Biocontrol Science and Technology (1996) 6, 235–245

CARFAX

Comparison of Bioassays to Measure Virulence of Different Entomopathogenic Nematodes

M. RICCI,[1] I. GLAZER,[2] J. F. CAMPBELL[3] AND R. GAUGLER[3]

[1]*Bio Integrated Technology srl, Pantalla, Perugia, I-06050 Italy;* [2]*Department of Nematology, ARO, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel;* [3]*Department of Entomology, Rutgers University, New Brunswick, NJ 08903, USA*

(*Received for publication 4 July 1995; revised manuscript accepted 13 January 1996*)

Five bioassays were compared for their usefulness to determine the virulence of four nematode strains. The objective of this study was to develop standard assays for particular nematode species. In all assays, the nematodes Steinernema feltiae *(strain UK),* S. riobravis, S. scapterisci *Argentina and* Heterorhabditis bacteriophora *HP88 were exposed to* Galleria mellonella *larvae. All bioassays except the sand column assay were conducted in multi-well plastic dishes. In the penetration rate assay, the number of individual nematodes invading the insect was determined after a 48-h exposure to 200 infective juveniles (IJs). In the one-on-one assay, each larva was exposed to an individual nematode for 72 h before insect mortality was recorded. In the exposure time assay, insect mortality was recorded after exposure to 200 IJs for variable time periods. The dose–response assay involved exposing larvae to different nematode concentrations over the range 1–200 IJs/insect and recording mortality every 24 h for a 96-h period. In the sand columns assay, insects were placed in the bottom of a plastic cylinder filled with sand. Nematodes were applied on top of the sand and insect mortality was determined after IJs had migrated through the cylinder. The highest mortality level in the sand column assay was obtained with IJs of* S. feltiae *followed by* H. bacteriophora*; treatments with* S. riobravis *and* S. scapterisci *produced low levels of insect mortality. In the other four assays,* S riobravis *was the most virulent, followed by* S. feltiae, H. bacteriophora and S. scapterisci. *In the exposure time assay, rapid mortality was achieved when the insects were exposed to* S. feltiae *and* S. riobravis. *For these nematode species, a gradual increase in the number of individuals which penetrated into cadavers was recorded. Conversely, the number of nematodes in the cadavers of insects infected by* H. bacteriophora *and* S. scapterisci *remained low during the entire exposure period. In this assay, exposing the insects to these nematodes resulted in a gradual increase in mortality. In the dose–response assay, complete separation among nematode species was obtained only after 48 h of incubation at a concentration of 15 IJs/insect. LD_{50} and LD_{90} values were calculated from dose–response assay data. However, these values did not indicate differences among the different nematode species. The present study demonstrated the variation in entomopathogenic nematode performance in different bioassays and supports the notion that one common bioassay cannot be used as a universal measure of virulence for all species and strains because nematodes differ in*

Correspondence to I. Glazer. Present address of J. F. Campbell, Department of Nematology, University of California, Davis, CA 96516, USA.

0958–3157/96/020235–11 $7.50

their behavior. Furthermore, particular assays should be used for different purposes. To select a specific population for use against a particular insect, assays that are more laborious but which simulate natural environmental conditions (e.g. the sand column assay) or invasion by the nematode (e.g. the penetration rate assay) should be considered. In cases where commercial production batches of the same nematode strains are compared, simple and fast assays are needed (e.g. the one-on-one and exposure time assays). Further studies are needed to determine the relationships between data obtained in each assay and nematode efficacy in the field.

Keywords: Heterorhabditis bacteriophora, Steinernema feltiae, Steinernema riobravis, Steinernema scapterisci, Galleria mellonella, *nematode*, *bioassay*, *virulence*,

INTRODUCTION

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae are considered to be effective agents for the biological control of insect pests (Georgis, 1992). Many qualities make them excellent biocontrol agents: they have a broad host range, possess the ability to search for hosts actively, present no hazard to mammals, and were made exempt from registration and regulation requirements by the US Environmental Protection Agency (EPA) (Gaugler, 1988; Georgis & Manweiler, 1994). At present, they are used commercially against soil-inhabiting pests (Georgis & Manweiler, 1994).

Insect mortality is caused by the nematode–bacterium complex (steinernematids with *Xenorhabdus* spp. and heterorhabditids with *Photorhabdus* spp.) (Akhurst & Boemare, 1990; Boemare *et al.*, 1993). The lack of fundamental knowledge concerning interactions between the nematode–bacterium complex and between the host and the environment has inhibited the development of a standard (universal) assay for measuring nematode–bacterium virulence. Virulence is defined as 'disease-producing' power (Tanada & Kaya, 1993).

With the expansion of commercial interest in entomopathogenic nematodes, the susceptibility of many economically important insect pests has been tested in a wide range of laboratory assays. The most commonly used bioassays consist of exposing the target insect to nematode infective juveniles (IJs) on filter paper (Kaya & Hara, 1980; Morris, 1985; Miller, 1989; Morris *et al.*, 1990; Glazer, 1992). Assuming that a positive relationship exists between nematode concentration and host mortality, probit analysis has been used to analyze data from dose–response tests and to calculate LD_{50} values (Morris *et al.*, 1990; Glazer, 1991). In order to simulate more closely environmental effects on the nematode–bacterium–insect complex, a series of sand- or soil-based bioassays have been developed (Bedding *et al.*, 1983; Molyneux, 1986; Fan & Hominick, 1991; Mannion & Jansson, 1993; Westerman, 1994). In these assays, either the mortality of the insect host or the 'invasion efficiency' (measured as the slope resulting from the linear regression of penetrating nematodes against the dose) was determined.

The studies which have attempted to determine the difference in virulence among nematode species have included one or, at most, two assays (Bedding *et al.*, 1983; Fan & Hominick, 1991; Glazer, 1991; Mannion & Jansson, 1993; Westerman, 1994). However, due to differences in nematode foraging strategies, one type of assay may not be suitable for all species (Grewal *et al.*, 1994). The few studies which were specifically aimed at the comparison of different assays included either a small number of assays (Sims *et al.*, 1992) or tested only one nematode species (Epsky & Capinera, 1993).

In a recent study, Caroli *et al.* (1996) used the penetration rate of a single dose of IJs to measure the virulence of six nematode species against five insect species. In that study, a low level of nematode penetration (1–5%) was recorded with the heterorhabditid species with four of the five insects tested. Therefore, it was suggested that the penetration rate assay may not be suitable for certain nematode species (Caroli *et al.*, 1996).

The objective of the present study was to ascertain the usefulness of different assays in determining the virulence of various nematode species. For this purpose, the virulence of

Steinernema feltiae UK strain, *S. riobravis* TX strain, *S. scapterisci* Argentina strain and *Heterorhabditis bacteriophora* HP88 strain was evaluated using *Galleria mellonella* larvae in five assays:

(1) Penetration rate (Caroli *et al.*, 1996);
(2) one-on-one (Miller, 1989);
(3) exposure time (Glazer, 1991);
(4) dose–response (Morris *et al.*, 1990);
(5) sand columns (Grewal *et al.*, 1994).

MATERIALS AND METHODS

Nematodes

The nematode species *S. feltiae*, *S. riobravis*, *S. scapterisci* and *H. bacteriophora* were reared in last instar *G. mellonella* according to the method of Woodring and Kaya (1988) at 25°C. Nematodes were stored at 10°C for 7–14 days before use. The nematodes in water suspensions were allowed to acclimate at ambient room temperature (21–23°C) for 24 h prior to exposure to insects.

Insects

Last instar larvae of the greater wax moth *G. mellonella* were obtained from Northern Bait Inc., Chetek, WI, USA. Insect larvae within the weight range 0.2–0.3 g were used throughout the study. The insects were stored at 6°C for 1 week and then allowed to acclimate at ambient room temperature for 24 h prior to exposure to nematodes.

Bioassays

In all bioassays except the sand column assay, one wax moth larva was exposed to IJs of the different nematode species in 1.5-cm diameter plates (24-well plates, Corning Cell Wells, Corning, NY, USA). Each well was padded with two filter paper discs (Whatman no. 1; 1.5 cm diameter). The nematodes were transferred to each well in a volume of 75 μl of distilled water. Control wells received water only. The plates were then incubated at 25°C in the dark. These particular plates were chosen because the wells have a relatively small volume. The insect is thus forced to be in close proximity to the nematodes (as opposed to the case with 5-cm diameter petri dishes which are commonly used in such assays). Each treatment consisted of 20 replicates. The different bioassays were tested in parallel each time. The experiments were repeated three times ($n = 60$). Different batches of nematodes and insects were used for each replication.

In the penetration rate assay (Caroli *et al.*, 1996) the insects were exposed to 200 IJs. Forty eight hours after inoculation the insect mortality was recorded, and the number of penetrated nematodes was determined in each host using the pepsin digestion procedure (Caroli *et al.*, 1996). In the one-on-one assay (Miller, 1989), individual nematodes were placed in each of the wells. Insect mortality was recorded 72 h post-inoculation. For the exposure time assay (Glazer, 1991), 200 IJs of each nematode species were used. The insects were exposed to nematodes for 0.5, 1, 2, 3, 6, 9 or 12 h. After each exposure time the insect larvae were rinsed in water to remove nematodes from the surface and then incubated until 48 h had passed from the beginning of the assay. The number of nematodes present in each cadaver was determined in each host by dissection. The dose–response assay (Morris *et al.*, 1990) included nematode concentrations of 0, 1, 5, 15, 50, 100, 200 IJs/well. Insect mortality was recorded 24, 48, 72 and 96 h post-inoculation.

The sand column assay (Grewal *et al.*, 1994) involved plastic pipes (5 cm diameter and 5 cm high) that had one opening covered with metal screens (1-mm hole size). The pipes were filled with sterile, moist (10% w/w) sand (particle size 0.05–0.1 mm diameter) to a depth of 4 cm. The cylinders were placed on top of lidless 5-cm diameter Petri dishes filled with 0.5 cm of moist sand. One *G. mellonella* larva was placed in the center of each Petri dish before it was covered

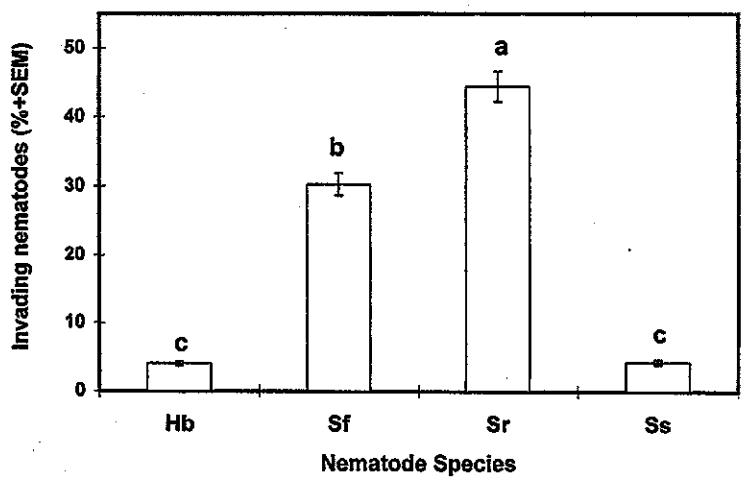


FIGURE 1. The penetration rate assay: the average percentage of nematodes counted in infected cadavers of *G. mellonella* larvae following 48 h of exposure to 200 IJs of *H. bacteriophora* HP88 (= Hb) *S. feltiae* UK (= Sf), *S. riobravis* TX (= Sr) and *S. scapterisci* Argentina (= Ss). Means with the same letter are not significantly different ($P < 0.05$, Tukey's multiple range test). SEM = standard error mean.

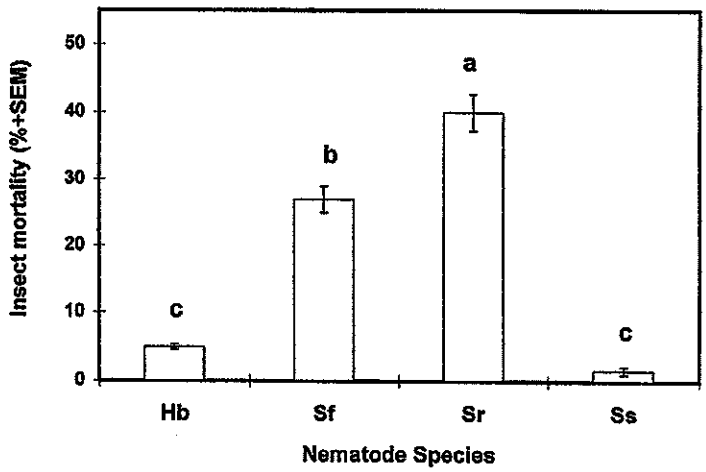


FIGURE 2. The percentage mortality of *G. mellonella* larvae following 72 h of exposure to individual IJs of *H. bacteriophora* HP88 (= Hb) *S. feltiae* UK (= Sf), *S. riobravis* TX (= Sr) and *S. scapterisci* Argentina (= Ss) in the one-on-one assay. Means with the same letter are not significantly different ($P < 0.05$, Tukey's multiple range test). SEM = standard error mean.

by the cylinder. The screen prevented vertical movement of the insect larva. One thousand IJs in 375 μl of distilled water were applied to the top of each sand column. The cylinders were covered with plastic lids and incubated at 25°C. Insect mortality was determined 48 h post-inoculation.

Data Analysis

Virulence data were analyzed using the general linear model procedure and Tukey's multiple range test (SAS Institute, 1987). Mortality data that were expressed as percentages were transformed to the arcsine of the square root and were analyzed using contingency tables and the

(a)

Insect mortality (%)

(b)

Number of Nematodes/ Cadaver

FIGURE 3. The
UK
foll
nem

χ^2 test. The let
probit analysis
significance.

RESULTS

In the penetra
cadavers infect
insects. The pe
of *S. riobravis*
of *H. bacterio*

A similar p
measured by i

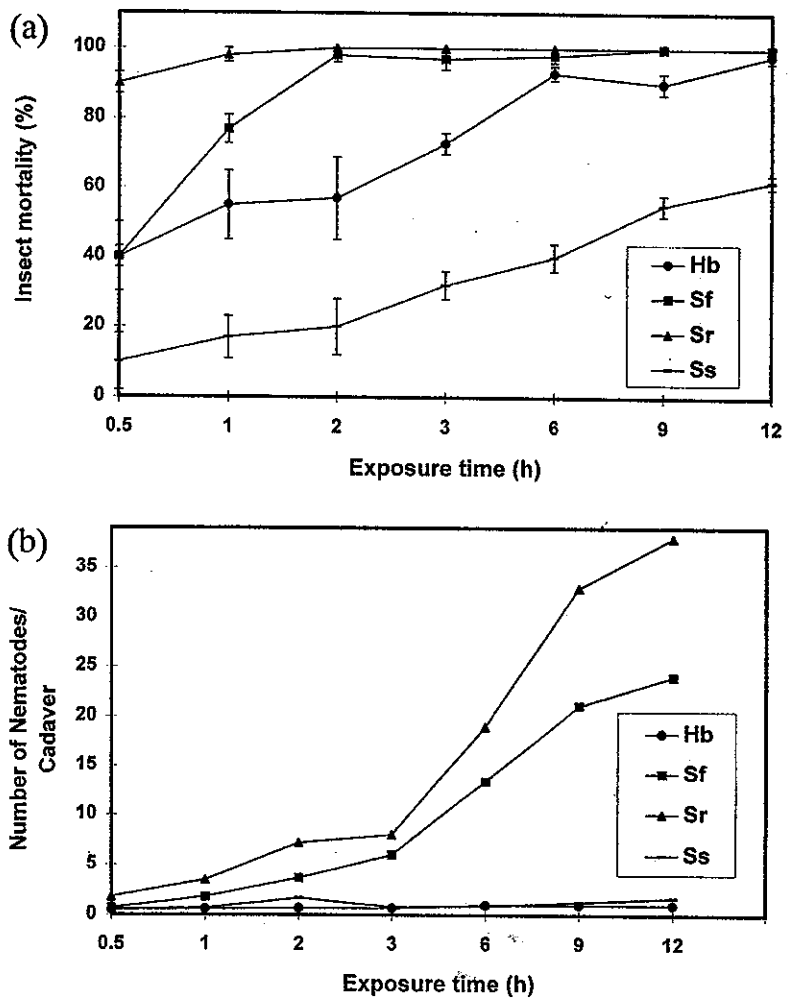


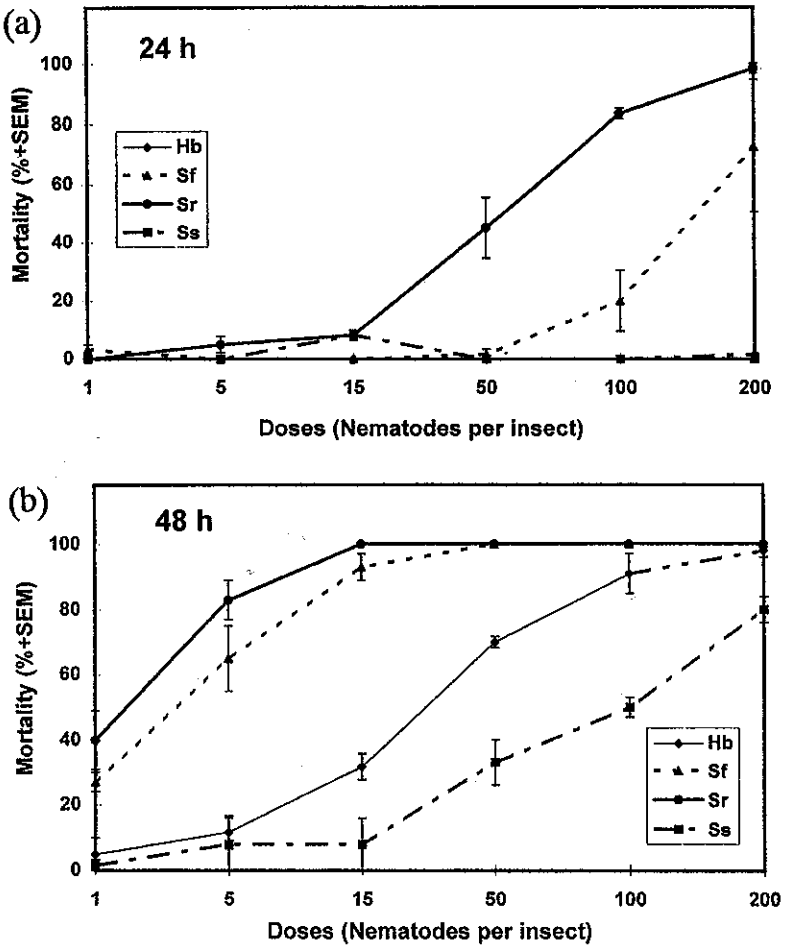
FIGURE 3. The effect of the exposure of *G. mellonella* larvae to 200 IJs of *H. bacteriophora* HP88 (= Hb) *S. feltiae* UK (= Sf), *S. riobravis* TX (= Sr) and *S. scapterisci* Argentina (= Ss) for different time periods, followed by 48 h of incubation at 25°C, on (a) percentage mortality and (b) the average number of nematodes found in the insect cadaver.

χ^2 test. The lethal dosages necessary to kill 50% of the population (LD_{50}) were calculated using probit analysis (SAS Institute, 1987). All comparisons were made at the 0.05 level of significance.

RESULTS

In the penetration rate assay, the highest proportion of nematodes was found in the insect cadavers infected with *S. riobravis* (44%) (Figure 1). A total of 30% of *S. feltiae* penetrated the insects. The penetration rates of *H. bacteriophora* and *S. scapterisci* were 10-fold lower than that of *S. riobravis*. No significant differences ($P > 0.05$) were found between the penetration levels of *H. bacteriophora* and *S. scapterisci*.

A similar pattern was observed in the one-on-one assay, where nematode virulence was measured by insect mortality after 48 h of exposure (Figure 2). The highest mortality was



obtained among insects exposed to one *S. riobravis* IJ followed by *S. feltiae*. Low mortality was observed among insects infected with *H. bacteriophora* and *S. scapterisci*, with no significant differences ($P > 0.05$) between them (Figure 2).

In the exposure time assay, complete mortality was achieved when the insects were exposed to nematodes from the species *S. riobravis* and *S. feltiae* for only 2 h (Figure 3(a)). In these treatments, the number of individuals that penetrated into the cadavers increased gradually as the time of exposure lengthened (Figure 3(b)). Exposing the insects to *H. bacteriophora* and *S. scapterisci* resulted in a gradual increase in mortality (Figure 3(a)), although 12 h of exposure to *S. scapterisci* was not suffficient to achieve 100% mortality. Despite the increase in mortality level, the number of nematodes in the cadavers of insects infected by these nematode species remained low during the entire exposure period (Figure 3(b)). Significant differences in insect mortality between the various treatments were obtained after 1 h.

In the dose–response assay, insect mortality increased over time at all dosages with the nematode species *S. riobravis* and *S. feltiae* (Figures 4(a)–(d)). Even as early as 24 h after inoculation, a gradual increase in mortality was recorded among insects with increasing numbers of IJs of *S. riobravis*. Mortality due to infection with *S. feltiae* was observed after 24 h only at concentrations of 100 and 200 IJs per insect (Figure 4(a)). Low insect mortaiity was recorded after the first 24 h of incubation with larvae exposed to *H. bacteriophora* and *S. scapterisci*.

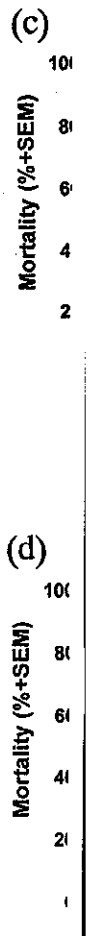


FIGURE 3. The
IJs o
Arge

After 48 h c
IJs/insect respe
nematode speci
given dose. Ad
mortality (Figu
however, contir
at the highest c

In the dose
obtained only a
treatments of n
ation between
showed the hig
between nemat

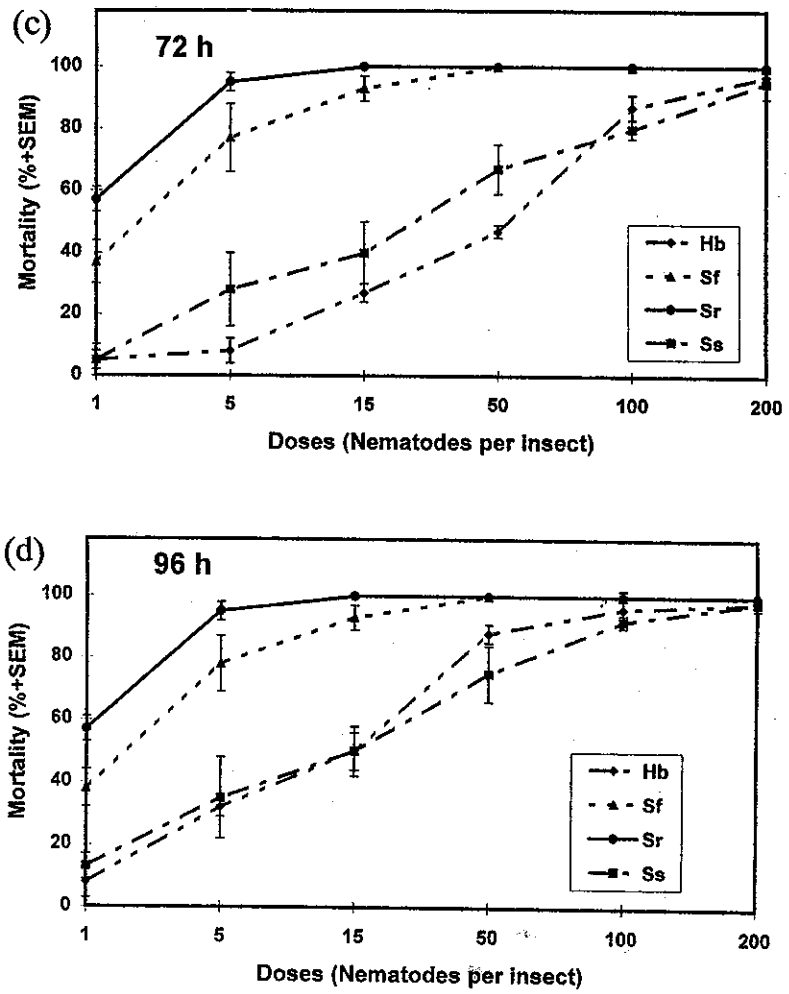


FIGURE 3. The percentage mortality of *G. mellonella* larvae following an exposure to different concentrations of IJs of *H. bacteriophora* HP88 (= Hb) *S. feltiae* UK (= Sf), *S. riobravis* TX (= Sr) and *S. scapterisci* Argentina (= Ss) in the dose–response assay for (a) 24 h, (b) 48 h, (c) 72 h or (d) 96 h of exposure.

After 48 h of incubation IJs of *S. riobravis* and *S. feltiae*, at concentrations of 15 and 50 IJs/insect respectively, were sufficient to achieve 100% insect mortality (Figure 4(b)). With these nematode species, 48 h of incubation was suffficient to obtain the highest mortality level at each given dose. Additional incubation time with the IJs of *S. riobravis* and *S. feltiae* did not increase mortality (Figures 4(a)–(d)). Insect mortality due to *H. bacteriophora* and *S. scapterisci*, however, continued to increase after 48 h of exposure, and complete mortality was recorded only at the highest dosage (Figures 4(c)and (d)).

In the dose–response assay, significant differences between all nematode species were obtained only after 48 h of incubation at a concentration of 15 IJs/insect (Figure 4(b)). All other treatments of nematode dosage–incubation time combinations provided varying levels of separation between species. In all cases where significant differences could be detected, *S. riobravis* showed the highest virulence followed by *S. feltiae*; this was similar to the differences found between nematode species in the penetration rate and the one-on-one assays.

Pergamon

International Journal for Parasitology, Vol. 27, No. 12, pp. 1517–1522, 1997

Printed in Great Britain
0020–7519/97 $17.00+0.00

PII: S0020–7519(97)00143–4

Migratory Behaviour and Desiccation Tolerance of Protostrongylid Nematode First-stage Larvae

AHARON SOLOMON,*§ ILAN PAPERNA,* ITAMAR GLAZER† and PHILIP U. ALKON‡

*Department of Animal Sciences, Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot 76100, Israel

†Department of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel

‡The Jacob Blaustein Institute for Desert Research, Mitrani Center for Desert Ecology, Ben-Gurion University of the Negev, Sede Boqer Campus 84990, Israel

(Received 17 March 1997; accepted 5 August 1997)

Abstract—Solomon A., Paperna I., Glazer I. & Alkon P. U. 1997. Migratory behavior and desiccation tolerance of protostrongylid nematode first-stage larvae. *International Journal for Parasitology* 27: 1517–1522. Migration of first-stage larvae (L1) from faeces to soil is a crucial stage in the life-history of protostrongylids transmitted via land snails. Migration of *Muellerius* cf. *capillaris* and a *Cystocaulus* sp. L1 from fresh Nubian ibex (*Capra ibex nubiana*) faeces (48–50% water content, W.C.) to substrate soils (at 100% r.h., 26°C) was measured experimentally using dry (3±1% W.C.), wet (31±0.43% W.C.) and flooded (48.4±2.45% W.C.) soils. The highest migration rates (90.4±1.6% migration) in both species occurred on flooded soils when the faecal pellet W.C. reached 90%. The next highest migration rates (43.2±3.6% migration, at 60% faecal W.C.) were on the wet soils and no migration occurred on dry soil or dry-substrate papers. Migration rates did not differ significantly ($P>0.05$) between species. Active *Theba pisana* were not infected by *M.* cf. *capillaris* L1 on dry infested soils, but were infected following rehydration of the same soils. By day 10, L1 of *M.* cf. *capillaris* demonstrated lower survival rates in water and in 97% and 76% r.h. (74.5%, 15.2% and 1.9%, respectively) than the *Cystocaulus* sp. (97.5%, 43.8%, 43.3%) and *Protostrongylus* sp. (97.9%, 43.2%, 23.8%, $P<0.05$). All three nematodes had a remarkably high survival rate (>99% overall survival, by day 10) when exposed directly to 0% r.h. at 23°C. Results demonstrate the ability of L1 to survive extreme desiccation through anhydrobiosis. Migration of L1 from faeces to soil can take place only during rains which coincide with peak activity of land snails in desert habitat. © 1997 Australian Society for Parasitology. Published by Elsevier Science Ltd.

Key words: protostrongylid first-stage larvae; migratory behaviour; desiccation tolerance; anhydrobiosis.

INTRODUCTION

Protostrongylidae (Nematoda: Metastrongyloidea) of small ruminants have a two-host life-cycle, with land snails as intermediate hosts. The first-stage larvae (L1) which are evacuated from the definitive host in its faeces, infect land snails and develop to the infective third-stage larvae (L3). The latter are ingested with the snail by grazing ruminants (Cabaret, 1984). L1 stages are often subjected to extreme desiccation in the faeces. The ability of protostrongylid L1 to survive for long periods in desiccated faeces under natural and laboratory conditions has already been reported (*Muellerius capillaris* for 3–27 weeks: Rose, 1957; Diez-Baños *et al.*, 1993; and *Protostrongylus stilesi* for 16 weeks: Forrester & Senger, 1963). Morrondo-Pelayo *et al.* (1992) showed that surviving *M. capillaris* L1 from faeces rapidly desiccated to 95% dry matter were less able to infect the land snail *Candidula intersecta* than the non-desiccated L1. Nothing is known about the abiotic conditions which stimulate migration of L1 from faecal pellets to the soil, or their subsequent ability to infect land snails. Cabaret *et al.* (1991) suggested that larvae abandoning the faeces have a better opportunity to infect crawling snails and

§To whom correspondence should be addressed. Fax: 972-8-9465763. e-mail: Solomon@agri.huji.ac.il.

slugs which are not attracted to faeces (Boag, 1983; Cabaret & Vendroux, 1986).

Protostrongylid infections in wild Nubian ibex (*Capra ibex nubiana*) populations in the desert region of Israel were studied with the aim of understanding how those parasites survive and are transmitted in an arid habitat (Solomon, 1995, M.Sc. Thesis, The Hebrew University of Jerusalem; Solomon *et al.*, 1996a, 1996b, 1996c). The present study describes the environmental factors that stimulate larval migration from the faecal pellets, their survival under different desiccation regimens and their ability to infect land snails after desiccation in soils.

MATERIALS AND METHODS

Faecal pellets collected from free-ranging Nubian ibex (*Capra ibex nubiana*) from the northern Negev desert, Israel, contained either *M.* cf. *capillaris* (Solomon *et al.*, 1996b) larvae alone or a mixture of *M.* cf. *capillaris*, a *Protostrongylus* sp., and a *Cystocaulus* sp. Because the Nubian ibex is a protected species we were unable to obtain adult nematodes of the three different species and their specific identity therefore remains undetermined. The collected fresh samples were placed in polyethylene bags. In the laboratory, after determining water contents (W.C.) of the faecal pellets, the samples were either retained humid in closed plastic bags under refrigeration (5°C), or allowed to dry at room temperature to 7–12% W.C. prior to refrigeration. Faeces stored at 5°C was used for all the experiments.

Loess Row soils (Ravikovitch, 1992) were collected for use as substrates from the study sites near Sede Boqer in the Negev desert. The soil was dug from a vegetation-free surface (up to 5-cm depth) and was autoclaved at 120°C for 30 min prior to use. Papers for use as substrates were dried at 62°C for 1 h. Faecal W.C. was determined by calculating weight loss (with an analytical balance at 0.001-g precision) after drying at 62°C for 24 h. Soil W.C. was determined after drying at 105°C for 24 h. Faeces, papers and soils were weighed again at the end of each experiment to determine ultimate water loss or gain. The numbers of L1 in soils and faecal samples were determined by the Baermann technique: 24 h after collection, larvae were centrifuged at 5000 *g* for 10 min. Tap water (200 or 500 μl) was added to the larval pellets, and the suspension was homogenised. Larval counts from each replicate were carried out on two or three 20–25 μl withdrawn samples. Arithmetic means of larval counts were adjusted to the final volume. Migration of larvae from the faeces to the substrate papers was determined by washing the substrates several times in running tap water and then counting the larvae (at $\times 25$ magnification).

For the desiccation treatments, relative humidity (r.h.) was controlled at 23°C or 26°C in a sealed desiccator with 60 ml saturated salt solutions of K_2SO_4 for 97% r.h., KCl for 85%, NaCl for 76%, and $MgCl_2\,6H_2O$ for 33% (Winston & Bates, 1960). Saturated atmosphere (100% r.h.) was established with 90 ml distilled water, and freshly prepared silica gel was used to obtain 0% r.h.

Migration of L1. Samples of 3 g of dry (7–12% W.C.) and fresh (48% W.C.) faeces, containing 400 *M.* cf. *capillaris* L1 per gram faeces, were placed in uncovered Petri dishes (5 cm diameter, 1.3 cm height) on ordinary papers and filter papers (Whatman No. 1), at 26°C and r.h. of 85%, 97% and 100% for 3 and 7 days. Fresh faeces containing *M.* cf. *capillaris* (50%) and *Cystocaulus* sp. (50%) (200 L1 per gram faeces), were placed in same-size uncovered Petri dishes containing dry (3 ± 1% W.C.), wet (30.9 ± 0.43% W.C.) and flooded (48 ± 2.45% W.C.) soils at 26°C, 100% r.h. for 24 h.

Infectivity of L1 to snails after desiccation in soil. Two-hundred *M.* cf. *capillaris* L1 extracted from faeces were pipetted onto the surface of sterilised wet soils (33–37% W.C.) in Petri dishes (9.8 cm diameter, 0.85 cm height). They were either left to dry gradually at an ambient atmosphere of 45–50% r.h., or left in 100% r.h. at 26°C for 3 days. The infectivity of the larvae from the different experimental groups was tested on snails. Adult *Theba pisana* (1.66 ± 0.22 cm diameter) were allowed to crawl for 6 h on the infested dry soils, wet soils and rehydrated soils. Following exposure, the snails were kept in open 700-ml plastic containers at 26°C and 45–50% r.h. All stored snails entered aestivation, and were examined 50 days later for larval infection in their foot as described by Solomon *et al.* (1996b).

Desiccation survival. For the desiccation experiments, 20 μl of distilled water containing 50 L1 was placed in an Eppendorf cover. The drop of water was allowed to dry at room temperature and the Eppendorf covers with the nematodes were immediately transferred to desiccators (since active clumping of L1 was not observed during drying, they were exposed to the different desiccation regimens as individuals). Desiccation treatments included (a) immediate exposures to 97%, 76%, 33% and 0% r.h.; or (b) initial exposure for 3 days at 97% r.h. prior to their exposure to 33% and 0% r.h. Survival of L1 under desiccation was compared with that of L1 kept in distilled water. Percentage L1 survival was determined by counting motile larvae following gentle probing with a hair probe, after rehydration in distilled water for 24 h.

Statistical analysis. Statistical analysis was performed with the SAS 6.04 software package. Because no L1 migration or infectivity to land snails was observed in dry soils, this treatment was not included in any of the analyses. Percentage L1 migration in the different experimental groups was compared by *t*-test following arcsine transformation. The numbers of larvae recovered in snails were compared over two treatments by the Wilcoxon two-sample test, using the normal approximation. In the desiccation experiment, χ^2 values were calculated to compare species survival rates.

RESULTS

Migration of L1

A saturated atmosphere (100% r.h.) did not induce migration of *M.* cf. *capillaris* and *Cystocaulus* sp. L1 from either fresh (50–60% W.C.) or dry (7–12% W.C.) faeces to dry soils (1.2–5.5% W.C.) during a 24-h exposure, or to paper substrates during 3 days of observation (Tables 1 and 2). Only one *M.* cf. *capillaris* larvae (0.06%, $n=1800$) was found when fresh faeces were left on substrate papers at 100% r.h. for 7 days. The water content of the paper beneath these faecal pellets was 19 ± 2.3% (Table 1). The highest migration rates of both species occurred when faeces were placed

Table 1—The percentage of remaining *Muellerius* cf. *capillaris* first-stage larvae in dry and fresh faeces at different relative humidities (r.h.) at 26°C

Exposure time (days)	r.h. (%)	% of water in faeces (mean ± S.D.)	% of water in the substrate papers (mean ± S.D.)	% of the remaining L1 in faeces (total L1 from all replicates)
3	85	11.4 ± 0.13[a] (12%)[b]	2.2 ± 1.5 (n = 6)	100% (7200)
	97	16.7 ± 2.06 (12%)	4.7 ± 0.8 (n = 6)	100% (7200)
	100	25.4 ± 1 (7%)	5 ± 0.2 (n = 4)	100% (4800)
	100	31.9 ± 1.2 (48%)	11 ± 1 (n = 4)	100% (1800)
7	85	13.09 ± 0.07 (12%)	2.5 ± 0.1 (n = 3)	100% (3600)
	97	16 ± 0.43 (12%)	5.2 ± 0.3 (n = 3)	100% (3600)
	100	27.2 ± 0.16 (7%)	5 ± 0.5 (n = 4)	100% (4800)
	100	38.2 ± 1.4 (48%)	19 ± 2.3 (n = 4)	99.94% (1800)

[a]% of water in faeces before treatment; [b]% of water in faeces after treatment.

Table 2—Migration of *Muellerius* cf. *capillaris* and *Cystocaulus* sp. first-stage larvae from fresh faeces (48% W.C.) to substrate soils with different water contents

Experimental group (% of water in soil) (mean ± S.E.)	% of water in faeces (mean ± S.E.)	% *Muellerius* cf. *capillaris*	% *Cystocaulus* sp. (mean ± S.E.)	% of total migration
Dry soil 3 ± 1%	25 ± 7.2%	0 (n = 3)	0	0
Wet soil 30.9 ± 0.43%	58.3 ± 2.75%	39.3 ± 6.7% (n = 4)	48.0 ± 5.9%	43.2 ± 3.6%
Flooded soil 48.4 ± 2.45%	87.3 ± 1.8%	91.5 ± 3.8% (n = 4)	88.5 ± 5.1%	90.4 ± 1.6%

on flooded soil and became completely wet (*t*-test: $t = 11.45$, d.f. = 6, $P < 0.05$) (Table 2). No significant differences in migration rate on wet or flooded soil were found between the two species (*t*-test: $t = -0.97$, d.f. = 6, and $t = 0.31$, $P \gg 0.05$, respectively) (Table 2).

Infectivity of L1 to snails after desiccation in soil

T. pisana allowed to crawl for 6 h on the dry soils containing desiccated *M.* cf. *capllaris* L1 were not infected. Infection of *T. pisana* occurred only after they crawled on continuously wet soil (37.3 ± 2.8% W.C.) or on rehydrated dry soil (from 1% to 38% W.C.). Snails exposed to the latter two treatments had similar rates of infection ($\chi^2 = 0.57$, d.f. = 1, $P > 0.05$) and larval recovery ($z = 1.46$, $P > 0.05$) (Table 3).

Desiccation survival

Survival rates of L1 from the three protostrongylid species at different r.h. are presented in Table 4. All L1 surviving desiccation had a smooth cuticle, and were motile or reacted to touch. Dead larvae had shrunken cuticles, and shrunken intestinal cells which were visible through the cuticle. By the end of the experiment (day 10), overall survival rates were significantly higher in the 33% and 0% r.h. treatments [100% ($n = 146$) and 99.4% ($n = 165$), respectively],

Table 3—Infectivity of *Muellerius* cf. *capillaris* first-stage larvae to *Theba pisana* from wet and desiccated soils

Experimental group (% of water content in soil) (mean ± S.E.)	No of larvae per snail (mean ± S.E.) Range (min–max L2)	% infected snails
Dry soil (0.97 ± 0.05%)	0	0% ($n=20$)
Rehydrated soil (37.5 ± 1.18%) ($n=4$)	5.9 ± 1.6% (0–28)	89% ($n=18$)
Wet soil (37.3 ± 2.8%)	3.6 ± 1.1 (0–23)	80% ($n=20$)

than in the 97% and 76% r.h. treatments [32.2% ($n=115$) and 19.2% ($n=125$), respectively] ($P < 0.05$).

Survival of L1 in water was higher than in the high r.h. treatments (90%, $n=142$) (overall $\chi^2=398.5$, d.f.$=4$, $P<0.05$). However, surviving larvae were sluggish, with shrunken, heavily vacuolated bodies. By the end of the experiment, a 3-day exposure to 97% r.h. had decreased survival rates of L1 subsequently exposed to 0% and 33% r.h. [27.3% ($n=132$) and 26.3% ($n=118$), respectively]. There were nificant differences among the three nematod directly exposed to 0% and 33% r.h. ($\chi^2=0$ $P \gg 0.05$ for 33% r.h.; $\chi^2=0.26$, d.f.$=2$, for 0% r.h.). In water and the 97% and treatments, *M.* cf. *capillaris* had the lowes rates among the three species ($\chi^2=18.1$ for water, $\chi^2=16.7$ for 97% r.h., $\chi^2=21.9$ for d.f.$=2$, $P<0.05$).

DISCUSSION

Gerichter (1950) reported the migration faeces immersed in water. Our results sho migration of free-living stages from faeca soil occurs only after faecal pellets become wet (at least 60% W.C.). Maximum occurred when the pellet's water conten 85% on a flooded soil. Rains in the nort desert cause flooding of the top soil due t teristic poor seepage capacity. Gerichter gested that larval exit from the faeces is als in heavy dews. Dew is a regular seasonal p in our study area in the Negev desert. I habitat, heavy dew and rains also stimula

Table 4—Survival rates of first-stage larvae of three protostrongylid species at different relative humidi

r.h. (%)	Time (days)	*M.* cf. *capillaris*	*Protostrongylus* sp.	*Cystocaulus* sp.	% of t
97	1	89.5% (57)	91.4% (35)[b]	100% (36)	9
	3	66.3% (89)	66.7% (45)	68.2% (44)	66.
	6	37.7% (61)	65.3% (52)	74.3% (35)	5
	10	15.2% (46)	43.2% (37)	43.8% (32)	32.
76	1	100% (62)	100% (42)	100% (35)	10
	3	100% (64)	96.7% (30)	100% (39)	99.
	6	45.5% (66)	58.7% (46)	85.3% (34)	58.
	10	1.9% (53)	23.8% (42)	43.3% (30)	19.
33	1	100% (74)	97.7% (44)	100% (29)	99
	3	100% (56)	100% (44)	100% (34)	1
	6	100% (73)	100% (44)	100% (36)	1
	10	100% (74)	100% (44)	100% (28)	1
33[a]	3 (6)	44.3% (61)	32.4% (68)	39.4% (33)	3
	7 (10)	24.4% (45)	22.9% (49)	31.6% (38)	2
0	1	98.8% (86)	100% (41)	100% (37)	9
	3	100% (56)	97.4% (39)	100% (45)	9
	6	100% (66)	100% (33)	100% (34)	1
	10	100% (80)	98.2% (57)	100% (28)	9
0[a]	3 (6)	36% (61)	29.3% (58)	25% (32)	3
	7 (10)	32.8% (64)	25% (44)	16.7% (24)	2
Control (water)	3	100% (73)	100% (39)	100% (32)	
	6	100% (53)	100% (43)	100% (30)	
	10	74.5% (55)	97.9% (47)	97.5% (40)	

[a]After an initial exposure to 97% r.h. for 3 days.
[b]Total first-stage larvae of three independent replicates.

