected from insect cadavers from Giza Governorate. The entomopathogenic fungi M. anisopliae isolates (C1, C2 and C3) were obtained from cadavers of the adult of red palm weevil Rhynchophorus ferrugineus

Inoculum preparation
Nematode:
Steinernema sp. was produced in G. mellonella larvae as described by Woodring and Kaya (1988).

To harvest of the infective Juveniles (IJs), a white trap consisted of an inverted Petri dish cover (60 mm diameter) placed inside a larger petri dish (150 X 15 mm) was used. Cadavers of G. mellonella were placed on a filter paper (Whatman no. 1, 110 mm diameter) in the Petri dish cover. The large Petri dish was filled with 100 ml distilled water. Cadavers were held on the trap for ten days to allow the development and migration of IJs into distilled water. Seven fresh G. mellonella last instar larvae were exposed to 2000 emerged IJs in a Petri dish lined with two moistened filter paper. Two to three days after exposure to the nematodes, the insects should die, they were then placed on white traps again to confirm nematode pathogenicity and complete Koch’s postulates (Pelczar and Reid, 1972; Poiner, 1975). The obtained IJs were stored in sterilized distilled water at 10°C (Woodring and Kaya, 1988).

Fungi:
The fungus M. anisopliae was grown on Sabourand Dextrose Yeast Agar medium containing 1% peptone, 0.2% yeast extract, 4% dextrose and 1.5 % agar in distilled water at 23 ±2 for at least 2 weeks. Conidiaospores were harvested and suspended in sterile distilled water containing 0.05 Tween 80 from 14 days old culture under sterile conditions. The conidial suspensions were adjusted to the desired spore counts by using Neubaur Haemocytometer. (Zayed et al., 2003)

Susceptibility of Schistocerca gregaria to entomopathogenic nematode and fungus:
Schistocerca gregaria nymphal stages were segregated from the gregarious stock colony at the beginning of the second instar and reared
under crowded condition in groups of 100 nymphs per cage. Five nymphs of the desert locust *S. gregaria* were equally confined in a large Petri dish (150 x 150 mm) furnished with filter papers. The dauer stage concentrations of *Stinernema* sp. were 500 and 1000 IJs/2 ml distilled water / nymph, and the spores concentrations of *M. anisopliae* were $10^6$ and $2.5 \times 10^6$. For each concentration, nematodes and fungus were applied to five large Petri dishes by spraying the suspension of nematodes or spores (nematodes or spores in distilled water) with an atomizer (hand sprayer) on the experimental nymphs or adults of the locust, the control experiments were done without treatment. The treated insects received the suspended nematodes or spores in 5 ml distilled water. Insect mortality was observed after treatment with nematodes and dead nymphs were dissected for the presence of the nematodes.

**Interaction of entomopathogenic nematode and fungus in vitro:**
Conidiospores of *M. anisopliae* (C1, C2 and C3) were harvested from two weeks old confluent agar cultures by flooding the surface with sterile distilled water and agitating gently. Resulting suspension was filtrated through cheesecloth to remove hyphal debris. Autoclaved glass slide was immersed in autoclave liquid PDA (40 – 50 °C) in order to form a medium film on the slide. One drop of spore suspension of fungal isolate suspension ($10^6$ spores / ml) was deposited on the agar film in the center of the glass slide, then one ml of nematode *Steinernema* sp. (AT4, ATs and SFN.27) suspension contain 500 IJs was added. Slides were incubated overnight (16h) at 26 ± 1°C and nearly 100% RH.

Rate of germination was assessed in one hundred spores counted on each slide. Five replicates were used for each fungi isolates. Only these spores which produced a germ tube that exceeded half of the diameter of the conidia were considered to be germinated.