195 dew nights between August and January, 42 rainy days between October and April) ov, 1971; Evenari, 1981; Hermony *et al.*, he period of the higher L1 output in ibex eptember–December) (Solomon *et al.*, 1996c) with the highest likelihood of either or both dew.

sults demonstrate survival of *M.* cf. *capillaris* ess soils desiccated to 1% W.C. After rehyf the dry infested soils, the infectivity of L1 vas equal to that of larvae kept continuously ils (37% W.C.). Larvae recovered in snails ter infection were all fully developed second-ae; they did not proceed to the third-stage nfected snails were aestivating. Snail aes-as been shown to delay completion of larval nt to the third-stage (Solomon *et al.*,

lity of the different protostrongylid species extreme desiccation (0% and 33% r.h.) at they survive in a state of anhydrobiosis. is well known in free-living and parasitic of several taxa exposed to desiccation (Van 65; Crowe, 1971; Womersley, 1987; Crowe).

ley (1987) described two strategies for o this state in nematodes: via slow or rapid n, which reflect adaptations of the different tic nematodes to divergent natural habi-

ehydration strategist such as *Pratylenchus* t be desiccated at 97% r.h. in order to r.h. (Glazer & Orion, 1983). Infective *richostrongylus colubriformis* can survive 0% r.h. (~100% survival after 24 h) only exposure to higher r.h. regimens (76% for or 48 h) (Allan & Wharton, 1990). An a rapid-dehydration strategist is the juvenile of the *Ditylenchus dipsaci*, which direct exposure to 0% r.h. (50% survival and is regarded as among the most desrant nematodes (Perry, 1977). Our results the studied protostrongylid free-living apable of rapid-dehydration by demceptional survival rates at 0% r.h. (total 9.4%, $n=165$, by day 10). The impling-term survival are a matter for subies. Our data also do not preclude the low-drying strategy in L1. This option lored in a subsequent study, currently d for publication.

) and Reguera-Feo *et al.* (1986) found al rates for *M. capillaris* at higher 0%, 76%, 100% r.h.) than at lower %, 30% r.h.). The discrepancy between their results and the present work may be explained by either differences among strains or even species of *Muellerius*, or discrepancies in methodology. Humidity regimens in the former studies were controlled with sulfuric acid and glycerin solutions, respectively. Wharton (1982) and Allan & Wharton (1990), using the method used here (salt solutions, according to Winston & Bates, 1960) with *T. colubriformis* third-stage larvae, also obtained significantly higher survival rates at 33% r.h. than in 76% or 97% r.h. The higher survival rates at low humidities may be explained by the successful induction of an anhydrobiotic state, enabling these larvae to survive dry conditions for long periods. The natural life span of the free-living larvae of the three nematode species studied is normally limited by aging, which explains their shorter longevity at high humidities. High humidity or storage in water reduced the survival of *M.* cf. *capillaris*, but not that of *Protostrongylus* or *Cystocaulus* spp. Cabaret *et al.* (1991) observed higher survival of *M. capillaris* as compared to *Neostrongylus linearis* and *Cystocaulus nigrescens* in desiccated faeces and attributed species differences to geographical distribution.

Our study suggests that the migratory behaviour of protostrongylid free-living stages during the rainy season in the Negev desert is a crucial factor in the infection process. In this habitat, wetting of the soil surface by rain or dew both stimulates larval migration from the faeces and activates land snails. This coincidental snail and nematode behaviour is instrumental in securing the nematode parasite's transmission. Larvae in soil which are denied from infecting the intermediate snail host, on the other hand, are capable of surviving by entering into anhydrobiosis through dry periods until the next favorable time for transmission.

Acknowledgements—We thank Dr Hillary Voet from the Faculty of Agriculture of the Hebrew University of Jerusalem for the statistical analysis. This study was partially supported by a fellowship to the first author from the Jacob Blaustein Institute for Desert Research, Mitrani Center for Desert Ecology, Sede Boqer Campus, Israel, and by an internal grant from the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

REFERENCES

Allan G. S. & Wharton D. A. 1990. Anhydrobiosis in the infective juveniles of *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidae). *International Journal of Parasitology* **20:** 183–192.

Boag D. A. 1983. The response of terrestrial snails to the presence of ungulate faeces, a source of nematode larvae (Metastrongyloidea: Protostrongylidae). *Canadian Journal of Zoology* **61:** 1852–1856.

Cabaret J. 1984. Sheep and goats: epidemiology of protostrongylid lungworm infections. *International Goat and Sheep Research* **2:** 142–152.

Cabaret J. & Vendroux P. 1986. The response of four terrestrial molluscs to the presence of herbivore faeces: its influence on infection by protostrongylids. *Canadian Journal of Zoology* **64:** 850–854.

Cabaret J., Risye Riseani S. & Baeza E. 1991. Survival of sheep and goat first stage protostrongylid larvae in experimental conditions: influence of humidity and temperature. *Journal of Helminthology* **65:** 201–207.

Crowe J. H. 1971. Anhydrobiosis: an unsolved problem. *American Naturalist* **105:** 563–573.

Crowe J. H., Hoekstra F. & Crowe L. M. 1992. Anhydrobiosis. *Annual Review of Physiology* **54:** 579–599.

Diez-Baños P., Cabaret J. & Morrondo-Pelayo P. 1993. Comparative survival of first-stage larvae of small lungworm *Muellerius capillaris* and *Neostrongylus linearis* of goats in alfalfa and ryegrass plots. *Veterinary Research* **24:** 266–271.

Evenari M. 1981. Ecology of the Negev desert, a critical review of our knowledge. In: *Developments in Arid Zone Ecology and Environmental Quality* (Edited by Shuval H. I.), pp. 1–33. Balaban ISS, Philadelphia, PA.

Forrester D. J. & Senger C. M. 1963. Effect of temperature and humidity on survival of first-stage *Protostrongylus stilesi* larvae. *Experimental Parasitology* **13:** 83–89.

Gerichter Ch.B. 1950. Studies of the lung nematodes of sheep and goats in Israel. *Refuah Veterinarit* **7:** 41–54.

Glazer I. & Orion D. 1983. Studies on anhydrobiosis of *Pratylenchus thornei*. *Journal of Nematology* **15:** 333–338.

Hermony I., Shachak M. & Abramsky Z. 1992. Habitat distribution in the desert snail *Trochoidea seetzenii*. *Oikos* **64:** 516–522.

Morrondo-Pelayo P., Diez-Baños P. & Cabaret J. 1992. Influence of desiccation of faeces on survival and infectivity of first stage larvae of *Muellerius capillaris* and *Neostrongylus linearis*. *Journal of Helminthology* **66:** 213–219.

Perry R. N. 1977. Desiccation survival of larval and adult stages of the plant parasitic nematode, *Ditylenchus dipsaci* and *D. myceliophagus*. *Parasitology* **74:** 139–148.

Ravikovitch S. 1992. *The Soils of Israel. Formation, Nature and Properties*. Hakibbuts Hameuchad, Tel-Aviv.

Reguera-Feo A., Rojo-Vazquez F. A. & Cordero-Del-Campillo M. 1986. On first-stage larvae resistance under controlled conditions of three species of nematodes of the family Protostrongylidae, Leiper, 1926. *Annales de Parasitologie Humaine et Comparee* **61:** 297–301.

Rose J. H. 1957. Observations on the bionomics of the free living first-stage larvae of the sheep lungworm *Muellerius capillaris*. *Journal of Helminthology* **31:** 17–28.

Solomon A., Paperna I. & Alkon U. P. 1996a. The suitability of *Trochoidea seetzenii* of different ages as snail intermediate hosts of *Muellerius* cf. *capillaris* (Nematoda: Protostrongylidae). *International Journal for Parasitology* **26:** 1317–1319.

Solomon A., Paperna I. & Markovics A. 1996b. The influence of aestivation in land snails on the larval development of *Muellerius* cf. *capillaris* (Metastrongyloidea: Protostrongylidae). *International Journal for Parasitology* **26:** 363–367.

Solomon A., Strubel I., Paperna I., Alkon U. P. & Markovics A. 1996c. Protostrongylids (Nematoda: Metastrongyloidea) and Coccidia (Apicomplexa: Eimeridea) infection in wild populations of the Nubian ibex (*Capra ibex nubiana*) in the Northern Negev desert, Israel. *Israel Journal of Medical Sciences* **32:** 1145.

Van Gundy S. D. 1965. Factors in survival of nematodes. *Annual Review of Phytopathology* **3:** 43–68.

Wharton D. A. 1982. The survival of desiccation by the free-living stages of *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidea). *Parasitology* **84:** 455–462.

Winston P. W. & Bates D. H. 1960. Saturated salt solutions for the control of humidity in biological research. *Ecology* **41:** 232–237.

Womersley C. 1987. A reevaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. In: *Vistas on Nematology* (Edited by Veech J. A. & Dickson D. W.). Society of Nematologists, Hyattsville, MD.

Yom-Tov Y. 1971. The biology of two desert snails *Trochoidea (Xerocrassa) seetzenii* and *Sphincterochila boissieri*. *Israel Journal of Zoology* **20:** 231–248.

Biocontrol Science and Technology (1997) 7, 499–512

CARFAX

Genetic Enhancement of Nematicide Resistance in Entomopathogenic Nematodes

I. GLAZER,[1] L. SALAME[1] AND D. SEGAL[2]

[1]*Department of Nematology, Agricultural Research Organization (ARO), The Volcani Center, Bet Dagan 50250, Israel;* [2]*Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel*

(*Received for publication 27 February 1997; revised manuscript accepted 25 May 1997*)

Entomopathogenic nematodes are highly effective bioinsecticides. Their efficacy may be reduced due to the various pesticides they encounter in the soil. These include insecticides as well as nematicides used against plant-parasitic nematodes. The purpose of this study was to examine the feasibility of genetic selection as a means of enhancing resistance of the entomopathogenic nematode Heterorhabditis bacteriophora *strain HP88 to the nematicides: Fenamiphos (an organophosphate), Oxamyl (a carbamate) and Avermectin (a biological product). Estimates of heritability* (h^2) *of resistance to the three nematicides were obtained from analysis of inbred lines derived from the base population. The heritability estimate for Fenamiphos was* $h^2 = 0.31$, *for Oxamyl* $h^2 = 0.71$ *and for Avermectin* $h^2 = 0.46$. *Five rounds of selection were performed. Thereafter, each line was divided into two: for one subline selection continued for six additional rounds. The other subline was reared without selection for the six additional rounds. After the eleventh round, resistance to the nematicides was examined as were several traits relevant to biocontrol efficacy including virulence, heat tolerance and reproduction potential. Selection resulted in an 8–9-fold increase in resistance to Fenamiphos and Avermectin and a 70-fold increase in resistance to Oxamyl. The enhanced resistance Oxamyl and Avermectin, and to a lesser extent to Fenamiphos, was stable and continued after selection was relaxed. No deterioration in traits relevant to biocontrol efficacy was observed in the selected lines as compared with the base population. The selected lines displayed enhanced cross-resistance towards some, but not all, of the nematicides tested. These results demonstrate that genetic selection can be used to enhance resistance of entomopathogenic nematodes to certain environmental stresses. The selected lines will be useful bioinsecticides in the context of integrated pest management.*

Keywords: *nematicides, resistance, environmental stresses, biological control,* Heterorhabditis bacteriophora, *genetic improvement*

E-mail: glazeri@netvision.net.il; Tel: 972 3 9683457; Fax: 972 3 9604180.

0958–3157/97/040499–14 $9.00

INTRODUCTION

Entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae have, in recent years, began to contribute significantly to the biological control of many insect pests. They are already widely used to control soil-inhabiting insects in citrus fruits, mushrooms, turfgrass, cranberries and ornamentals (Georgis & Manweiler, 1994). The infective juvenile (IJ) stage of these nematodes is capable of actively searching for and parasitizing target hosts in the soil. After penetrating the insect, the IJ releases into the host's hemocoel the symbiotic bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) carried in its intestine. The bacteria multiply, killing the host within 24–48 h (Akhurst & Boemare, 1990; Boemare *et al.*, 1993). The nematodes complete two to three life cycles in the host and the progeny IJs leave the cadaver in search of a new host carrying the bacteria with them.

Several attributes make these nematodes attractive bioinsecticides. First, they have a wide host range. Second, their virulence is very high, and in some cases provides a level of insect control equivalent to that of chemical insecticides. Third, technology is available for mass production of these nematodes for commercial use. Finally, entomopathogenic nematodes are not pathogenic to mammals or plants, and have therefore been exempted from registration and regulation requirements by the US Environmental Protection Agency.

While searching for target hosts in agricultural soils the IJs encounter a variety of substances that could hamper their activity (Kaya, 1990; Kaya & Koppenhöfer, 1996). These include residues of various fungicides, herbicides and insecticides (Georgis & Manweiler, 1994). The IJs can withstand many such chemicals which are commonly used in cultivated soils (Hara & Kaya, 1982, 1983; Rovesti *et al.*, 1989; Rovesti & Deseö, 1990; Ishibashi & Takii, 1993). This resistance has been attributed mainly to the thick protective cuticle of the IJs, and to their closed oral and anal openings (Mráček *et al.*, 1981) which prevent penetration of these chemicals into the nematode. However, several insecticides as well as nematicides used against plant-pathogenic nematodes were found to be highly toxic to steinernematid and heterorhabditid IJs in laboratory assays (Hara & Kaya, 1982, 1983; Rovesti *et al.*, 1989; Rovesti & Deseö, 1990). Thus, exposure of IJs in the soil to the organophosphates Mevinphos, Fenamiphos and Trichlorofon, or the carbamates Carbofuran, Methomyl, and Oxamyl, may reduce the persistence and activity of these nematodes as biological control agents.

Genetic selection has been suggested as a means to enhance the tolerance of entomopathogenic nematodes to environmental extremes (Kaya, 1985; Gaugler, 1988b). The feasibility of the genetic approach has been demonstrated subsequently as increasing nematode host-finding activity (Gaugler *et al.*, 1989, 1991) and infectivity (Tomalak, 1994), but the selected lines did not reach practical use. Successful genetic selection depends on the presence of genetic diversity for the particular trait in the population (heritability, h^2) (Glazer *et al.*, 1991).

The goal of the present study was to enhance tolerance to the nematicides Fenamiphos, Oxamyl and Avermectin in the commercial strain *Heterorhabditis bacteriophora* HP88. To that end, heritability (h^2) for nematicidal resistance in this population was estimated. Second, the population was selected for tolerance to these chemicals. Third, the stability of the enhanced nematicidal resistance in the selected lines was determined and their performance characterized with respect to several key traits relevant to biocontrol efficacy.

MATERIALS AND METHODS

Nematode Rearing

The HP88 strain of *H. bacteriophora* was originally isolated from a single scarab larva (*Phyllophaga* sp.) in Logan, UT, USA in 1982 (Poinar & Georgis, 1990). Since then, the

strain has been reared continuously *in vivo* or *in vitro*. For this study, the population of this strain was obtained from Ecogen, Inc. (Langhorne, PA, USA). The nematodes were reared in last-instar larvae of the greater Wax moth *Galleria mellonella* at 25°C according to the method of Woodring and Kaya (1988). Hereafter, this nematode population will be referred to as the 'base population'.

Nematicides

Three chemicals, representing different classes of nematicides, were used: Fenamiphos (FE), Oxamyl (OX) and Avermectin (AV). Their specifications are given in Table 1.

Toxicity Bioassay

The effect of the different nematicides on nematode viability was determined by placing suspensions of ca. 10 000 IJs in distilled water in 5-cm diameter Petri dishes containing various concentrations of each of the chemicals dissolved in water. The nematicides were used in their commercial formulations. Their final concentrations were calculated as percentage of the final volume (% v:v). The dishes were sealed with parafilm to avoid rapid evaporation and incubated at 25°C in the dark. After 48 h of exposure to the nematicide, a sample of 1 ml of the nematode suspension was withdrawn from each dish for assessment of nematode viability. This sample was transferred to a new Petri dish containing 14 ml of distilled water, and was further incubated in this diluted solution for 24 h (25°C, in the dark). Thereafter, nematode viability was recorded by observing the motility and response to probing of at least 200 individuals. Each assay (nematicide/concentration) consisted of five replicates (five Petri dishes). Control treatments consisted of exposing the nematodes to distilled water.

Heritability Estimates

The genetic diversity of the resistance to the three nematicides was determined for the *H. bacteriophora* HP88 population by comparing the resistance of 16 inbred lines in the toxicity assay just described. The inbred lines were each initiated according to Glazer *et al.* (1991) from a single hermaphrodite, and each of the 10 subsequent generations was derived from a single hermaphrodite of the previous generation. This inbreeding regime resulted in highly homogeneous inbred lines (>95% homozygosity). Approximately 3000 IJs from each of the 16 inbred lines were exposed for 48 h to the following concentrations of the three nematicides: 0.025, 0.35 and 0.015% v:v for FE, OX and AV respectively. These concentrations were chosen because, in a preliminary dose–response experiment, they resulted in approximately 50% mortality of the base population. The nematode viability of the 16 inbred lines was monitored as described earlier and the data were used to estimate h^2 according to Glazer *et al.* (1991).

Selection Regime

IJs ($N = 30\,000$) from the base population were exposed to the nematicides at concentrations of 0.05, 0.75 and 0.025% v:v for FE, OX and AV respectively. These concentrations were chosen because, in a preliminary experiment, they resulted in approximately 10% viability of the base population. This level of viability was chosen because it represented a strong selection pressure, yet resulted in enough survivors to avoid loss of variability by genetic drift. Small numbers of survivors create a bottleneck that often reduces genetic variability (Hartl & Clark, 1989). Following 48 h of incubation with the nematicide (25°C, in the dark) a 1-ml sample was withdrawn for measurement of viability, as described earlier. In order to recover the survivors from the rest of the nematode suspension, it was concentrated with vacuum on a disc of filter paper (diameter = 5 cm). The disc was then laid on a nylon sieve (60 mesh/200-m pore size) which was placed over distilled water in a Petri dish. Under these conditions, the viable nematodes crawled into the water, washing off the nematicide from their surface, and were separated from the dead ones. The surviving

TABLE 1. Properties of the nematicides used in the present study

Nematicide group	Common name	Abbreviation	Compound	Commercial name	Active ingredient (% v:v)	Manufacturer
Organophosphate	Fenamiphos	FE	Ethyl 3-methyl-4-[methylthio] phenyl [1-methylethyl] phosphoramidate,	Nemacure	40	Bayer, Germany
Carbamate	Oxamyl	OX	Methyl *N'*,*N'*-dimethyl-*N*-[(methyl carbamoyl) oxy]-1-thiooxamimidate	Vydate	24	Du Pont, Geneva, Switzerland
Biological product	Avermectin	AV	AV. B_1, macrocyclic lacton derived from the mycelia of *Streptomyces avermitilis* (Arena *et al.*, 1995)	Vertimec	1.8	Merck, Rahway, NJ, USA

IJs were concentrated again on a fresh filter paper, and were placed in a Petri dish containing 10 larvae of the host *G. mellonella*. The Petri dish was incubated for a period of 48 h at 25°C in the dark, during which time the IJs that had survived the nematicide infected the insect larvae, killed them and initiated reproduction in the cadavers. To allow further reproduction of the nematodes, the 10 insect cadavers were placed in 'White traps' (Woodring & Kaya, 1988) and incubated under the same conditions for 12–14 days. During this period, the nematodes usually complete three life cycles in the host and, when resources in the host become limiting, the progeny IJs leave the cadaver. Emerging IJs were collected in 'White traps' (Woodring & Kaya, 1988) and subjected to another round of selection by exposing them to the same concentration of the nematicide. Nematode mortality was determined by the toxicity assay described earlier, and the surviving IJs were recultured for the next round of selection. As a control in each round of selection, IJs from the base population were similarly subjected to the toxicity assay. Each round of selection comprised five replicates.

After the fifth round of selection, when enhancement was detected in the resistance to the three nematicides, each of the three lines selected was divided into two sublines, one selected as before and the other maintained with selection relaxed.

Sublines for continued selection. The selected sublines were subjected to six additional rounds of selection, each followed by a toxicity assay. For the FE- and AV-selected sublines the concentration of the nematicide was the same as in the first five rounds of selection. However, the OX line had reached 100% resistance at the fifth round of selection, therefore, the concentration of OX was increased three-fold to 2.25% for the six additional rounds of selection. This concentration yielded 10% survival in a dose–survival experiment performed on the OX line after the fifth round of selection.

After the eleventh round of selection, we evaluated whether key traits relevant to biocontrol efficacy had been compromised during the selection process. To do that, the performance of each of the three sublines selected was examined in the following bioassays: heat tolerance, virulence and reproduction. The performance of the sublines was compared to that of the non-selected base population. The details of the bioassays are described in the following.

Tests were also carried out to determine whether resistance after 11 rounds of selection to one nematicide had any effect on resistance to the other two. To answer this, the three selected sublines were assayed for resistance to all three nematicides. The unselected base population served as a control.

Sublines for relaxed selection. The sublines exposed to relaxed selection pressures were reared for six additional rounds under conditions similar to those of the 'selected' sublines, but with distilled water substituted for the nematicide solution. After the eleventh round, resistance was assayed in the 'relaxed-selection' sublines, contemporaneously with the tests of the selected sublines and the base population. Each experiment consisted of six replicates and the experiment was performed twice.

Traits Relevant to Biocontrol Efficacy

Heat tolerance. The assay was established according to Shapiro *et al.* (1996). For each selected subline, a 0.2-ml suspension containing approximately 3000 IJs was placed in a 20-ml glass scintillation vial containing 5 ml of distilled water. Prior to the addition of nematodes, all vials were incubated in a water-bath shaker at 37°C. Thus, the nematodes were immediately introduced to high temperature. They were held in the water-bath shaker, at 70 rpm and 37°C, for 2 h after which 1 ml of the nematode suspension was placed in a 5-cm diameter Petri dish containing 9 ml of distilled water at room temperature (25°C). After 24 h incubation at 25°C, survival was recorded as described for the toxicity assay. Each treatment consisted of five replicates and the experiment was performed twice.

Virulence. An 'exposure time assay' (Ricci *et al.*, 1996) was used. Briefly, IJs from each of the three selected sublines were placed together with one last-instar *G. mellonella* larva in a 1.5-cm diameter well of 24-well plates (Corning Cell Wells, Corning, NY, USA). Each well was padded with two discs of filter paper (diameter = 1.5 cm; Whatman no. 1). The nematodes, at a concentration of 150 IJs per insect, were transferred to each well in a volume of 0.5 ml distilled water. Control wells contained insect larvae that received water only. The insects were exposed to the nematodes for 1, 2, 4 or 8 h. After each exposure time, the insect larvae were rinsed in water to remove nematodes from their surfaces, and then incubated at 25°C in the dark until 48 h after the beginning of the assay. For each treatment (subline × exposure time), 12 insect larvae were used and the experiment was performed three times. Lethal exposure periods necessary to kill 50% of the insect population (LE_{50}) were calculated using probit analysis (SAS, 1985).

Reproduction potential. The assay was performed according to Shapiro *et al.* (1996). For each of the three selected sublines, 10 last-stadium larvae of *G. mellonella* were exposed to 200 IJs in Petri dishes (diameter = 5 cm) padded with moist filter paper and incubated at 25°C, in the dark. After 72 h, dead insects were transferred to 'White traps' (Woodring & Kaya, 1988) and the number of progeny IJs emerging from each cadaver was recorded. The experiment was performed twice.

Data analysis. An arcsine transformation was used on data presented in percentages. The data were then subjected to analysis of variance (ANOVA). If significant differences were detected among the effects of treatments, means were compared using Tukey's multiple-range test at $a = 0.05$.

RESULTS

Heritability Estimates from Inbred Lines

Considerable variability was observed between inbred lines in their ability to withstand exposure to the different nematicides (Figure 1). For example, for AV there was a 26-fold difference between the most and the least resistant lines (Figure 1(c)). A smaller difference was observed in the resistance of the various lines to FE (Figure 1(a)) or OX (Figure 1(b)).

None of the inbred lines tested was highly resistant to all three nematicides. Some of the lines showed higher resistance levels to one or two of the nematicides and a moderate level to the other nematicides (e.g. lines 6B, 9A, 7I; Figure 1). Other lines showed moderate or low resistance for the different chemicals (e.g. lines 6C, 8; Figure 1). Only line 7 showed low resistance for all three nematicides.

The heritabilities were for FE, $h^2 = 0.31$ (Figure 1(a)), for OX, $h^2 = 0.71$ (Figure 1(b)) and for AV, $h^2 = 0.46$ (Figure 1(c)). These are broad sense h^2s, which include interaction variance. Selectable genetic variance is therefore lower than these estimates.

Selection

Substantial enhancement in resistance was achieved for all three nematicides after 11 rounds of selection (Figure 2). Resistance to FE increased gradually throughout the entire selection period (Figure 2(a)). A high level of resistance was recorded after 11 rounds of selection (Figure 2(a)), and remained constant for an additional three rounds of selection (data not shown). The most rapid enhancement was observed for OX resistance (Figure 2(b)). After five rounds of selection, OX resistance was elevated 5.7-fold. Therefore, the selection regime was intensified by increasing the concentration of OX to which the nematodes were exposed. Even under this higher OX dosage, resistance increased 13-fold from the fifth to the eleventh round of selection (Figure 2(b)). No nematodes from the base population survived under the intensified selection regime (Figure 2(b)).

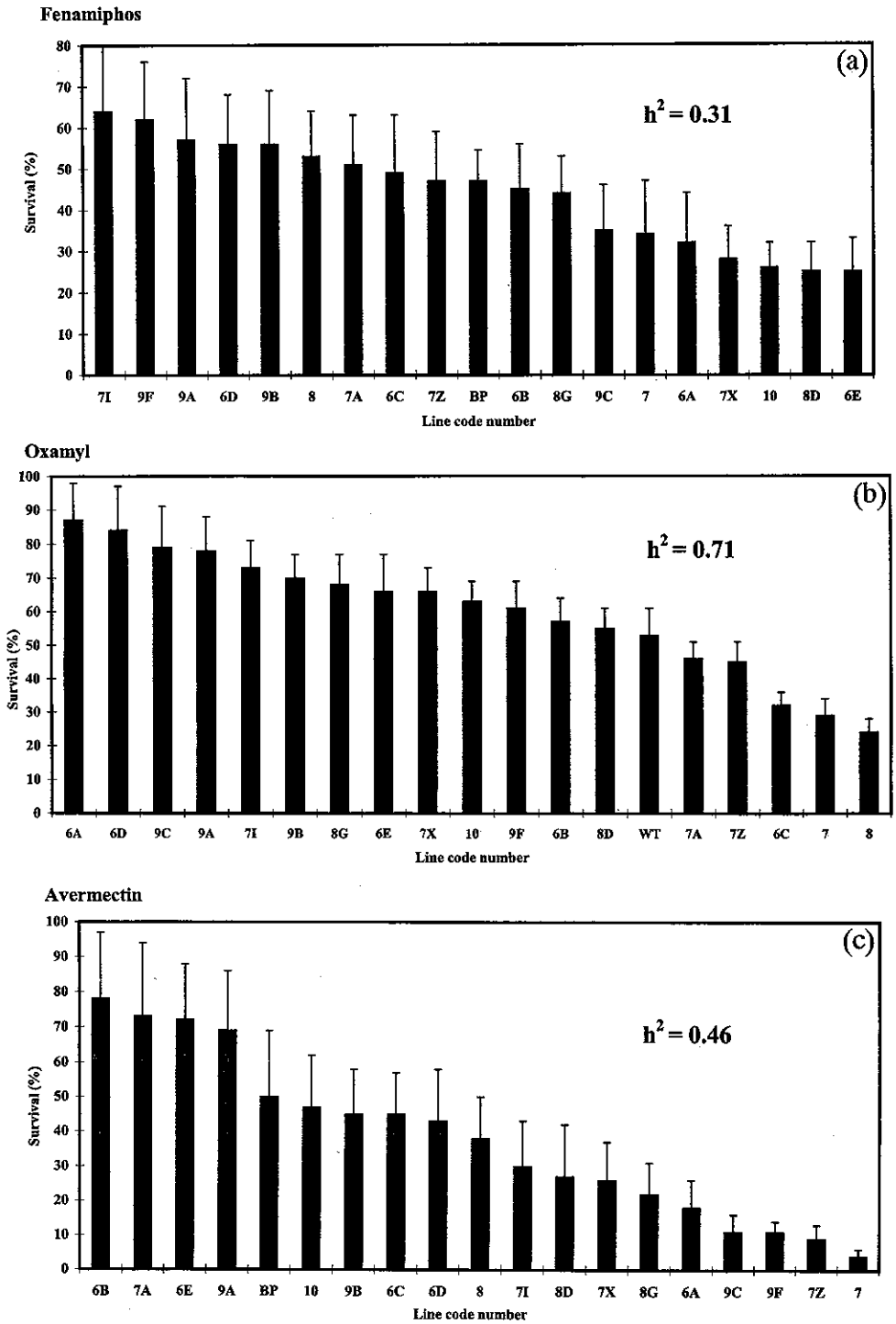


FIGURE 1. Survival of infective juveniles from inbred lines of the base population of *H. bacteriophora* HP88 following 42 h exposure to different nematicides. (a) Fenamiphos (0.025% v/v). (b) Oxamyl (0.35% v/v). (c) Avermectin (0.015% v/v). BP is the base population. The corresponding estimates of heritability (h^2) calculated from these data are shown.

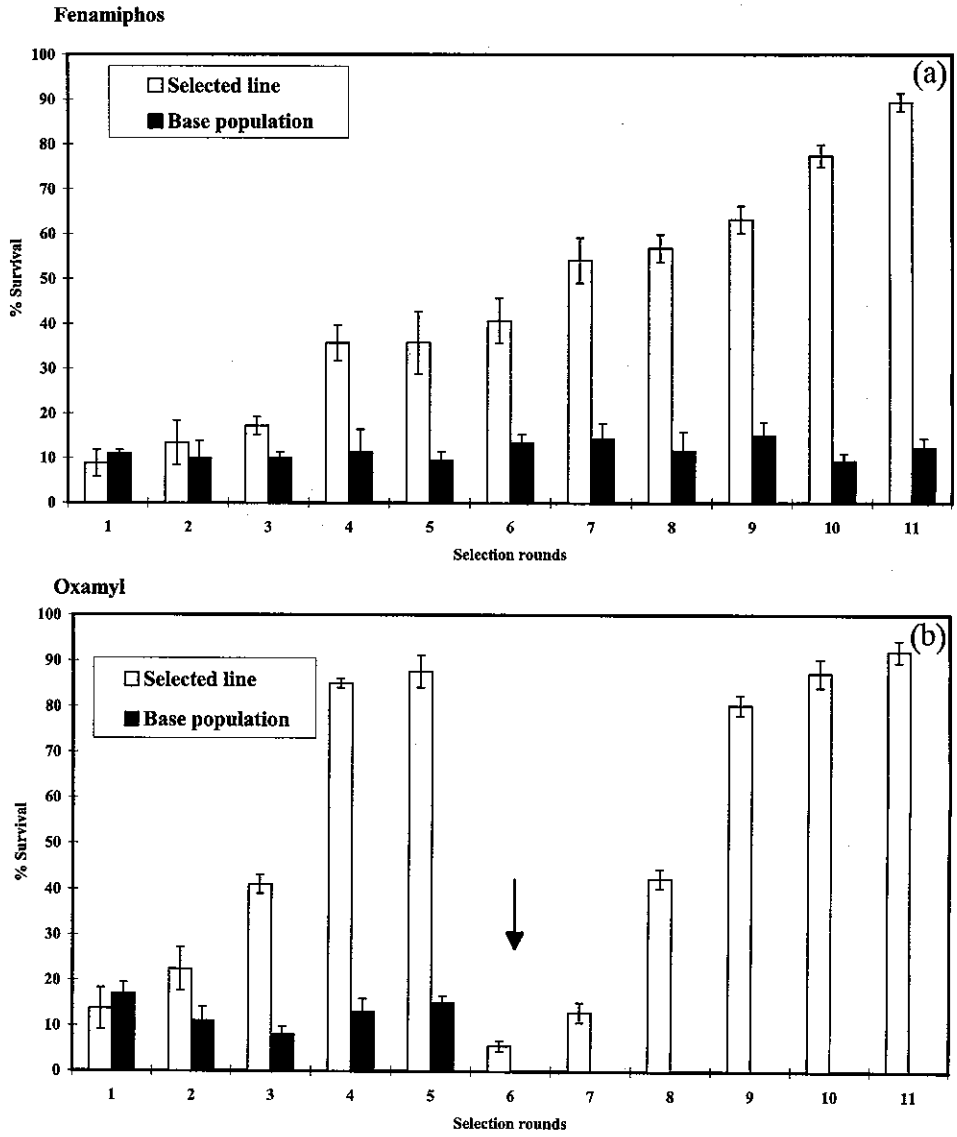


FIGURE 2

The AV selection line achieved high levels of survival after five rounds of selection. The level of resistance did not increase over the additional six rounds of selection (Figure 2(c)).

In the AV and OX relaxed-selection sublines, no reduction in nematicide resistance was observed (Figure 3). The FE relaxed-selection subline became less resistant, but its survival in the toxicity assay was still significantly higher ($P < 0.05$) than the survival of the base population.

Cross-resistance

The OX and AV selected sublines were more resistant to FE than was the base population (Figure 4). The FE selected subline had enhanced resistance to OX but not to AV. No cross-resistance to OX and AV was observed (Figure 4).

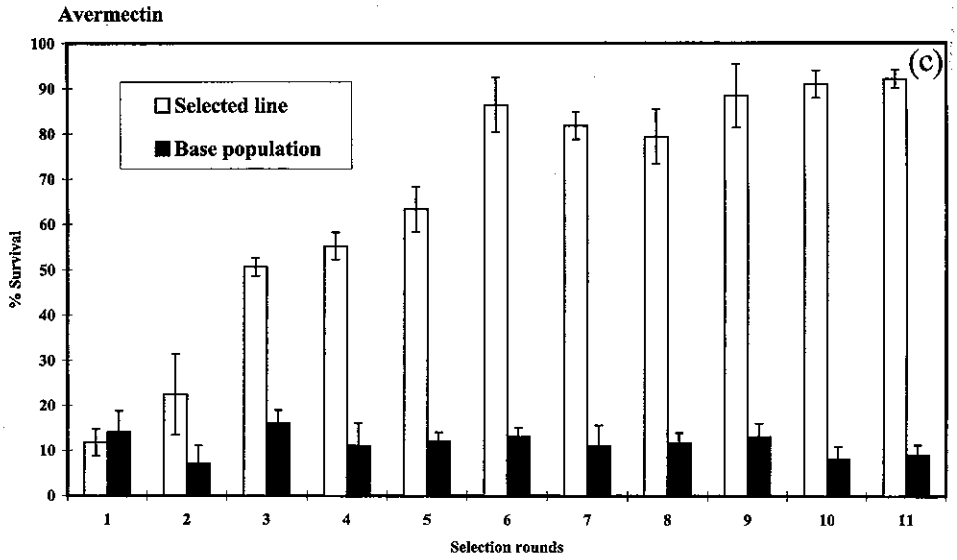


FIGURE 2. Selection for nematicide resistance. Nematicide resistance of the survivors after each round of selection. (a) Resistance to Fenamiphos (0.05% v/v). (b) Resistance to Oxamyl (0.75% v/v). (c) Resistance to Avermectin (0.025% v/v). For Oxamyl, selection was enhanced after the fifth round by increasing the concentration of the nematicide three-fold (2.25% v/v, arrow).

Traits Related to Biocontrol Efficacy

After the eleventh round of selection, we tested the selected sublines for three key traits relevant to biocontrol efficacy: heat tolerance, virulence and reproduction. For all three traits tested, no reduction was recorded in the selected sublines as compared with the base population (Table 2). In the reproduction assay, the average number of progeny in the sublines selected for OX and AV resistance was significantly higher ($P < 0.05$) than in the base population.

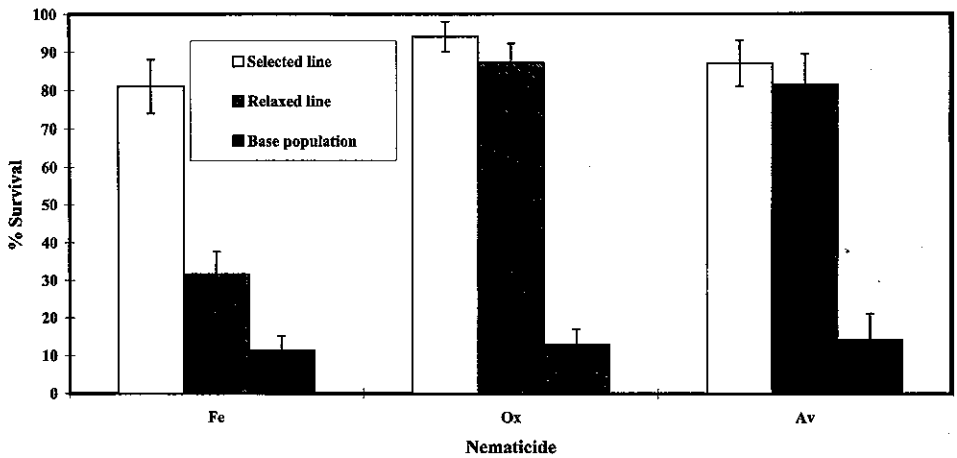


FIGURE 3. Stability of the enhanced nematicide resistance. Resistance to the nematicide was compared, after the eleventh round of selection, for the selected subline, the relaxed-selection subline and the base population.

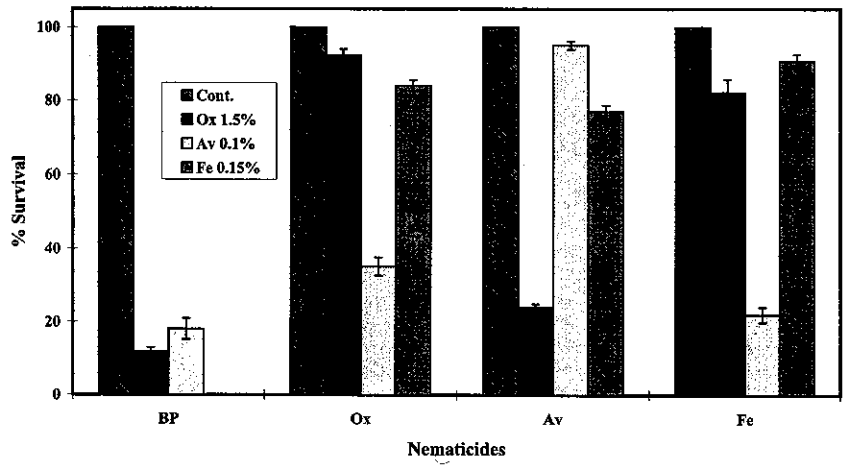


FIGURE 4. Cross-resistance to nematicides: resistance of the selected lines towards the nematicides they were not selected against was examined after the eleventh round of selection.

DISCUSSION

For a selection programme to be of practical value, either heritability for a desired trait must be moderately high or a very large base population must be employed. In the present study, the broad sense h^2 estimates obtained from an analysis of inbred lines were sufficiently high ($h^2 > 0.3$) to suggest that selection would work. Upon selection, the rate of enhancement in nematicidal resistance was correlated to the h^2 estimate for the corresponding nematicide, higher for OX and lower for FE (Figures 1(a)–(c)). These findings suggest that heritability estimates may have a predictive value regarding selection for beneficial traits in entomopathogenic nematodes.

Selective breeding has been utilized previously for the enhancement of biocontrol efficacy of entomopathogenic nematodes, focusing primarily on increasing efficacy. For example, Gaugler *et al.* (1989, 1991) and Gaugler and Campbell (1991) selected *Steinernema carpocapsae* for enhancement of host-finding ability in laboratory conditions. The enhanced

TABLE 2. Performance of nematicide-selected lines in traits relevant to biocontrol efficacy

Nematode population	Heat tolerance (% survival ± SEM)	Reproduction potential (IJs/cadaver ± SEM) (×1000)	Virulence			
			LE_{50} (h)[a]	CI(95%) (h)[b]	LE_{90} (h)[a]	CI(95%) (h)[b]
BP[c]	43.2 ± 3.5ab[d]	138.3 ± 26.4b	4.7	2.6–7.1	11.6	7.4–15.3
FE	50.6 ± 4.7a	181.0 ± 35.9ab	5.1	3.8–8.5	12.3	8.1–14.8
OX	37.5 ± 6.9b	203.4 ± 31.6a	5.5	4.0–6.5	10.5	6.8–14.3
AV	44.8 ± 5.1ab	207.5 ± 33.8a	3.9	2.4–6.4	13.0	7.8–16.3

[a] LE_{50} = lethal exposure period necessary to kill 50% of the insect population.
[b] CI = confidence interval.
[c] BP = base population.
[d] Values bearing the same letters are not significantly different, $a < 0.05$.

trait was subsequently shown to be due to the increased sensitivity of the nematodes to CO_2 released by the target insect (Gaugler *et al.*, 1991). Tomalak (1994) used selection, under more natural conditions, to enhance infectivity of *S. feltiae* against sciarid flies in mushroom houses. Heterorhabditids (Griffin & Downes, 1994) and Steinernematids (Grewal *et al.*, 1996a) were successfully selected for increased infectivity at low temperatures. Grewal *et al.* (1996b) also selected *H. bacteriophora* for enhanced heat tolerance. The present study demonstrates that genetic selection can also be applied to enhance nematode resistance to chemical nematicides.

The retention of the resistance to OX and AV observed after removal of the selection pressure is a significant finding relative to some previous attempts at the genetic improvement of beneficial traits in entomopathogenic nematodes. In those reports, rapid loss of the improved trait was observed after selection was relaxed (e.g. Burman & Pye, 1980; Gaugler *et al.*, 1989). For the FE-resistant line, a similar loss of resistance was noted after six rounds of rearing without exposure to FE (Figure 3). Thus, for FE resistannce, five rounds of selection were not sufficient to reach fixation of the resistance alleles. The loss of FE resistance during relaxed selection could reflect overdominance for FE resistance, negative genetic correlations with fitness characters, or linkage disequilibrium. The two latter phenomena are often cited as explanations for rebound during relaxed selection and for plateau (Hartl & Clark, 1989). The problem of linkage disequilibrium can be overcome by further selection, allowing time for recombination to produce more favorable genotypes. The first two causes of blocked selection may be overcome, at least partially, by the accumulation of modifiers.

It has been argued that such reversion of beneficial traits may not be important for the genetic improvement of entomopathogenic nematodes if they are only applied as short-term (inundative) biocontrol agents (Gaugler, 1988a). Recent studies, however, have indicated the potential for employing entomopathogenic nematodes in inoculative approaches (Parkman *et al.*, 1994). If entomopathogenic nematodes are used for long-term pest control, then stability of improved beneficial traits will be advantageous. Furthermore, large volume commercial production of entomopathogenic nematodes requires multiple generations to be reared in mass quantities and stored for variable periods of time. Accordingly, trait stability will be an important factor in the commercial production of genetically improved nematodes regardless of the application approach.

Two of the nematicides used in the present study, the carbamate Oxamyl and the organophosphate Fenamiphos, are commonly used antihelmintics. However, their mode of action is unknown. Resistance to OX and FE in plant-parasitic nematodes has been reported in the field (MacDonald, 1976; Smolik, 1978) and in the laboratory (Yamashita & Viglierchio, 1986), but its mechanism is unknown. Avermectin, however, is a more recently introduced nematicide. Its mode of action has been studied in some detail, particularly in the free-living rhabditid nematode *Caenorhabditis elegans* which is used to screen for new nematicides. AV has been shown to act by disrupting the GABA-mediated transmission of nerve signals (Campbell, 1989). The AV receptor has been suggested to be a glutamate-gated chloride channel (Arena *et al.*, 1995). AV-resistant nematode strains have been reported but the mechanism of resistance remains to be elucidated (Novàk & Vanék, 1992; Arena *et al.*, 1995). The relatively rapid response of the nematode population may indicate that these mechanisms are simple and exist in natural populations.

It is interesting that in the unselected populations of the nematode there appears to be no cross-resistance between lines, i.e. nematode inbred lines most resistant to one nematicide are not most resistant to another (Figures 1(a)–(c)). The cross-resistance observed among the selected lines (Figure 3) is hard to explain in the absence of detailed molecular information on the mechanism of action of these three nematicides. Since the mouths and the anuses of the IJs are closed, the nematicides probably penetrate through the cuticle (Mraček *et al.*, 1981). The cross-resistance could, therefore, reflect a general reduced permeability of the nematode cuticle. This hypothesis awaits anatomical and biochemical tests.