**Effect of the combination of entomopathogenic nematode and entomopathogenic fungus on *S. gregaria*:**
*Stinernema* sp. (AT4) at concentration of 60, 125, 250 and 500 IJs/ml and *M. anisopliae* (C3) at concentration of $10^5$, $10^6$, $2.5 \times 10^6$ and $10^7$ spores / ml were employed. All possible combination tests between the two pathogens were made. Using 2 ml from each fungal spores and infective juveniles were suspended in sterile distilled water. Equal volumes of sterile distilled water were used as control experiment (Zayed et al., 2003). Third nymphal instar and adult stage of *S. gregaria* in Petri dishes prepared with whatman filter moisture paper were exposed to 2 ml conidio spores + 2 ml from IJs by using small hand sprayer. Both pathogens at all possible concentrations were tested simultaneously and in sequential combinations where insects were exposed to nematodes 1 – 2 days after fungus. The percentage mortality of 3rd nymphal instar and adult stage of *S. gregaria* were assessed daily for five days after treatment. Dead *S.gregaria* nymphs and / or adults were arranged on a white trap to collected juveniles and / or recirculation to fungus spores from cadaver. The cadavers produced nematodes and / or fungus after 15 days, were recorded.
RESULTS

Susceptibility of 3rd instar nymphal and adult stage of S. gregaria to entomopathogenic nematode and fungus:

The obtained results in (Table 1) showed that, the mortality of 3rd nymphal instar and adult stage began to appear two days after treatment at high concentration (1000 IJs) then, the mortality increased to reach its maximum 4 days after treatment. The same data showed LT_{50} values of Steinernema sp (AT4) when tested against 3rd instar and adult stage. The isolate AT4 caused high mortality in shortest time (3.7 and 3.8 days) for 3rd instar and adult at 500 IJs/ml concentration respectively. The obtained results in (Table 2) showed that, the mortality of 3rd instar and adult stage began to appear two days after treatment at low and high concentrations (10^5 and 2.5X10^6 spores/ml) then, the mortality increased to reach its maximum 4 days after treatment. The data in (Table 2) showed LT_{50} values of M. anisopliae (C3) when tested against 3rd nymphal instar and adult stage, which caused high mortality in shortest time (2.4 and 2.5 days) at 10^5 spores/ml concentration respectively.

Table (1): Percentage mortality and LT_{50} values (days) of third instar and adult stage of the desert locust S. gregaria treated with steinernema sp. (AT4) at concentrations 500 and 1000 infective juvenile/ml

<table>
<thead>
<tr>
<th>Stage</th>
<th>% mortality and LT_{50} at indicated concentration</th>
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<tr>
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<td>% mortality 1day 2days 3days LT_{50} 1day 2days 3days LT_{50}</td>
</tr>
<tr>
<td>3rd instar</td>
<td>0        0       60     3.7       0   20   100  3.3</td>
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<tr>
<td>adult</td>
<td>0        0       60     3.8       0   40   100  3.4</td>
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Table (2): Percentage mortality and LT_{50} values (days) of third instar and adult stage of the desert locust S. gregaria treated with M. anisopliae, (C3) at concentrations 10^5 and 2.5x10^6 spores/ml

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<th>Stage</th>
<th>% mortality and LT_{50} at indicated concentration</th>
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<tr>
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<td>% mortality 1day 2days 3days LT_{50} % mortality 1day 2days 3days LT_{50}</td>
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<tr>
<td>3rd instar</td>
<td>0        20     100    2.4       0    60    100  1.8</td>
</tr>
<tr>
<td>adult</td>
<td>0        20     100    2.5       0    20    40   2.5</td>
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</table>

Interaction of entomopathogenic nematode and entomopathogenic fungus in vitro:

Tests were conducted to quantify the effect of Steinernema sp, isolates SFN, ATs and AT4 on germination rates of conidiospores of the fungus M. anisopliae isolates, C1, C2 and C3. These obtained results in fig. (1) showed that the isolates SFN and ATs inhibited the germination of conidiospores of M. anisopliae while isolate C3 was not inhibited by isolate AT4 Steinernema sp. The germination rate of conidiospores of M. anisopliae isolate C3 was 97.8% when combined with isolate AT4 of Steinernema sp.
Fig.(1): Germination percentage of *Metarhizium anisopliae* isolates compatible with *Steinernema* spp. isolates