Often, selection for one trait results in a deterioration of other aspects of fitness which were not selected for (Hartl & Clark, 1989). The retention of biocontrol efficacy could also be due to the relatively large size of the population used at each round of selection. The study was aimed at 10–15% survivors, and of these a few hundred infected *G. mellonella* and reproduced, producing hundreds of thousands of progeny which served as the population for the next round of selection (see Materials and Methods). Presumably, these numbers allowed for retention of sufficient genetic variation in the selected lines with respect to the three parameters of efficacy. This biocontrol efficacy remains to be confirmed in field tests.

The importance of maintaining a high degree of genetic heterogeneity in the population has been emphasized in previous studies on the genetic selection of entomopathogenic nematodes (Gaugler *et al.*, 1989; Griffin & Downes 1994; Tomalak, 1994). Molecular approaches for estimating the degree of genetic variation in a population are readily applicable to entomopathogenic nematodes (Hashmi *et al.*, 1996; Shapiro *et al.*, 1997b). Applying them to the base population prior to the initiation of the selection, to verify that it is heterogeneous, would be desirable.

The nematicide-resistant lines produced in the present study add to the list of *H. bacteriophora* strains with enhanced beneficial traits. Controlled genetic crosses, facilitated by available genetic markers, are now feasible in this species (Koltai *et al.*, 1994) for transferring beneficial traits from one strain to another (Shapiro *et al.*, 1997a) and could be used to try to combine several beneficial traits into one 'superior' strain.

Entomopathogenic nematodes are used in conventional agriculture particularly in high-value crops (Georgis & Manweiler, 1994). There the use of various nematicides against harmful plant-parasitic nematodes is widespread. Therefore, the increase obtained in nematicide resistance of the selected lines may enhance the biocontrol efficacy of these entomopathogenic nematodes in the field, enabling their use to be expanded in the context of integrated pest management.

ACKNOWLEDGEMENTS

The authors are grateful to John Ringo for critical reading of the manuscript. This research was supported by the Binational Agricultural Research Development Fund, Grant IS-2099-92C.

REFERENCES

AKHURST, R.J. & BOEMARE, N.E. (1990) Biology and taxonomy of *Xenorhabdus*, in *Entomopathogenic Nematodes in Biological Control* (GAUGLER, R. & KAYA, H.K, Eds) CRC Press, Boca Raton, FL, pp. 75–90.

ARENA, J.P., LIU, K.K., PARESS, P.S., FRAZIER, E.G., CULLY, D.F., MROZIK, H. & SCHAEFFER, J.M. (1995) The mechanism of action of avermectins in *Caenorhabditis elegans*: Correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. *Journal of Parasitology* **81,** 286–294.

BOEMARE, N.E., AKHURST, R.J. & MOURANT, R.G. (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae) symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescence* to a new genus, *Photorhabdus* gen. nov. *Journal of Bacteriology* **43,** 249–255.

BURMAN, M. & PYE, A.E. (1980) *Neoaplectana carpocapsae*: Movements of nematode populations on a thermal gradient. *Experimental Parasitology* **49,** 258–265.

CAMPBELL, W.C. (1989) *Ivermectin and Abamectin.* Springer, New York.

GAUGLER, R, (1988a) Entomogenous nematodes and their prospects for genetic improvement, in *Biotechnological Advances in Invertebrate Pathology and Cell Culture* (MARAMOROSCH, K., Ed.) Academic Press, New York, pp. 457–484.

GAUGLER, R, (1988b) Ecological considerations in the biological control of soil-inhabiting insects with entomopathogenic nematodes. *Agriculture Ecosystem and Environment* **24,** 351–360.

GAUGLER, R. & CAMPBELL, J.F. (1991) Selection for enhanced hostfinding to scarab larvae (Coleoptera: Scarabaeidae) in the entomopathogenic nematode *Stein ernema carpocapsae. Environmental Entomology* **20,** 700–706.

GAUGLER, R., CAMPBELL, J.F. & MCGUIRE, T. (1989) Selection for hostfindings in *Steinernema feltae*. *Journal of Invertebrate Pathology* **54,** 363–372.

GAUGLER, R., CAMPBELL, J.F. & GUPTA, P. (1991) Characterization and basis of enhanced host-finding in a genetically improved strain of *Steinernema carpocapsae*. *Journal of Invertebrate Pathology* **57,** 234–241.

GEORGIS, R. & MANWEILER, S.A. (1994) Entomopathogenic nematodes: A developing biocontrol technology, in *Agricultural Zoology Reviews* (EVANS, K., Ed.) Intersept, Andover pp. 63–94.

GLAZER, I., GAUGLER, R. & SEGAL, D. (1991) Genetics of the entomopathogenic nematode *Heterorhabditis bacteriophora* strain HP88: The diversity of beneficial traits. *Journal of Nematology* **23,** 224–233.

GREWAL, P.S., GAUGLER, R. & WANG, Y. (1996a) Enhanced cold tolerance of the entomopathogenic nematode *Steinernema feltiae* through genetic selection. *Annals of Applied Biology* **126,** 335–341.

GREWAL, P.S., GAUGLER, R. & SHUPE, C. (1996b) Rapid changes in thermal sensitivity of entomopathogenic nematodes in response to selection at temperature extremes. *Journal of Invertebrate Pathology* **68,** 65–73.

GRIFFIN, C.T. & DOWNES, M.J. (1994) Selection of *Heterorhabditis* sp. for improved infectivity at low temperatures, in *Genetics of Entomopathogenic Nematode–Bacterium Complex*, COST 812 Workshop, Maynooth, Ireland. EUR 15681 EN Report, European Commission, pp. 143–151.

HARA, A.H. & KAYA, H.K. (1982) Effects of selected insecticides and nematicides on the *in vitro* development of the entomogenous nematode *Neoaplectana carpocapsae*. *Journal of Nematology* **14,** 86–91.

HARA, A.H. & KAYA, H.K. (1983) Development of the entomogenous nematode *Neoaplectana carpocapsae* (Rhabditida: Steinernematidae), in insecticide-killed beet armyworm (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **76,** 429–426.

HARTL, D.L. & CLARK, A.G. (1989) *Principles of Population Genetics*, 2nd Edn. Sinauer Assoc., Sunderland, MA.

HASHMI, G., GLAZER. I. & GAUGLER, R. (1996) Molecular characterization of entomopathogenic nematodes using RAPD markers. *Fundamental and Applied Nematology* **19,** 399–406.

ISHIBASHI, N. & TAKII, S. (1993) Effects of insecticides on movement, nictation, and infectivity of *Steinernema carpocapsae*. *Journal of Nematology* **25,** 204–213.

KAYA, H.K. (1985) Entomogenous nematodes for insect control in IPM systems, in *Biological Control in Agricultural IPM Systems* (HOY, M. & HERZOG, D. C., Eds) Academic Press, New York pp. 283–302.

KAYA, H.K. (1990) Soil ecology, in *Entomopathogenic Nematodes in Biological Control* (GAUGLER, R. & KAYA, H.K., Eds) CRC Press, Boca Raton, FL, pp. 93–115.

KAYA, H.K. & KOPPENHÖFER, A.M. (1996) Effect of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol Science and Technology* **6,** 357–371.

KOLTAI, H., GLAZER, I. & SEGAL, D. (1994) Phenotypic and genetic characterization of two new mutants of *Heterorhabditis bacteriophora*. *Journal of Nematology* **26,** 32–39.

MACDONALD, D.H. (1976) Effects of continued application of aldicarb to greenhouse rose beds infested with *Pratylenchus hamatus*. *Journal of Nematology* **8,** 293–294.

MRÁČEK, Z., CIERDIN, S. & WEISER, J. (1981). Head and cuticular structures of some species in the family Steinernematidae (Nematoda). *Nematologica* **27,** 443–448.

NOVÀK, J. & VANĚK, Z. (1992) Screening for new generation of antihelminthic compounds. *In vitro* selection of the nematode *Caenorhabditis elegans* for ivermectin resistance. *Folia Microbiologia* **37,** 237–238.

PARKMAN, J.P., FRANK, J.H., NGUYEN, K.B. & SMART, G.C. (1994) Inoculative release of *Steinernema scapterrisci* (Rhabditida: Steinernematidae) to suppress pest mole crickets (Orthoptera: Gryllotalpidae) on golf courses. *Environmental Entomology* **23,** 1331–1337.

POINAR, G.Q. JR. & GEORGIS, R. (1990) Characterization and field application of *Heterorhabditis bacteriophora* strain HPB8 (Heterorhabditidae: Rhabditida). *Reviue de Nematologie* **13,** 387–393.

RICCI, M., GLAZER, I. & GAUGLER, R. (1996) Comparison of laboratory bioassays to measure virulence of different entomopathogenic nematodes. *Biocontrol Science and Technology* **6,** 235–245.

ROVESTI, L. & DESEÖ, K.V. (1990) Compatibility of chemical pesticides with entomopathogenic nematodes, *Steinernema carpocapsae* Weiser and *Steinernema feltiae* (Nematoda, Steinernematidae). *Nematologica* **36,** 237–245.

ROVESTI, L., TAGLIENTE, F., DESEÖ, K.V. & HEINZPETER, E.W. (1989) Compatibility of pesticides with the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar (*Nematoda*: *Heterorhabditidae*). *Nematologica* **34,** 462–476.

SAS (1985) *SAS User Guide*, Version 5. SAS Institute, Cary, NC.

SELVAN, S., GAUGLER, R. & LEWIS, E. (1993) Biochemical energy reserves of entomopathogenic nematodes. *Journal of Parasitology* **79,** 167–172.

SHAPIRO, D.I., GLAZER, I. & SEGAL, D. (1996) Trait stability and fitness of the heat tolerant entomopathogenic nematode *Heterorhabditis bacteriophora* IS5 strain. *Biological Control* **6,** 238–244.

SHAPIRO, D.I., GLAZER, I. & SEGAL, D. (1997a) Genetic improvement of heat tolerance in *Heterorhabditis bacteriophora* through hybridization. *Biological Control* **8,** 153–159.

SHAPIRO, D.I., GLAZER, I. & SEGAL, D. (1997b) Genetic diversity in wild and laboratory populations of *Heterorhabditis bacteriophora* as determine by RAPD-PCR. *Fundamental and Applied Nematology* (in press).

SMOLIK, J.D. (1978) Influence of previous insecticidal use on ability of carbofuran to control nematode populations in corn and effect on corn yield. *Plant Disease Report* **62,** 95–99.

TOMALAK, M. (1994) Selective breeding of *Steinernema feltiae* (Filipjev) (Nematoda: Steinernematidae) for improved efficacy in control of a mushroom fly, *Lycoriella solani* Winnertz (Diptera: Sciaridae). *Biocontrol Science and Technology* **4,** 187–198.

WOODRING, J.L. KAYA, H.K. (1988) *Steinernematid and Heterorhabditid Nematodes: A Handbook of Techniques*. Southern Cooperative Series, Fayetteville, AR.

YAMASHITA, T.T. & VIGLIERCHIO, D.R. (1986) The behavior of nonfumigant nematicide-stressed, unstressed and wild populations of *Pratylenchus vulnus* cultured on grapevines and bean plants. *Revue de Nematologie* **9,** 267–276.

Fundam. appl. Nematol., 1997, **20** (6), 581-585

Genetic diversity in wild and laboratory populations of *Heterorhabditis bacteriophora* as determined by RAPD-PCR analysis

David I. SHAPIRO*°, Itamar GLAZER* and Daniel SEGAL**

**Department of Nematology, Volcani Center, Bet Dagan, 50-250 Israel, and **Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel.*

Accepted for publication 14 February 1997.

Summary – Genetic variation in laboratory reared biocontrol agents may be reduced due to founder effect, inbreeding, and selection. We used random amplified polymorphic DNA (RAPD-PCR) to compare genetic variation in two strains of *Heterorhabditis bacteriophora*. One strain (IS5) was recently isolated from the field and the other strain (HP88) has been reared under laboratory conditions for over 10 years. For each strain, fifteen inbred lines were generated by eight cycles of selfing of a single hermaphrodite (reaching > 90 % homozygosity). Genomic DNA from each of the inbred lines was screened with fourteen decamer primers. Genetic variation was calculated based on average percentage similarity of DNA banding patterns and cluster analysis. The level of within population variation detected did not differ significantly between the two strains.

Résumé – ***Diversité génétique chez des populations sauvages et des populations élevées au laboratoire d'*Heterorhabditis bacteriophora *telle que révélée par l'analyse en RAPD-PCR*** – La variabilité génétique d'agents de contrôle biologique élevés au laboratoire peut avoir des causes limitées à l'effet d'établissement, les croisements internes et la sélection. Les auteurs ont utilisé l'analyse en RAPD-PCR pour comparer la variabilité génétique de deux souches d'*Heterorhabditis bacteriophora*, l'une (souche IS5) récemment isolée du champ, l'autre (HP 88) élevée au laboratoire depuis 10 ans. Pour chaque souche, quinze lignées consanguines ont été produites grâce à huit cycles d'autofécondation d'un seul hermaphrodite (atteignant ainsi plus de 90 % d'homozygotie). L'ADN génomique de chaque lignée consanguine a été testé à l'aide de quatorze amorces décamères. La variabilité génétique a été calculée en se fondant sur la moyenne des pourcentages de similarité des profils d'ADN et sur une analyse en grappes. Les niveaux de variabilité interne de chacune des populations ne diffèrent pas significativement entre eux.

Key-words : biological control, diversity, entomopathogenic nematodes, genetic variation, RAPD.

Organisms that reproduce under non-natural conditions may lose genetic variation (Hartl & Clark, 1989). Such a phenomenon may lead to a decline in fitness. For example, inbreeding has been reported to cause yield reductions in crop plants and losses in fecundity and survivability in rats (Hartl & Clark, 1989). Similarly, reduction in genetic variation during laboratory rearing of biological control agents can jeopardize the use of these organisms in pest control. For example, genetic changes in laboratory-reared parasitic Hymenoptera have been reported to cause deficiencies in fecundity and longevity (Geden *et al.*, 1992), and in host acceptance and suitability (Van Bergeijk *et al.*, 1989). Furthermore, conservation of genetic variation in biological control agents is required for the success of genetic improvement programs involving artificial selection (Gaugler, 1988).

Soil dwelling nematodes in the genus *Heterorhabditis* have many promising traits as biological control agents including wide host ranges, easy storage, non-toxicity to mammals, host seeking ability, and a symbiosis with a pathogenic bacterium (*Photorhabdus luminescens*) which enables the nematodes to kill their hosts rapidly (Georgis & Manweiler, 1994). The nematodes generally enter their host as infective juveniles and complete two to three life cycles before exiting (Poinar, 1990). The first generation adults of *H. bacteriophora* are exclusively hermaphroditic and subsequent generations are mixed automictic and hermaphroditic forms (Strauch *et al.*, 1994; Koltai *et al.*, 1995).

The genetic variation in *Heterorhabditis* spp. has been hypothesized to be low due to hermaphroditic reproduction (Downes & Griffin, 1996). The objec-

° *Present address: Integrated BioControl Systems, Inc., Lawrenceburg, IN 47025, USA.*

tive of the present study was to evaluate genetic variation in two strains of the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar: the recently isolated IS5 strain and the HP88 strain, which has been reared under laboratory conditions for over 10 years.

Materials and methods

NEMATODES

Genetic variation was characterized in two strains of *H. bacteriophora*. The HP88 strain of *H. bacteriophora* was originally isolated from a single scarab larva (*Phyllophaga* sp.) in Logan, Utah, USA in 1982 (Poinar & Georgis, 1990). Since that time the strain has been reared continuously *in vivo* or *in vitro* (Friedman, 1990). We obtained our population of this strain from Ecogen, Inc. (Langhorne, PA, USA).

The IS5 strain was isolated from the Negev desert region of Israel in 1994 (Glazer *et al.*, 1996). The nematodes were recovered from soil samples using larvae of the greater wax moth, *Galleria mellonella* (L.) in a live baiting technique (Fan & Hominick, 1991). After the recovery of the first sample of this isolate from that site, and the initial characterization of its heat tolerance ability (Glazer *et al.*, 1996), fifteen additional soil samples were taken from the same spot. The founding population of IS5 used for the present study was constructed by pooling the nematodes from these samples. The IS5 nematodes were then reared in *G. mellonella* larvae for six passages at 30 °C using standard laboratory protocols (Woodring & Kaya, 1988). Morphological examination and measurements of the adults and infective juveniles from IS5 did not distinguish this new isolate from other *H. bacteriophora* nematodes (Glazer *et al.*, 1996). Further evidence that IS5 is a strain of *H. bacteriophora* was recently obtained when we demonstrated, using genetic markers, that nematodes from IS5 and the HP88 strain of *H. bacteriophora* can cross breed to produce fertile progeny (Shapiro *et al.*, 1997).

CREATION OF INBRED LINES

The genetic variation of the nematodes was evaluated by creating fifteen inbred lines for each of the strains. Creation of inbred lines was based on the procedures described in Glazer *et al.* (1991). Briefly, infective juveniles were surface sterilized and inoculated onto Nematode Growth Medium (Brenner, 1974) in 50 cm Petri dishes. Prior to nematode inoculation, the medium had been seeded with symbiotic bacteria (*P. luminescens*). After approximately 72 h of incubation at 25 °C each of the resulting nematode progeny, which were exclusively hermaphrodites, was transferred to new medium (one nematode per Petri dish). This process was repeated seven times resulting in inbred lines that were highly (> 95 %) genetically homogeneous (Hartl & Clark, 1989). To obtain sufficient numbers of nematodes for DNA extraction, nematodes from the final round of selfing were twice passed through *G. mellonella* larvae.

DNA EXTRACTION AND PCR ANALYSIS

DNA extraction was based on protocols described by Hashmi *et al.* (1996). Approximately one million adult nematodes were crushed in liquid nitrogen and then digested in 10 ml extraction buffer for 1 hour at 50 °C (Hashmi *et al.*, 1996). Protein was removed by phenol extractions and DNA was precipitated in ethanol.

The DNA from each inbred line was analyzed using random amplified polymorphic DNA markers (RAPD) (Welsh & McClelland, 1990; Williams *et al.*, 1990). Total volume for each reaction was 20 µl. Each reaction mixture contained 2 µl of Promega (Madison, WI) 10X assay buffer (500 mM KCl, 100 mM Tris-HCl, and 1 % Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 1.5 µM decamer primer, 0.5 units Taq DNA polymerase (Promega), and 20 ng DNA. The following fourteen Operon (Alameda, CA, USA) primers were chosen because they were previously shown to be suitable for RAPD analysis of *Heterorhabditis* DNA (Hashmi *et al.*, 1996): A01, A02, A03, A13, A18, C06, C09, C11, C12, HO4, H15, H18, H19, and S16.

The mixtures were placed in a MJ Research Mini-Cycler programmed as follows: 94 °C for 1 min, 40 °C for 2 min, and 72 °C for 3 min, then 39 cycles of 94 °C for 1 min, 40 °C for 1.5 min, and 72 °C for 2 min. The PCR products were subjected to gel electrophoresis in 1.2 % agarose gels. The DNA banding patterns (stained with Ethidium Bromide) were photographed using a #88-5 Polaroid camera.

DATA ANALYSIS

Average percentage similarities in band sharing were used to compare the degree of genetic variation between strains (Shapiro *et al.*, 1991; Rus-Kortekaas *et al.*, 1994). A similarity matrix was generated for the analysis of each primer with each strain. These matrices were based on the percentage of shared bands in all pair-wise comparisons. The equation $2cxy/(Tx + Ty) \times 100$ (where Cxy is the number of common bands and Tx and Ty are the total number of bands in that particular pair-wise comparison) was used to generate the similarity matrices (Nei & Li, 1979). The average percent similarities from each matrix was subjected to a T test to determine if differences in genetic variation between the strains were statistically significant. The analysis was paired according to primers so that average variation between strains was compared

tive of the present study was to evaluate genetic variation in two strains of the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar: the recently isolated IS5 strain and the HP88 strain, which has been reared under laboratory conditions for over 10 years.

Materials and methods

NEMATODES

Genetic variation was characterized in two strains of *H. bacteriophora*. The HP88 strain of *H. bacteriophora* was originally isolated from a single scarab larva (*Phyllophaga* sp.) in Logan, Utah, USA in 1982 (Poinar & Georgis, 1990). Since that time the strain has been reared continuously *in vivo* or *in vitro* (Friedman, 1990). We obtained our population of this strain from Ecogen, Inc. (Langhorne, PA, USA).

The IS5 strain was isolated from the Negev desert region of Israel in 1994 (Glazer *et al.*, 1996). The nematodes were recovered from soil samples using larvae of the greater wax moth, *Galleria mellonella* (L.) in a live baiting technique (Fan & Hominick, 1991). After the recovery of the first sample of this isolate from that site, and the initial characterization of its heat tolerance ability (Glazer *et al.*, 1996), fifteen additional soil samples were taken from the same spot. The founding population of IS5 used for the present study was constructed by pooling the nematodes from these samples. The IS5 nematodes were then reared in *G. mellonella* larvae for six passages at 30 °C using standard laboratory protocols (Woodring & Kaya, 1988). Morphological examination and measurements of the adults and infective juveniles from IS5 did not distinguish this new isolate from other *H. bacteriophora* nematodes (Glazer *et al.*, 1996). Further evidence that IS5 is a strain of *H. bacteriophora* was recently obtained when we demonstrated, using genetic markers, that nematodes from IS5 and the HP88 strain of *H. bacteriophora* can cross breed to produce fertile progeny (Shapiro *et al.*, 1997).

CREATION OF INBRED LINES

The genetic variation of the nematodes was evaluated by creating fifteen inbred lines for each of the strains. Creation of inbred lines was based on the procedures described in Glazer *et al.* (1991). Briefly, infective juveniles were surface sterilized and inoculated onto Nematode Growth Medium (Brenner, 1974) in 50 cm Petri dishes. Prior to nematode inoculation, the medium had been seeded with symbiotic bacteria (*P. luminescens*). After approximately 72 h of incubation at 25 °C each of the resulting nematode progeny, which were exclusively hermaphrodites, was transferred to new medium (one nematode per Petri dish). This process was repeated seven times resulting in inbred lines that were highly (> 95 %) genetically homogeneous (Hartl & Clark, 1989). To obtain sufficient numbers of nematodes for DNA extraction, nematodes from the final round of selfing were twice passed through *G. mellonella* larvae.

DNA EXTRACTION AND PCR ANALYSIS

DNA extraction was based on protocols described by Hashmi *et al.* (1996). Approximately one million adult nematodes were crushed in liquid nitrogen and then digested in 10 ml extraction buffer for 1 hour at 50 °C (Hashmi *et al.*, 1996). Protein was removed by phenol extractions and DNA was precipitated in ethanol.

The DNA from each inbred line was analyzed using random amplified polymorphic DNA markers (RAPD) (Welsh & McClelland, 1990; Williams *et al.*, 1990). Total volume for each reaction was 20 µl. Each reaction mixture contained 2 µl of Promega (Madison, WI) 10X assay buffer (500 mM KCl, 100 mM Tris-HCl, and 1 % Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 1.5 µM decamer primer, 0.5 units Taq DNA polymerase (Promega), and 20 ng DNA. The following fourteen Operon (Alameda, CA, USA) primers were chosen because they were previously shown to be suitable for RAPD analysis of *Heterorhabditis* DNA (Hashmi *et al.*, 1996): A01, A02, A03, A13, A18, C06, C09, C11, C12, HO4, H15, H18, H19, and S16.

The mixtures were placed in a MJ Research Mini-Cycler programmed as follows: 94 °C for 1 min, 40 °C for 2 min, and 72 °C for 3 min, then 39 cycles of 94 °C for 1 min, 40 °C for 1.5 min, and 72 °C for 2 min. The PCR products were subjected to gel electrophoresis in 1.2 % agarose gels. The DNA banding patterns (stained with Ethidium Bromide) were photographed using a #88-5 Polaroid camera.

DATA ANALYSIS

Average percentage similarities in band sharing were used to compare the degree of genetic variation between strains (Shapiro *et al.*, 1991; Rus-Kortekaas *et al.*, 1994). A similarity matrix was generated for the analysis of each primer with each strain. These matrices were based on the percentage of shared bands in all pair-wise comparisons. The equation $2cxy/(Tx + Ty) \times 100$ (where Cxy is the number of common bands and Tx and Ty are the total number of bands in that particular pair-wise comparison) was used to generate the similarity matrices (Nei & Li, 1979). The average percent similarities from each matrix was subjected to a T test to determine if differences in genetic variation between the strains were statistically significant. The analysis was paired according to primers so that average variation between strains was compared

for each primer and variation among primers was not a factor.

Additionally, differences in genetic variation were illustrated with a cluster analysis using the average linkage method (Anon., 1985). The cluster analysis used genetic distance (1-similarity) which was calculated from similarity matrices. If the average distances form less clusters in one strain than in the other then the strain with less clusters contains less genetic variation.

Results

RAPD-PCR analysis resulted in banding patterns (Fig. 1) that were reproducible. The total number of bands produced from fourteen primers was 74 and 61 for the IS5 and HP88 strains, respectively. After screening with eleven of the fourteen primers, DNA from a few of the IS5 inbred lines was used up and the nematodes were no longer available. Therefore the last three primers (A01, A02, and A03) were screened with less than fifteen inbred lines for each strain (Table 1).

Occasionally DNA from a particular inbred line would not produce any PCR product. If this result was repeated then the inbred line was scored as having no bands in common with the other lines. The absence of PCR products occurred in less than 2 % of the reactions and never more than twice with any primer.

The average percent similarities calculated for each primer varied from 68 %-100 % and 40.9 %-100 % for HP88 and IS5, respectively (Table 1). Statistical analysis indicated that the overall average similarity was not significantly different among the inbred lines of HP88 and IS5 (T= 2.02, df=14, α = 0.05).

The degree of genetic variation is also depicted in the results of the cluster analysis (Table 2). The degree of genetic variation may be determined by the number of clusters at specific average genetic distances. The number of clusters differ between IS5 and HP88 in only three of the eleven distances illustrated (Table 2).

Discussion

We found the genetic heterogeneity of a recently isolated population of *H. bacteriophora* to be similar to a population that has been reared in the laboratory for a long period. Thus, in this case considerable genetic variation has been retained in the HP88 population even after an extended period of reproduction under laboratory conditions. This conclusion is supported

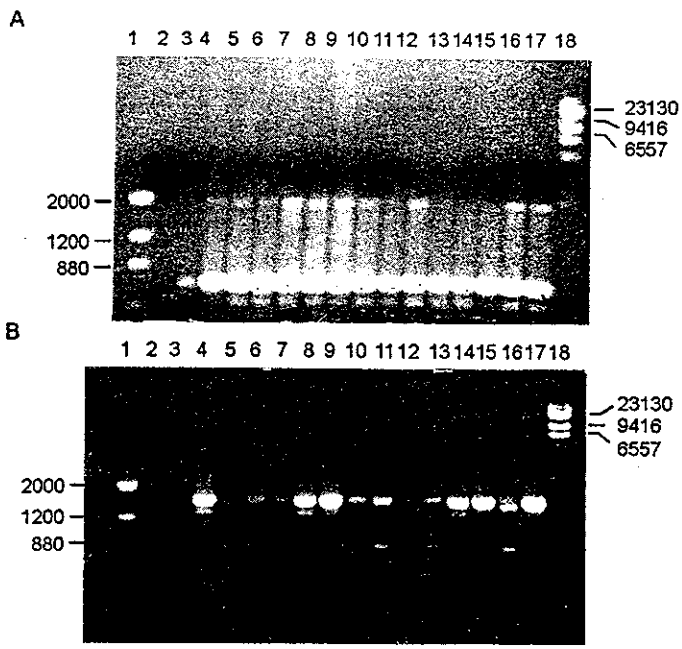


Fig. 1. *Examples of PCR products using Operon primer A13 and DNA from fifteen inbred lines of the HP88 (A) and IS5 (B) strains of* Heterorhabditis *sp. (Lane 1: Low weight DNA mass ladder (Giborcol, Inc.); Lane 2: A control containing the PCR mix minus DNA; Lanes 3-17: The fifteen inbred lines A-O; Lane 18: Lambda DNA cut with HindIII).*

Table 1. Average percent similarity in band sharing.

Operon primer	n	Nematode strain	
		HP88	IS5
C09	15	95.9	81.8
H18	15	81.4	81.1
A13	15	95.9	81.6
A18	15	77.6	100
C06	15	83.4	85.9
C11	15	86.1	86.7
C12	15	81.8	84.3
H04	15	93.0	58.0
H15	15	95.1	80.5
H19	15	68.0	68.7
S16	15	100	65.8
A01	12	69.9	65.1
A02	14	81.0	40.9
A03	14	87.6	84.9
Overall mean ± SE		85.5 ± 2.5	76.1 ± 3.84

DNA from n inbred lines of two strains of *Heterorhabditis* were subjected to RAPD-PCR analysis; subsequently, the average percent similarity was calculated from all pair-wise comparisons.
SE = Standard error of the mean.

Table 2. Number of clusters at various average genetic distances.

Distance	Nematode strain	
	HP88	IS5
1.0	1	1
0.9	2	2
0.8	2	2
0.7	2	2
0.6	2	2
0.5	3	2
0.4	3	3
0.3	3	8
0.2	7	14
0.1	14	15
0.08	15	15

DNA from n inbred lines of two strains of *Heterorhabditis* were subjected to RAPD-PCR analysis; subsequently, cluster analysis was made using the average linkage method.
Distance = 1 – similarity.

by a previous genetic study in which we have shown that an appreciable degree of genetic heterogeneity exists in the HP88 strain with respect to various traits such as desiccation tolerance, heat tolerance, host finding, and UV resistance (Glazer *et al.*, 1991). The genetic heterogeneity in HP88 can thus be used for selection studies (Glazer *et al.*, 1997). Better understanding of the source of genetic heterogeneity in a population requires information on the history of its development. The background information about the isolation of HP88 and about IS5 is rather limited, but the former apparently has passed through in a considerably narrower "bottle-neck" then the latter (see materials and methods).

Downes and Griffin (1996) hypothesized that genetic variation within populations of *Heterorhabditis* would be extremely low due to hermaphroditism. Therefore they suggested that the nematodes may be considered clonal, or nearly clonal, organisms. Further, a relatively limited dispersal combined with the low genetic variability would result in populations that are highly adapted to local environmental conditions. A high degree of specialization is indeed evident in the heat tolerant strain IS5 (Glazer *et al.*, 1996) and in cold tolerant isolates from Ireland (Griffin & Downes, 1991).

Attempts to characterize within population variation of entomopathogenic nematodes have heretofore included studies that analyzed variation in several phenotypic traits only (Glazer *et al.*, 1991). This is the first study to use molecular markers to characterize overall within population variation for an entomopathogenic nematode. More studies will be needed to determine if genetic variation of biological control agents decreases when they are reared under laboratory conditions over a long period. Verification of a loss in diversity can best be obtained by monitoring the genetic variation in a single population from the time it is collected until sufficient time has elapsed to make a decrease in genetic variation plausible.

Hopper *et al.* (1993) assert that the risk of loss in genetic diversity due to drift may be negligible as long as the populations size is not too small; furthermore, they argue that problems due to inbreeding in amphimictically reproducing biocontrol agents tend to be minimal. However, this conclusion is based on studies, with beneficial insects used for biological control, in which a relatively few number of generations were observed and mating was exclusively amphimictic (Hopper *et al.*, 1993). Because of their short generation time (about 5 days) and hermaphroditic reproduction *Heterorhabditis* spp. are likely to be more susceptible to problems of reduction of genetic diversity than other biocontrol agents.

Acknowledgements

The authors thank Liora Salame, Tali Zitman-Gal, and Svetlana Uretsky for technical assistance, and Dr. Uri Lavi for helpful advice and a critical review of an earlier draft of

this manuscript. Software to generate percent similarity from band sharing data was created by Charles Sharman. Valuable assistance in statistical analysis was provided by Dr. Abraham Gnizi. David I. Shapiro gratefully acknowledges support from the Fulbright Foundation and the United States - Israel Education Foundation. This research was funded in part by the Binational Agricultural Research Development Fund, grant No. IS-2099-92C This article was a contribution from the Agriculture Research Organization, Bet Dagan Israel, No. E-2094, 1996.

References

ANON. (1985). *SAS Users Guide: Version 5.* Cary, NC, USA, SAS Institute.

BRENNER, S. (1974). The genetics of *Caenorhabditis elegans. Genetics*, 77: 71-94.

DOWNES, M. J. & GRIFFIN, C. T. (1996). Dispersal behaviour and transmission strategies of the entomopathogenic nematodes *Heterorhabditis* and *Steinernema* (Rhabditida: Heterorhabditidae and Steinernematidae). *Biocontr. Sci. Technol.*, 6: 347-356.

FAN, X. & HOMINICK, W. M. (1991). Efficiency of the *Galleria* (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. *Revue Nématol.*, 14: 381-387.

FRIEDMAN, M. J. (1990). Commercialization and application technology. *In*: Gaugler R. & Kaya H. K. (Eds). *Entomopathogenic nematodes in biological control.* Boca Raton, FL, USA, CRC Press: 153-172.

GAUGLER, R. (1988). Entomogenous nematodes and their prospects for genetic improvement. *In*: Maramorosch K. (Ed.). *Biotechnology in invertebrate pathology and cell culture.* New York, NY, USA, Academic Press: 457-484.

GEDEN, C. J., SMITH, L., LONG, S. J. & RUTZ, D. A. (1992). Rapid deterioration of searching behavior, host destruction, and fecundity of the parasitoid *Muscidifurax raptor* (Hymenoptera: Pteromalidae) in culture. *Ann. ent. Soc. Am.*, 85: 179-187.

GEORGIS, R. & MANWEILER S. A. (1994). Entomopathogenic nematodes: a developing biological control technology. *Agric. Zool. Rev.*, 6: 63-94.

GLAZER, I., GAUGLER, R. & SEGAL, D. (1991). Genetics of the nematode *Heterorhabditis bacteriophora* strain HP88: the diversity of beneficial traits. *J. Nematol.*, 23: 324-333.

GLAZER, I., KOZODOI, E., HASHMI, G. & GAUGLER, R. (1996). Biological characteristics of the entomopathogenic nematode *Heterorhabditis* sp. IS-5: a heat tolerant nematode from Israel. *Nematologica*, 42: 481-492.

GLAZER, I., SALAME, L. & SEGAL, D. (1997). Genetic enhancement of nematicide resistance of entomopathogenic nematodes. *Biocontr. Sci. Technol.* (in press).

GRIFFIN, C. T. & DOWNES, M. J. (1991). Low temperature activity in *Heterorhabditis* sp. (Nematoda: Heterorhabditidae). *Nematologica*, 37: 83-91.

HARTL, D. L. & CLARK, A. G. (1989). *Principles of population genetics, 2nd ed.* Sunderland, MA, USA, Sinauer Assoc., 682 p.

HASHMI, G., GLAZER, I. & GAU, R. (1996). Molecular comparisons of entomopathogenic nematodes using Random Amplified Polymorphic DNA (RAPD) markers. *Fundam. appl. Nematol.*, 19: 399-406.

HOPPER, K. R., ROUSH, R. T. & POWELL, W. (1993). Management of genetics of biological-control introductions. *A. Rev. Ent.*, 38: 27-51.

KOLTAI, H., GLAZER, I. & SEGAL, D. (1995). Reproduction of the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar, 1976: hermaphroditism *vs* amphimixis. *Fundam. appl. Nematol.*, 18: 55-61.

NEI, M. & LI, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc natn. Acad. Sci. USA*, 74: 5267-5273.

POINAR, G. O., Jr (1990). Biology and taxonomy of Steinernematidae and Heterorhabditidae. *In*: Gaugler R. & Kaya H. K. (Eds). *Entomopathogenic nematodes in biological control.* Boca Raton, FL, USA, CRC Press: 23-62.

POINAR, G. O. JR & GEORGIS, R. (1990). Characterization and field applications of the HP88 strain of *Heterorhabditis bacteriophora. Revue Nématol.*, 13: 387-393.

RUS-KORTEKAAS, W., SMULDERS, M. J. M, ARENS, P. & VOSMAN, B. (1994). Direct comparison of levels of genetic variation in tomato detected by a GACA-containing microsatellite probe and by random amplified polymorphic DNA. *Genome*, 37: 375-381.

SHAPIRO, D. I., FUXA, J. R., BRAYMER, H. D. & PASHLEY, D. P. (1991). DNA restriction polymorphism in wild isolates of *Spodoptera frugiperda* nuclear polyhedrosis virus. *J. Invert.. Pathol.*, 58: 96-105.

SHAPIRO, D. I., GLAZER, I. & SEGAL, D. (1997). Genetic improvement of heat tolerance in *Heterorhabditis bacteriophora* through hybridization. *Biol. Control.*, 8: 153-159.

STRAUCH, O., STOESSEL, S. & EHLERS, R. U. (1994). Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis. Fundam. appl. Nematol.*, 17: 575-582.

VAN BERGEIJK, K. E., BIGLER, F., KAASHOEK, N. K. & PAK, G. A. (1989). Changes in host acceptance and host suitability as an effect of rearing *Trichogramma maidis* on a factitious host. *Ent. exp. & appl.*, 52: 229-238.

WELSH, J. & MCCLELLAND, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.*, 18: 7213-7218.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. & TINGEY, S. V. (1990). DNA polymorphisms amplified by primers are useful as genetic markers. *Nucleic Acids Res.*, 18: 6531-6535.

WOODRING, J. L. & KAYA, H. K. (1988). *Steinernematid and heterorhabditid nematodes: a handbook of techniques.* Fayetteville, AK, USA, Southern Cooperative Series Bulletin: 30 p.

Evaluation of Entomopathogenic Nematodes for Biocontrol of the European Corn Borer, *Ostrinia nubilalis*, on Sweet Corn in Israel

D. Ben-Yakir,[1] D. Efron,[2] M. Chen[1] and I. Glazer[3]

The potential of entomopathogenic nematodes for biological control of the European corn borer (ECB), *Ostrinia nubilalis* (Hübner), was evaluated under laboratory, screenhouse and field conditions. The 'All' and 'Mexican' strains of *Steinernema carpocapsae* (Weiser) and the 'HP88' strain of *Heterorhabditis bacteriophora* Poinar were compared in both dose response assays (5, 50 and 500 infective juveniles [IJ] per petri dish containing five 5th-instar ECB eggs; 72 h of incubation) and exposure time assays (3, 6 and 9 h of incubation). In the dose response assays the highest rates of ECB killing resulted from infestation with the Mexican strain of *S. carpocapsae.* In the exposure time assays there were no significant differences between the killing rates of the three nematode strains. Sweet corn plants (*Zea mays* var. *saccharata*) grown in a screenhouse, were infested with ECB neonates and 4 days later sprayed with a suspension of the Mexican strain of *S. carpocapsae* (50,000 IJ per plant). The number of ECB larvae found on treated corn plants after one week was significantly (P=0.05) lower (3- to 5-fold) than the number found on untreated plants. Similar treatment in the field significantly reduced the rate of economic ear damage from 20% to 5%.
KEY WORDS: Entomopathogenic nematodes; *Heterorhabditis bacteriophora; Steinernema carpocapsae; Ostrinia nubilalis;* European corn borer; biological control; sweet corn.

INTRODUCTION

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is a major pest of corn, *Zea mays* L., in Europe and North America (17) and also in Israel (6,23,25). ECB is difficult to control by contact insecticides because the larvae bore into plant tissues shortly after hatching (17). In addition, pesticide residues in food are becoming increasingly unacceptable to consumers. These constraints have encouraged the search for biological control methods for this pest.

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are parasites that carry mutualistically associated entomopathogenic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) in their intestines (2,7). The infective juveniles (IJ) actively seek and invade insects (21). Infected insects die of septicemia following the release of the symbiotic bacteria by the invading nematodes (1). These nematodes are used

Contribution from the Agricultural Research Organization. No. 2260-E, 1997 series. Received Aug. 4, 1997; received in final form Dec. 29, 1997; http://www.phytoparasitica.org posting March 4, 1998.
[1]Dept. of Entomology, ARO, The Volcani Center, Bet Dagan 50250, Israel [Fax: +972-3-9604180; e-mail: glazeri@netvision.net.il].
[2]Dept. of Entomology, The Hebrew University of Jerusalem, Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot 76100, Israel.
[3]Dept. of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel.

as biological control agents for soil-inhabiting insect pests (11,19); attempts to use them to control foliage-feeding insects have usually produced disappointing results (18). This is probably because the foliar environment has low humidity, high temperatures and intense sunlight, which are unfavorable conditions for nematode survival (5). However, insects were successfully controlled by nematodes above ground when they resided in protected sites on plants (5). Neonate larvae of ECB often migrate to leaf axils, and remain in these protected and moist sites for a few days (8). Therefore, we also tested the ability of the nematodes to control them in those niches on corn plants.

Previous studies have indicated the possibility of using entomopathogenic nematodes to control corn pests. In laboratory tests, ECB was killed effectively by the DD-136 strain of *Steinernema carpocapsae* (Weiser). However, these nematodes have failed to control overwintering larvae in cornstalk debris under field conditions (22). When nematodes of the 'All' and 'Mexican' strains of *S. carpocapsae* were sprayed on ears of sweet corn infested with the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and the corn earworm, *Helicoverpa zea* (Boddie), the pest populations were reduced by 53% compared with untreated plants (27). The 'Agriotos' strain of *S. feltiae (=carpocapsae)* gave 92% control of the Asian corn borer, *Ostrinia furnacalis* (Guenée) (16), which is a close relative of ECB.

In this study we first selected the most effective nematode strain for ECB control in the laboratory testing. For this purpose the All and Mexican strains of *S. carpocapsae* and the 'HP88' strain of *Heterorhabditis bacteriophora* Poinar were used. Then we evaluated the performance of the most effective nematode on infested plants under various environmental conditions.

MATERIALS AND METHODS

Insects and nematodes

ECB larvae were reared on a meridic diet as described by Melamed-Madjar and Raccah (24). We used 5th instar larvae (17 days old; head width 1.6±0.2 mm) for the laboratory studies and eggs at the black-head stage (shortly before hatching) for the on-plant studies. The Mexican and All strains of *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae), and the HP88 strain of *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), were originally obtained from "biosys" (Columbia, MD, USA) The nematodes were reared on an artificial medium according to a method described by Bedding (3). In all tests, infective juveniles (IJ) of nematodes were suspended in deionized water to obtain the desired concentration. Deionized water alone was applied to the control groups. During laboratory studies, insects and nematodes were kept at 25±2°C and 16:8 L:D in 5-cm-diam petri dishes. After infestation, petri dishes were sealed with parafilm to maintain a humid environment.

Dose-response assay

Nematode suspensions with concentrations of 10, 100 and 1000 IJ/ml were prepared. Ten treatments (including control) were carried out, in three replicates (= petri dishes) per treatment with five ECB larvae per replicate. One-half ml of suspension was applied to a 5-cm-diam filter-paper disc (Whatman no. 1) placed in a petri dish (*i.e.*, 5, 50 and 500 IJ/dish). ECB larvae were added shortly after that. The insects were inspected and dead

larvae were recorded and removed every 24 h for 3 days. The entire assay was repeated three times.

Exposure-time assay

ECB larvae were exposed to nematodes (500 IJ/dish) for periods of 3, 6 and 9 h (ten treatments, six replicates, five ECB larvae per replicate). At the end of each exposure period, ECB larvae from two petri dishes of each treatment were rinsed and transferred to clean petri dishes. Insect mortality was recorded 48 h after initial exposure. The entire assay was repeated three times. Dead ECB larvae from the third run were kept under moist conditions for 3 days, then dissected individually in deionized water. The numbers of nematodes in these larvae were determined 20 h after dissection with the aid of a stereoscopic microscope.

Screenhouse and greenhouse assays

Sweet corn (*Zea mays* var. *saccharata* (Sturter) Bailey, cv. 'Jubilee') plants were grown in large planters (150×40×40 cm, ten plants/planter) in a screenhouse (white screen, 30 mesh) during June through September 1993 (trials 1 and 2), or in 10-*l* planting pots in a greenhouse during October 1994 to January 1995 (trial 3). Plants at the late whorl stage were infested with 100 ECB eggs on a piece of waxed paper which was attached to the underside of the second-from-top leaf. Four days after infestation, plants were sprayed with a suspension of the Mexican strain of *S. carpocapsae* (5000 IJ/ml) in water from the top downwards, using a hand sprayer. Each plant was sprayed with 10 ml at dusk, to provide better survival conditions for the nematodes (two treatments including control, 10–13 plants per treatment). All plants grown together in a planter received the same treatment. Six days after spraying, plants were dissected and the numbers of ECB larvae found on individual plants were recorded. Only a very small portion of the larvae developed in the tassel. Those larvae were omitted because they were unlikely to cause any damage to the plant.

Field trial

Small experimental plots (8×15 m) of sweet corn were planted at Bet Dagan on May 1 (trial 1) and June 5 (trial 2), 1995. Tasseling dates were June 19 and July 16, respectively. Plants were grown under conditions similar to those in commercial fields (irrigated and enriched with 300 nitrogen units/ha; 60,000 plants/ha). There were four plots for each treatment, arranged in a random block design. One week after tasseling, ten plants were randomly selected in each plot and each plant was infested with 40 ECB eggs at the black-head stage (*i.e.*, within 24 h of hatching). Eggs (on a piece of waxed paper) were attached to either the underside of the second leaf above the ear (trial 1) or to the ear itself (trial 2). Four days after infestation, the ears on each plant were sprayed with a 10 ml suspension of the Mexican strain of *S. carpocapsae* (5,000 IJ/ml) in aqueous solution of 6% Folicote (Asia-Riesel, Ramat Gan, Israel) and 0.05% Tween 80 (Sigma Chemicals, St. Louis, MO, USA), using a hand sprayer. Nematodes were sprayed again, at dusk, to provide better conditions for their survival. Control plants were sprayed with 10 ml of the aqueous solution of 6% Folicote and 0.05% Tween 80. At harvest time, the percent economic damage (for processing corn, *i.e.*, damage to the body of the ear excluding the top 2 cm) was determined for each group.

Statistical analysis

Data obtained during laboratory studies were normalized by an arcsine of square root transformation. The quantity (dose) of IJ per dish was subjected to logarithmic transformation. The significance of differences in strain effect were determined by analysis of variance (ANOVA); in on-plant larval mortality, by Student's t-test; and in economic damage, using a single factor ANOVA. The significance level for all analyses was $P<0.05$. Treatments were ranked using Duncan's multiple range test (9).

RESULTS

Dose-response assay

The three different experiments were considered as randomized blocks. Statistical analysis of the overall study showed that the Mexican strain of *S. carpocapsae* caused a significantly higher death rate than the two other strains (df 2, 80; F=9.1; $P<2\times10^{-4}$). In the control treatment, none of the larvae died. The differences among the ECB mortality rates caused by the three strains were most noticeable after 48 h of exposure (Fig. 1).

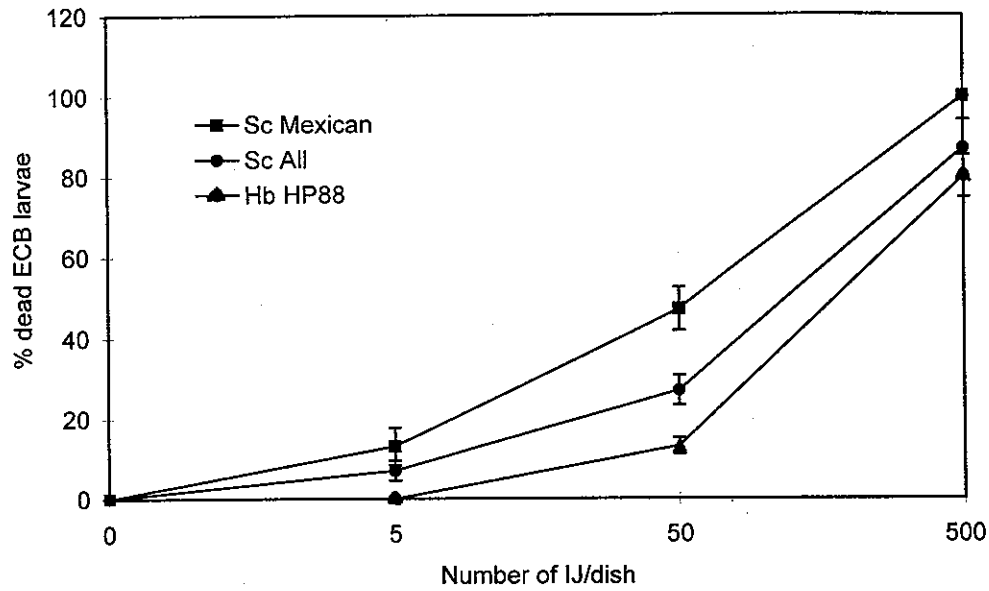


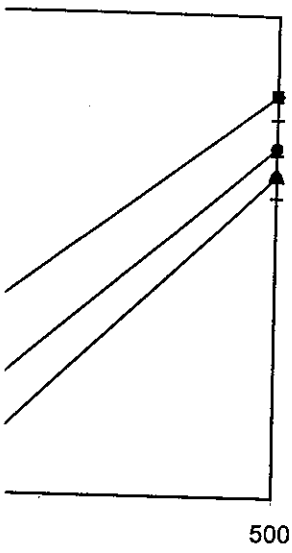
Fig. 1. Percent of dead European corn borer (ECB) larvae (mean of three petri dishes, five larvae per dish ±SEM as bars) 48 h after exposure to infective juveniles (IJ) of entomopathogenic nematodes (*Steinernema carpocapsae,* Sc; *Heterorhabditis bacteriophora,* Hb) at various doses. Data were normalized by an arcsine of square root transformation.

Exposure-time assay

Data obtained from the three experiments were combined, since no significant differences were found among them (Fig. 2). No significant differences were found in ECB killing rates among the three nematode strains. Overall, ECB larval death rate was

by an arcsine of square
subjected to logarithmic
re determined by analysis
s t-test; and in economic
all analyses was $P<0.05$.

nized blocks. Statistical
S. carpocapsae caused a
); F=9.1; $P<2\times10^{-4}$). In
mong the ECB mortality
f exposure (Fig. 1).



petri dishes, five larvae per
omopathogenic nematodes
various doses. Data were

l, since no significant
ferences were found in
B larval death rate was

significantly higher after 9 h exposure to the nematodes than after 3 or 6 h exposure. The number of nematodes found in dead ECB larvae ranged between 0 and 44, and no relationship could be established between these numbers and the time of exposure.

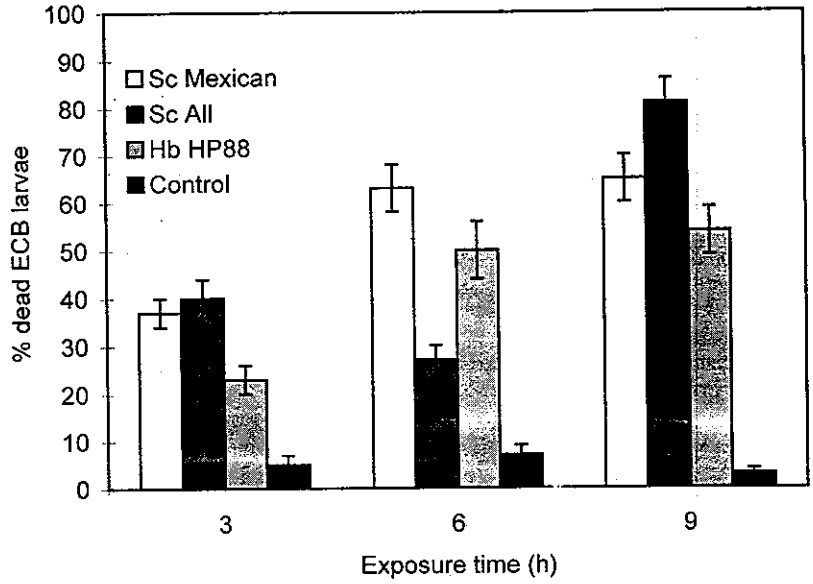


Fig. 2. Percent of dead European corn borer (ECB) larvae (mean of two petri dishes, five larvae per dish; SEM as bars) after various exposure times to entomopathogenic nematodes (*Steinernema carpocapsae*, Sc; *Heterorhabditis bacteriophora*, Hb) at an infestation rate of 500 infective juveniles per petri dish. Data were normalized by an arcsine of square root transformation.

Screenhouse and greenhouse assays

The number of ECB larvae found on corn plants that had been sprayed with nematode suspension was significantly lower in all three trials than that found on the control plants (Table 1). In trial 3 approximately half of the ECB larvae developed on the tassel, which was not sprayed with nematodes. Therefore, these larvae were not included in the analysis of the results.

TABLE 1. Mean number (±SD) of European corn borer larvae per plant[z] 6 days after the plants were sprayed with a suspension (5000 IJ/ml) of the Mexican strain of *Steinernema carpocapsae* nematodes in water

Treatment	Trial 1	Trial 2	Trial 3[y]
Nematodes	2.8 (±1.8) a[x]	3.4 (±3.3) a	1.9 (±1.6) a
Water	7.9 (±4.3) b	18.7 (±6.1) b	3.3 (±2.1) b

[z] n=10–13 plants; each plant was infested with 100 ECB eggs 4 days before spraying.
[y] Larvae that developed in the tassel were not included.
[x] Within columns, means followed by different letters are significantly different (Student's t-test, P=0.05).

Field trial

Infestation directly on the ear resulted in greater economic damage than infestation on the second leaf above the ear. In both trials, treatment of the ear with nematodes reduced the economic damage significantly (Table 2).

TABLE 2. Mean percent (±SD) of economic damage caused by European corn borer larvae per plot[z] at harvest time, after the ears were sprayed with a suspension (5000 IJ/ml) of the Mexican strain of *Steinernema carpocapsae* nematodes in aqueous solution of 6% Folicote and 0.05% Tween 80

Treatment	Trial 1[y]	Trial 2[x]
Nematodes	0.0 (±0.0) a	5.3 (±6.1) a[w]
Water	6.8 (±5.0) a	19.7 (±5.7) b

[z] n=four plots, ten plants per plot; each plant was infested with 40 ECB eggs 4 days before spraying.
[y] Eggs were attached to the second leaf above the ear.
[x] Eggs were attached to the ear itself.
[w] Within columns, means followed by different letters are significantly different (Student's t-test, P=0.05).

DISCUSSION

The efficacy of various nematode species or strains for controlling a particular insect pest may differ significantly (4,10,20). Efficacy is influenced by the rate of IJ penetration into the insect, the time it takes to release the symbiotic bacteria, and the virulence of the latter (12). In the present study, when ECB was exposed to various dosages of nematodes in the laboratory for 48 h, the Mexican strain of *S. carpocapsae* killed the greatest number of larvae. Although direct relationships were found between results obtained in dose-response and exposure-time assays in studies of the effect of nematodes on other lepidopteran pests (13,26), in the present study no such relationships were found. This may be due to the fact that exposing ECB for up to 9 h (as compared with the 48 h of exposure during the dose-response assay) was insufficient to distinguish among the killing effects of the three nematode strains tested. Indeed, within the exposure-time assay, ECB larval death rate gradually increased and was significantly higher after 9 h of exposure than after 3 or 6 h.

Since the Mexican strain of *S. carpocapsae* had given the best control of ECB in the dose-response assay and had been shown to be more tolerant to low relative humidity conditions than the other strains tested here (14,15), it was chosen for the on-plant assay, aimed at controlling ECB neonates during the tasseling to green silk stages of the corn plant. These neonates usually feed in the leaf axil (8). The corn leaves act as funnels, directing applied fluids such as nematode suspensions to the axil. Therefore, the likelihood of contact between the nematodes and young ECB larvae is high. Because ECB larvae remain in this vulnerable site for only a few days after hatching (17), accurate timing of application is essential for effective control. The effectiveness of control also depends on the length of time the nematodes can remain infective on the plant (residual effect). He *et al.* (16) reported that under field conditions in China, entomopathogenic nematodes remained infective on corn plants for 5 to 7 days. Richter and Fuxa (27) obtained similar results in Louisiana (USA) when they applied nematodes to ears of sweet corn in the field.

In this study, application of the Mexican strain of *S. carpocapsae* to the corn ears under field conditions reduced the economic damage significantly. In both trials treatment

reduced the ear damage to less than 5.5% which is the maximum level of damage accepted by the processing plants. This reduction was achieved despite adverse weather conditions for the nematodes (mid summer) and a relatively high infestation rate.

This has been a pilot study to decide whether additional research on this control method is warranted. The results of our study, together with previous reports, indicate that entomopathogenic nematodes may be used successfully for the control of ECB. Additional studies are needed for developing commercial methods of application and evaluation of economic feasibility.

ACKNOWLEDGMENTS

The authors are grateful to the late Professor David Rosen, Department of Entomology, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem; and to Liora Salame, Department of Nematology, ARO, The Volcani Center, for their generous assistance and advice. This study was partly supported by the Israel Ministry of Agriculture through the Chief Scientist's Fund, and by the Corn Growers' Association, as Project No. 131-0762.

REFERENCES

1. Akhurst, R.J. (1983) *Neoaplectana* species: specificity of association with bacteria of the genus *Xenorhabdus*. *Exp. Parasitol.* 55:258-263.
2. Akhurst, R.J. and Boemare, N.E. (1990) Biology and taxonomy of *Xenorhabdus*. pp. 75-87. *in:* Gaugler, R. and Kaya, H.K. [Eds.] Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, USA.
3. Bedding, R.A. (1981) Low cost, *in vitro* mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. *Nematologica* 27:109-114.
4. Bedding, R.A., Molyneux, A.S. and Akhurst, R.J. (1983) *Heterorhabditis* spp., *Neoaplectana* spp. and *Steinernema kraussei*: Interspecific and intraspecific differences in infectivity for insects. *Exp. Parasitol.* 55:249-257.
5. Begley, J.W. (1990) Efficacy against insects in habitats other than soil. pp. 215-231. *in:* Gaugler, R. and Kaya, H.K. [Eds.] Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, USA.
6. Ben-Yakir, D. and Chen, M. (1995) The phenology of corn borer on sweet corn in Israel. *XVI Symp. International Working Group on Ostrinia* (Volos, Greece), 1993, pp. 53-57.
7. Boemare, N.E., Akhurst, R.J. and Mourant, R.G. (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Fotorhabdus* gen. nov. *Int. J. Syst. Bacteriol.* 43:249-255.
8. Coll, M. and Bottrell, D.G. (1991) Microhabitat and resource selection of the European corn borer (Lepidoptera: Pyralidae) and its natural enemies in field corn. *Environ. Entomol.* 20:526-533.
9. Duncan, D.B. (1955) Multiple range and multiple F tests. *Biometrics* 11:1-42.
10. Forschler, B.T. and Nordin, G.L. (1988) Comparative pathogenicity of selected entomogenous nematodes to the hardwood borers, *Prionoxystus robiniae* (Lepidoptera: Cossidae) and *Megacyllene robiniae* (Coleoptera: Cerambicidae). *J. Invertebr. Pathol.* 52:343-347.
11. Georgis, R. (1992) Present and future prospects for entomopathogenic nematode products. *Biocontrol Sci. Technol.* 2:83-99.
12. Glazer, I. and Navon, A. (1990) Activity and persistence of entomogenous nematodes used against *Heliothis armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 83:1795-1800.
13. Glazer, I. (1992) Measures for evaluation of entomopathogenic nematode's infectivity to insects. pp. 195-200. *in:* Gommers, F.J. and Mass, P.W.Th. [Eds.] Nematology from Molecule to Ecosystem. European Society of Nematologists, Dundee, Scotland.

14. Glazer, I. (1992) Invasion rate as a measure of infectivity of steinernematid and heterorhabditid nematodes to insects. *J. Invertebr. Pathol.* 59:90-94.
15. Glazer, I., Klein, M., Navon, A. and Nakache, Y. (1992) Comparison of efficacy of entomopathogenic nematodes combined with antidesiccants applied by canopy against three cotton pests (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 85:1636-1641.
16. He, K., Zhou, D. and Yang, H. (1991) Biological control of Asian corn borer with entomopathogenic nematode, *Steinernema feltiae* Agriotos. *Chinese J. Biol. Control* 7:1-6 (Chinese, with English summary).
17. Hudon, M., LeRoux, E.J. and Harcourt, D.G. (1989) Seventy years of European corn borer (*Ostrinia nubilalis*) research in North America. *Agric. Zool. Rev.* 3:53-96.
18. Kaya, H.K. (1985) Entomogenous nematodes for insect control in IPM systems. pp. 283-302. *in:* Hoy, M.A. and Herzog, D.C. [Eds.] Biological Control in Agricultural IPM Systems. Academic Press, New York, NY.
19. Klein, M.G. (1990) Efficacy against soil-inhabiting insect pests. pp. 195-214. *in:* Gaugler, R. and Kaya, H.K. [Eds.] Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, USA.
20. Kondo, E. and Ishibashi, N. (1988) Histological and SEM observations on the invasion and succeeding growth of the entomopathogenic nematode *Steinernema feltiae* (Str. DD-136) in *Spodoptera litura* (Lepidoptera: Noctuidae) larvae. *Appl. Entomol. Zool.* 23:88-96.
21. Lewis, E.E., Gaugler, R. and Harrison, R. (1993) Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. *Can. J. Zool.* 71:765-769.
22. Lewis, L.C. and Raun, E.S. (1978) Laboratory and field evaluation of the DD-136 strain of *Neoaplectana carpocapsae* for control of the European corn borer, *Ostrinia nubilalis. Iowa J. Res.* 52:391-396.
23. Melamed-Madjar, V. (1990) Status of *Chilo agamemnon* Bles. in Israel and the probable reasons for the decrease in its populations. *Insect Sci. Appl.* 11:541-545.
24. Melamed-Madjar, V. and Raccah, B. (1979) The transstadial and vertical transmission of a granulosis virus from the corn borer *Sesamia nonagrioides. J. Invertebr. Pathol.* 33:259-264.
25. Melamed-Madjar, V. and Tam, S. (1980) A field survey of changes in the composition of corn borer populations in Israel. *Phytoparasitica* 8:201-204.
26. Ricci, M., Glazer, I. and Gaugler, R. (1996) Entomopathogenic nematode infectivity assay: Comparison of laboratory bioassays. *Biocontrol Sci. Technol.* 6:235-245.
27. Richter, A.R. and Fuxa, J.R. (1990) Effect of *Steinernema feltiae* on *Spodoptera frugiperda* and *Heliothis zea* (Lepidoptera: Noctuidae) in corn. *J. Econ. Entomol.* 83:1286-1291.

Interaction of Entomopathogenic Nematodes (*Steinernematidae*) With Selected Species of Ixodid Ticks (Acari: Ixodidae)

KATHERINE M. KOCAN,[1] MARSHA S. PIDHERNEY,[1] EDMOUR F. BLOUIN,[1] P. L. CLAYPOOL,[2] MICHAEL SAMISH,[3] AND ITAMAR GLAZER[4]

J. Med. Entomol. 35(4): 514–520 (1998)

ABSTRACT Entomopathogenic nematodes, currently used for biological control of various insect pests, were tested for their ability to penetrate and kill replete females of several species of ticks including *Dermacentor variabilis* (Say), *Rhipicephalus sanguineus* (Latreille), *Amblyomma maculatum* Koch, and *A. cajennense* (F.). These species were found to be susceptible to the entomopathogenic nematodes, *Steinernema feltiae* (Filipjev) or *S. riobravus* (Cabanillas & Poinar), shown in previous studies in our laboratory to be attracted to and kill replete *A. americanum*. *S. riobravus* killed *D. variabilis* (96%), *R. sanguineus* (89%), *A. maculatum* (24%), and *A. cajennense* (88%), and *S. feltiae* killed *D. variabilis* (91%) and *R. sanguineus* (71%). Of the ticks that survived, mean egg mass weights were significantly lower than those of the unexposed controls. When nematode-exposed ticks were examined with light microscopy, nematodes were found to have entered ticks but did not multiply or produce subsequent generations of infective juveniles. The nematodes were separated from surrounding tissues by a clear space, suggesting that they produced protective compounds. Bacteria, thought to be symbiotes released from the nematodes, multiplied initially in the hemocoel of the tick and subsequently were found throughout the degenerating tick tissues. These bacteria eventually filled the tick and appeared to be the cause of tick death. Nematode guts were filled with the bacteria, suggesting that the bacteria were a food source. When ticks were exposed to nematodes while feeding on cattle, partially engorged females were most susceptible to the nematodes. Tick mortality and reduced egg production resulted when the ticks had fed 6 and 9 d before nematode exposure but not when ticks were exposed after 3 d of feeding. Exposure of feeding female ticks demonstrated that the nematodes were able to penetrate tick orifices other than via the hypostome, which was embedded in the bovine epidermis for the duration of the feeding process.

KEY WORDS *Steinernema feltiae*, *Steinernema riobravois*, ixodid ticks, interaction, entomopathogenic nematodes, biological control

ENTOMOPATHOGENIC NEMATODES OF the families Steinernematidae and Heterorhabditidae have been used for biological control of several important insect pests of plants. These 2 families of facultative parasitic rhabditoids are characterized by their association with symbiotic bacteria of the genus *Xenorhabdus* (Akhurst 1980, 1983). The nematode 3rd-stage juvenile, which is infective for insect larvae, carries this bacterial symbiont monoxenically in a specialized vesicle located in the foregut (Poinar and Thomas 1966). The infective juvenile seeks the insect host by a chemotactic response (Gaugler et al. 1980) and penetrates the host through natural body openings (Akhurst 1986, Marcek et al. 1988). After the nematodes have entered the hemocoel of the insect host, the *Xenorhabdus* symbiotic bacteria are released into the hemolymph where they proliferate, and the host dies from septicemia, usually within 24–48 h. The bacteria release antibiotic-like compounds, thus creating a stable habitat for proliferation of the *Xenorhabdus* by inhibiting growth of other microorganisms. The symbiotic bacteria also serve as a food source for the developing nematodes. After 2–3 generations of nematodes have been produced, the infective juveniles develop and emerge from the insect cadaver. Thousands of infective juveniles that will search for a new host can be produced from a single insect larva in ≈2–3 wk (Poinar 1986).

Ticks are important pests of animals worldwide. They serve as vectors of several animal diseases including *Anaplasma*, *Babesia*, *Cowdria*, *Ehrlichia*, and *Theileria* (Sonenshine 1993). They also transmit important human zoonotic diseases such as Lyme disease, Rocky Mountain spotted fever, relapsing fever, and Q fever (Sonenshine 1993). In addition to the transmission of pathogens, ticks often provoke anemia, toxicosis, weight loss, hide damage, and tick paralysis. Annual worldwide losses from ticks and their pathogens have been conservatively estimated at $7 billion (U.S.). Tick control has been effected primarily by

[1] Department of Anatomy, Pathology and Pharmacology, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK 74078.
[2] Department of Statistics, Oklahoma State University, Stillwater, OK 74078.
[3] Division of Parasitology, Kimron Veterinary Institute, Bet-Dagan 50250, Israel.
[4] Department of Nematology, the Volcani Center ARO Institute, Bet-Dagan 50250, Israel.

0022-2585/98/0514–0520$02.00/0

application of chemicals which often produce undesirable effects. Acaricides require stringent application regimes, and ticks develop resistance to them. The residual chemicals often cause environmental pollution, may cause toxic reactions in domestic and wild animals, and the expense of this labor-intensive control method is enormous (Norval et al. 1992).

Recent studies have shown that engorged females of some tick species are susceptible to infection with entomopathogenic nematodes. Samish and Glazer were the first to demonstrate in laboratory studies that fully engorged *Boophilus annulatus* (Say) ticks were highly susceptible to infection by nematodes of the families Steinernematidae and Heterorhabditidae (Glazer and Samish 1993; Samish and Glazer 1991, 1992) Mauléon et al. (1993) also demonstrated that the nematodes would kill replete *B. annulatus* but found that *Amblyomma variegatum* (F.) and *B. microplus* (Canestrini) were resistant when exposed to the nematodes. *Steinernema carpocapsae* and *S. glaseri* were found to be pathogenic for the tick vector of Lyme disease, *Ixodes scapularis* (Say) (Zhioua et al. 1995). Engorged female ticks appeared to be the stage most susceptible to penetration and killing by the nematodes.

Studies in our laboratory were undertaken to test the susceptibility of selected tick species of importance in the United States to entomopathogenic nematodes. Initial studies demonstrated that replete female *Amblyomma americanum* (L.), lone star tick, were killed by several species of entomopathogenic nematodes including *Steinernema glaseri, S. riobravus* (Cabanillas & Poinar), *S. carpocapsae, S. feltiae,* and *Heterorhabiditis bacteriophora,* Poinar (Kocan et al. 1998). Of these nematode species studied, *S. riobravus* and *S. feltiae* were chosen for subsequent studies because they killed a larger percentage of ticks and were most notably attracted to the replete females.

In the studies reported herein, *S. riobravus* and *S. feltiae* nematodes were further tested for their ability to kill replete females of several tick species including *A. maculatum* Koch, *A. cajennense* (F.), *Dermacentor variabilis* (Say), and *Rhipicephalus sanguineus* (Latreille). Studies were then conducted using microscopy to define the interaction between the nematodes and ticks, to determine the mechanism by which the nematodes killed ticks and to determine whether they reproduce within ticks as they do in larval insect hosts. Finally, we tested whether *S. riobravus* would penetrate and kill *A. americanum* females while they were feeding on cattle.

Materials and Methods

Nematode Species and Laboratory Propagation. Two species of entomopathogenic nematodes, *S. riobravus* and *S. feltiae,* were obtained from stocks maintained at the Department of Entomology, Rutgers University, NJ, and the Department of Nematology, Volcani Center, ARO Institute, Israel. *Galleria mellonella* L. larvae (Northern Bait, Chetek, WI) were exposed to nematodes on moistened filter paper in large petri dishes at an exposure dose of 5,000 nematodes per 50 moth larvae. Forty-eight h after exposure, the moth larvae cadavers were placed in small petri dish glued to the bottom of a larger petri dish that was filled with distilled water. As the infective juvenile nematodes emerged from the cadavers and migrated to the water, they were collected and stored at 7°C in plastic tissue culture flasks until used for experiments. The nematodes were used for experiments within 6 wk of production.

Ticks. Ticks used for these studies—*A. americanum, A. cajennense, A. maculatum, D. variabilis* and *R. sanguineus*—were reared at the Centralized Tick Rearing Facility, Department of Entomology, Oklahoma State University. Larval and nymphal ticks were fed on rabbits and used as adults. Adult females were partially fed or allowed to feed to repletion on sheep, after which they were collected and used for nematode exposure experiments using detached ticks.

Exposure of Various Species of Engorged Female Ticks to Entomopathogenic Nematodes. Entomopathogenic nematodes were tested for their ability to kill replete ticks of the species *A. cajennense, A. maculatum, D. variabilis,* and *R. sanguineus.* Engorged ticks were placed individually in plastic petri dishes (60 by 15 mm) that contained 15 g of moistened, sifted sand, exposed to 5,000 nematodes (Kocan et al. 1998), and the dishes were then covered and placed in a humidity chamber (98% RH). Replete ticks of each species were exposed to the same volume of distilled water as used for the nematode exposures in the same environment (petri dishes filled with moistened sand) to serve as controls. *D. variabilis* and *R. sanguineus* were tested with *S. riobravus* and *S. feltiae,* whereas *A. maculatum* and *A. cajennense* were tested with only *S. riobravus.* Ticks were observed daily for the presence of nematodes and changes in appearance until the control ticks had completed oviposition or the exposed ticks died (or both). The effect of the nematodes on ticks was judged in comparison with unexposed control ticks by determining the percentage of ticks that died as evidenced by a change to a black color associated with putrification, and by calculating the average egg mass weights which were statistically analyzed and compared with that of unexposed control ticks using *t*-tests for 2 independent samples.

Microscopy Studies of Partially Fed Ticks Exposed to Nematodes. Partially fed male and female *D. variabilis* were exposed to *S. riobravus* nematodes and fixed at various times after exposure to determine if and when the nematodes entered the ticks. Unfed male and female ticks were placed on a sheep and allowed to feed for 3 d. The ticks were then removed, washed, and divided into groups of 10. Females of similar size were selected. Ten pairs (male–female) were placed in individual petri dishes (60 by 15 mm) containing moistened sand. Before the addition of the ticks, *S. riobravus* (5,000 infective juveniles per 1 ml distilled water) were added to each dish. The petri dishes were covered and placed in a humidity chamber. Five pairs (male–female) were collected at 8 h, 1, 2, 3, 4, 5, and 6 d after exposure, cut in half separating the right and

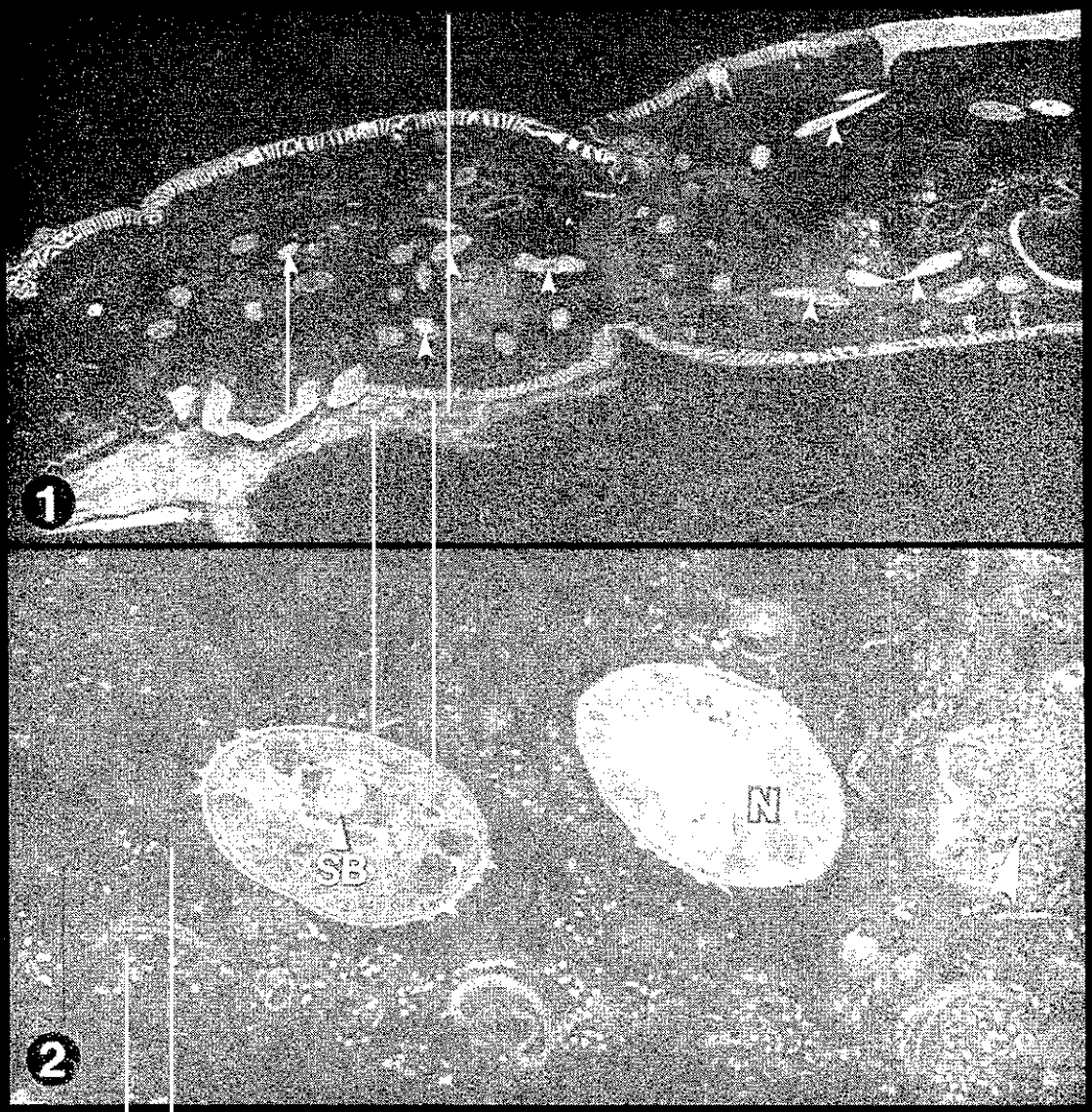


Fig. 1. Photomicrograph of cross sections of nematodes (small arrows) in a partially fed *D. variabilis* female fixed 24 h after exposure to *S. riobravus* (250×).

Fig. 2. Photomicrograph of 2 nematode cross sections (N) in a tick 24 h after exposure to *S. riobravus*. In one cross section, a packet of symbiotic rickettsia (SB) is visible in the foregut. Clusters of bacteria (large arrow), presumably released from the nematodes and multiplying with the tick, are present in the hemocoel (2,500×).

left sides, fixed in cold 2% glutaraldehyde in 0.2 M sodium cacodylate buffer, and postfixed in 2% sodium cacodylate buffer–osmium tetroxide. Subsequently, the fixed tissues were dehydrated in a graded series of ethanol and infiltrated with epoxy resin. Semithin sections (1.0 μm) were cut, stained with Mallory's stain (Richardson et al. 1960), and examined with light microscopy for the presence of nematodes.

Exposure of Ticks to Nematodes While Feeding on Cattle. Orthopedic stockinettes (20-cm lengths) were glued to the side of a calf to serve as cells for tick feeding. The unglued end of the stockinette was fastened with a rubber band to prevent escape of ticks and allowed for daily observation of the feeding ticks. In 3 trials, *A. americanum* ticks (25 male–female pairs) were placed in each of 4 cells. In trials 1 and 3, the feeding ticks were exposed to *S. riobravus* infective juveniles nematodes in distilled water (250,000 per cell) on days 3, 6, or 9 postinfestation. Feeding ticks in trial 2 were exposed on days 3 or 9 postinfestation. Control ticks were exposed to an equal volume of water as used for the nematode exposures. In trial 1,

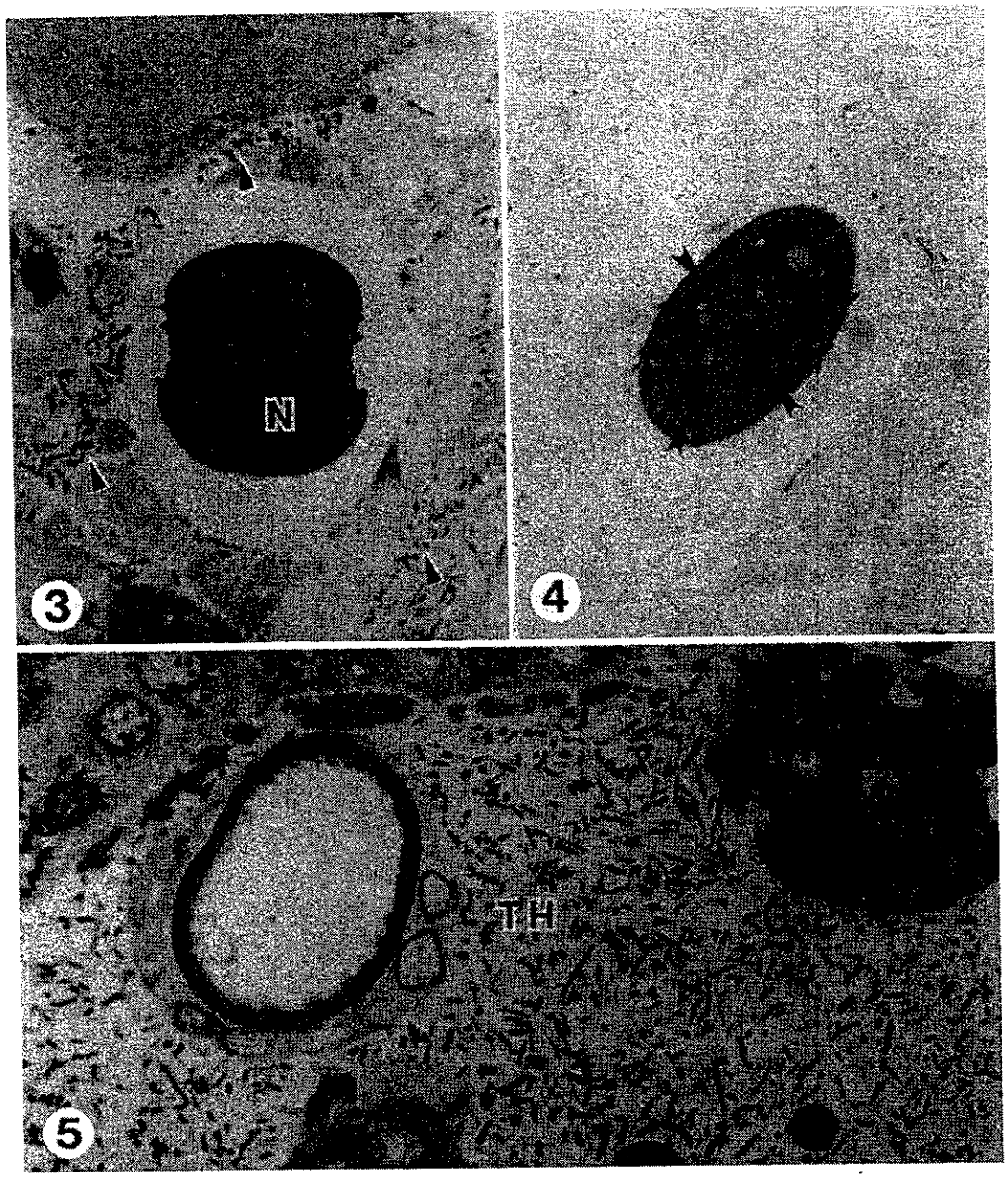


Fig. 3. Photomicrograph of a nematode cross section (N) within a tick that is separated from the surrounding tissues by a clear space. Bacteria (arrows) are present in the tick hemocoel (2,500×).

Fig. 4. Photomicrograph of bacteria (arrows) filling the nematode gut that were presumably ingested during feeding (2,500×).

Fig. 5. Photomicrograph of symbiotic bacteria within the tick hemocoel (TH) adjacent to degenerated tick tissues (2,500×).

water-soaked cotton balls were added to the cells to maintain a moist environment. In trials 2 and 3, pieces of moistened sponges were added for the same purpose. The cotton balls and sponge pieces in the tick feeding cells were moistened daily. Female ticks were allowed to feed to repletion on the cattle, after which they were collected and placed in individual petri dishes to oviposit. The ticks were observed daily for signs of nematode infection as described previously. Egg mass weights of the nematode-exposed ticks that oviposited were compared statistically with those of the controls. For each experiment, these comparisons

Table 1. Exposure of replete female ticks to the entomopathogenic nematodes *S. riobravus* and *S. feltiae*

Nematode species[a]	No. ticks[a]	No. nematodes/ exposure dish	No. dead ticks (%)	Mean egg mass wt, g (*n*)
		Dermacentor variabilis		
S. riobravus	45	5,000	43 (96)	0.003 (12)*
Controls[b]	25	0	0 (0)	0.14 (25)
S. feltiae	45	5,000	41 (91)	0.05 (36)*
Controls	25	0	0 (0)	0.19 (25)
		Rhipicephalus sanguineus		
S. riobravus	45	5,000	40 (89)	0.01 (6)*
Controls	25	0	0 (0)	0.09 (25)
S. feltiae	45	5,000	32 (71)	0.10 (32)**
Controls	25	0	0 (0)	0.14 (25)
		Amblyomma maculatum		
S. riobravus	50	5,000	12 (24)	0.27 (40)*
Controls	25	0	0 (0)	0.58 (24)
		Amblyomma cajennense		
S. riobravus	50	5,000	44 (88)	0.05 (16)*
Controls	25	0	1 (4)	0.22 (25)

*, $P < 0.001$; **, $P < 0.003$ (2 independent sample *t*-test).
[a] Ticks placed individually in sand-filled petri dishes.
[b] Ticks were exposed to the same volume of water used for the nematode exposures to serve as controls.

were made using a 1-way analysis of variance (ANOVA) procedure. If differences were detected, then the Dunnett test was used to compare the mean for each treatment level with the mean for the controls.

Results

Exposure of Replete Ticks to Entomopathogenic Nematodes. All species of ticks tested—*D. variabilis*, *R. sanguineus*, *A. maculatum*, and *A. cajennense*—proved to be susceptible to *S. riobravus* and *S. feltiae* when exposed in petri dishes filled with sifted, moistened sand. In the ticks that oviposited, mean egg mass weights of the nematode-exposed ticks were significantly less than those of the associated unexposed controls for each tick species ($P < 0.01$ or 0.03) (Table 1). When *D. variabilis* were exposed to *S. riobravus*, 96% of the ticks died, whereas *S. feltiae* killed 91% of the replete females. Similarly, *S. riobravus* killed 89% of the *R. sanguineus*, whereas *S. feltiae* killed 71%. When *A. maculatum* and *A. cajennense* were exposed to *S. riobravus*, the tick mortality rate was 24 and 88%, respectively.

Microscopy Studies of Partially Fed Ticks Exposed to Nematodes. Nematode larvae were observed in male and female *D. variabilis* with light microscopy at 24 h postexposure and throughout the remaining collection times (Figs. 1 and 2). The number of nematodes could only be estimated. One nematode may have been observed in several cross sections. The greatest number of nematode cross sections were observed in ticks collected 3 and 4 d postexposure. The number of nematodes per tick did not appear to increase. The nematodes did not reproduce within ticks as they do in insect larvae. Infective juveniles were not produced and therefore did not emerge from the nematode-exposed ticks. Symbiotic bacteria were observed in a small packet in the nematode foregut (Fig. 2) in a cross-section of a tick collected at 24 h postexposure. Bacteria were present in the hemocoel and tissues of all nematode-exposed ticks (Figs. 2, 3, 5). They were observed 1st in the tick hemocoel (Figs. 2, 3, 5) and later were seen throughout degenerating tick tissues. A clear space often was seen separating the nematode from the surrounding tissues and bacteria (Figs. 3 and 4). Nematode guts were filled with bacteria that presumably served as a source of food for the nematodes (Fig. 4).

Exposure of Ticks to Nematodes While Feeding on Cattle. In trial 1 (Table 2), in which feeding ticks were exposed to nematodes in cells moistened by cotton balls, no tick mortality was observed, and the egg mass weights between the control and exposure groups were not significantly different. However, in trials 2 and 3 in which moisture within cells was maintained by pieces of sponge, mortality and reduced egg mass weights were observed in ticks that were exposed to nematodes in the later stages of feeding. In trial 2, 60% mortality occurred in ticks exposed on day 9 of feeding with significantly reduced egg mass weights ($P < 0.0001$). In trial 3, tick mortality was observed in the 6- and 9-d postinfestation exposure groups, and the egg masses in both of these groups were significantly different from the controls ($P < 0.001$) (Table 2). Female ticks, therefore, were more susceptible to penetration by nematodes after a 6-d feeding period when they were enlarged.

Discussion

Many insect species have been found to be susceptible to the entomopathogenic nematodes (Poinar 1986), whereas the nematodes do not appear to harm mammals or most beneficial insects. Mass-rearing techniques have been developed for commercial pro-

Table 2. Exposure of female *A. americanum* to the entomopathogenic nematode *S. riobravus* while feeding on cattle

Days ticks fed until exposed to nematodes[a]	No. replete ticks[a]	No. ticks dead (%)	Mean egg mass wt, g
Trial 1[b]			
Day 3	24	0 (0)	0.42
Day 6	23	0 (0)	0.38
Day 9	25	0 (0)	0.37
Control	25	0 (0)	0.44
Trial 2[c]			
Day 3	24	5 (20)	0.39
Day 9	29	17 (60)	0.24*
Control	25	0 (0)	0.44
Trial 3[c]			
Day 3	25	0 (0)	0.41
Day 6	19	26 (5)	0.29**
Day 9	21	20 (5)	0.29**
Control	24	0 (0)	0.42

*, $P < 0.0001$; **, $P < 0.001$ (1-way ANOVA followed by the Dunnett test).