Effect of the combination of entomopathogenic nematode and fungus on *S. gregaria*:

The effect of combination of indigenous isolates of entomopathogenic nematode *Steinernema* sp. (AT4), and entomopathogenic fungus *M. anisopliae* (C3) against 3rd nympal instar and adult stage of *S. gregaria* was investigated. Both pathogens were tested simultaneously and in different sequential combination at different concentrations.

The obtained results showed that the effect of combination varied according to concentrations of pathogens, stage of tested *S. gregaria* and sequential combinations.

The obtained results in (Table 3) showed that, the mortality of 3rd nympal instar and adult stage of *S. gregaria* were zero% one day after treated simultaneously at low concentrations of both *M. anisopliae* isolate C3 ($10^5$ spores / ml) and *Steinernema* sp. AT4 (60IJs). However, the mortality was increased when the combination of both pathogens were used at high concentrations. Mortality of 3rd nympal instar reached 100% one day after being treated simultaneously with the two pathogens at high concentration of nematode (500 IJs) and low concentration of fungus ($10^5$ spores/ ml), while the adult mortality were 20% and 80% after one and two days respectively. The mortality of 3rd instar reached 60% and 100% one day and two days after treated simultaneously at high concentrations of fungus ($10^5$ spores / ml) and low concentration of nematode (60IJs/ ml) respectively, while the adult mortalities were 80% and 100% respectively. The use of sequential combination (nympal and adult were exposed to nematode 1 – 2 days after treatment with the fungus) increased the mortality of 3rd instar and adult stage of *S. gregaria* at all concentrations.

Mortalities of 3rd nympal instar and adult stage of *S. gregaria* were 60% one day after treated simultaneously at high concentration of fungus ($10^5$ spores / ml) and low concentration of nematode (60IJs).
Table (3): Percentage mortality of third instar nymphal and adult stage of *S. gregaria* treated with nematode *Steinernema* sp. (AT4) and fungus *M. anisopliae* (C3) simultaneously and in sequential (insect exposed to nematode 1-2 days after fungus)

<table>
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<tr>
<th>Dual exposure</th>
<th>Fungal concentrations spores/ml</th>
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<th>60 L/s/ml</th>
<th>125 L/s/ml</th>
<th>250 L/s/ml</th>
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<td>0 day</td>
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- 0 day: simultaneously
- 1 day and 2 days: insect exposed to nematode 1-2 days after treatment with the fungus
- @ corrected mortality
The mortality increased to reach 100% and 80% for 3rd nympal instar and adult stage respectively when treated sequentially (insects exposed to nematode 1 – 2 days after fungus). The LT50 (days) was affected as a result of using both pathogens in combinations simultaneously or sequentially. In simultaneous combination, the LT50 values for 3rd nympal instar and adult stage decreased when either one or nematode and fungus were used at high concentrations. The LT50 of 3rd nympal instar and adults were 1.4 and 2.1 days in simultaneous treatment at low concentration of both nematode (60 IJs) and fungus (10^5 spores / ml), respectively decreased to 0.9 day when fungal concentration increased to 10^7 spores / ml. However, LT50 values of nympal and adult stage of S. gregaria decreased to 0.9 and 1.4 days, respectively when nematode concentration increased to 500 IJs (Table 4). In sequential combination, the LT50 values of 3rd nympal instar and adult stage decreased when nymphs and adults were exposed to nematode one and two days after fungus compared with simultaneously combination.