[a] 250,000 infective juveniles were used to expose feeding ticks in each cell; unexposed controls received the same volume of water as used for the nematode exposures.

[b] Moisture in tick feeding cells maintained by the addition of moistened cotton balls.

[c] Moisture in tick feeding cells maintained by the addition of moistened pieces of sponge.

duction of the nematodes (Bedding 1981), enabling their use as a biological control agent of several insect pests including Japanese beetles, Mediterranean fruit flies, and white grubs (Gaugler and Kaya 1990). The nematodes have been used commercially for control of insect pests in both agricultural and domestic settings in several countries (United States, western Europe, Japan, and Australia (Georgis 1990). Nematodes can be dispersed either by spraying from the ground or air or by introduction into irrigation systems (Georgis 1990).

These studies were undertaken to test if the entomopathogenic nematodes would penetrate and kill ticks, and subsequently propagate within the tick cadavers as they do in insect larvae. The results obtained herein confirmed the findings of other studies (Glazer and Samish 1993; Samish and Glazer 1991, 1992; Mauléon et al. 1993; Zhioua et al. 1995; Kocan et al. 1998) by demonstrating that replete female ticks were susceptible to being killed by entomopathogenic nematodes. Based on a previous study in which we tested the susceptibility of *A. americanum* (Kocan et al. 1998) to 5 species of nematodes, we chose *S. riobravus* and *S. feltiae* for these studies on replete females of several species of ixodid ticks of economic importance in the United States because both nematode species killed a high percentage of ticks and caused a significant reduction in the egg production compared with the untreated controls. Furthermore, these 2 species of nematodes clearly were attracted to the ticks and clustered around the mouthparts and over the spiracle and genital orifices (Kocan et al. 1998).

All replete females tested in our laboratory—*D. variabilis, R. sanguineus, A. americanum, A. maculatum* and *A. cajennense*—were susceptible to the various strains of entomopathogenic nematodes. The nematodes, therefore, did not appear to be specific for any given tick species. However, in a study reported by Mauléon et al. (1993), only 1 of 3 species of replete female ticks were affected when exposed to 17 species of entomopathogenic nematodes, including *S. feltiae* but not *S. riobravus*. We found that maintaining moisture in petri dishes or in tick feeding cells was necessary for interaction between nematodes and ticks, but other factors, yet to be defined, also may be involved in facilitating nematode killing of ticks.

The microscopy studies clearly demonstrated that the nematodes penetrated ticks. The bacteria that proliferated within the tick cadaver and appeared to be the cause of tick death were morphologically similar to symbiotic bacteria that were retained in the foregut of the nematodes. Subsequent studies are needed to ascertain the identity of the bacteria and to confirm that the bacteria that multiplied in the ticks were the same as the bacteria found symbiotically in the nematode foregut.

Many nematodes in cross-sections of ticks were surrounded by a clear space that separated them from surrounding tissues and bacteria. We speculated that the nematodes may release compounds that protect them against detrimental host responses.

The nematodes did not appear to multiply in ticks or complete their life cycle by producing subsequent generations of infective juveniles that exit the cadaver in search of new hosts. The number of nematodes appeared to be maximum at days 3 and 4 postexposure and were decreased in ticks collected later postexposure. Therefore, if entomopathogenic nematodes were adapted for biological control of ticks, populations would not be self-sustaining in nature, requiring repeated applications of infective juveniles. In insect hosts, entomopathogenic nematodes produced several generations of nematodes which eventually resulted in production of infective juveniles that left the insect cadaver in search of new hosts.

It was difficult to assess the immediate death of the ticks because replete females are quiescent. Several days were required before the ticks degenerated, turned black, and acquired a putrid odor, providing evidence that they were dead. However, bacteria were distributed throughout the hemocoel by 24 h postexposure and appeared to increase over time. Bacteria were subsequently observed in degenerating tick tissues. By day 9 postexposure, most tick tissues were necrotic and could not be identified. Bacteria or nematodes were not observed in the unexposed control ticks. We speculate that tick death occurs within 24 h postexposure because nematode-exposed ticks, later determined to be dead based on their appearance and odor, did not proceed to oviposit, which usually occurred in the unexposed control ticks within a few days after becoming replete.

Nematodes penetrated ticks that were feeding on cattle, which provided evidence that they can either enter ticks via orifices other than the hypostome or penetrate through the thinner cuticle of engorging females. We confirmed that the female ticks in the 3

trials remained attached during feeding and, therefore, the hypostome, being embedding in the bovine host skin, was not available as a site for nematode penetration. Interestingly, ticks were susceptible to nematode penetration only when they were exposed after 6 and 9 d of feeding. The ticks were enlarged by this point in feeding, which may have resulted in changes in orifice size or function that allowed for nematode penetration. Exposed ticks fed to repletion and tick mortality or reduced egg production (or both) was not evident for several days after the replete ticks were placed in petri dishes. Nematodes did not appear to be as effective at killing ticks feeding on cattle as they were they were in petri dishes.

Moisture was found to be an important factor in the on-cattle exposures. In trial 1, where moisture was not effectively maintained via moistened cotton balls placed in the tick feeding cells, ticks were not killed by the nematodes nor was egg production significantly affected. However, when water-soaked pieces of sponge were placed in the tick cells in trials 2 and 3, nematodes penetrated and killed a percentage of ticks in the 3- and 9-d exposure groups; the subsequent egg production in these exposed ticks was significantly reduced. Dependence on moisture may be an important limiting factor in the ability of entomopathogenic nematodes to be effective biological control agents of ticks.

Acknowledgments

Technical assistance was provided by Tiffany Frietz, who was a participant in the Native Americans in Biological Sciences, a summer research program for high school students funded by the Howard Hughes Medical Institute, Grant No. 71192-515501. This research was sponsored by the Binational Agricultural Research and Development Fund (BARD), Project No. IS-2156-92.

References Cited

Akhurst, R. J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J. Gen. Microbiol. 121: 303–309.

1983. *Neoaplectana* species: specificity of association with bacteria of the genus *Xenorhabdus*. Exp. Parasitol. 55: 258–263.

1986. Controlling insects in soil with entomopathogenic nematodes, pp. 265–267. *In* R. A. Samson, J. M. Vlak, and D. Peters [eds.], Fundamental and applied aspects of invertebrate pathology. Foundation of the Fourth International Colloquim. of Invertebrate Pathology, Wageningen, The Netherlands.

Bedding, R. A. 1981. Low cost, in vitro mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. Nematologica 27: 109–114.

Gaugler, R., and H. K. Kaya. 1990. Entomopathogenic nematodes in biological control. CRC, Boca Raton, FL.

Gaugler, R., L. LeBeck, B. Nakagaki, and G. M. Boush. 1980. Orientation of the entomogenous nematode *Neoplectana carpocapsae* to carbon dioxide. Environ. Entomol. 9: 649–652.

Georgis, R. 1990. Formulation and application technology, pp. 173–194. *In* R. Gaugler and H. K. Kaya [eds.], Entomopathogenic nematodes in biological control. CRC, Boca Raton, FL.

Glazer, I., and M. Samish. 1993. Suitability of *Boophilus annulatus* replete female ticks as hosts of the nematode *Steinernema carpocapsae*. J. Invertebr. Pathol. 61: 220–222.

Kocan, K. M., E. F. Blouin, M. S. Pidherney, P. L. Claypool, M. Samish, and I. Glazer. 1998. Entomopathogenic nematodes as a potential biological control method for ticks. Ann. N.Y. Acad. Sci. (in press).

Marcek, Z., R. Hanzal, and D. Kodrik. 1988. Site of penetration of juvenile steinernematids and heterorhabditids (Nematoda) into the larvae of *Galleria mellonella* (Lepidoptera). J. Invertebr. Pathol. 52: 477–478.

Mauléon, H., N. Barre, and S. Panoma. 1993. Pathogenicity of 17 isolates of entomophagous nematodes (Steinernematidae and Heterorhabditidae) for the ticks *Amblyomma variegatum* (Fabricius), *Boophilus microplus* (Canestrini) and *Boophilus annulatus* (Say). Exp. Appl. Acarol. 17: 831–838.

Norval, R.A.I., B. D. Perry, and A. S. Young. 1992. The epidemiology of theileriosis in Africa. Academic, New York.

Poinar, G. O., Jr. 1979. Nematodes for biological control of insects. CRC, Boca Raton, FL.

1986. Entomogenous nematodes, pp. 95–121. *In* B. D. Franz [ed.], Biological plant and health protection. Fischer, Stuttgart.

Poinar, G. O., Jr., and G. M. Thomas. 1966. Significance of *Achromobacter nematophilus* Poinar and Thomas (Achromobacteriaceae: Eubacteriales) in the development of the nematode DD136 (*Neoaplectana* sp., Steinernematidae). Parasitology 56: 385–390.

Richardson, K. C., L. Jarrett, and E. H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35: 313–323.

Samish, M., and I. Glazer. 1991. Killing ticks with parasitic nematodes of insects. J. Invertebr. Pathol. 58: 281–282.

1992. Infectivity of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to female ticks of *Boophilus annulatus* (Arachnida: Ixodidae). J. Med. Entomol. 29: 614–618.

Sonenshine, D. E. 1993. Biology of ticks, vol. 2. Oxford University Press, New York.

Zhioua, E., R. A. Lebrun, H. S. Ginsberg, and A. Aeschlimann. 1995. Pathogenicity of *Steinernema carpocapsae* and *S. glaseri* (Nematode: Steinernematidae) to *Ixodes scapularis* (Acari: Ixodidae). J. Med. Entomol. 32: 900–905.

Received for publication 31 July 1997; accepted 26 December 1997.

J. Parasitol., 84(4), 1998 p. 802–805

DESICCATION TOLERANCE OF *MUELLERIUS* CF. *CAPILLARIS* (NEMATODA: PROTOSTRONGYLIDAE) FIRST-STAGE LARVAE

Aharon Solomon, P. Ilan, and G. Itamar*
Department of Animal Sciences, Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot 76100, Israel

ABSTRACT: *Muellerius* cf. *capillaris* is the most common lung worm of wild Nubian ibex (*Capra ibex nubiana*) in the northern Negev desert, Israel. The capacity of the free-living stages (L1) of the parasite to survive extreme desiccation was tested under 2 different dehydration regimes at 23 C: rapid dehydration through direct exposure to 0% relative humidity (RH), and a slow dehydration regime of preconditioning at 33% RH for 7 days prior to exposure to 0% RH for a further 21 days. In direct exposures to 0% and 33% RH, by day 11 survival rates of L1 were significantly higher than when stored in water and in 97% RH ($P < 0.05$). The slow dehydration regime enhanced the survival of L1 up to 10-fold by day 28 as compared with direct exposure to 0% RH. The same mean numbers of larvae were recovered from the land snail *Theba pisana* infected with L1 exposed for 21 days at 33% RH and from *T. pisana* infected with nondesiccated L1 ($P > 0.1$). L1 surviving after 21 days of desiccation at 0% RH were, on the other hand, less infective to *T. pisana*. The percentage of such postdesiccated L1 reaching infective stage (L3) was, however, the same as that of the control group. The ability of *M.* cf. *capillaris* L1 to survive anhydrobiosis and retain infectivity to land snails after extreme desiccation enables their coexistence with the Nubian ibex in desert habitat.

Free-living and parasitic nematodes of several taxa enter into a state of anhydrobiosis when exposed to desiccation (Van Gundy, 1965; Crowe, 1971; Glazer and Orion, 1983; Womersley, 1987, 1990; Allan and Wharton, 1990; Crowe et al., 1992). In this unique physiological state, dehydrated organisms can survive for long periods and resume activity upon rehydration when water becomes available in the surrounding environment. Womersley (1987) described 2 strategies of entering into a state of anhydrobiosis in nematodes, i.e., via slow or rapid dehydration. These strategies reflect evolved adaptations of different anhydrobiotic nematodes to divergent natural habitats.

Muellerius cf. *capillaris* (Nematoda: Protostrongylidae) is the most common lung worm infecting Nubian ibex (*Capra ibex nubiana)* in the northern Negev desert of Israel (Solomon, 1995; Solomon, Strubel et al., 1996). Protostrongylid nematodes have a 2-host life cycle, with land snails as intermediate hosts. The free-living stages (L1) of *M.* cf. *capillaris,* which are released in the feces to the arid environment, must often withstand extreme desiccation inside feces or, after migrating out of feces, in the soil (Solomon et al., 1997). In preliminary results we demonstrated the remarkable capacity of *M.* cf. *capillaris* L1 to survive anhydrobioisis after rapid dehydration at 0% relative hummidity (RH) (100% survival after 10 days; Solomon et al., 1997). The present study compares survival of L1 after rapid dehydration and slow dehydration and examines the ability of larvae to become established and develop to the infective stage (L3) in their land snail host following these treatments.

MATERIALS AND METHODS

Collection of feces

Fecal samples were collected from naturally infected Nubian ibexes from the northern Negev desert, Israel. From each observed defecating animal, fresh fecal samples were immediately collected and placed in polyethylene bags. In the laboratory, fecal samples were allowed to dry at room temperature before being refrigerated (5 C). First-stage larvae (L1) were extracted by the Baermann technique from the stored feces. Only fecal samples containing L1 conforming with *M.* cf. *capillaris* (Solomon, Paperna, and Markovies, 1996) were selected for the experiments.

Desiccation trials

Relative humidity was controlled in sealed desiccators with 60 ml of the saturated salt solutions at 23 C as follows: K_2SO_4 for 97% RH, $MgCl_2 \cdot 6H_2O$ for 33% RH (Winston and Bates, 1960), and fresh silica gel for 0% RH. Relative humidity values were confirmed by overnight hygrometer (Lambrecht, Berlin Germany) measurements. L1 (150 ± 10) in 10 µl distilled water were placed in an Eppendorf cap and allowed to dry at room temperature, then transferred immediately to desiccators at the different RHs. Survival of L1 was tested, first, after exposure to 0%, 33%, or 97% RH for 1, 3, 6, and 11 days and, subsequently, after exposure to 33% RH for 7 days and then to 0% RH for a further 21 days. Concurrently with the second experiment, L1 survival was tested after exposure to 0% and 33% RH on days 1, 7, 14, 21, and 28. In each time interval, survival rates of L1 were determined from subsamples containing 50 larvae after 24 hr of incubation in distilled water at 23 C. Survival of the various desiccated groups of L1 was compared with L1 kept in distilled water at 23 C. Living larvae were recognized by either their active movement or their response to stimulation (gentle prodding with a hair probe).

Recovery of desiccated L1s

Recovery times of desiccated L1, which were determined by monitoring larval motility (n = 45) over a period of 24 hr, were compared for the different RH regimes.

Infectivity of L1 to snails after desiccation

Known numbers of L1 desiccated at 33% and 0% RH for 7, 14, or 21 days were rehydrated in distilled water for 24 hr. Active adults of *Theba pisana* were pipetted with a 250-L1 dose in 20 µl of distilled water via their opercular opening by placing them on their shell apex and leaving them in this position for 20 min. Infected snails were fed on fresh lettuce and kept at 23 C as described elsewhere (Solomon, Paperna, and Markovics, 1996). Control snails were inoculated with nontreated L1 from stored feces. Larval load and stages of development in the snails were determined 30 days postinoculation by squashing the snails' feet between 2 heavy glass slides as described by Cabaret and Morand (1990).

Statistical analysis

Statistical analysis was performed with the SAS 6.04 software package. To compare survival curves, we fitted a linear curve of survival by time for each treatment, after performing a logit transformation on the survival data ($\log[p/1 - p]$) with p = percentage of surviving larvae. The model was fitted by a general linear model with the repeated mea-

Received 17 November 1997; revised 11 March 1998; accepted 11 March 1998.
* Department of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel.

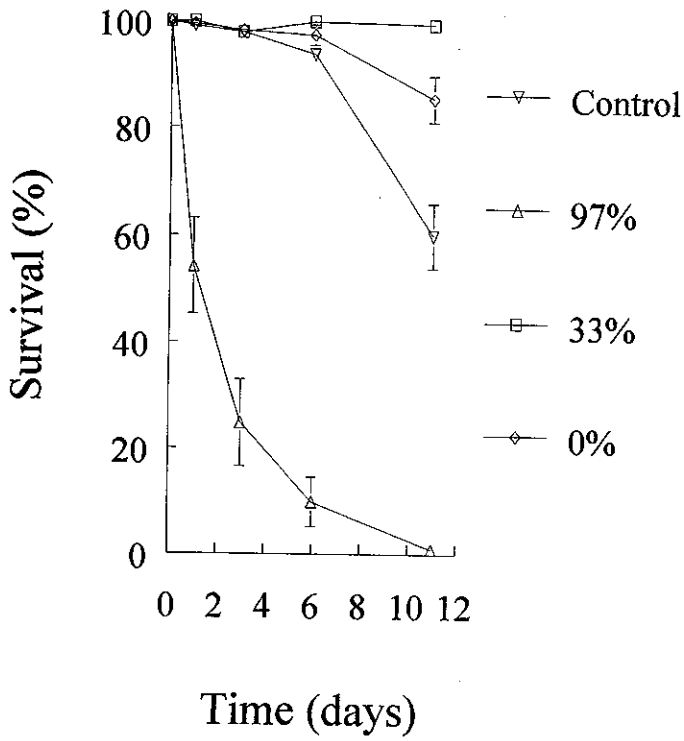


FIGURE 1. Survival curves of *Muellerius* cf. *capillaris* L1 at different relative humidity (RH) levels and in distilled water (control) at 23 C (n = 4). Error bars show ±SE.

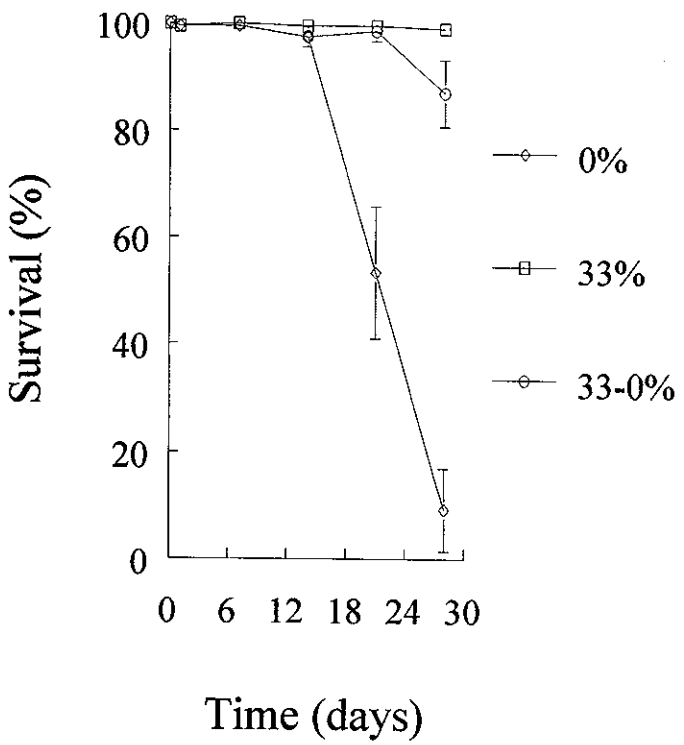


FIGURE 2. Survival curves of *Muellerius* cf. *capillaris* L1 after dehydration at 33% and 0% RH and after preconditioning at 33% RH for 7 days prior to exposure to 0% RH at 23 C (n = 4). Error bars show ±SE.

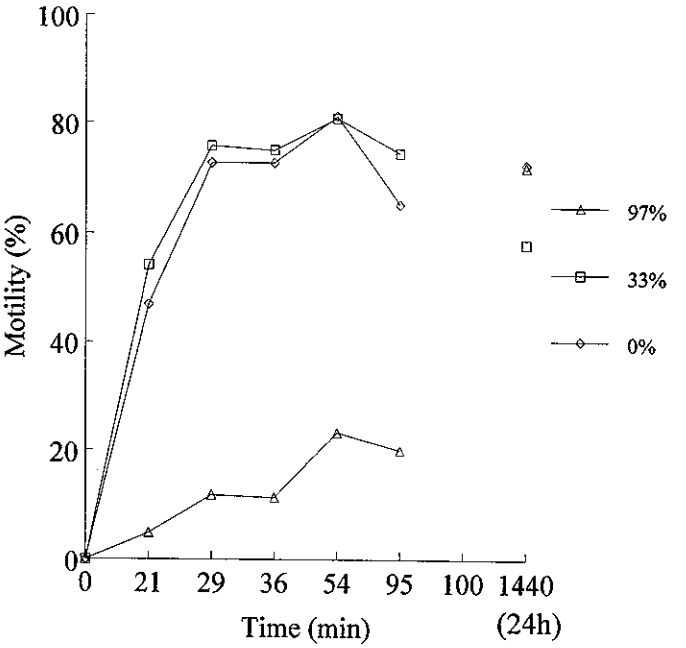


FIGURE 3. Recovery time (as reflected by motility) of *Muellerius* cf. *capillaris* L1 after 24 hr of exposure to 97%, 33%, and 0% RH at 23 C (n = 45).

surements from each desiccator. The slopes of all the curves were compared by an *F*-test, and in pairs, by *t*-test. In addition, the percentages of nematode survival at the end of each experiment were compared by 1-way ANOVA after arcsine transformation. Chi-square values were calculated to compare specific relative frequencies of the different larval stages. Mean numbers of larvae recovered in snails were compared over 3 treatments by the Kruskal–Wallis test. Pairs of treatments were thereafter compared by the Wilcoxon 2-sample test, and significance levels were adjusted for simultaneity by the Bonferroni correction.

RESULTS

Desiccation trials

Survival rate of L1 on day 11 in 33% RH was significantly higher (99.5 ± 0.5% [mean ± SE]) than that of L1 in 0% RH (85.5 ± 4.4%), water (59.9 ± 9%), or 97% RH (1 ± 1%) (F = 94.64, df = 3, $P \ll 0.05$; Fig. 1). Although survival in water was better than in the 97% RH treatment, larvae surviving in water were sluggish and had shrunken bodies.

Slopes of survival curves of L1 after dehydration at 0% and 33% RH and after a slow dehydration of 7 days in 33% RH prior to exposure to 0% RH were significantly different (F = 23.3, df = 2, $P \ll 0.05$; Fig. 2). Due to the experimental design, a comparison and statistical analysis of all 3 treatment groups was relevant only from day 14. By day 28, the survival rate of L1 pretreated in 33% RH was 10-fold higher (86.9 ± 3.1%) than that of L1 exposed directly to 0% RH (9.25 ± 3.9%). Overall survival rate was still highest in the 33% RH treatment (99 ± 0.55%; F = 143.96, df = 2, $P < 0.001$).

Recovery of desiccated L1

Recovery times of L1 maintained for 24 hr in 33% and 0% RH were the same and were shorter than that of L1 kept in 97% RH (Fig. 3). After 54 min, 81% (n = 45) of the observed larvae from the 33% and 0% RH treatments were active com-

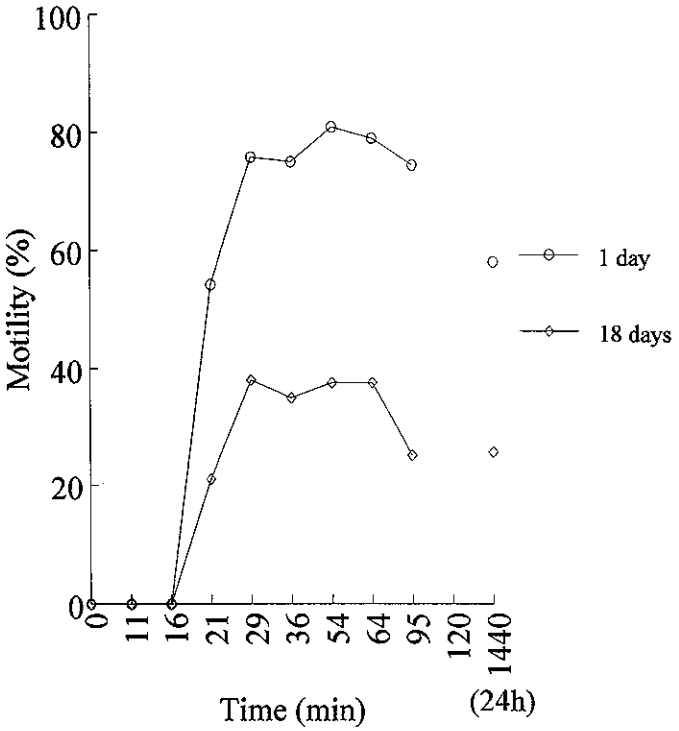


FIGURE 4. Recovery time (as reflected by motility) of *Muellerius* cf. *capillaris* L1 after 1 and 18 days of exposure to 33% RH at 23 C (n = 45).

pared with only 23.3% of the larvae from the 97% RH treatment. In the latter, recovery was delayed, reaching 72% after 24 hr. After 54 min of rehydration, larvae from 0% and 33% RH became half-moon-shaped, inactive, or slow, with movements restricted to the anterior part of the body, but retained vitality (100% survival). The lower percentage of active larvae observed in 0% and 33% RH treatments after the 54-min counts is the result of the above-described behavioral change. Survival of L1 exposed directly to 33% RH for 24-hr and for 18 days remained the same (100 ± 0% and 98.5 ± 0.95%, respectively). However, the recovery time of L1 after a 24 hr exposure to 33% RH was much shorter than that of L1 left for 18 days (81% vs. 40% motility after 54 min; Fig. 4).

Infectivity of L1 to snails after desiccation

The rate of development of L1 after been exposed to 0% RH for 7, 14, or 21 days was the same as in the control group (overall chi-square = 2.23, df = 3, $P > 0.1$; Table I). L1 kept at 33% RH also developed at the same rate for the first 7 days; later, however, larval development was somewhat delayed (overall chi-square = 17.85, df = 3, $P < 0.05$; Table I).

The mean numbers of larvae recovered from snails infected with nontreated L1 (16.6 ± 3.8) and with L1 desiccated for 21 days in 33% RH (15.2 ± 3.25) did not differ ($P > 0.1$), but fewer larvae (4 ± 1.34) were recovered from snails infected with L1 after 21 days of desiccation in 0% RH ($P < 0.05$; Table I).

DISCUSSION

Our present work demonstrates the unusual capacity of *M.* cf. *capillaris* L1 to survive a rapid-dehydration stress for short

TABLE I. Development of *Muellerius* cf. *capillaris* L1 after exposure to 0% or 33% relative humidity in the land snail *Theba pisana* on day 30 postinfection.

Treatment	Period of exposure (days)	% L1 (n)*	% L2 (n)*	% L3 (n)	No. larvae/snail (mean ± SE) (n)†
Control	—	0% (0)	84.5% (98)	15.5% (18)	16.6 ± 3.8 (7)
33% RH	7	1.2% (1)	82.8% (72)	16% (14)	12.4 ± 2.6 (7)
33%	14	0.6% (1)	96.4% (164)	3% (5)	20.1 ± 6.2 (7)
33%	21	0% (0)	92.1% (70)	7.9% (6)	15.2 ± 3.3 (5)
0% RH	7	0% (0)	78.5% (51)	21.5% (14)	9.3 ± 1.6 (7)
0%	14	1.6% (1)	86.7% (52)	11.7% (7)	8.6 ± 1.8 (7)
0%	21	4.1% (0)	79.2% (19)	16.7% (4)	4 ± 1.3 (6)

* Total number of larvae from all snails examined shown in parentheses.
† Number of snails examined shown in parentheses.

periods. Womersley (1987) suggested that nematodes from dry habitats must be preadapted to withstand rapid water loss at the cellular level. However, subjecting larvae to 0% RH does not have to imply that these animals have experienced rapid dehydration at the cellular level. For longer periods of desiccation, however, the slower dehydration regime (preconditioning at 33% RH for 7 days) improved L1 survival at 0% RH by 10-fold relative to the fast-dehydration treatment. Womersley (1987, 1990) and Barrett (1991) reviewed the importance of slow rates of water loss in free-living and phytoparasitic nematodes in modulating metabolic and biochemical processes crucial for the successful induction of a state of anhydrobiosis. However, the physiological mechanisms mediating the induction of anhydrobiosis in protostrongylid nematodes remain unknown.

The desiccation regimes used in our experiments are the most extreme dehydration stresses known to be tolerated by any animal-parasitic nematodes undergoing anhydrobiosis. For example, infective stages (L3) of the drought-resistant nematode *Trichostrongylus colubriformis* can survive exposure to 0% RH (~100% survival after 24 hr) only after initial exposure to higher RH regimes (76% for 72 hr, 33% for 48 hr; Allan and Wharton, 1990). The recovery process of *M.* cf. *capillaris* L1 from desiccation (81% after 29 min) under similar experimental conditions (33% RH, 20–23 C) is also much faster than that of L3 of *T. colubriformis* (~85% after 24 hr; Allan & Wharton, 1990). Recovery of *M.* cf. *capillaris* L1 from prolonged anhydrobiosis (18 days in 33% RH) is suggested to have a high energy cost, which may explain the observed reduced motility of the surviving nonfeeding L1.

The experiments described here also confirm our preliminary observations of lower survival rates of *M.* cf. *capillaris* L1 in high humidity and when stored in water (Solomon et al., 1997).

We suggest that long-term anhydrobiosis spares the nematode from the necessity of wasting storage resources, whereas the nonfeeding L1 would otherwise be limited by its energy storage and aging.

We demonstrated that after 21 days of desiccation on an exposed surface at 0% RH, L1 were less infective to the land snail *T. pisana* than were their nondesiccated counterparts; this recalls the findings of Morrondo-Pelayo et al., (1992) that surviving *M. capillaris* L1 from feces containing 95% dry matter were less infective (to the land snail *Candidula intersecta)* than nondesiccated L1. We also found that similar proportions of postdesiccated (at 0% RH) and control larvae reached the infective stage in the exposed snail host.

Our data strongly suggest that the remarkable capacity of *M.* cf. *capillaris* free-living stages to survive desiccation is one of the main factors involved in the successful transmission of protostrongylid nematodes in desert habitat.

ACKNOWLEDGMENTS

We thank Jacques Cabaret from I.N.R.A, Station de Pathologie Aviaire et de Parasitologie, Ecology des Parasites, Nouzilly, France, for his helpful comments on an earlier draft of this manuscript and Hillary Voet from the Faculty of Agriculture of the Hebrew University of Jerusalem for the statistical analysis. This study was supported by an internal grant from the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

LITERATURE CITED

ALLAN, G. S., AND D. A. WHARTON. 1990. Anhydrobiosis in the infective juveniles of *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidae). International Journal of Parasitology **20:** 183–192.

BARRETT, J. 1991. Anhydrobiotic nematodes. Agricultural Zoology Reviews **4:** 161–176.

CABARET, J., AND S. MORAND. 1990. Single and dual infections of the land snail *Helix aspersa* with *Muellerius capillaris* and *Alloionema appendiculatum* (Nematoda). Journal of Parasitology **76:** 579–580.

CROWE, J. H. 1971. Anhydrobiosis: An unsolved problem. American Naturalist **105:** 563–573.

———, F. HOEKSTRA, AND L. M. CROWE. 1992. Anhydrobiosis. Annual Review of Physiology **54:** 579–599.

GLAZER, I., AND D. ORION. 1983. Studies on anhydrobiosis of *Pratylenchus thornei.* Journal of Nematology **15:** 333–338.

MORRONDO-PELAYO, P., P. DIEZ-BANOS, AND J. CABARET. 1992. Influence of desiccation of faeces on survival and infectivity of first stage larvae of *Muellerius capillaris* and *Neostrongylus linearis.* Journal of Helminthology **66:** 213–219

SOLOMON, A. 1995. Transmission of lung worm infections in wild populations of the Nubian ibex (*Capra ibex nubiana*) in the northern Negev desert. M.Sc.Agr. Thesis. University of Jerusalem, Jerusalem, Israel, 59 p. [In Hebrew.]

———, I. PAPERNA, AND A. MARKOVICS. 1996. The influence of aestivation in land snails on the larval development of *Muellerius* cf. *capillaris* (Metastrongyloidea: Protostrongylidae). International Journal for Parasitology **26:** 363–367.

———, ———, I. GLAZER, AND U. P. ALKON. 1997. Migratory behavior and desiccation tolerance of protostrongylid nematode first-stage larvae. International Journal for Parasitology **27:** 1517–1522.

———, I. STRUBEL, I. PAPERNA, U. P ALKON, AND A. MARKOVICS. 1996. Protostrongylids (Nematoda: Metastrongyloidea) and Coccidia (Apicomplexa: Eimeridea) infection in wild populations of the Nubian ibex (*Capra ibex nubiana*) in the northern Negev desert, Israel. Israel Journal of Medical Sciences **32:** 1145.

VAN GUNDY, S. D. 1965. Factors in survival of nematodes. Annual Review of Phytopathology **3:** 43–68.

WINSTON, P. W., AND D. H. BATES. 1960. Saturated salt solutions for the control of humidity in biological research. Ecology **41:** 232–237.

WOMERSLEY, C. 1987. A reevaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. *In* Vistas on nematology, J. A. Veech and D. W. Dickson (eds.). Society of Nematologists, Hyattsville, Maryland, p. 165–173.

WOMERSLEY, C. 1990. Dehydration survival and anhydrobiotic potential. *In* Entomophatogenic nematodes in biological control, R. Gaugler and H. K. Kaya (eds.). CRC Press, Boca Raton, Florida, p. 117–137.

J. Parasitol., 84(4), 1998 p. 802–805

DESICCATION TOLERANCE OF *MUELLERIUS* CF. *CAPILLARIS* (NEMATODA: PROTOSTRONGYLIDAE) FIRST-STAGE LARVAE

Aharon Solomon, P. Ilan, and G. Itamar*
Department of Animal Sciences, Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot 76100, Israel

ABSTRACT: *Muellerius* cf. *capillaris* is the most common lung worm of wild Nubian ibex (*Capra ibex nubiana*) in the northern Negev desert, Israel. The capacity of the free-living stages (L1) of the parasite to survive extreme desiccation was tested under 2 different dehydration regimes at 23 C: rapid dehydration through direct exposure to 0% relative humidity (RH), and a slow dehydration regime of preconditioning at 33% RH for 7 days prior to exposure to 0% RH for a further 21 days. In direct exposures to 0% and 33% RH, by day 11 survival rates of L1 were significantly higher than when stored in water and in 97% RH ($P < 0.05$). The slow dehydration regime enhanced the survival of L1 up to 10-fold by day 28 as compared with direct exposure to 0% RH. The same mean numbers of larvae were recovered from the land snail *Theba pisana* infected with L1 exposed for 21 days at 33% RH and from *T. pisana* infected with nondesiccated L1 ($P > 0.1$). L1 surviving after 21 days of desiccation at 0% RH were, on the other hand, less infective to *T. pisana*. The percentage of such postdesiccated L1 reaching infective stage (L3) was, however, the same as that of the control group. The ability of *M.* cf. *capillaris* L1 to survive anhydrobiosis and retain infectivity to land snails after extreme desiccation enables their coexistence with the Nubian ibex in desert habitat.

Free-living and parasitic nematodes of several taxa enter into a state of anhydrobiosis when exposed to desiccation (Van Gundy, 1965; Crowe, 1971; Glazer and Orion, 1983; Womersley, 1987, 1990; Allan and Wharton, 1990; Crowe et al., 1992). In this unique physiological state, dehydrated organisms can survive for long periods and resume activity upon rehydration when water becomes available in the surrounding environment. Womersley (1987) described 2 strategies of entering into a state of anhydrobiosis in nematodes, i.e., via slow or rapid dehydration. These strategies reflect evolved adaptations of different anhydrobiotic nematodes to divergent natural habitats.

Muellerius cf. *capillaris* (Nematoda: Protostrongylidae) is the most common lung worm infecting Nubian ibex (*Capra ibex nubiana)* in the northern Negev desert of Israel (Solomon, 1995; Solomon, Strubel et al., 1996). Protostrongylid nematodes have a 2-host life cycle, with land snails as intermediate hosts. The free-living stages (L1) of *M.* cf. *capillaris,* which are released in the feces to the arid environment, must often withstand extreme desiccation inside feces or, after migrating out of feces, in the soil (Solomon et al., 1997). In preliminary results we demonstrated the remarkable capacity of *M.* cf. *capillaris* L1 to survive anhydrobioisis after rapid dehydration at 0% relative hummidity (RH) (100% survival after 10 days; Solomon et al., 1997). The present study compares survival of L1 after rapid dehydration and slow dehydration and examines the ability of larvae to become established and develop to the infective stage (L3) in their land snail host following these treatments.

MATERIALS AND METHODS

Collection of feces

Fecal samples were collected from naturally infected Nubian ibexes from the northern Negev desert, Israel. From each observed defecating animal, fresh fecal samples were immediately collected and placed in polyethylene bags. In the laboratory, fecal samples were allowed to dry at room temperature before being refrigerated (5 C). First-stage larvae (L1) were extracted by the Baermann technique from the stored feces. Only fecal samples containing L1 conforming with *M.* cf. *capillaris* (Solomon, Paperna, and Markovies, 1996) were selected for the experiments.

Desiccation trials

Relative humidity was controlled in sealed desiccators with 60 ml of the saturated salt solutions at 23 C as follows: K_2SO_4 for 97% RH, $MgCl_2 \cdot 6H_2O$ for 33% RH (Winston and Bates, 1960), and fresh silica gel for 0% RH. Relative humidity values were confirmed by overnight hygrometer (Lambrecht, Berlin Germany) measurements. L1 (150 ± 10) in 10 µl distilled water were placed in an Eppendorf cap and allowed to dry at room temperature, then transferred immediately to desiccators at the different RHs. Survival of L1 was tested, first, after exposure to 0%, 33%, or 97% RH for 1, 3, 6, and 11 days and, subsequently, after exposure to 33% RH for 7 days and then to 0% RH for a further 21 days. Concurrently with the second experiment, L1 survival was tested after exposure to 0% and 33% RH on days 1, 7, 14, 21, and 28. In each time interval, survival rates of L1 were determined from subsamples containing 50 larvae after 24 hr of incubation in distilled water at 23 C. Survival of the various desiccated groups of L1 was compared with L1 kept in distilled water at 23 C. Living larvae were recognized by either their active movement or their response to stimulation (gentle prodding with a hair probe).

Recovery of desiccated L1s

Recovery times of desiccated L1, which were determined by monitoring larval motility (n = 45) over a period of 24 hr, were compared for the different RH regimes.

Infectivity of L1 to snails after desiccation

Known numbers of L1 desiccated at 33% and 0% RH for 7, 14, or 21 days were rehydrated in distilled water for 24 hr. Active adults of *Theba pisana* were pipetted with a 250-L1 dose in 20 µl of distilled water via their opercular opening by placing them on their shell apex and leaving them in this position for 20 min. Infected snails were fed on fresh lettuce and kept at 23 C as described elsewhere (Solomon, Paperna, and Markovics, 1996). Control snails were inoculated with nontreated L1 from stored feces. Larval load and stages of development in the snails were determined 30 days postinoculation by squashing the snails' feet between 2 heavy glass slides as described by Cabaret and Morand (1990).

Statistical analysis

Statistical analysis was performed with the SAS 6.04 software package. To compare survival curves, we fitted a linear curve of survival by time for each treatment, after performing a logit transformation on the survival data ($\log[p/1 - p]$) with p = percentage of surviving larvae. The model was fitted by a general linear model with the repeated mea-

Received 17 November 1997; revised 11 March 1998; accepted 11 March 1998.
* Department of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel.

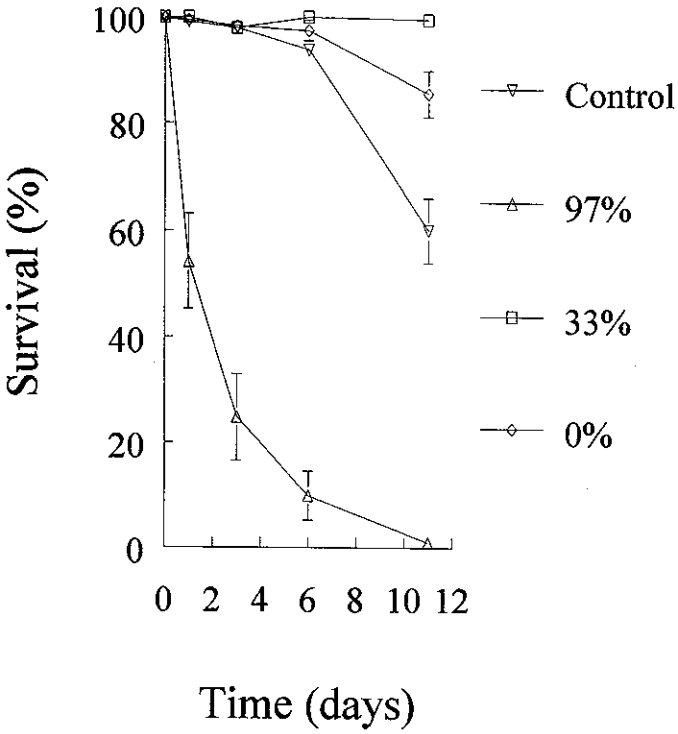


FIGURE 1. Survival curves of *Muellerius* cf. *capillaris* L1 at different relative humidity (RH) levels and in distilled water (control) at 23 C (n = 4). Error bars show ±SE.

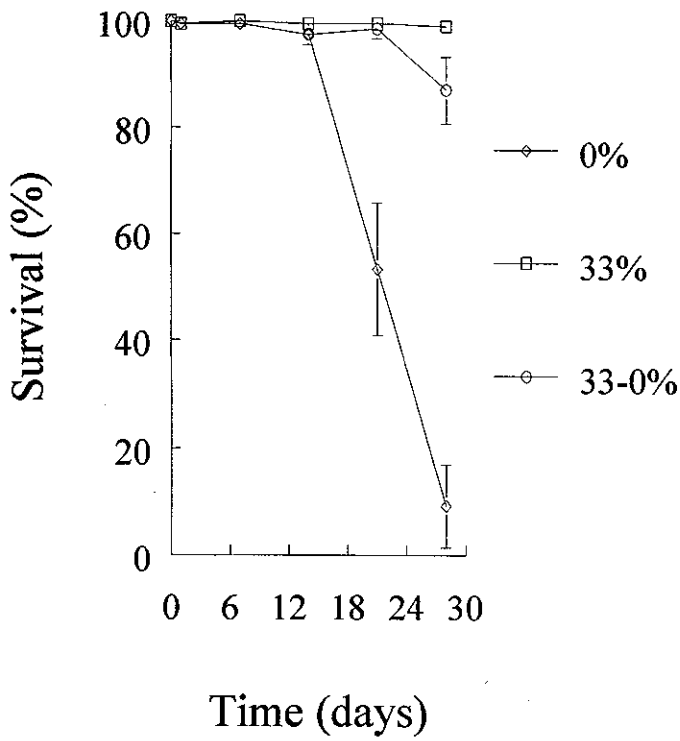


FIGURE 2. Survival curves of *Muellerius* cf. *capillaris* L1 after dehydration at 33% and 0% RH and after preconditioning at 33% RH for 7 days prior to exposure to 0% RH at 23 C (n = 4). Error bars show ±SE.

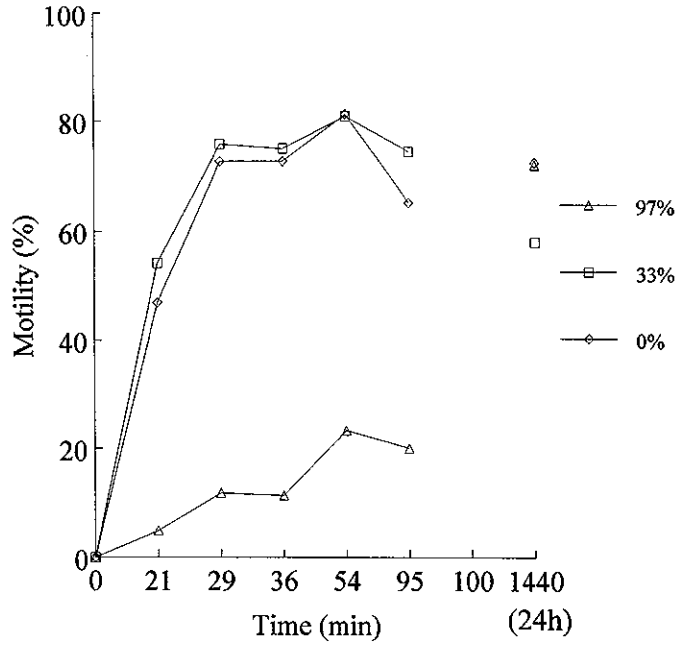


FIGURE 3. Recovery time (as reflected by motility) of *Muellerius* cf. *capillaris* L1 after 24 hr of exposure to 97%, 33%, and 0% RH at 23 C (n = 45).

surements from each desiccator. The slopes of all the curves were compared by an *F*-test, and in pairs, by *t*-test. In addition, the percentages of nematode survival at the end of each experiment were compared by 1-way ANOVA after arcsine transformation. Chi-square values were calculated to compare specific relative frequencies of the different larval stages. Mean numbers of larvae recovered in snails were compared over 3 treatments by the Kruskal–Wallis test. Pairs of treatments were thereafter compared by the Wilcoxon 2-sample test, and significance levels were adjusted for simultaneity by the Bonferroni correction.

RESULTS

Desiccation trials

Survival rate of L1 on day 11 in 33% RH was significantly higher (99.5 ± 0.5% [mean ± SE]) than that of L1 in 0% RH (85.5 ± 4.4%), water (59.9 ± 9%), or 97% RH (1 ± 1%) (F = 94.64, df = 3, $P \ll 0.05$; Fig. 1). Although survival in water was better than in the 97% RH treatment, larvae surviving in water were sluggish and had shrunken bodies.

Slopes of survival curves of L1 after dehydration at 0% and 33% RH and after a slow dehydration of 7 days in 33% RH prior to exposure to 0% RH were significantly different (F = 23.3, df = 2, $P \ll 0.05$; Fig. 2). Due to the experimental design, a comparison and statistical analysis of all 3 treatment groups was relevant only from day 14. By day 28, the survival rate of L1 pretreated in 33% RH was 10-fold higher (86.9 ± 3.1%) than that of L1 exposed directly to 0% RH (9.25 ± 3.9%). Overall survival rate was still highest in the 33% RH treatment (99 ± 0.55%; F = 143.96, df = 2, $P < 0.001$).

Recovery of desiccated L1

Recovery times of L1 maintained for 24 hr in 33% and 0% RH were the same and were shorter than that of L1 kept in 97% RH (Fig. 3). After 54 min, 81% (n = 45) of the observed larvae from the 33% and 0% RH treatments were active com-

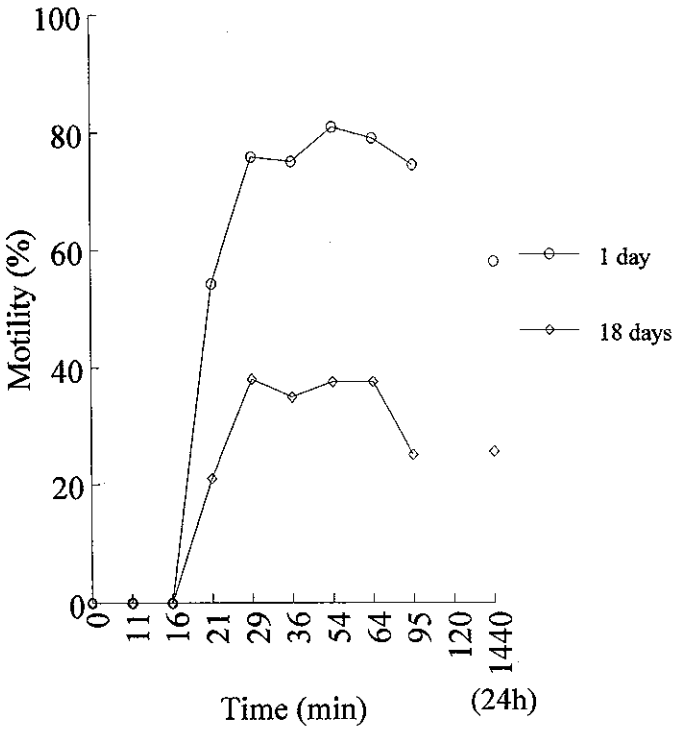


FIGURE 4. Recovery time (as reflected by motility) of *Muellerius* cf. *capillaris* L1 after 1 and 18 days of exposure to 33% RH at 23 C (n = 45).

pared with only 23.3% of the larvae from the 97% RH treatment. In the latter, recovery was delayed, reaching 72% after 24 hr. After 54 min of rehydration, larvae from 0% and 33% RH became half-moon-shaped, inactive, or slow, with movements restricted to the anterior part of the body, but retained vitality (100% survival). The lower percentage of active larvae observed in 0% and 33% RH treatments after the 54-min counts is the result of the above-described behavioral change. Survival of L1 exposed directly to 33% RH for 24-hr and for 18 days remained the same (100 ± 0% and 98.5 ± 0.95%, respectively). However, the recovery time of L1 after a 24 hr exposure to 33% RH was much shorter than that of L1 left for 18 days (81% vs. 40% motility after 54 min; Fig. 4).

Infectivity of L1 to snails after desiccation

The rate of development of L1 after been exposed to 0% RH for 7, 14, or 21 days was the same as in the control group (overall chi-square = 2.23, df = 3, $P > 0.1$; Table I). L1 kept at 33% RH also developed at the same rate for the first 7 days; later, however, larval development was somewhat delayed (overall chi-square = 17.85, df = 3, $P < 0.05$; Table I).

The mean numbers of larvae recovered from snails infected with nontreated L1 (16.6 ± 3.8) and with L1 desiccated for 21 days in 33% RH (15.2 ± 3.25) did not differ ($P > 0.1$), but fewer larvae (4 ± 1.34) were recovered from snails infected with L1 after 21 days of desiccation in 0% RH ($P < 0.05$; Table I).

DISCUSSION

Our present work demonstrates the unusual capacity of *M.* cf. *capillaris* L1 to survive a rapid-dehydration stress for short

TABLE I. Development of *Muellerius* cf. *capillaris* L1 after exposure to 0% or 33% relative humidity in the land snail *Theba pisana* on day 30 postinfection.

Treatment	Period of exposure (days)	% L1 (n)*	% L2 (n)*	% L3 (n)	No. larvae/snail (mean ± SE) (n)†
Control	—	0% (0)	84.5% (98)	15.5% (18)	16.6 ± 3.8 (7)
33% RH	7	1.2% (1)	82.8% (72)	16% (14)	12.4 ± 2.6 (7)
33%	14	0.6% (1)	96.4% (164)	3% (5)	20.1 ± 6.2 (7)
33%	21	0% (0)	92.1% (70)	7.9% (6)	15.2 ± 3.3 (5)
0% RH	7	0% (0)	78.5% (51)	21.5% (14)	9.3 ± 1.6 (7)
0%	14	1.6% (1)	86.7% (52)	11.7% (7)	8.6 ± 1.8 (7)
0%	21	4.1% (0)	79.2% (19)	16.7% (4)	4 ± 1.3 (6)

* Total number of larvae from all snails examined shown in parentheses.
† Number of snails examined shown in parentheses.

periods. Womersley (1987) suggested that nematodes from dry habitats must be preadapted to withstand rapid water loss at the cellular level. However, subjecting larvae to 0% RH does not have to imply that these animals have experienced rapid dehydration at the cellular level. For longer periods of desiccation, however, the slower dehydration regime (preconditioning at 33% RH for 7 days) improved L1 survival at 0% RH by 10-fold relative to the fast-dehydration treatment. Womersley (1987, 1990) and Barrett (1991) reviewed the importance of slow rates of water loss in free-living and phytoparasitic nematodes in modulating metabolic and biochemical processes crucial for the successful induction of a state of anhydrobiosis. However, the physiological mechanisms mediating the induction of anhydrobiosis in protostrongylid nematodes remain unknown.

The desiccation regimes used in our experiments are the most extreme dehydration stresses known to be tolerated by any animal-parasitic nematodes undergoing anhydrobiosis. For example, infective stages (L3) of the drought-resistant nematode *Trichostrongylus colubriformis* can survive exposure to 0% RH (~100% survival after 24 hr) only after initial exposure to higher RH regimes (76% for 72 hr, 33% for 48 hr; Allan and Wharton, 1990). The recovery process of *M.* cf. *capillaris* L1 from desiccation (81% after 29 min) under similar experimental conditions (33% RH, 20–23 C) is also much faster than that of L3 of *T. colubriformis* (~85% after 24 hr; Allan & Wharton, 1990). Recovery of *M.* cf. *capillaris* L1 from prolonged anhydrobiosis (18 days in 33% RH) is suggested to have a high energy cost, which may explain the observed reduced motility of the surviving nonfeeding L1.

The experiments described here also confirm our preliminary observations of lower survival rates of *M.* cf. *capillaris* L1 in high humidity and when stored in water (Solomon et al., 1997).

We suggest that long-term anhydrobiosis spares the nematode from the necessity of wasting storage resources, whereas the nonfeeding L1 would otherwise be limited by its energy storage and aging.

We demonstrated that after 21 days of desiccation on an exposed surface at 0% RH, L1 were less infective to the land snail *T. pisana* than were their nondesiccated counterparts; this recalls the findings of Morrondo-Pelayo et al., (1992) that surviving *M. capillaris* L1 from feces containing 95% dry matter were less infective (to the land snail *Candidula intersecta)* than nondesiccated L1. We also found that similar proportions of postdesiccated (at 0% RH) and control larvae reached the infective stage in the exposed snail host.

Our data strongly suggest that the remarkable capacity of *M.* cf. *capillaris* free-living stages to survive desiccation is one of the main factors involved in the successful transmission of protostrongylid nematodes in desert habitat.

ACKNOWLEDGMENTS

We thank Jacques Cabaret from I.N.R.A, Station de Pathologie Aviaire et de Parasitologie, Ecology des Parasites, Nouzilly, France, for his helpful comments on an earlier draft of this manuscript and Hillary Voet from the Faculty of Agriculture of the Hebrew University of Jerusalem for the statistical analysis. This study was supported by an internal grant from the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

LITERATURE CITED

ALLAN, G. S., AND D. A. WHARTON. 1990. Anhydrobiosis in the infective juveniles of *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidae). International Journal of Parasitology **20:** 183–192.

BARRETT, J. 1991. Anhydrobiotic nematodes. Agricultural Zoology Reviews **4:** 161–176.

CABARET, J., AND S. MORAND. 1990. Single and dual infections of the land snail *Helix aspersa* with *Muellerius capillaris* and *Alloionema appendiculatum* (Nematoda). Journal of Parasitology **76:** 579–580.

CROWE, J. H. 1971. Anhydrobiosis: An unsolved problem. American Naturalist **105:** 563–573.

———, F. HOEKSTRA, AND L. M. CROWE. 1992. Anhydrobiosis. Annual Review of Physiology **54:** 579–599.

GLAZER, I., AND D. ORION. 1983. Studies on anhydrobiosis of *Pratylenchus thornei.* Journal of Nematology **15:** 333–338.

MORRONDO-PELAYO, P., P. DIEZ-BANOS, AND J. CABARET. 1992. Influence of desiccation of faeces on survival and infectivity of first stage larvae of *Muellerius capillaris* and *Neostrongylus linearis.* Journal of Helminthology **66:** 213–219

SOLOMON, A. 1995. Transmission of lung worm infections in wild populations of the Nubian ibex (*Capra ibex nubiana*) in the northern Negev desert. M.Sc.Agr. Thesis. University of Jerusalem, Jerusalem, Israel, 59 p. [In Hebrew.]

———, I. PAPERNA, AND A. MARKOVICS. 1996. The influence of aestivation in land snails on the larval development of *Muellerius* cf. *capillaris* (Metastrongyloidea: Protostrongylidae). International Journal for Parasitology **26:** 363–367.

———, ———, I. GLAZER, AND U. P. ALKON. 1997. Migratory behavior and desiccation tolerance of protostrongylid nematode first-stage larvae. International Journal for Parasitology **27:** 1517–1522.

———, I. STRUBEL, I. PAPERNA, U. P ALKON, AND A. MARKOVICS. 1996. Protostrongylids (Nematoda: Metastrongyloidea) and Coccidia (Apicomplexa: Eimeridea) infection in wild populations of the Nubian ibex (*Capra ibex nubiana*) in the northern Negev desert, Israel. Israel Journal of Medical Sciences **32:** 1145.

VAN GUNDY, S. D. 1965. Factors in survival of nematodes. Annual Review of Phytopathology **3:** 43–68.

WINSTON, P. W., AND D. H. BATES. 1960. Saturated salt solutions for the control of humidity in biological research. Ecology **41:** 232–237.

WOMERSLEY, C. 1987. A reevaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. *In* Vistas on nematology, J. A. Veech and D. W. Dickson (eds.). Society of Nematologists, Hyattsville, Maryland, p. 165–173.

WOMERSLEY, C. 1990. Dehydration survival and anhydrobiotic potential. *In* Entomophatogenic nematodes in biological control, R. Gaugler and H. K. Kaya (eds.). CRC Press, Boca Raton, Florida, p. 117–137.

Biocontrol Science and Technology (1998) 8, 429–437

An Edible-to-insects Calcium Alginate Gel as a Carrier for Entomopathogenic Nematodes

A. NAVON,[1] SARA KEREN,[1] LIORA SALAME[2] AND I. GLAZER[2]

Departments of [1]Entomology and [2]Nematology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel

(*Received for publication 23 July 1997; revised manuscript accepted 20 March 1998*)

A carrier for entomopathogenic nematodes based on an edible-to-insects calcium alginate gel was developed. The alginate system was produced by external setting through an interaction between an aqueous sodium alginate mixture and calcium ions under acidic conditions. Sodium hexa-metaphosphate was used to control gel formation. Yeast extract used in the gel as a phagostimulant for Spodoptera littoralis *larvae improved the insect's relative consumption rate and digestibility. The nematodes in the gel effectively controlled the larvae in a 24-h leaf bioassay, although nematode survival in the gel was ~50%. Gels subjected to 31% relative humidity (RH) prior to larval feeding became desiccated and were inedible to insects. However, gels at 61% RH supported larval feeding, although the water loss from the gel due to evaporation from 200–400-mg gel cubes at this humidity exceeded 50%. The gel might be a useful delivery system for nematodes against insects infesting the plant canopy in greenhouses.*

Keywords: *Calcium alginate gel, hydrophilic carrier edible to insects,* Spodoptera littoralis, *larvae feeding stimulant, nutritional indices, relative humidity, entomopathogenic nematodes,* Steinernema riobrave

INTRODUCTION

To date, entomopathogenic nematode have been effective mostly against soil-inhabiting pests (Kaya, 1990), as in the soil, the nematodes are protected from detrimental sunshine effects, such as desiccation and ultraviolet (UV) radiation. Although nematode activity on plant foliage in sheltered crops was possible to some extent (Begley, 1990), the outdoor activity of the infective juveniles (IJs) on the leaf was limited to several hours—a situation that is economically unacceptable. Numerous formulations have been developed to improve the nematode activity on the crop and in storage (Georgis, 1990), and techniques have been developed to increase nematode efficacy on the crop. Entomopathogenic nematode strains that tolerated desiccation better than others were selected (Glazer & Navon, 1990), and antidesiccants in the aqueous nematode mixtures improved persistence (Glazer *et al.*, 1992). Furthermore, application technologies were developed for the nematodes in aqueous

Correspondence to: A. Navon. Tel: 972 3 9683111; Fax: 972 3 960418; E-mail: navona@netvision.net.il.

0958-3157/98/030429-09 $9.00

TABLE 1. Composition of the calcium alginate gel

No.	Material	Source	%
1.	Sodium alginate Keltone LV[a]	Kelco Division, Merck & Co., NJ, USA	2.0
2.	Calcium carbonate (precipitated)	Merck, Darmstadt, Germany	2.4
3.	Sodium hexa metaphosphate	Sigma, St Louis, MO, USA	0.4
4.	Citric acid monohydrate (crystals)	Merck	0.7
5.	Yeast extract	Sigma	0.25
6	Methyl *para*-hydroxy benzoate	Sigma	0.05
7.	Distilled water	—	94.2

[a]LV = low viscosity.

mixtures, with adjuvants to improve their deposition on the plant (Lello *et al.*, 1996) and their tolerance to temperature extremes (Georgis & Manweiler, 1994).

A different approach to formulations of entomopathogenic nematodes was based on immobilizing the IJs in hydrophilic colloids. Encapsulation of entomopathogenic nematodes in calcium alginate gels by means of external gel settings (Kaya & Nelson, 1985; Kaya *et al.*, 1987) and other hydrophylic colloids (Patel & Vorlop, 1994) were used to protect the IJs from desiccation and UV effects. The efficacy of these microcapsules in pest control was based on nematode migration from the gel to infest the insect, as the gel was not edible to agricultural pests. For these and other reasons, the alginate encapsulation technologies were not recommended for the control of defoliators on the crop.

The need to develop a carrier for entomopathogenic nematodes that would support IJ activity and persistence on the plant canopy is of prime importance. A start in this direction was made by embedding the IJs in an alginate gel that was edible to insects. The idea was that insects would be infested with nematodes via feeding from a carrier in which IJs were entrapped. The gelling technique was based on an external setting at room temperature in which sodium alginate interacts with calcium ions under acid conditions to produce an irreversible gel (King, 1982). This gelling procedure was used previously to substitute agar in insect diets (Navon, 1985). The nematodes that were evenly distributed in the gel matrix by mixing become immobilized at gel setting. In the present work, the alginate gel system was developed as a carrier for the nematodes that was edible to insects. The activity of the nematode *Steinernema riobrave* in the alginate gels against *Spodoptera littoralis* Boisduval larvae at different combinations of gel weight and relative humidities (RHs) was also studied.

MATERIALS AND METHODS

Nematode Colony

The entomopathogenic nematode *S. riobrave* was obtained from the laboratory of R. Gaugler (Rutgers University, NJ, USA). The nematodes were reared on last-instar larvae of the greater wax moth (*Galleria mellonella*) at 25°C according to the methods of Woodring and Kaya (1988). Nematodes were stored in distilled water at 10°C for 7–14 days and then allowed to acclimatize at ambient temperature (23–25°C) for 24 h before use.

Insect Colony

S. littoralis larvae had been reared for the previous 3 years on a premix diet based on the soya bean–wheat germ diet 'Manduca Premix–Heliothis Premix' (Stonefly Industries Inc., Bryan, TX, USA) under conditions of 16:8 h (light:dark) photophase, 25±2°C and 60–80% RH.

Nematode Embedding in the Alginate Gel

The composition of the calcium alginate gel is given in Table 1. The gel was prepared as follows. Sodium alginate, methyl *para*-hydroxybenzoate, yeast extract, sodium hexa

metaphosphate and calcium carbonate were dissolved in distilled water by stirring with a laboratory stirrer type RZR (Heidolph, Kelheim, Germany) until a homogeneous solution was obtained. Then *S. riobrave* nematodes, concentrated in 1 ml of water, were added to the alginate solution at a dose of 500 IJs/g and mixed in. Finally, citric acid dissolved in 4 ml of water was mixed with the alginate mixture for 15 s. Gel setting was complete within 20–30 min. The ion sequestrant, sodium hexa metaphosphate, was needed to slow down the reaction between the alginate and calcium ions, thereby avoiding premature gelling when the citric acid was mixed in. The nematodes were homogeneously dispersed in the gel. They were observed moving freely on the gel surface. Inside the gel, nematodes were immobilized by the colloid matrix.

Nematode Survival in the Alginate Gel

Gels with nematodes were placed in Petri dishes in the insect rearing room. The nematodes were counted in the gel at zero time, and after 24 and 48 h. A gel sample was taken out from the 12-mm thick layer using a cork borer (diameter 5 mm). The gel was macerated with scalpels and the nematodes were counted under a binocular $\times 40$ magnification.

Leaf Bioassays with the Nematode–Gel Preparation

Leaves of *Ricinus communis* grown on the Bet Dagan Experimental Farm were used in the bioassays. Leaf sizes ranged from 50 to 100 cm^2. Single leaves were placed in 200-ml plastic cups with a hole in the bottom. The cups were placed on a rigid plastic grid. The leaf petiole was inserted through the hole and immersed in water. Leaves remained fresh for 3 days. In each cup, one mature *S. littoralis* larva (weight: 214–275 mg; length: 18–24 mm; head capsule width: 2.10–2.55 mm) was placed with a nematode–alginate gel cube weighing approximately 200 mg. The gel combinations on the leaves were:

(1) without yeast extract;
(2) with yeast extract;
(3) with yeast extract and nematodes; and
(4) without gel (control).

One sixth-instar larva was placed in each cup (200 ml). Five cups in five replicates were used, each with a different date (=25 larvae/treatment). The bioassay period was 48 h. The experiments were conducted in a rearing room (16:8 h (light:dark) photophase at 25 ± 2°C and 60–80% RH).

Larval mortality was recorded. Each dead larva was introduced into a Petri dish with 5 ml of saline solution. the body of the larva was cut into pieces to free the nematodes from the insect; nematodes were counted and their sex ratio was determined. Larval weight gain was recorded in gels without nematodes. Results were analyzed with *t*-test or analysis of variance (ANOVA), followed by the Student–Newman–Keuls test for rating means.

Bioassays of Insect Feeding Stimulants on Styropor Lamellae

Yeast extract (Sigma, St Louis, MO, USA) was tested for feeding stimulation on Styropor (foamed polystyrene) lamellae (Meisner *et al.*, 1970; Navon *et al.*, 1987). The lamellae were dipped in a 3% aqueous methanolic suspension of 3% sucrose and 1% yeast extract. Surplus liquids were shaken off the lamellae. The control was 3% sucrose only. The lamellae were dried in a hood at room temperature and put in a 9-cm sterile plastic Petri dish. They were kept separate using a plastic strip (8 cm long $\times$ 0.7 cm wide) attached to the bottom of the Petri dish. The larvae could move freely between the lamellae. The bioassays were conducted in a rearing room equipped with a 16:8 h (light:dark) photophase at 25 ± 2°C and 60–80% RH.

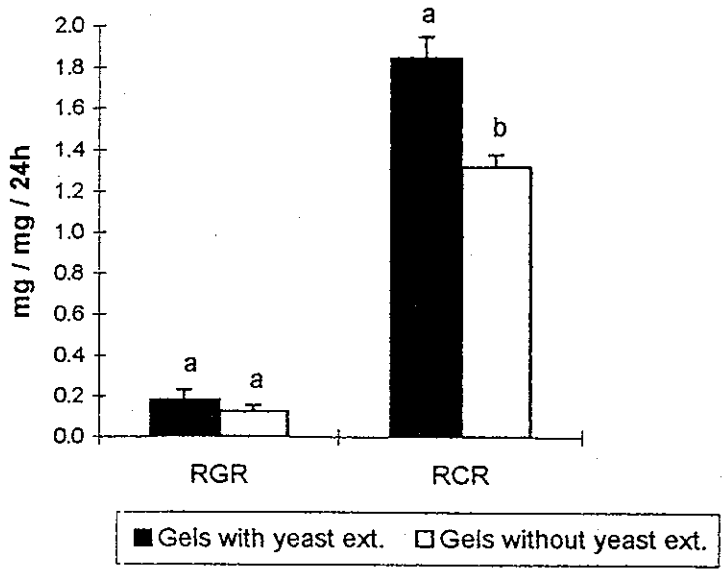


FIGURE 1. RGR and RCR of *S. littoralis* sixth-instar larvae fed on calcium alginate gel containing yeast extract. Bars represent ± SE. Different letters above the bars within the same nutritional index indicate statistical differences at $P = 0.05$ (Student's t-test).

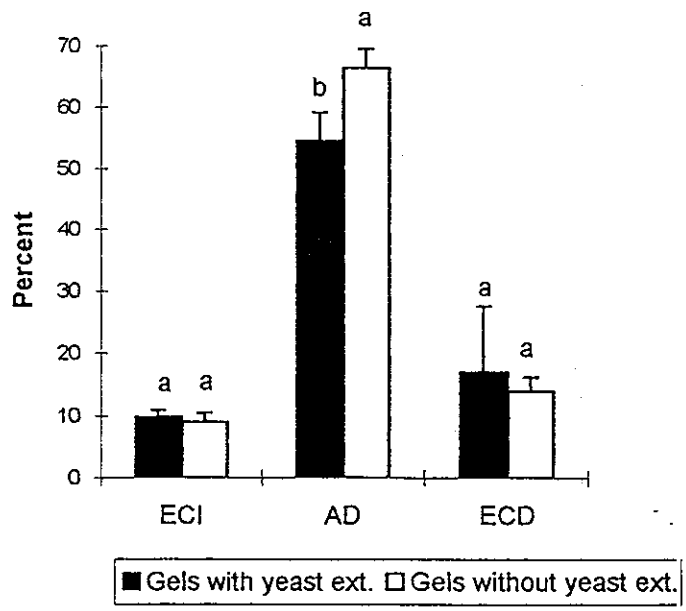


FIGURE 2. ECI, AD and ECD calculated from feeding of *S. littoralis* larvae on alginate gels containing yeast extract. Bars represent ± SE. Different letters above the bars within the same nutritional index indicate statistical differences at $P = 0.05$ (Student's t-test).

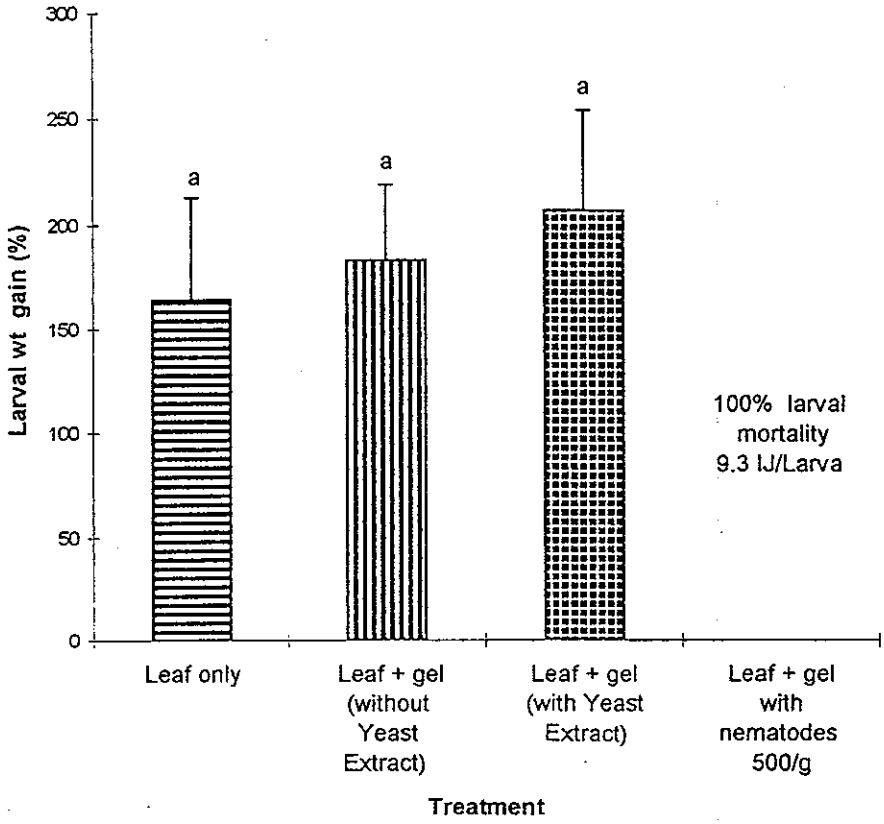


FIGURE 3. Weight gain of *S. littoralis* larvae in a 48-h leaf bioassay with gels containing yeast extract and nematode combinations. Bars represent ± SE (ANOVA followed by Student–Newman–Keuls test).

Dietary Bioassay—Effect of Yeast Extract in the Alginate Gel

Sixth-instar larvae of *S. littoralis* weighing 252 ± 4.9 mg standard error (SE) were offered singly, in disposable plastic Petri dishes with a calcium alginate cube weighing 1100 ± 120 mg,with and without yeast extract (Sigma). There were four replicates of 10 larvae each. The feeding period was 24 h. The bioassay conditions were as described above. Larvae and alginate gels were weighed before the start of the bioassay and 24 h later, at which time faecal pellets also were weighed. The calculations of the nutritional indices were based on fresh weight. Water loss from the gels due to evaporation did not exceed 5% of the initial gel weight, and this reduction in weight was compensated for in the gel consumption calculations. The definitions of the nutritional indices were as follows:

- relative consumption rate (RCR) = food consumed/initial larval weight/24 h;
- relative growth weight (RGR) = larval weight gain/initial larval weight/24 h;
- efficiency of conversion of ingested food to body mass (ECI) = (larval weight gain/food consumed) × 100;
- approximate digestibility (AD) = ((food consumed − weight of faeces)/diet consumed) × 100;
- efficiency of conversion of digested food (ECD) = larval weight gain/(food consumed − weight of faeces) × 100.

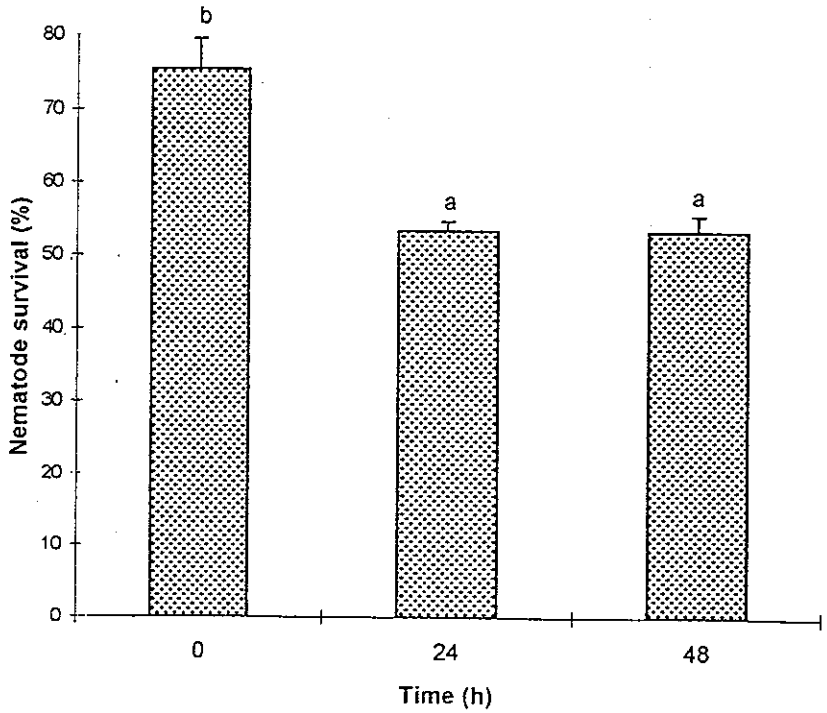


FIGURE 4. Nematode survival in an alginate gel at 0, 24 and 48 h. Bars represent ± SE. Different letters above the bars indicate statistical differences at $P = 0.05$ (ANOVA followed by Student–Newman–Keuls test).

Larval Feeding on Alginate Gels at Different RHs

RH levels of 31, 61 and 90% were prepared in 1-l glass jars, each containing 100 ml of KOH (Merck, Darmstadt, Germany) aqueous solution (Peterson, 1953). The alginate cubes were placed in a 5-cm diameter plastic Petri dish suspended from the lid of the jar. Three jars were used for each RH and gel exposure was replicated four times. The jar was closed tightly with a lid and parafilm bands. Alginate gel cubes of different weights were exposed to these RHs for 24 h, after which time they were offered to sixth-instar *S. littoralis* larvae (weight 190–240 mg) in disposable 9-cm Petri dishes for 48 h. Larval weight gain was recorded after 24 and 48 h and analyzed by the Student's *t*-test.

In a separate experiment, alginate gel cubes weighing 100–400 mg were exposed to these humidities for 24 h. Gel weights were then recorded and the loss of water due to evaporation was calculated.

RESULTS

Figure 1 shows that the yeast extract in the gel increased the RCR to about 25% higher than the control without the yeast extract ($P < 0.05$). The RGR values were low because the gel did not contain the major nutrients needed to increase the insect body mass.

Figure 2 shows that the yeast extract improved AD, as this nutritional index was based on food consumption which was significantly higher ($P < 0.05$) than that in the control (Figure 1). The other indices, ECI and ECD, did not differ as a result of the yeast extract in the gel, probably because they reflected the insignificant differences in weight gain or RGR (Figure 1).

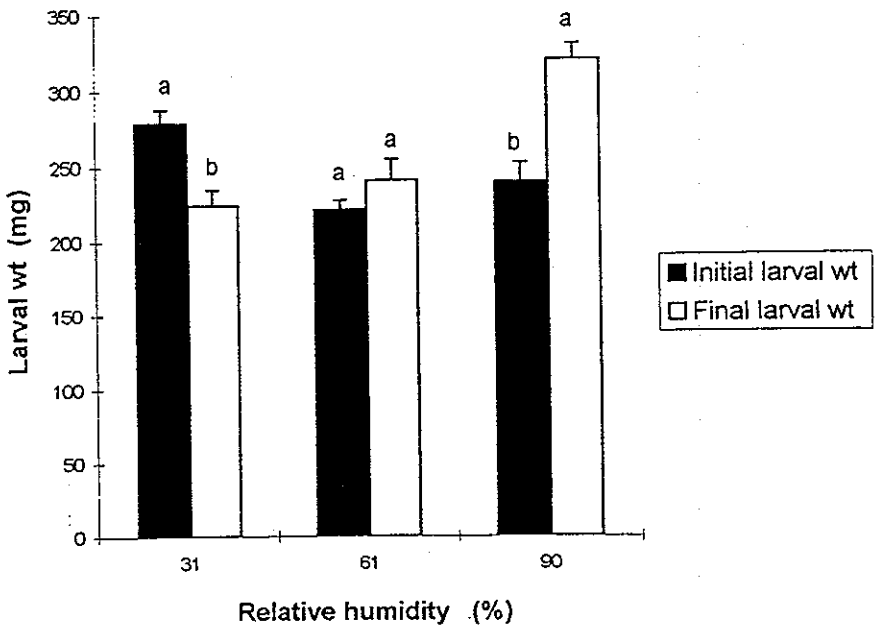


FIGURE 5. Weights of *S. littoralis* larvae offered alginate gels that had been exposed to different RHs for 24 h prior to feeding by the larvae. Bars represent ± SE. Different letters above the bars within the same RH indicate statistical differences at $P = 0.05$ (Student's t-test).

Gels containing yeast extract that were bioassayed with leaves (Figure 3) resulted in improved larval weight gain, although this change was not significant. The nematodes in the gel with the yeast extract caused mortality of all *S. littoralis* larvae. Dead larvae contained an average of 9.3 IJs/larva, and the sex ratio of the nematodes was close to one. Larvae offered the nematode–gel product did not consume the leaves, whereas the control larvae consumed 60–80% of the leaf area. In addition to leaf feeding, it was observed that the control larvae also fed on the gel without nematodes.

Nematode survival tests showed that the IJ count at zero time was 75%, and that within 48 h nematode survival in the gel was reduced to ~50% (Figure 4). The nematode survival at 24 and 48 h was similar, and the reason for the drop in survival between 0 and 24 h remains unknown.

Figure 5 shows that gels exposed for 24 h to 31% RH at 25 ± 2°C prior to larval feeding did not support larval weight gain. This was, however, slightly increased at 61% RH and significantly elevated ($P < 0.05$) at 90% RH. These differences in larval weight gain reflected the availability of the gel to larvae. The gel held at 31% RH became dry, and was therefore inedible to the insects; the gel at 61% RH shrank but was still edible. At 90% RH, the larvae could feed freely on the gels. These changes in gels were due to differences in water loss as a result of evaporation at the different RHs (Figure 6). At 31% RH, 75% of the water was lost from a 400-mg gel cube. At 61% RH the gel weight was reduced by one half. At 90% RH, only ~10% of the water was lost from the gel.

DISCUSSION

Embedding or encapsulation of entomopathogenic nematodes in hydrophilic gels is a promising formulation for improving IJ persistence during exposure to unfavourable conditions, such as sunlight (Gaugler & Boush, 1978) and desiccation (Dutky, 1959). The

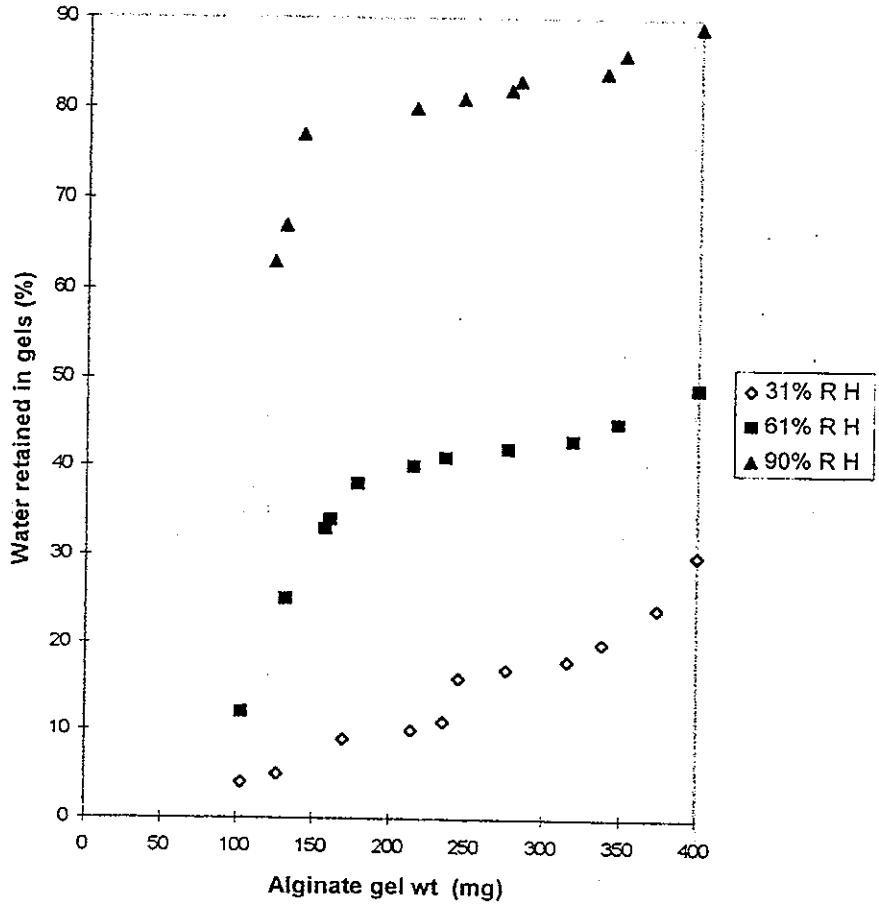


FIGURE 6. Water retained in gel cubes (weight 100–400 mg) which were exposed to different RHs for 48 h.

novel approach to the gel system in this work was based on making the gel edible to larvae. Yeast extract effectively increased the RCR (Figure 1) and AD (Figure 2) of *S. littoralis* mature larvae in feeding bioassays, and was therefore used in the nematode–gel formulation. Nematodes in gels readily infested and killed the larvae in leaf bioassays (Figure 3), although nematode survival was reduced to 50% after 48 h (Figure 4). It seems that RHs between 60 and 90% protected the gel from desiccation. Therefore, the gel remained edible to the insects, although it lost water due to evaporation (Figures 5 and 6). Previous studies (Glazer & Navon, 1990; Glazer *et al.*, 1992) had also shown 60% RH as the lower limit for a reasonable level of nematode survival. However, in those studies nematodes were exposed to low RHs for 2–12 h, whereas in the present study the gel formulation supported nematode viability for as long as 48 h.

To date, alginate gels have been based on the external setting of sodium alginate drops, so that the gelling process locked the nematodes within the middle of the capsule. This delivery system of nematodes was thus based on nematode migration from the capsules (Kaya & Nelson, 1985). This release of nematodes from the alginate gel was difficult and took several days (Capinera *et al.*, 1988). Alginate encapsulation of nematodes with plant seeds was developed to improve protection of the roots from insect damage and to improve nematode release from the capsule at seed germination (Kaya *et al.*, 1987). In the gel used

in the present study, the nematodes were uniformly dispersed within the gel matrix; larvae that fed on the gel were therefore readily infested with nematodes. Low RHs (below 60%) did not support gel availability to insects. Flakes of 100–200 mg may be suitable for insect control on the plant canopy at 60–90% RH and 25 ± 2°C. These conditions are common in greenhouses throughout most of the year. Therefore, the alginate gels developed in this work could be used on the plant canopy of sheltered crops to control lepidopterous larvae as well as other pests. Recently, foliar application of entomopathogenic nematodes by spraying showed various levels of effectiveness (Lello *et al.*, 1996). Methods to disperse the nematodes in gel capsules on the plant or to produce the gel on the plant using suitable spraying machines are being investigated.

ACKNOWLEDGEMENT

This is a contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, no. 2235-E 1997 series.

REFERENCES

BEGLEY, J.W. (1990) Efficacy against insects in habitats other than soil, in: *Entomopathogenic Nematodes in Biological Control* (GAUGLER, R. & KAYA, H.K., Eds) CRC, Boca Raton, FL, pp. 215–231.

CAPINERA, J.L., PELISSIER, D., MENOUT, G.S. & EPSKI, N.D. (1988) Control of black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae), with entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae). *Journal of Invertebrate Pathology* **52**, 427–435.

DUTKY, S.R. (1959) Insect microbiology. *Advances in Applied Microbiology* **1**, 175–200.

GAUGLER, R. & BOUSH, G.M. (1978) Effects of ultraviolet radiation and sunshine on the entomopathogenic nematode *Neoplectana carpocapsae*. *Journal of Invertebrate Pathology* **32**, 291–296.

GEORGIS, R. (1990) Formulation and application technology, in: *Entomopathogenic Nematodes in Biological Control* (GAUGLER, R. & KAYA, H.K., Eds) CRC, Boca Raton, FL, pp. 173–194.

GEORGIS, R. & MANWEILER, S.A. (1994) Entomopathogenic nematodes: a developing biocontrol technology, in: *Agricultural Zoology Reviews* (EVANS, K., Ed.) Intercept, Andover, pp. 63–94.

GLAZER, I. & NAVON, A. (1990) Activity and persistence of entomopathogenic nematodes tested against *Heliothis armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **83**, 1795–1800.

GLAZER, I., KLEIN, M., NAVON, A. & NAKACHE, Y. (1992) Comparison of efficacy of entomopathogenic nematodes combined with antidesiccants applied by canopy sprays against three cotton pests (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **85**, 1636–1641.

KAYA, H.K. (1990) Soil ecology, in *Entomopathogenic Nematodes in Biological Control* (GAUGLER, R. & KAYA, H.K., Eds) CRC, Boca Raton, FL, pp. 215–231.

KAYA, H.K. & NELSON, C.E. (1985) Encapsulation of steinernematid and heterorhabditid nematodes with calcium alginate: a new approach for insect control and other applications. *Environmental Entomology* **14**, 572–574.

KAYA, H.K., ANNION, C.M., BURKANDO, T.M. & NELSON, C.E. (1987) Escape of *Steinernema feltiae* from alginate capsules containing tomato seeds. *Journal of Nematology* **19**, 278–291.