Table (4): The LT50 values (days) of third nympal and adult stage of S. gregaria treated with nematode Steinernema sp. (AT4) and fungus M. anisopliae (G3) simultaneously and in sequential (insect exposed to nematodes 1-2 days after treated with the fungus)

<table>
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<th>nematode concentrations (IJs/ ml)</th>
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- 0 day: simultaneously
- 1 day and 2 days: insect exposed to nematode 1-2 days after fungus
DISCUSSION

The present study confirms that additive or synergistic interaction between the entomopathogenic nematode *Steinernema* sp. and fungus *M. anisopliae* was observed in the laboratory. The interaction of two pathogens in vitro showed that the *Steinernema* sp. isolates ATs and SFN.27 inhibit conidiospores germination of all fungal tested isolates (C1, C2 and C3), while nematode isolate AT4 did not inhibit conidiospores germination of fungus isolate C3. This finding may refer to nematode bacteria production of a wide range of metabolites, some of which have antimicrobial properties (Forst and Nealson 1996) and anti fungal properties (Li et al. 1995; McInerney et al., 1991), Also Barbercheck and Kaya (1990), mentioned that the primary forms of *X. nematophilus* and *X. luminescens* inhibited the growth of *B. bassiana* blastospores in vitro. The results showed that the effect of combination varied according to the concentration of pathogens, tested stage of *S. gregaria* and type of treatment (simultaneously and sequential). Simultaneous and sequential combinations increased percentage mortality and decreased the period of lethal infection at treated 3rd nymphal instar and adult stage of *S. gregaria*. The host period of lethal infection was shortest in sequential exposure compared to simultaneous. These refer to exposure to fungus at first. Ansari et al., (2004) reported that the grubs *Hopla philantus* are better controlled if larvae are first exposed to fungus *M. anisopliae*, then nematode and that may indicate that the fungus acts as a stressor making the grubs more susceptible to the nematode when these are sequential added. A stressor can increase the insect’s susceptibility to nematode, and in combination with another antagonist may be useful in biological control program (Steinhaus 1958; Thurston et al., 1994). The results showed that the pathogens development in the same host were affected by relative time of infection, nematode concentrations, fungus concentrations and tested stages of insect. In the experiments with dual infection, nematode development and progeny production was the best in high concentration of nematode and simultaneously combination on 3rd nymph instar, while the lowest with high concentration of fungi. These results are in agreement with those of Ansari 2004, who found that, the combination between *M. anisopliae* and *S. glaseri* killed *Hopla philanthus* larvae and produced nematodes with no effect of fungus on nematode progeny. However, the fungal development was the best in high concentration of fungus and sequential combination on adult stage of *S. gregaria*. This demonstrated that nematodes are only compatible with low concentration of *M. anisopliae*. A possible explanation for this difference is that higher concentration of *M. anisopliae* increases the probability for the insect to get in contact with the conidia. Upon their attachment to the cuticle of the insect, conidia, germinate and penetrate into the body where they utilize the body content (Butt et al., 1995). It is reasonable to assume that this process is sped up at higher concentrations of the fungus. This may influence the multiplication of the symbiotic bacteria of the nematodes and consequently the production of nematode progeny. In addition, various toxins produced by entomopathogenic fungi (Vey et al.,
2001) may have inhibited the nematode or their symbiotic bacteria. Barbercheck and Kaya (1990) mentioned that, the interactions between pathogens in a single host are relevant to application strategies. Dual infection with fungus and nematode can result in a more rapid period lethal infection than in singly infected hosts, and this could be an advantage if these pathogens are applied inunadventically. However, antagonistic interaction between fungus and nematode in a single host and their potential effects on pathogen recycling and population dynamics in the soil may be disadvantages to inoculative release.

REFERENCES


التأثير المشترك للنematoda المرضية للحشرات

ـ Steinernema sp. ~

ـ M. anisopliae

فترة المرضية للحشرات

ـ organism

ـ T. ATs and SFN

ـ C1 and C2

ـ Steinernema sp.

ـ فاصل الزمن

ـ anisopliae

ـ AT4

ـ C3

ـになった مسار

ـ organism

ـ T. ATs and SFN

ـ C1 and C2

ـ Steinernema sp.