KING, A.H. (1982) Brown seaweed extracts (alginates), in *Food Hydrocolloids*, Vol. 2 (GLICKSMAN, M., Ed.) CRC Press, Boca Raton, FL, pp. 115–188.

LELLO, R., PATEL, M.N., MATTHEWS, G.A. & WRIGHT, D.J. (1996) Application technology of entomopathogenic nematodes against foliar pests. *Crop Protection* **15**, 567–574.

MEISNER, J., ASCHER, K.R.S. & KAMHI, J. (1970) Feeding stimulants for the larva of the Egyptian cotton leafworm, *Spodoptera littoralis* Boisd. I. Assaying the larval response to extracts of several host plants and to some pure substances with the Styropore method. *World Review of Pest Control* **9**, 104–118.

NAVON, A. (1985) *Spodoptera littoralis*, in *Handbook of Insect Rearing* (SINGH, P. & MOORE, R.F., Eds) Elsevier, Amsterdam, pp. 469–475.

NAVON, A., MEISNER, J. & ASCHER, K.R.S. (1987) Feeding stimulant mixtures for *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **80**, 990–993.

PATEL, A.V. & VORLOP, K.D. (1994) Entrapment of biological control agents applied to entomopathogenic nematodes. *Biotechnological Techniques* **8**, 569–574.

PETERSON, A. (1953) *A Manual of Entomological Techniques*. Eduards Brothers Inc., MI, p. 350.

WOODRING, J.L. & KAYA, H.K. (1988) *Steinernematid and Heterorhabditid Nematodes: a Handbook of Techniques*. Arkansas Agricultural Experimental Station, Fayetteville, AR.

Genetic Approaches for Enhancing Beneficial Traits in Entomopathogenic Nematodes

Daniel SEGAL[1] and Itamar GLAZER[2]

Genetics is a powerful means for improving crops and livestock. In recent years we and others have utilized a variety of genetic approaches aimed at enhancing beneficial traits in entomopathogenic nematodes (EPN). We review here some of the approaches we have taken for enhancing tolerance of *Heterorhabditis bacteriophora* to environmental extremes, in particular heat. Selection for heat tolerance proved to be effective, but was associated in deterioration of reproductive potential. On the other hand selection for resistance to nematicides was very effective, lasted when the selection pressure was removed, and did not compromise other parameters of biocontrol efficacy. Screening for natural isolates resulted in the identification of a heterorhabditid heat tolerant strain IS-5. Using genetic markers and cross hybridization we demonstrated that the trait was dominant and transferable to the commercial strain HP88 without concomitant reduction in biocontrol efficacy. Mutagenesis is useful for generating mutants displaying either desired beneficial traits or marker mutations. We demonstrate the utility of the latter. Finally, genetic engineering is a most promising tool for enhancing beneficial traits in EPN. The success in genetic transformation of EPN opens the way for generating transgenic nematodes carrying genes conferring resistance to various environmental extremes, most notably heat shock genes.

INTRODUCTION

Various strategies of genetic improvement have been used successfully for enhancing desired traits in many species of crops and livestock. These have included screening and isolation of populations exhibiting the desired traits, artificial selection, cross hybridization, and more recently genetic manipulations such as mutagenesis and genetic engineering (4, 8). Yet, until recently for entomopathogenic nematodes (EPN) the general notion has been that "genetics may be the most neglected area ...", and that "a major obstacle to genetic improvement effort is the lack of fundamental understanding of the genetic basis of traits relevant to efficacy, and their underlying genetic architecture" (20).

During the past several years we and others have embarked upon a systematic endeavor for genetic improvement of EPN (1, 8). Here we review several genetic approaches that we have used in an attempt to enhance beneficial traits, in particular heat tolerance, in the EPN *Heterorhabditis bacteriophora*. Detailed accounts of these studies can be found elsewhere (9, 10,

[1] Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel-Aviv 69978, Israel.
[2] Department of Nematology, Institute of Plant Protection, Volcani Center, Bet Dagan 50250, Israel.

11, 21, 22, 26, 27, 28, 32, 33).

The sensitivity of EPN to environmental extremes is a major cause preventing them from reaching their full biocontrol potential (20). Heat intolerance of the infective juveniles (IJs) may be the most important factor restricting their application on exposed surfaces such as foliage, and in relatively warm habitats typical of the Mediterranean and southern Europe (12, 19). Temperature influences the rate at which food reserves (lipids, proteins, and carbohydrates), are utilized by nematodes (25), and it affects their motility, survival, infectivity, development and reproduction (3, 12, 19). Unfortunately, the temperature range for activity of EPN is narrow (18-28°C) and the infectivity and persistence of most species is significantly reduced above 30°C (19). Storage of the nematodes in the ambient temperatures of warehouses also requires considerable heat tolerance.

Our studies have focused on the EPN *Heterorhabditis bacteriophora* because it is a good candidate for genetic improvement studies. The life cycle of *H. bacteriophora* has been described (22, 32). A mutagenesis protocol and several mutants that may act as genetic markers have been developed (21, 33). The occurrence of *H. bacteriophora* as both hermaphroditic and amphimictic forms is conducive to genetic studies (22, 29). Hermaphrodites can be used to maintain homozygous lines whereas amphimictic individuals can be crossed to transfer genetic markers and exchange genetic material. We have used for these studies the HP88 strain of *H. bacteriophora*. This strain is commonly used for field experiments as well as for commercial application.

RESULTS AND DISCUSSION

Selection

Successful genetic selection depends on the presence of genetic variation for the particular trait in the population (heritability, h^2). To evaluate the genetic diversity for heat tolerance in the HP88 strain we have generated from it several dozens inbred lines and compared their performance in a heat tolerance assay (survival of a 6 hours exposure to 37°C). The heritability value in this population was very high ($h^2=0.98$) suggesting that selection could be an effective approach for enhancing its heat tolerance (9).

Accordingly, we attempted to select this population for heat tolerance by exposure of the IJs to 37°C for 6 hours, after which we recorded viability, collected the survivors, allowed them to reproduce in larvae of the wax moth *Galleria mellonella*, and subjected their offspring to another round of exposure to the high temperature. We repeated this procedure for 4 cycles. In addition we evaluated the reproductive potential of the survivors by counting the number of IJs that resulted from each *G. mellonella* cadaver at every cycle.

The results showed that selection is indeed an effective means for improving heat tolerance in this population. Heat tolerance increased already two fold over 4 cycles. However, concomitant with the increase in heat tolerance the selected population experienced a dramatic decrease (over 10 fold) in its reproductive capacity.

Deterioration in aspects of fitness which have not been selected for is a common observation in selection experiments. It has been suggested that avoiding bottlenecks during the selection regimen, by ensuring a large population of survivors at each cycle of selection, might reduce this problem (18, 20). In a subsequent study aimed at enhancing nematicide resistance (11) these considerations were taken into account and we could better demonstrate the power of selection.

In that study we first estimated heritability in the HP88 population for resistance to the three nematicides Fenamiphos, Oxamyl, and Avermectin. The heritability values were relatively high (h^2=0.37, 0.71, and 0.46, respectively). Consequently, we performed selection for 11 rounds and it resulted in 8-9-fold increase in resistance to Fenamiphos and Avermectin, and 70-fold to Oxamyl. The enhanced resistance to Oxamyl and Avermectin, and to a lesser extent to Fenamiphos, was stable and lasted after selection was relaxed for 5 cycles. No deterioration in traits relevant to biocontrol efficacy, including virulence, heat tolerance, and reproductive potential, was observed in the selected lines as compared to the base population (11).

Screening for isolates

A survey was conducted in the arid Negev desert in Israel for natural populations of EPN that would exhibit enhanced tolerance to high temperatures. The heat tolerance assay involved measuring survival after exposure to 37°C for 6 hours. Various isolates, and *H. bacteriophora* HP88 as a control, were thus tested and several of them were found to display increased heat tolerance. The heterorhabditid isolate, IS-5, was markedly more heat tolerant and was used for further experiments bearing on genetic improvement outlined below. Details of the characteristics of IS-5 can be found elsewhere (10).

Cross hybridization

Cross breeding is an effective means for combining several desired traits into a single 'superior' strain. We therefore tested whether the trait of heat tolerance could be transferred by crossing from the IS-5 to the commercial HP88 strain. Crosses were performed on petri plates between single hermaphrodites from the HP88 strain and 4-6 IS-5 males (27).

Ideally, crosses would have only been performed between amphimictic nematodes because all progeny would result from outcrossing. However, it is impossible to distinguish hermaphrodites from amphimictic females in *H. bacteriophora* based on morphological characteristics only (29). We therefore carefully controlled conditions that would favor recovery of amphimictic HP88 females and facilitate outcrossing (22, 29). Even if a hermaphrodite was present in an attempted cross, outcrossing was favored because male sperm have a competitive advantage over hermaphroditic sperm (2). A more rigorous criterion that outcrossing has taken place and the offspring are hybrids rather than products of self fertilization was sought by the use of a marker mutant. The dumpy mutant (*H-dpy-2*), induced by EMS in HP88 nematodes, is autosomal and recessive, and is readily distinguishable in its morphology from the normal phenotype (21). Offspring with a normal (*i.e.* non-dumpy) phenotype from a cross between IS-5 males and homozygous mutant (*H-dpy-2*) amphimictic females (or hermaphrodites) can result only from outcrossing.

Offspring were produced when IS-5 males were mated with amphimictic females (or hermaphrodites) from the HP88 strain (♂ IS-5 x ♀ HP cross) and with the *H-dpy-2* mutant (♂ IS-5 x ♀ HPM cross). In the latter cross approximately 70% of the offspring had normal (non-dumpy) phenotype, hence they must have been hybrids (indicated IM) and genotypically heterozygous (*e.g.* D/d, where D represents the normal and d the recessive *H-dpy-2* mutant alleles, respectively). By extrapolation we presume that the majority of the offspring in the ♂ IS-5 x ♀ HP cross were hybrids too. Further confirmation that the non-dumpy offspring of the ♂ IS-5 x ♀ HPM cross were indeed hybrids (D/d) was obtained from backcrossing them to *H-dpy-2*

mutant amphimictic females (or hermaphrodites). If the non-dumpy offspring of the ♂ IS-5 x ♀ HPM cross were indeed hybrids (*D/d*) the backcross (♂ IM x ♀ HPM, genotypically *D/d* x *d/d*) would be expected to produce half non-dumpy (*D/d*) and half dumpy (*d/d*) offspring. A ratio of 1 : 1 of dumpy : non-dumpy offspring was indeed observed in this backcross (27), indicating that the offspring of the ♂ IS-5 x ♀ HPM cross, and by extrapolation also of the ♂ IS-5 x ♀ HP cross, were hybrids. We next tested whether the heat tolerance trait has been transferred to the offspring of these outcrosses. Survival rate, in the heat tolerance assay, of the hybrid offspring of both crosses was similar to that of the IS-5 strain, indicating that in both cases the trait has been successfully transferred by cross hybridization. The similarity in the degree of survival of the hybrids and the parental IS-5 strain suggests that the heat tolerance trait is dominant. Heat tolerance was assayed also for the population of offspring of the back-cross (♂ IM x ♀ HPM). Since half of this population comprises hybrids (*D/d*) and half mutants (*d/d*), the overall heat tolerance of this population is expected to be an average between the degrees of tolerance of IS-5 and of HPM. The results confirmed this expectation. Similar results were obtained for the heat tolerance of the population of offspring of a comparable ♂ IH x ♀ HP backcross. Taken together the results of these crosses indicate that the heat tolerance trait of IS-5 is genetically based, behaves as a dominant trait (presumably monogenic), and can be transferred by cross hybridization (27). This work also demonstrates the usefulness of marker mutations.

Characteristics of efficacy of hybrids

To determine if other parameters of efficacy have been compromised by the cross hybridization we compared the virulence, reproduction, and storage capacity of the hybrid nematodes (IH resulting from the ♂ IS-5 x ♀ HP cross, and IM resulting from the ♂ IS-5 x ♀ HPM cross) with the parental strains IS-5 and HP88 (27). Both the IH and IM hybrids have acquired increased virulence at high temperatures relative to the parental HP88 strain. The IS-5, IH, and IM populations caused mortality of *G. mellonella* at a higher rate than HP88 at 32°C. All nematode populations examined caused larval mortality at a faster rate in 32°C than at 25°C. Crossing IS-5 with the HP88 strain or the mutant (HPM) did not compromise reproductive potential of the progeny. The IS-5, IH, and IM populations exhibited sensitivity to cold storage relative to the HP88 strain. The IS-5, IH, and IM populations survived significantly longer at 25°C than at 10°C. After four weeks of storage at the survival of IS-5 and IH was still greater than 50%, whereas the survival of HP88 (and of IM) was significantly lower.

CONCLUSIONS

Our studies reviewed herein on genetic improvement of heat tolerance in *H. bacteriophora* clearly illustrate the feasibility of this approach for enhancing beneficial traits in EPN. Together with studies by our colleagues on selection (*e.g.* 5, 6, 7, 13, 14, 30), and on mutagenesis for behavioral and morphological marker mutants (*e.g.* 24, 31), as well as for beneficial mutants (*e. g.* 23), they constitute a solid starting point for a systematic application of genetic techniques for improving the efficacy of EPN. We hope that our and our colleagues' success will encourage other researchers to join this exciting endeavor which will require a concerted effort of several groups. Our attempts to apply selection for enhancing heat tolerance of the HP88 strain of *H.*

bacteriophora underscores the importance of ensuring a wide genetic variation in the population to be selected. Molecular approaches for estimating the degree of genetic variation in a population, *e.g.* RAPD analysis, are now readily applicable to EPN (*e.g.* 16, 28).

Natural isolates have been a rich source for genetic variants in domestication of crops and livestock. The work on the natural isolate IS-5 demonstrates the importance of collecting and preserving the rich genetic diversity available in nature, with particular emphasis on sampling nematodes from as many ecologically and geographically diverse sites as possible (18). Mutagenesis aimed at induction of beneficial mutations is also a viable approach for genetic improvement as illustrated by the recent generation of a desiccation tolerant mutant of *H. megidis* (23). Our work on IS-5 also emphasizes the importance of marker mutations for the genetic analysis of genetically enhanced traits. It is hoped that more marker mutations will be generated in EPN.

The study of IS-5 constitutes the first demonstration that a genetically improved beneficial trait can be transferred between strains, and paves the way for generating 'superior' strains by combining desired traits from different strains.

The recent accomplishment of genetic transformation in EPN (15, 17) opens an exciting avenue of using the power of genetic engineering for improvement of efficacy in EPN. It is encouraging to note that transgenic *H. bacteriophora* carrying supernumerary copies the *hsp70* gene from *C. elegans* displayed enhanced heat tolerance (17). It is likely that field release of genetically modified transgenic EPN will meet less opposition if the engineered genes were their own. Our work on IS-5 calls therefore for isolation of *H. bacteriophora* genes encoding heat shock proteins, comparison of their expression in HP88 vs. IS-5. Experiments along these lines are in progress in our lab. This should be followed by *in vitro* manipulation of these genes and their reintroduction into the nematode for generating transgenic *H. bacteriophora* whose heat tolerance can be carefully controlled and which will be environmentally acceptable. We are also identifying genes involved in sex determination in *H. bacteriophora*, with the long-term goal of increasing the proportion of hermaphrodites in the population. This could increase the reproductive potential of these nematodes when mass reared in fermentors, since the yield will not depend on male-female encounters which are infrequent in liquid culture.

ACKNOWLEDGMENTS

Work described in this paper was supported in part by the Binational Agricultural Research Development Fund (BARD), grants US-1588-88 and IS-2099-92C, and by the Israel-France Agricultural Biotechnology Fund (project 4434).

LITERATURE CITED

1) Burnell, A. M. & Dowds, B. C. A. (1996) The genetic improvement of entomopathogenic nematodes and their symbiont bacteria: Phenotypic targets, genetic limitations and an assessment of possible hazards, Biocontr. Sci. Tech. **6**, 435-447.

2) Dix, I., Koltai, H., Glazer, I. & Burnell, A. M. (1994) Sperm competition in mated first generation hermaphroditic females of HP 88 strain of *Heterorhabditis* (Nematoda: Heterorhabditidae) and progeny sex ratios in mated and unmated females. Fund. Appl. Nematol. **17**, 17-27.

3) Dunphy, G. B. & Webster, J. M. (1986) Temperature effects on the growth and virulence of

Steinernema feltiae strains and *Heterorhabditis heliothidis*. J. Nematol. **18**, 270-272.
4) GAUGLER, R. (1987) Entomogenous nematodes and their prospects for genetic improvement. In: *Biotechnology in Invertebrate Pathology and Cell Culture.* (MARAMOROSCH, K., ed.) Academic Press, San Diego, CA, 457-484.
5) GAUGLER, R., CAMPBELL, J. F. & MCGUIRE, T. R. (1989) Selection for host-finding in *Steinernema feltiae*. J. Invertebr. Pathol. **54**, 363-372.
6) GAUGLER, R., CAMPBELL, J. F. & MCGUIRE, T. R. (1990) Fitness of a genetically improved entomopathogenic nematode. J. Invertebr. Pathol. **56**, 106-116.
7) GAUGLER, R., GLAZER, I., CAMPBELL, J. F. & LIRAN, N. (1994) Laboratory and field evaluation of an entomopathogenic nematode genetically selected for improved host-finding. J. Invertebr. Pathol. **63**, 68-73.
8) GAUGLER, R. & HASHMI, S. (1996) Genetic engineering of an insect parasite. In: *Genetic Engineering* 18. (SETLOW, J. K. ed.) Plenum Press, New York, 135-155.
9) GLAZER, I., GAUGLER, R. & SEGAL, D. (1991) Genetics of the nematode *Heterorhabditis bacteriophora* strain HP88: the diversity of beneficial traits. J. Nematol. **23**, 324-333.
10) GLAZER, I., KOZODOI, E., HASHMI, G. & GAUGLER, R. (1996) Biological characteristics of the entomopathogenic nematode *Heterorhabditis bacteriophora* IS-5: a heat tolerant nematode from Israel. Nematologica **42**, 481-492.
11) GLAZER, I., SALAME, L. & SEGAL, D. (1997) Genetic enhancement of nematicide resistance in entomopathogenic nematodes. Biocont. Sci. Technol. **7**, 499-512.
12) GREWAL, P. S., SELVAN, S. & GAUGLER, R. (1994) Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. J. Therm. Biol. **19**, 245-253.
13) GREWAL, P. S., GAUGLER, R. & WANG, Y. (1996) Enhanced cold tolerance of the entomopathogenic nematode *Steinernema feltiae* through genetic selection. Ann. Appl. Biol. **126**, 335-341.
14) GRIFFIN, C. T. & DOWNES, M. J. (1994) Selection of *Heterorhabditis* sp. for improved infectivity at low temperatures. In: *Genetics of Entomopathogenic Nematode-Bacterium Complexes.* (BURNELL, A. M., EHLERS, R.-U. & MASSON, J. P., eds.) European Comission Publication EUR 15681 EN, Luxembourg, 143-151.
15) HASHMI, S., HASHMI, G. & GAUGLER, R. (1995) Genetic transformation of an entomopathogenic nematode. J. Invertebr. Pathol. **66**, 293-296.
16) HASHMI, G., GLAZER, I. & GAUGLER, R. (1996) Molecular comparisons of entomopathogenic nematodes using Random Amplified Polymorphic DNA (RAPD) markers. Fund. Appl. Nematol. **19**, 399-406.
17) HASHMI, S., HASHMI, G., GLAZER, I. & GAUGLER, R. (1998) Thermal response of *Heterorhabditis bacteriophora* transformed with *Caenorhabditis elegans hsp70* encoding gene. J. Exp. Zool. **281**, 164-170.
18) HOPPER K. R., ROUSH, R. T. & POWELL, W. (1993) Management of genetics of biological-control introductions. Annu. Rev. Entomol. **38**, 27-51.
19) KAYA, H. K. (1990) Soil Ecology. In: *Entomopathogenic nematodes in biological control* (GAUGLER, R. & KAYA, H. K., eds.) CRC Press, Boca Raton, Florida, 93-115.
20) KAYA, H. K. & GAUGLER, R. (1993) Entomopathogenic nematodes. Annu. Rev. Entomol. **38**, 181-206.
21) KOLTAI, H., GLAZER, I. & SEGAL, D. (1994) Phenotypic and genetic characterization of two new mutants of *Heterorhabditis bacteriophora*. J. Nematol. **26**, 32-39.
22) KOLTAI, H., GLAZER, I. & SEGAL, D. (1995) Reproduction of the entomopathogenic nematode *Heterorhabditis bacteriophora* POINAR, 1976: hermaphroditism *vs* amphimixis. Fund. Appl. Nematol. **18**, 55-61.
23) O'LEARY, S. A. & BURNELL, A. M. (1997) The isolation of mutants of *Heterorhabditis megidis* (Strain UK211) with increased desiccation tolerance. Fund. Appl. Nematol. **20**, 197-205.

24) RAHIMI, F. T., MCGUIRE, T. R. & GAUGLER, R. (1993) Morphological mutant in the nematode *Heterorhabditis bacteriophora*. J. Hered. **84**, 475-478.
25) SELVAN, S., GAUGLER, R. & LEWIS, E. E. (1993) Biochemical energy reserves of entomopathogenic nematodes. J. Parasitol. **79**, 167-172.
26) SHAPIRO, D. I., GLAZER, I. & SEGAL, D. (1996) Trait stability and fitness of the heat tolerant entomopathogenic nematode *Heterorhabditis bacteriophora* IS-5 strain. Biol. Contr. **6**, 238-244.
27) SHAPIRO, D., GLAZER, I. & SEGAL, D. (1997a) Genetic improvement of heat tolerance in *Heterorhabditis bacteriophora* through hybridization. Biol. Contr. **8**, 153-159.
28) SHAPIRO, D., GLAZER, I. & SEGAL, D. (1997b) Genetic diversity in wild and laboratory populations of *Heterorhabditis bacteriophora* as determined by RAPD-PCR analysis. Fund. Appl. Nematol. **20**, 581-585.
29) STRAUCH, O., STOESSEL, S. & EHLERS, R. U. (1994) Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*. Fund. Appl. Nematol. **17**, 575-582.
30) TOMALAK, M. (1994a) Selective breeding of *Steinernema feltiae* (FILIPJEV) (Nematoda: Steinernematidae) for improved efficacy in control of a mushroom fly, *Lycoriella solani* WINNERTZ (Diptera: Sciaridae). Biocontr. Sci. Technol. **4**, 187-198.
31) TOMALAK, M. (1994b) Phenotypic and genetic characterization of dumpy infective juvenile mutant *Steinernema feltiae* (Nematoda: Steinernematidae). Fund. Appl. Nematol. **17**, 485-495.
32) ZIONI, S., GLAZER, I. & SEGAL, D. (1992a) Life cycle and reproductive potential of the nematode *Heterorhabditis bacteriophora* strain HP88. J. Nematol. **24**, 352-358.
33) ZIONI, S., GLAZER, I. & SEGAL, D. (1992b) Phenotypic and genetic analysis of a mutant of *Heterorhabditis bacteriophora* strain HP88. J. Nematol **24**, 359-364.

Nematology, 1999, Vol. 1(1), 61-68

Desiccation survival of the entomopathogenic nematode *Steinernema feltiae*: induction of anhydrobiosis

Aharon SOLOMON [1], Ilan PAPERNA [1] and Itamar GLAZER [2]

[1] *Department of Animal Sciences, Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot 76100, Israel;*
[2] *Department of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel*

Accepted for publication: 23 March 1998

Summary – The present study describes different desiccation tolerance traits of three strains of *Steinernema feltiae* (IS-6, IS-15, and N8). A slow dehydration regime (pre-conditioning at 97% relative humidity [RH] for 3 days at 23°C) induced a quiescent anhydrobiosis state in all strains, which enabled them to survive at lower humidities (75% and 85% RH). The IS-6 strain isolated from the Negev desert region of Israel exhibited the best desiccation tolerance. The second best tolerance was observed in the IS-15 strain isolated from Galilee, in the northern part of Israel. The poorest tolerance was exhibited by the N8 strain, which was obtained from Germany. The higher desiccation tolerance of the IS-6 and IS-15 strains was associated with a dispersal response of the aggregated infective juveniles (IJs) at the slow dehydration regime. This allowed the coiled IJs to enter into anhydrobiosis individually, whereas the IJs of the N8 strain remained clumped together. In the present study, the IS-6 strain was chosen to determine the optimal conditions for induction into, and recovery from, anhydrobiosis. A high correlation ($r = 0.875$, $P < 0.05$) was found between the survival of individual IJs at 85% RH and the initial numbers of IJs (ranging from 70 to 7700) in the pre-conditioned clump. The same recovery rates of pre-conditioned IJs exposed to 85% RH over a period of 12 days were obtained with either direct immersion in distilled water or immersion in distilled water after 24 h exposure to 100% RH. No significant differences in virulence and ability to penetrate *Tenebrio molitor* larvae were observed between non-desiccated IJs and rehydrated IJs that had been pre-conditioned and desiccated for 5 days at 85% RH.

Résumé – ***Survie en état de déshydratation du nématode entomopathogène* Steinernema feltiae: *induction de l'anhydrobiose*** – La présente étude décrit différents aspects de la tolérance à la dessiccation chez trois souches de *Steinernema feltiae* (IS-6, IS-15 et N8). Une déshydratation lente — préconditionnement à une humidité relative (RH) de 97% pendant 3 jours à 23°C — induit un stade de quiescence anhydrobiotique chez toutes les souches, ce qui les rend capables de survivre à des humidités faibles (RH 75% et 85%). La souche IS-6 isolée dans le désert du Negev (Israël) fait montre de la meilleure tolérance à la dessiccation. Vient ensuite la souche IS-15 isolée en Galilée (partie nord d'Israël). La plus faible tolérance est celle de la souche N8 provenant d'Allemagne. La tolérance plus élevée à la dessiccation des souches IS-6 et IS-15 est associée à une réaction de séparation des juvéniles infestants (IJ) dans les agrégats lors de la déshydratation lente. Cette séparation est suivie d'une entrée en anhydrobiose des séparés, enroulés sur eux-mêmes, tandis que les IJ de la souche N8 restent agglomérés. La souche IS-6 a été choisie pour déterminer les conditions optimales induisant l'anhydrobiose et la sortie de ce stade physiologique. Une forte corrélation ($r = 0,875$, $P < 0,05$) a été observée entre la survie des IJ séparés à une RH de 85% et la présence d'agrégats (comptant 70 à 7700 IJ) au moment du préconditionnement initial. Les mêmes taux de reviviscence sont obtenus, soit par immersion directe dans l'eau distillée, soit par exposition à une RH de 100% avant immersion dans l'eau. Aucune différence significative dans la virulence et la capacité à pénétrer les larves de *Tenebrio molitor* n'a été observée entre les IJ desséchées, après le préconditionnement pendant 5 jours à une RH de 85% et les IJ n'ayant pas subi ce traitement.

Keywords: anhydrobiosis, desiccation tolerance, entomopathogenic nematodes, *Galleria mellonella*, *Steinernema feltiae*, *Tenebrio molitor*.

Infective juveniles (IJ) of steinernematid and heterorhabditid nematodes are currently used as biological control agents, but their sensitivity to desiccation and other environmental constraints reduces their efficacy in the field (Kaya & Gaugler, 1993). Poor stability in storage is another factor limiting nematode expansion beyond niche markets (Friedman, 1990). Anhydrobiosis is a general term for a reversible physiologically arrested state of dormancy that results from the absence of water. The following traits broadly define the characteristics of anhydrobiotes (Barrett, 1991): true anhydrobiotes can lose up to 95-98% of their body water and, while in anhydrobiosis, they have virtually no metabolism, thereby conserving energy. Nematodes in anhydrobiotic state are highly resistant to extreme environmental conditions (Demeure & Freckman, 1981). Entomopathogenic nematodes are ca-

pable only of a shallow level of dormancy described as quiescent anhydrobiosis (Womersley, 1990).

In the present study, we compared the desiccation tolerance of three different strains of *Steinernema feltiae*, we determined the anhydrobiosis induction conditions affecting the desiccation survival of one strain (IS-6), and we evaluated the influence of desiccation on infectivity and virulence of this entomopathogenic nematode.

Materials and methods

NEMATODE CULTURE

In the present study, IJs of three different strains of *Steinernema feltiae*, IS-6, IS-15, and N8, were studied. The IS-6 strain was isolated from soil in a citrus orchard in Negev, a semi-arid region in Israel. The IS-15 strain was isolated from soil in an olive orchard in Galilee, in the northern part of Israel. The N8 strain was obtained from the laboratory of Dr. R.-U. Ehlers, Raisdorf (North of Hamburg), Germany. The Israeli isolates were recovered from soil samples using the last-instar larvae of the greater wax moth, *Galleria mellonella*, in a live baiting technique (Fan & Hominick, 1991). Nematodes were reared in larvae of *G. mellonella* as described by Dutky *et al.* (1964). The emerging IJs were stored for 3-4 weeks in 250 ml distilled water in culture flasks at 5°C prior to being used in the experiments.

DESICCATION SURVIVAL

Comparison of desiccation tolerance of S. feltiae *strains*

Relative humidity (RH) levels were controlled in sealed desiccators with 60 ml of saturated salt solutions at 23°C as follows: K_2SO_4 for 97% RH, KCl for 85% RH, and NaCl for 75% RH (Winston & Bates, 1960). Relative humidity of 100% was established with 90 ml distilled water. For the desiccation experiments, 20 µl of distilled water containing 660 ± 50 IJs of the various strains were placed on the surface of the cap of an Eppendorf tube. Excess water was removed with a tip of a filter paper (Whatman No. 1). Under these conditions, the nematodes aggregated in a clump on the Eppendorf cap. Caps were placed on Petri dishes (5 cm diameter) and immediately transferred to the desiccators with the various RH. The desiccation treatments included: *i*) fast dehydration, *i.e.*, direct exposure to 85% or 75% RH for 3 days and *ii*) slow dehydration, *i.e.*, initial exposure for 3 days at 97% RH (pre-conditioning treatment) followed by exposure to 85% or 75% RH for 3 more days.

Nematodes were rehydrated by exposure to 100% RH for 24 h, followed by immersion in distilled water for another period of 24 h. Nematode survival was determined daily following rehydration. Viability was scored by counting active IJs or by motility after gentle prodding with a hair probe under an inverted microscope. Each treatment was replicated three times.

Factors affecting desiccation survival of the IS-6 strain

The behavioural response of aggregated IJs in the slow dehydration regime was monitored after 4, 20, 44, and 72 h. Samples were taken from the desiccators and nematode behaviour was observed under the inverted microscope. Numbers of coiled *vs* non-coiled individual IJs were determined in clumps of 70 ± 5, 1550 ± 50 and 7700 ± 500 IJs and distribution of nematodes was recorded. Three replicates were examined for each desiccation time. The behavioural response to 97% RH was also monitored in the IS-15 and N8 strains, but was not quantified.

The effect of the initial clump size (70 ± 5, 600 ± 25, and 7700 ± 500 IJs) on IJ survival was determined after conditioning the different clumps at 97% RH for 3 days followed by exposure to 85% RH at 23 ± 1°C over a period of 9 days. Each treatment was replicated five times.

A possible correlation between desiccation survival of IJs and the initial pre-conditioned clump size was determined in a different experiment, by exposing six clumps of different sizes (70 ± 5, 660 ± 50, 1660 ± 36, 4400 ± 300, 5200 ± 390, and 7700 ± 500 IJ) to 85% RH for a fixed period of 3 days. The various recovery rates were determined as described above. Each treatment was replicated five times.

To determine the effect of the rehydration regime on nematode recovery from the anhydrobiotic state, IJs were either rehydrated directly in distilled water overnight or exposed to 100% RH for 24 h prior to immersion in distilled water at 23 ± 1°C. In this experiment, 4400 ± 300 IJs were used to create the initial clump of each subsample. Each treatment was replicated five times.

Effect of desiccation on the IS-6 infectivity and pathogenicity

Rehydrated IJs from a desiccation treatment (pre-conditioning followed by 5 days exposure to 85% RH) and non-desiccated IJs were exposed to the last-instar larvae of the meal-worm *Tenebrio molitor* in 100 ml plastic containers filled with 11g moist vermiculite (30% water content). Each container received 14 400 ± 3025 IJs and thirteen *T. molitor* larvae. Ten containers for each treatment

were incubated at 23 ± 1°C. Nematode-free containers with insect larvae were used as controls. Total mortality of the insects was recorded 72 h after exposure to the nematodes. Ten cadavers from each treatment were dissected under an inverted microscope after 48 h and the numbers of nematodes that had invaded the insect were counted.

SEM examination of desiccated IJs of the IS-6 strain

For observation with scanning electron microscopy (SEM), slowly- and quickly-dehydrated IJs as described above were coated directly with gold as described by Crowe *et al.* (1978) and examined with a Jeol ISM-5140C.

STATISTICAL ANALYSES

Statistical analyses were performed using the SAS 6.04 software package. To compare survival curves, a linear curve of survival by time was fitted for each treatment, after performing a logit transformation on the survival data ($\log[p/1-p]$) with p = percentage of surviving IJs. The model was fitted by GLM, using the repeated measurements from each desiccator. The slopes of the curves were compared overall by a F-test and in pairs by T-tests. In addition, the percentages of survival at the end of each experiment were compared by one-way Anova after arcsin transformation. In the 75% RH treatment, as the change in survival rates over time did not fit the logit model, we used a two-way analysis of variance with repeated measurements after arcsin transformation. The same test was used to compare recovery rates of IJ in the different rehydration regimes. The correlation between desiccation survival of IJ and initial clump size was measured by the Pearson correlation coefficient. A semi-logarithmic regression was fitted to the survival rate after arcsin transformation. After back-transforming the regression equation, a prediction equation was determined for percentage of survival. Mean numbers of nematodes recovered in the insect cadaver were compared over two treatments by the Wilcoxon-Mann-Whitney two-sample test.

Percentage of insect survival in the pathogenicity experiments was compared by one-way Anova after arcsin transformation. Chi-square values were calculated to compare coiling rates of IJ for different clump sizes.

Results

DESICCATION SURVIVAL

Desiccation survival of the different S. feltiae *strains*

When IJ were exposed directly to a fast-dehydration regime, complete mortality was recorded with all three strains after 24 h exposure. However, after a pre-conditioning treatment, all strains survived at 75% and 85% RH. Survival curves of pre-conditioned IJ of the different strains at 85% RH at 23°C are presented in Fig. 1. By day 3, the N8 strain exhibited the poorest survival rate (24.7 ± 9.3% SE, n = 3), compared to the IS-6 and IS-15 strains (63.3 ± 4.7% and 52.3 ± 1.7%, n = 3, respectively) ($F = 8.76, d.f. = 2, P < 0.05$). The IS-6 strain was the only strain that survived 48 to 72 h exposure at 75% RH ($F = 12.9, d.f. = 2, P < 0.05$) (Fig. 2).

Factors affecting desiccation survival of the IS-6 strain

When clumps of 1550 and 7700 IJ were exposed to 97% RH for 3 days, they exhibited a characteristic pattern of behaviour: after 4 h exposure, they were active and aggregated in a ball-shaped formation. After 20 h exposure, they started to disperse from the clump on the exposed surface; by this time only 5% (n = 147) of IJ from both clumps were found coiled. After 44 h exposure, 64.4% (n = 720) and 33% (n = 210) of the IJ from the large (7700 IJ) and small clumps (1550 IJ), respectively, were found individually coiled (Chi-square = 66.3, $d.f. = 1$, $P < 0.05$). By the end of the pre-conditioning period (72 h), clumps were disassembled with 70% (n = 943) and 50% (n = 212) coiled IJ in the large and small clump, respectively (Chi-square = 41.95, $d.f. = 1$, $P < 0.05$). When groups of 70 IJs were exposed to the slow dehydration regime, no dispersal response similar to that in the larger clumps was observed. After 72 h, these IJs were found dried-up on the surface touching each other with a significant lower percentage of coiling (34%) than in the larger clumps (Chi-square = 64.7, $d.f. = 2$, $P < 0.05$).

Although this pattern of behaviour was quantified only in the IS-6 strain, it was also observed in the IS-15 strain, but not in the N8 strain. IJs of the N8 strain remained clumped together after the pre-conditioning treatment and were exposed to the lower RH as clumps and not as individuals.

SEM observation of IJs of the IS-6 strain in the pre-conditioned clump (after 44 h) revealed a mass of coiled IJs with shrunken bodies and prominent cuticular folds (Fig. 3A, B). When the clumps were directly exposed to a fast desiccation regime (85% RH), the IJs were found

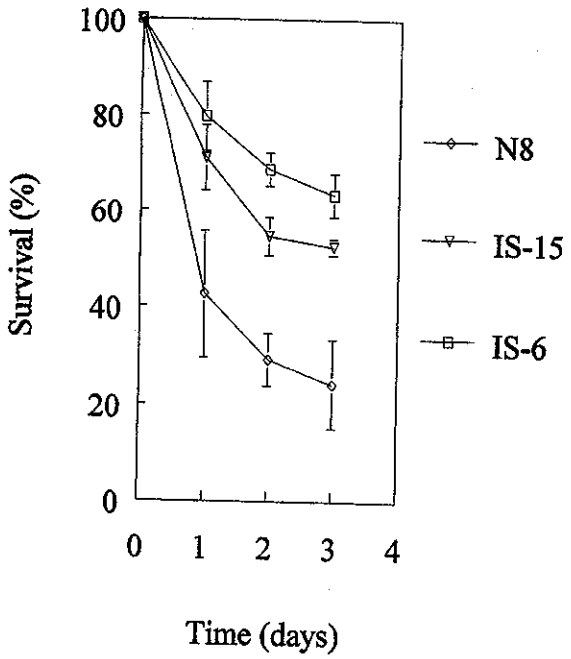


Fig. 1. *Survival curves of pre-conditioned infective juveniles (IJ) of three different strains of* Steinernema feltiae *(N8, IS-15, and IS-6) in 85% RH at 23°C (n = 3; error bars show ± S.E.; initial clump size of 660 ± 50 IJ).*

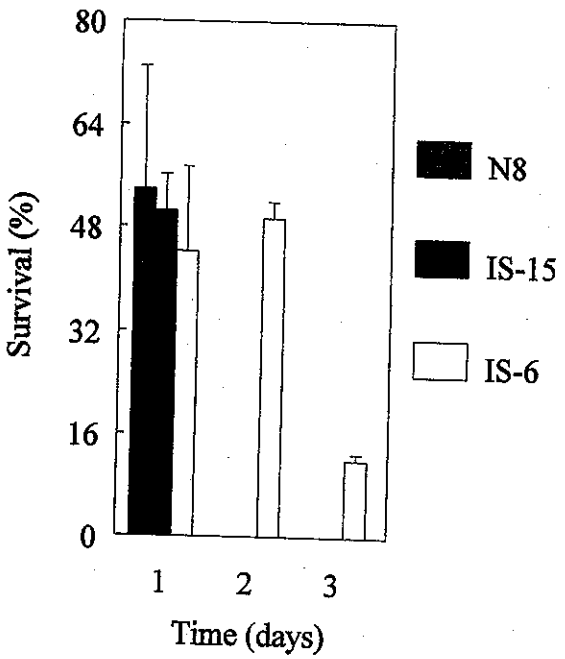


Fig. 2. *Survival rates of pre-conditioned IJ of three different strains of* Steinernema feltiae *(N8, IS-15, IS-6) in 75% RH at 23°C (n = 3; error bars = ± S.E.; initial clump size of 660 ± 50 IJ).*

blotted on the surface after 4 h. They were not coiled and had smooth cuticles (Fig. 3C, D). An individually coiled IJs in a quiescent anhydrobiosis state at the end of the pre-conditioning treatment is presented in Fig. 4.

Survival curves of pre-conditioned IJs of IS-6 from clumps of different sizes following exposure to 85% RH at 23°C are presented in Fig. 5. Slopes of survival curves of IJs from the different clumps were significantly different from each other ($F = 39.98$, $d.f. = 2$, $P < 0.001$). By day 9, the highest survival rate of IJs was observed in the larger clump (7700 IJ, 62.4 ± 8.9% SE), compared to lower survival rates of IJs in the groups of the smaller clump (660 and 70 IJ) (20 ± 4.9% and 13.7 ± 3.9% $n = 5$, respectively) ($F = 16.5$, $d.f. = 2$, $P < 0.001$).

A high positive correlation ($r = 0.875$, $P < 0.05$) was found between the successful survival of individual IJs at 85% RH and the initial size of the pre-conditioned clumps (Fig. 6).

With pre-conditioned IJs desiccated at 85% RH over a period of 12 days, the same recovery rates were found when the nematodes were rehydrated directly in distilled water or pre-exposed to 100% RH for 24 h prior to immersion in water at 23°C ($F = 0.92$, $d.f. = 1$, $P > 0.05$) (Fig. 7).

Effect of desiccation on IS-6 infectivity and pathogenicity

No significant differences were observed between post-desiccated and non-desiccated IJs in their virulence to *T. molitor* larvae, 93.6 ± 2.6% mortality *vs* 95.8 ± 3% ($F = 4.41$, $d.f. = 1$, $P > 0.05$), and 0% mortality of the control group. Also, the same numbers of post-desiccated and non-desiccated nematodes penetrated the insect cadaver (104 ± 26 SE *vs* 117 ± 17 [n = 10], $P > 0.05$).

Discussion

The present study demonstrates that a quiescent anhydrobiosis state can be induced in different strains of *S. feltiae* after exposure to 97% RH for 72 h. These findings are in agreement with previous results for other steinernematid species (Popiel *et al.*, 1989; Womersley, 1990) and demonstrate the importance of the slow dehydration rate as a prerequisite for induction of anhydrobiosis. Womersley (1990) classified steinernematid nematodes as slow dehydration strategists, as they inhabit the upper soil profile where the rates of water loss are moderate. Patel *et*

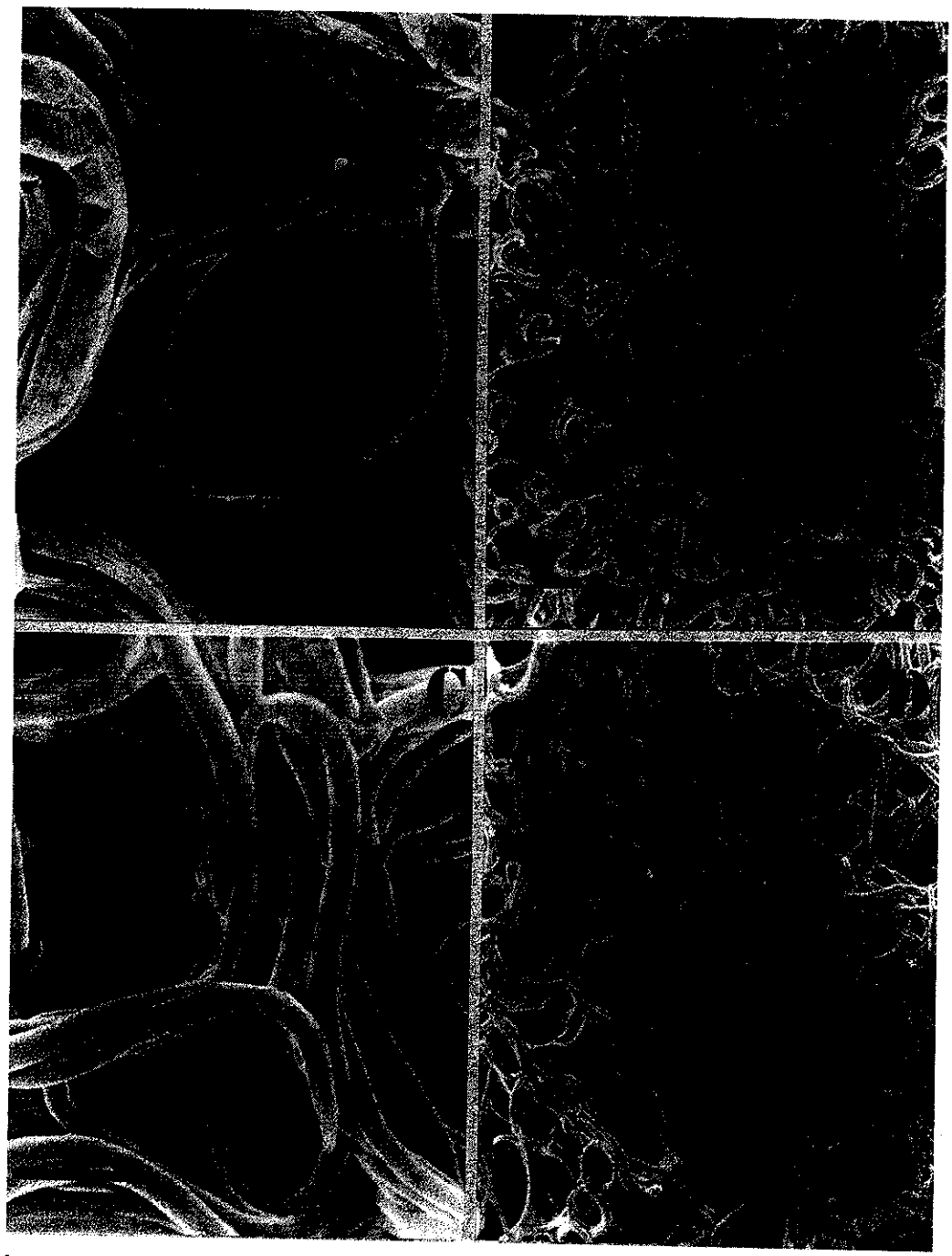


Fig. 3. *SEM observation of* Steinernema feltiae *IS-6 strain. A: Details of coiled IJ in the pre-conditioned clump (at 97% RH; the coiled IJ has a shrunken body and prominent cuticular folds); B: Formation of a clump of coiled IJ during the pre-conditioning treatment; C: Details of rapidly desiccated IJ at 85% (notice the non-coiled IJ with the smooth cuticles); D: Rapidly desiccated clump of IJ at 85% RH (Scale bars: A = 10 μm; B-D = 100 μm).*

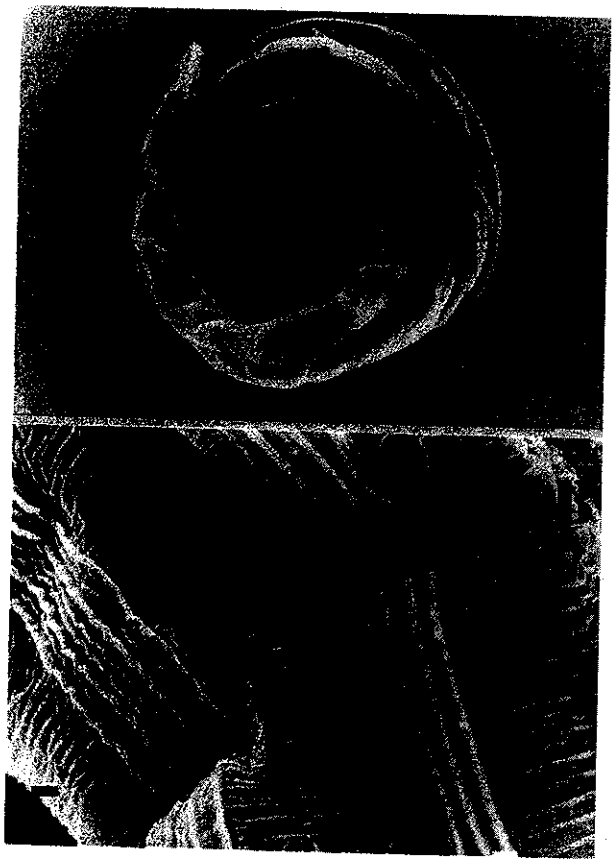


Fig. 4. *SEM observation of* Steinernema feltiae *IS-6 strain. A: Individual coiled IJ in a quiescent anhydrobiosis state; B: Details of the shrunken cuticle and prominent cuticular folds (Scale bars: A = 10 μm; B = 1 μm).*

al. (1997) improved the success of survival of *S. feltiae* at 80% RH by allowing them to dry slowly on 1% agarose. Womersley (1987, 1990) and Barrett (1991) reviewed the importance of slow rates of water loss in different species of nematodes in modulating metabolic and biochemical processes crucial for the successful induction of a state of anhydrobiosis.

The present study showed that strains differ in their ability to survive desiccation. The IS-6 strain, which was obtained from a desert region in Israel, exhibited the highest desiccation tolerance, indicating adaptation to the harsh environmental constraints in the desert habitat. In addition, a difference in the behavioural response to desiccation was also observed among these strains. The higher desiccation tolerance of the IS-6 and IS-15 strains was associated with a dispersal response at 97% RH and entrance into anhydrobiosis as coiled individuals, whereas poor desiccation tolerance was characteristic of the N8

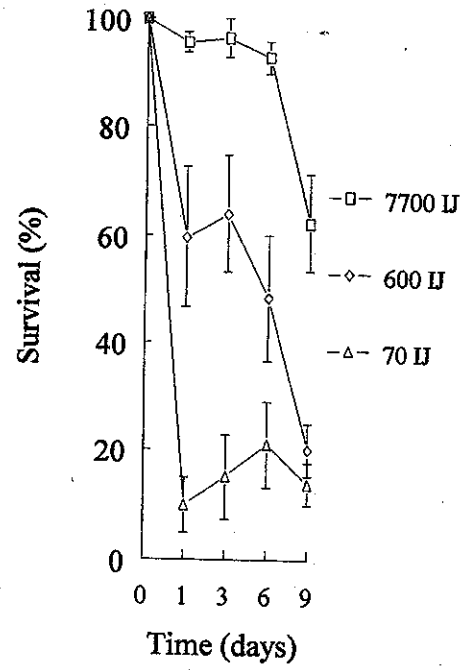


Fig. 5. *Survival curves of pre-conditioned IJs of* Steinernema feltiae *IS-6 strain originating from clumps of different sizes after exposure to 85% RH at 23°C (n = 5; error bars = ± S.E.).*

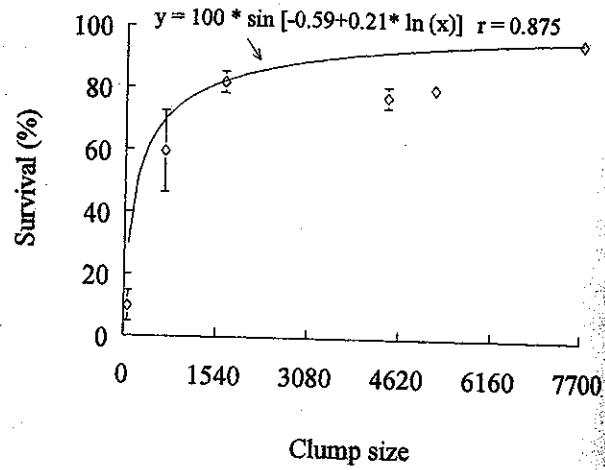


Fig. 6. *Survival rates of pre-conditioned IJs of* Steinernema feltiae *IS-6 originating from clumps of six different sizes after 3 days of exposure to 85% RH at 23°C (n = 5; error bars = ± S.E.).*

strain, which remained clumped. The ability to disperse may be attributed either to physiological differences, such as synthesis of trehalose or stress proteins, or to intrinsic mechanisms for control of water loss, which are probably slower in the IS-6 and IS-15 strain and faster in the strain

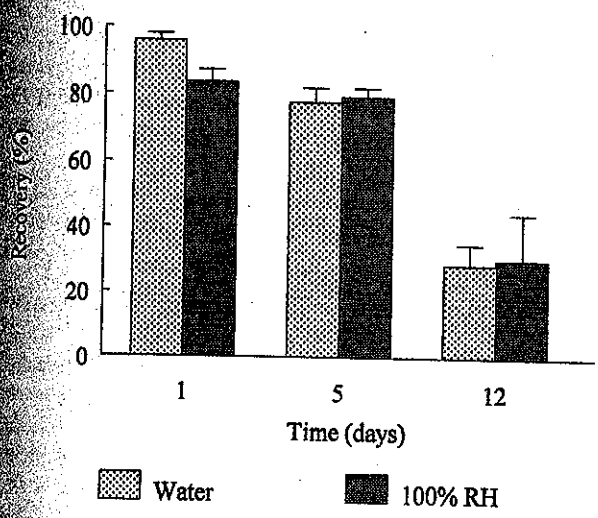


Fig. 7. Recovery of pre-conditioned desiccated IJs (at 85% RH at 23° C) of the IS-6 strain at different rehydration regimes (n = 5; error bars = ± S.E.; initial clump size of 4400 ± 300 IJ).

with the poorer desiccation tolerance. However, these assumptions need to be verified.

Patel *et al.* (1997) and Menti *et al.* (1997) presented evidence of differences in rates of water loss between species and strains of steinernematid nematodes, which were related to survival rates. Those studies, however, were conducted under direct exposure to lower relative humidity regimes (0-80% RH).

We found that coiling of individual IS-6 IJs was proportional to the increase in dehydration time at the 97% RH treatment. Patel *et al.* (1997) also observed large proportions of coiled *S. feltiae* (UK76 strain) in slow drying conditions on 1% agarose. The physical response of coiling is thought to reduce the surface area of the nematode body exposed to the dry environment and thus to reduce the rate of water loss by evaporation (Bird & Buttrose, 1974). Our results demonstrated a high positive correlation between the initial clump size of pre-conditioned IJs of IS-6 strain and desiccation survival. Furthermore, a significantly higher percentage of individually coiled IJs was observed in the larger clumps at the end of the pre-conditioning treatment. This result strongly suggests that clump size affects coiling behaviour and survival of IJs in dry conditions.

Although the IS-6 strain clumps dispersed completely, we suggest that clump formation is important for the reduction of the rates of water loss. As the initial clump size increases, the nematode surface area exposed is reduced, which slows the rate of water loss by evaporation from the 'ball formation' exposed to the dry environment. This gives the IJs the time they need to undergo the physiological changes that are necessary to withstand low relative humidity conditions. Ishibashi *et al.* (1987) also demonstrated that an increase (by weight) in the IJ clump size enhanced the success of survival of *S. carpocapsae* (DD-136), but numbers of nematodes were not given. It is expected that under natural conditions, when IJs migrate out of infected insect cadavers and are exposed to lower relative humidity in the soil environment, they will aggregate in clumps. Whether such a clump formation truly occurs among entomopathogenic nematodes under natural field conditions has yet to be determined.

The structural changes (shrunken bodies and prominent cuticular folds) observed in the desiccated coiled IJs of the IS-6 strain have already been described in other species of plant-parasitic nematodes entering into anhydrobiosis (Crowe & Crowe, 1982; Glazer & Orion, 1983). The smooth cuticles of the non-coiled quick-dried IJs of the IS-6 strain observed here were also described in quick-dried *Aphelenchus avenae* nematodes (Crowe & Crowe, 1982).

Although it has been shown that desiccation and rehydration processes have a high energy cost (Storey *et al.*, 1982), we have demonstrated here that virulence and invasion ability of nematodes were not hampered by the anhydrobiotic process. Our present data imply, indirectly, that the symbiotic bacteria are not affected by the low relative humidity conditions inside the desiccated IJs. Kung *et al.* (1991) demonstrated a significant decrease in pathogenicity of *S. carpocapsae* and *S. glaseri* to *Galleria mellonella* after 4 days of direct exposure to 90% RH. This regime was probably too stressful for the IJs and resulted in a reduction in their pathogenicity.

In the present study, gradual rehydration of desiccated IJs at 100% RH, prior to immersion in water, did not affect their survival. When anhydrobiotic dry organisms are rehydrated directly in water, their cell membranes can undergo a lipid phase transition (from gel phase to liquid crystalline) and may be expected to leak their contents during rehydration (Womersley, 1981; Crowe & Crowe, 1992), which affects the survival of the organisms. Thus, we expected that gradual rehydration at 100% RH would avoid such lipid phase transition and enhance the viability of the IJs. The present results can be explained by the fact that the pre-conditioning treatment was sufficient to induce the physiological changes needed for protection from the low relative humidity condition. One of those changes is an increase in trehalose levels, as reported in other anhydrobiotic nematodes during the dehydration process (Womersley, 1987, 1990; Crowe & Crowe, 1992).

In preliminary studies we also found an increase in trehalose level in the IS-6 strain during the pre-conditioning treatment (Solomon & Glazer, unpubl.). Trehalose can preserve the liquid crystalline phase of dry lipids and therefore prevent the cell leakage described above (Crowe & Crowe, 1992).

We suggest that accurate induction conditions of a quiescent anhydrobiotic state in entomopathogenic nematodes, is a key factor in future research on the physiological adaptations involved in desiccation tolerance. Womersley (1981) recommended investigating these physiological adaptations in a particular species of anhydrobiotic nematode composed of different strains adapted to divergent natural habitats.

Acknowledgements

We thank Mrs. Salame Liora for technical assistance and Dr Hillary Voet from the Faculty of Agriculture of the Hebrew University of Jerusalem for the statistical analysis. The study was supported by an internal grant from the Faculty of Agriculture of the Hebrew University of Jerusalem, Israel.

References

BARRETT, J. (1991). Anhydrobiotic nematodes. *Agricultural Zoology Review* 4, 161-176.

BIRD, A.F. & BUTTROSE, M.S. (1974). Ultrastructural changes in the nematode *Anguina tritici* associated with anhydrobiosis. *Journal of Ultrastructural Research* 48, 177-189.

CROWE, J.H. & CROWE L.M. (1982). Inducing of anhydrobiosis: membrane changes during drying. *Cryobiology* 19, 317-328.

CROWE, J.H. & CROWE, L.M. (1992). Membrane integrity in anhydrobiotic organisms: Towards a mechanism for stabilizing dry cells. In: Somerso, G.N., Osmond, C.B. & Bolis, C.L. (Eds). *Water and Life*. Berlin & Heidelberg, Germany, Springer-Verlag. pp. 87-103.

CROWE, J.H., LAMBERT, D.T. & CROWE, L. M. (1978). Ultrastructural and freeze fracture studies on anhydrobiotic nematodes. In: Crowe, J.H. & Clegg J.S. (Eds). *Dry biological systems*. New York, USA, Academic Press. pp. 23-51.

DEMEURE, Y. & FRECKMAN, D.W. (1981). Recent advances in the study of anhydrobiotic nematodes. In: Zuckerman, B.M. & Rohde, R.A. (Eds). *Plant parasitic nematodes, Vol. 3*. New York, USA, Academic Press. pp. 205-226.

DUTKY, S.R., THOMPSON, J.V. & CANTWELL, G. E. (1964). A technique for the mass propagation of the DD-136 nematode. *Journal of Insect Pathology* 5, 417-422.

FAN, X. & HOMINICK, W.M. (1991). Efficiency of the *Galleria* (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. *Revue de Nématologie* 14, 381-387.

FRIEDMAN, M.J. (1990). Commercial production and development. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control*. Boca Raton, FL, USA, CRC Press. pp. 153-157.

GLAZER, I. & ORION, D. (1983). Studies on anhydrobiosis of *Pratylenchus thornei*. *Journal of Nematology* 15, 333-338.

ISHIBASHI, N., TOJO, S. & HATATE, H. (1987). Desiccation survival of *Steinernema feltiae* str. DD-136 and possible desiccation protectants for foliage application. In: Ishibashi, N. (Ed.). *Recent advances in biological control of insect pests by entomogenous nematodes in Japan*. Tokyo, Japan, Ministry of Education. pp. 139-144.

KAYA, H.K. & GAUGLER, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology* 38, 181-206.

KUNG, S.P., GAUGLER, R. & KAYA, H.K. (1991). Effects of soil temperature, moisture and relative humidity on entomopathogenic nematode persistence. *Journal of Invertebrate Pathology* 57, 242-249.

MENTI, H., WRIGHT, D.J. & PERRY, R.N. (1997). Desiccation survival of populations of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the UK. *Journal of Helminthology* 71, 41-46.

PATEL, M.N., PERRY, R.N. & WRIGHT, D.J. (1997). Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae). *International Journal for Parasitology* 27, 61-70.

POPIEL, I., GLAZER, I. & VASQUES, E.M. (1989). Desiccation survival of *Steinernema feltiae* infective juveniles. *Journal of Nematolology* 21, 580.

STOREY, R.M.J., GLAZER,I. & ORION, D. (1982). Lipid utilization by starved anhydrobiotic individuals of *Pratylenchus thornei*. *Nematologica* 28, 373-378.

WINSTON, P.W. & BATES, D.H. (1960). Saturated salt solutions for the control of humidity in biological research. *Ecology* 41, 232-237.

WOMERSLEY, C. (1981). Biochemical and physiological aspects of anhydrobiosis. *Comparative Biochemistry and Physiology* 70, 669-678.

WOMERSLEY, C. (1987). A reevaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. In: Veech, J.A. & Dickson, D.W. (Eds). *Vistas on nematology*. Hyattsville, MD, USA, Society of Nematologists. pp. 165-173.

WOMERSLEY, C. (1990). Dehydration survival and anhydrobiotic potential. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control*. Boca Raton, FL, USA, CRC Press. pp. 117-137.

Biocontrol Science and Technology (1999) 9, 259–266

Susceptibility of Sap Beetles (Coleoptera: Nitidulidae) to Entomopathogenic Nematodes

I. GLAZER,[1] L. SALAME,[1] S. GOLDENBERG[2] AND D. BLUMBERG[2]

[1]*Department of Nematology, ARO, Volcani Center, Bet Dagan, 50250, Israel;*
[2]*Department of Entomology, ARO, Volcani Center, Bet Dagan, 50250, Israel*

(*Received for publication 19 August 1998; revised manuscript accepted 13 December 1998*)

Nitidulid beetles (Coleoptera) are considered to be serious pests of date palms throughout the world. They attack ripe fruit, causing it to rot, and damage is reflected in both reduced yield and lower fruit quality. The present study was aimed at an evaluation of the susceptibility to different sap beetles to entomopathogenic nematodes. We further tested nematode efficacy in pots filled with soil infested by third instar larvae of the two beetle species. In Petri dish assay, mortality levels of Carpophilus humeralis *and* C. hemipterus *exposed to* Heterorhabditis *sp. IS-5 strain indicated that the latter is less susceptible to nematode infection. Exposure of both sap beetle species to different nematode strains gave moderate levels of mortality (35–65%) with the heterorhabditid strains HP88, IS-5 and IS-25. The IS-12 strain of* Heterorhabditis *sp. showed poor virulence (<35% mortality) against larvae of* C. humeralis *as well as larvae and pupae of* C. humipterus. *The nematode species* S. riobrave *showed moderate virulence (35–65%) mortality to larvae and pupae of* S. humeralis *as well as to larvae of* C. hemipterus. *Exposure of* C. hemeralis *to different concentrations of* Heterorhabditis *sp. IS-5 in pots containing soil resulted in high mortality (>65%). In contrast, the lower concentrations (500 and 1000 nematodes/pot) caused low mortality (35%) of* C. hemipterus. *Other heterorhabditid strains caused 95–100% mortality of* C. humeralis *in pot assay. The HP88 strain of* H. bacteriophora *and the Tx strain of* Steinernema riobrave *showed poor effectiveness. Incubation of different nematode strains with the* C. humeralis *larvae at high temperature (32°C) resulted in an increase in insect mortality with the IS-12 and IS-21 strains. Reduced mortality was recorded with the HP88 strain treatment at the higher temperature. The IS-5 and IS-12 strains were equally effective in all three soil types tested, whereas the IS-19 strain was more effective in the Almog type soil than in the others.*

Keywords: *sap beetles, entomopathogenic nematodes, biological control, date orchards,* Carpophilus hemipterus, Carpophilus humeralis, Heterorhabditis bacteriophora, Heterorhabditis *sp.*, Steinernema riobrave

INTRODUCTION

Nitidulid beetles (Coleoptera) are considered serious pests of date palms throughout the world (Carpenter & Elmer, 1978; Lepesme, 1947, Lindgren & Vincent, 1953), including

Correspondence to: I. Glazer. Tel: +972 3 968 3334; Fax: +972 3 960 4180; E-mail: glaseri@netvision.net.il.

ISSN 0958-3157 (print)/ISSN 1360–0478 (online)/99/020259–08

Israel (Kehat *et al.*, 1974; 1976; 1983). Four species of nitidulids infest dates in Israel, of which two, *Carpophilus hemipterus* L. and *C. humeralis* F. were investigated in the present work. *Carpophilus hemipterus* is found in Israel throughout the autumn, winter and spring; while *C. humeralis* predominates during the summer (Kehat *et al.*, 1983). They attack ripe fruit, causing it to rot, and damage is reflected in both reduced yield and lower fruit quality. The beetles gnaw at the fruit and lay their eggs therein. The larvae develop inside the fruit and when fully grown they leave the fruit and enter the soil for pupation (Barnes & Lindgren, 1946). The control of these pests in date plantations is therefore of great economic importance. Although an integrated control program for successful pest management was established in Israel (Kehat *et al.*, 1974; 1976) nitidulid beetles still have to be controlled by insecticides. Repeated applications of chemicals, which are required in order to obtain satisfactory control, may upset the biological balance in the plantations, consequent upon which outbreaks of new pests may occur (Kehat *et al.*, 1974).

Entomopathogenic nematodes are receiving a great deal of attention due to their use as alternative measures to chemicals for insect biological control, as reviewed by Kaya and Gaugler (1993), Georgis and Manweiler (1994) and Martin (1997). The infective juveniles (IJs) of these nematodes search for suitable insect hosts; when a host has been located, they penetrate into the hemocoel by mechanical and enzymatic means (Abuhatab *et al.*, 1995). Shortly after entry the nematodes release symbiotic bacteria. After the nematode and its bacterial partner overcome the immune system of susceptible insects (Dunphy & Thurston, 1990), they quickly cause the death of the host. The nematodes feed upon the rapidly multiplying bacteria, mature, mate, and produce two or more generations within the insect cadaver before emerging as infective juveniles in search of fresh hosts.

The impressive attributes of nematodes as biological insecticides including ease of mass culture, ease of application, high lethality to diverse insect pests, and safety (Kaya & Gaugler, 1993) have resulted in a rapid increase of interest. Consequently, nematodes are already produced commercially on four continents, and are successfully applied against soil-inhabiting insects including various coleopteran pests, like weevils (e.g. the black vine weevil and citrus weevil) and white grubs of Scarabaeidae (Georgis & Manweiler, 1994; Martin, 1997).

Vega *et al.* (1994) examined the susceptibility of the sap beetle *C. hemipterus* to different steinernematid nematodes in Petri dish bioassay in order to evaluate the potential use of these biological control agents against this pest in date palm orchards. The present study was aimed at an evaluation of the susceptibility of another sap beetle *C. humeralis* as well as a determination of the virulence of heterorhabditis nematode strains. We further tested nematode efficacy in pots filled with soil infested by third instar larvae of the two beetle species.

MATERIALS AND METHODS

Insect

The nitidulids were reared on an artificial diet used for *Drosophila* spp. (Demerec & Kaufman, 1965), as described in detail by Blumberg *et al.* (1985). Third instar larvae (8–10 mm in length) and pupae of *C. hemipterus* or *C. humeralis* were used for the experiments. All the experiments were incubated, unless indicated differently, at 28 ± 2°C in the dark.

Nematodes

The origin of the nematode species tested in the present study is detailed in Table 1.

The various steinernematid and heterorhabditid strains used in the present study were reared at 25°C in the last instar of *Galleria mellonella* according to the method of Kaya and Stock (1997). After storage at 10°C for 7–14 days, they were allowed to acclimate before use at an ambient room temperature (21–23°C) for 24 h.

TABLE 1. The origin of the nematode species tested in the present study

Nematode species	Strain	Code	Obtained from
Steinernema riobrave	Texas	Sr TX	Dr Gaugler, Rutgers University, NJ, USA
Heterorhabditis bacteriophora	HP88	Hb HP88	Dr Gaugler, Rutgers University, NJ, USA
Heterorhabditis sp.	IS-5	H IS-5	Isolated from soil of Avocado orchard, Nir-Itzhak, Negev region, Israel
Heterorhabditis sp.	IS12	H IS-12	Isolated from date orchard, Neot Smadar, Arava region, Israel
Heterorhabditis sp.	IS-19	H IS-19	Isolated from date orchard, Almog, Jordan valley, Israel
Heterorhabditis sp.	IS-21	H IS-21	Isolated from date orchard, Yitav, Jordan valley, Israel
Heterorhabditis sp.	IS-23	H IS-23	Isolated from date orchard, Mitzpe-Shalem, Dead Sea region, Israel
Heterorhabditis sp.	IS-25	H IS-25	Isolated from date orchard, Kalia, Dead Sea region, Israel
Heterorhabditis sp.	IS-19	H IS-29	Isolated from citrus orchard, Pardes-Hana, Israel

Petri Dish Assays

The effect of different nematode concentrations on the mortality of the sap beetle larvae and pupae was determined first in a Petri dish bioassay. Infective juveniles (IJs) of the various nematode strains were placed in 5-cm diameter (plastic) Petri dishes padded with filter paper (Whatman No. 1) in a 0.5 ml distilled water suspension at 0, 40, 200, 1000 or 5000 IJs per Petri dish. Each dish contained 10 third instar larvae. Ten dishes were used for each treatment. The dishes were incubated at 25°C in the dark. Insect mortality was recorded after 48 h. Control treatment consisted of nematode-free dishes with 0.5 ml distilled water.

Comparison of the virulence between different nematode strains was done by exposing third instar larvae of the sap beetles to different heterorhabditid nematodes and one steinernematid. The nematodes were applied onto moist filter paper in a Petri dish at a concentration of 1000 IJs per dish. All other experimental conditions were similar to the procedure described above.

Pot Bioassays

Nematode suspensions were added to plastic pots (200 cc) filled with 170 cc moistened soil (8% water w/w). Most experiments were conducted with sandy soil from the Volcani Center. The nematodes were added onto the soil surface at different concentrations, in 1 ml of water suspension. Approximately 1 h after nematode application 20 sap beetle larvae were placed onto the soil surface. The pots were placed at 28°C in the dark. Mortality of larvae, pupae, and/or emergence of adults were determined after 10–12 days from the beginning of the experiment. Each treatment consisted of five replicates. Control treatments consisted of nematode-free pots infested by beetle larvae.

Using this system, the following experiments were conducted: (a) the effects of nematode concentrations were determined by exposing larvae of *H. humeralis* and *H. hemipterus* to 0, 500, 1000, 1500 and 2000 IJs/pot; (b) the efficacy of different nematode strains was evaluated by exposing 1000 IJs per pot of various strains to larvae of *H. humeralis*; (c) effect of high temperature was determined by exposing larvae of *H. humeralis* beetle at 28 and 32°C to different nematode strains applied at a concentration 1000 IJs per pot; (d) effect of soil texture on nematode activity was tested by exposing larvae of *H. humeralis* to 1000 IJs per pot of different nematode strains in pots which contained three different soils; Volcani (a sandy soil), Almog and Yitav (sandy loam soils which were obtained from date orchards in the Jordan Valley at the Eastern part of Israel). Nematode-free soils infested by the larvae were used as control.

Statistical Analysis

An arcsin transformation was used on the mortality data which is presented in percentages. The significance between treatments was determined using Student–Newman–Keuls (SNK) multiple range test at $P < 0.05$.

RESULTS

For both sap beetle species investigated in the present work, susceptibility to nematode infection was referred as poor (when the mortality level was lower than 35%), moderate (35–65%) and high (above 65%).

Petri Dish Assays

The effect of different nematode concentrations on larval mortality of both *C. humeralis* and *C. hemipterus* was initially tested with the *Heterorhabditis* sp. IS-5 strain (Figure 1). Nematode concentrations above 40 IJs per dish resulted in mortality rate of *C. humeralis* larvae significantly higher ($P < 0.05$) than the control treatment, ranging from $56 \pm 4.5\%$ to $65 \pm 3.5\%$, with no significant differences between them ($P > 0.05$). As for larvae of *C. hemipterus*, nematode concentrations between 40 to 1000 IJs per dish caused mortality ranging from $27 \pm 11\%$ to $40 \pm 7.5\%$, with no significant differences ($P > 0.05$) between the treatments. Only at 5000 IJs / dish, the mortality level of *C. hemipterus* was significantly higher ($P < 0.05$) than at the lower nematode concentrations. This was similar to the mortality level recorded with *C. humeralis*.

For both insect species, moderate levels of mortality were recorded with the heterorhabditid strains HP88, IS-5 and IS-25 among the larvae and pupae (Figure 2). Whereas, the IS-12 strain of *H. bacteriophora* and the Texas strain of *S. riobrave* displayed varying levels of virulence. A moderate mortality level was recorded only for the pupal stage of *C. humeralis* exposed to the IS-12 strain (similarly to the other heterorhabditid strains tested here (Figure 2(b)). This heterorhabditid strain showed poor virulence against larvae of *C. humeralis* as well as larvae and pupae of *C. hemipterus*. The nematode species, *S. riobrave*, showed moderate virulence to larvae and pupae of *C. humeralis* as well as to larvae of *C. hemipterus*. This nematode displayed poor virulence against the larval stage of *C. hemipterus*.

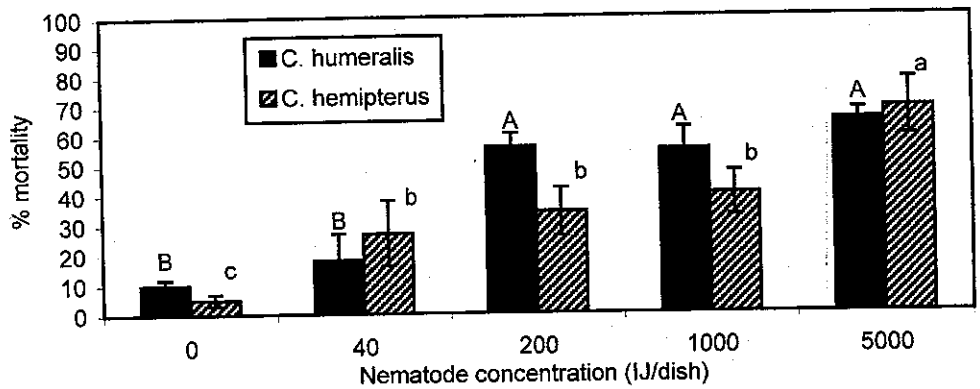


FIGURE 1. Effect of different concentrations of the nematode *Heterorhabditis* sp. IS-5 on the mortality of the sap beetles larvae in Petri dishes. Bars on columns represent ± standard deviation of the mean. Columns marked with the same letter, for each insect species, are not statistically different at $P = 0.05$ according to SNK multiple range test.

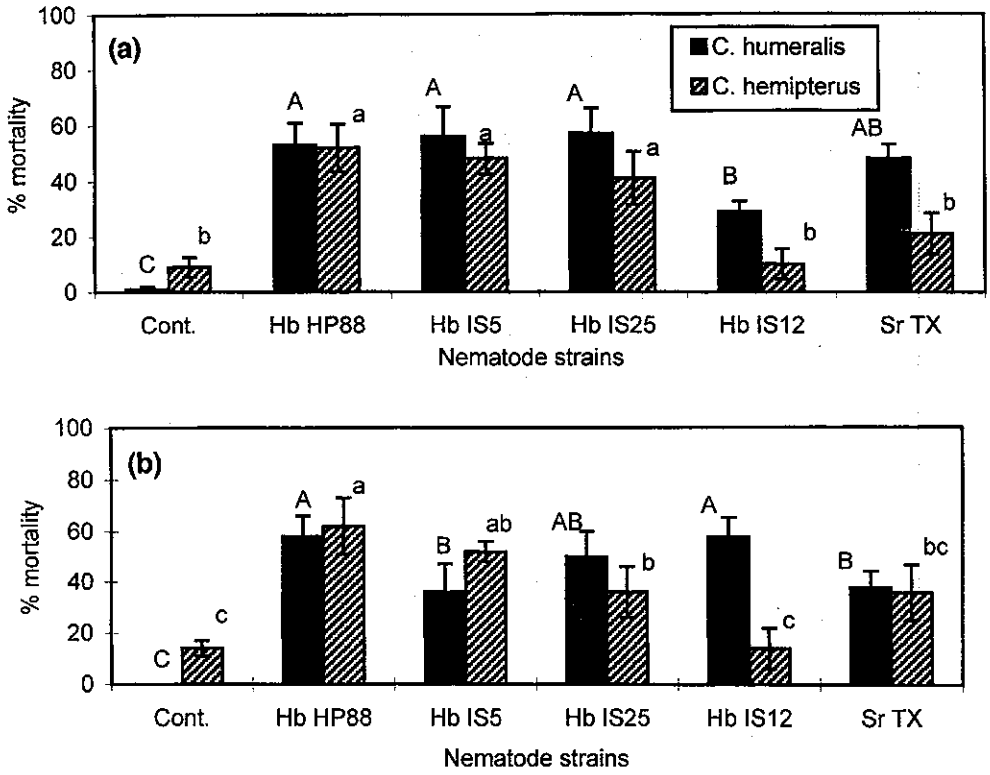


FIGURE 2. Effect of different nematode strains on the mortality of sap beetles larvae (a) and pupae (b) in Petri dishes. IS-# = heterorhabditis strains recently isolated in Israel. Bars on columns represent ± standard deviation of the mean. Columns marked with the same letter, for each insect species, are not statistically different at $P = 0.05$ according to SNK multiple range test.

Pot Assays

In soil assays, all *Heterorhabditis* sp. IS-5 concentrations resulted in high mortality of *C. humeralis* (Figure 3), whereas, the lower concentrations (500 and 1000 IJs / pot) caused low mortality of *C. hemipterus*. High mortality was obtained only by the higher concentrations. Therefore, we further focused our experiments on *C. humeralis* only.

Most of the heterorhabditid strains caused almost 100% or 100% mortality of *C. humeralis* (Figure 4). The IS-12 strain caused moderate mortality (67 ± 11%) and the HP88 strain showed poor effectiveness (19 ± 7.8%). Treatments with the Tx strain of *S. riobrave* also produced low mortality.

Incubation of different nematode strains with *C. humeralis* larvae at high temperature (32°C) resulted in a significant ($P < 0.05$) increase in insect mortality with the IS-12 and IS-21 strains (Figure 5). Reduced mortality was recorded with the HP88 strain treatment at the higher temperature. The IS-5 and IS-12 strains were equally effective in all three soil types tested, whereas the IS-19 strain was more effective in the Almog type soil (Figure 6).

DISCUSSION

Vega *et al.* (1994) demonstrated that larvae of *C. hemipterus* are susceptible to infection by steinernematid nematodes. In the present study, we confirm these findings but have further demonstrated that another sap beetle species, *C. humeralis*, is susceptible to nematode

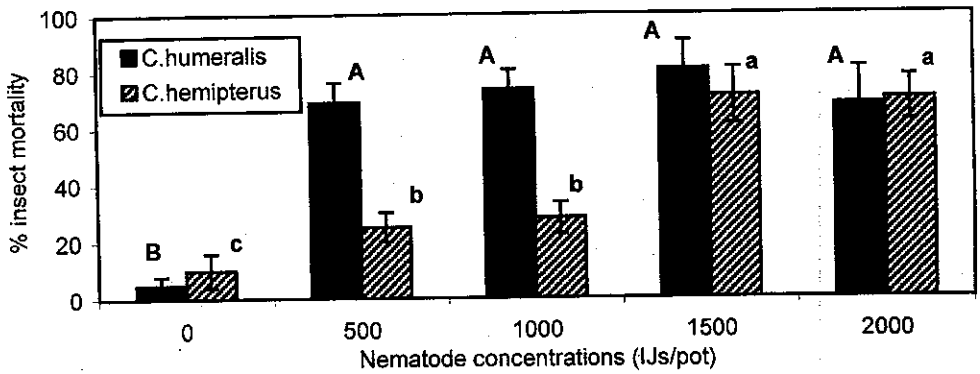


FIGURE 3. Effect of different concentrations of the nematode *Heterorhabditis* sp. IS-5 on the mortality of the sap beetles in soil. Bars on columns represent ± standard deviation of the mean. Columns marked with the same letter, for each insect species are not statistically different at $P = 0.05$ according to SNK multiple range test.

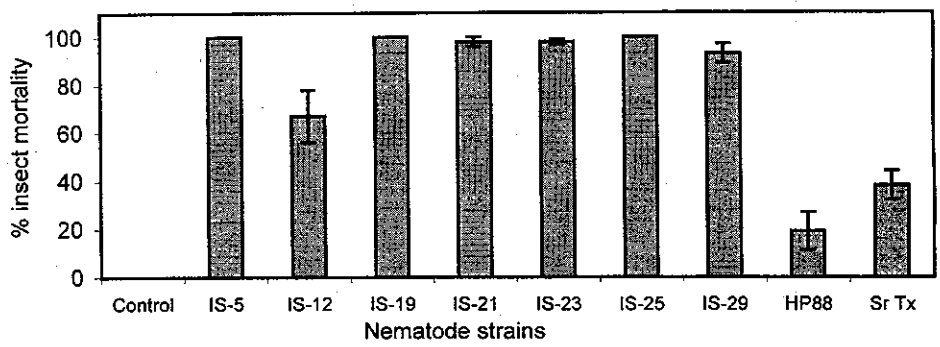


FIGURE 4. Effect of different nematode strains on the mortality of the sap beetle *Carpophilus humeralis* in soil. IS-# = heterorhabditid strains recently isolated in Israel. Bars on columns represent ± SE of the mean.

infection. Furthermore, the data obtained in both Petri dishes and pot assays, suggest that *C. hemipterus* is less susceptible than *C. humeralis* to nematode infection. We also demonstrated that the pupae of both sap beetles are susceptible to nematode infection. These findings imply that nematode treatments against the sap beetles could be effective for both the stages that are found in the soil habitat under the date palm trees. Other beetle developmental stages were not tested, since they can not be found in the proximity of the natural habitat of the nematodes, the soil.

Substantial differences in virulence were observed among the various nematode strains tested in the present study. The Tx strain of *S. riobrave* showed poor virulence both in the Petri dish and pot assays as compared to most of the heterorhabditid strains. The *S. riobrave* Tx nematode was the most virulent species among the ones tested by Vega *et al.* (1994). Our findings indicate that the two sap beetles tested here, and perhaps other species, are more susceptible to infection by heterorhabditid nematodes than to steinernematids. These findings are in agreement with previous findings that heterorhabditid species are more virulent to Coleopteran insects than steinernematids (Kaya, 1990).

Variation in virulence was also observed among the different heterorhabditid strains. Some of the highly virulent strains were recently isolated from soils of date palm orchards

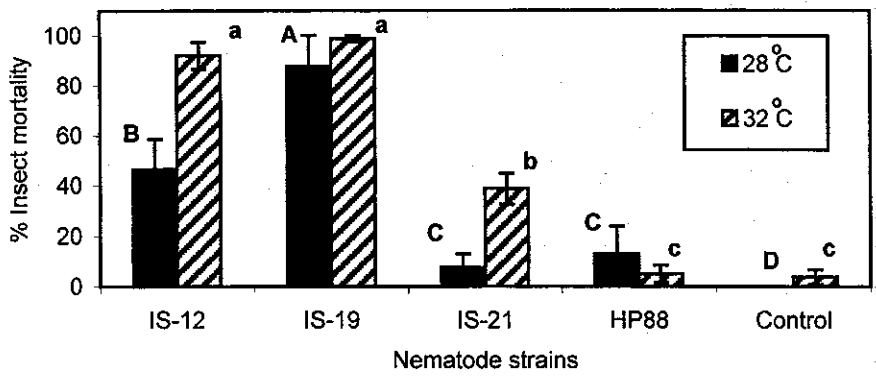


FIGURE 5. Effect of different nematode strains on the mortality of the sap beetle *Carpophilus humeralis* in soil under two temperate conditions. IS-#-heterorhabditid strains recently isolated in Israel. Bars on columns represent ± standard deviation of the mean. Columns marked with the same letter, for each temperature, are not statistically different at $P = 0.05$ according to SNK multiple range test.

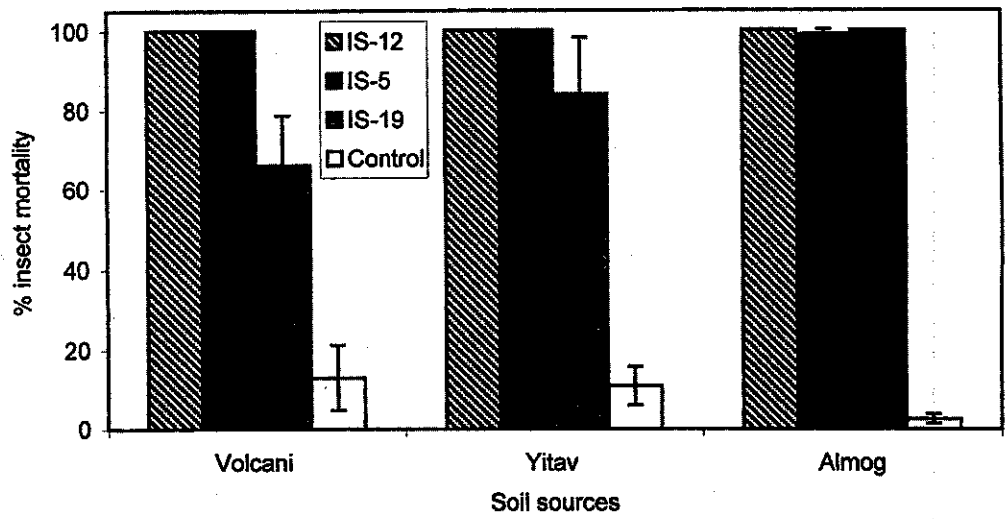


FIGURE 6. Effect of different soil types on the mortality of the sap beetle *Carpophilus humeralis* in sand. IS-# = heterorhabditid strains recently isolated in Israel. Bars on columns represent ± standard deviation of the mean.

in arid regions of Israel (Table 1). These strains are probably highly-adapted to the natural habitat of the sap beetles and will be more effective under field conditions than the laboratory-maintained strains. This notion is further emphasized by the results obtained in the experiment where nematode efficacy was compared at two temperatures. The efficacy of the nematode strains, which were isolated from soils of date palm orchards in hot regions, was increased at the higher temperature. In contrast, the efficacy of the commercial strain was reduced under the same conditions. This is particularly important for the *C. humeralis* species which prevails during the hot summer (Kehat *et al.*, 1983).

Another factor that might influence nematode efficacy is the soil type. We showed in the present study that the IS-19 strain was more effective in the soil type of its origin (Almog;

Table 1). It is well known that entomopathogenic nematodes are most effective in aerated and moist soils (Georgis & Manweiler, 1994). However, prior to field tests it may be important to validate the activity of candidate nematode strains in soil samples typical of the date orchard region.

In conclusion, the findings of the present study strongly suggest that entomopathogenic nematodes, particularly heterorhabditids, have high potential for use as control agents for sap beetles in date palm orchard. The entomopathogenic nematodes should be considered for use as part of integrated pest management approach in the date palm orchard, where they reduce the beetle larval population. Other biological means should be considered for these insect stages, which live above ground, including *Bacillus thuringiensis* or parasitic wasps (Blumberg *et al.*, 1984; Williams *et al.*, 1984). Further studies, under natural conditions, are needed to evaluate the commercial utilization of these biological control agents.

REFERENCES

Abuhatab, M., Selvan, S. & Gaugler, R. (1995) Role of protease in penetration of insect gut by the entomopathogenic nematode *Steinernema glaseri* (Nematoda: Steinernematidae) *Journal of Invertebrate pathology* **66,** 125–130.

Barnes, D.F. & Lindgren, D.L. (1946) The beetles infestation in dates. *Annual Report of Date Grower's Institute* **23,** 34–35.

Blumberg, D., Demeter, M., Kehat, M. & Williams, R.N. (1984) Biological studies of *Zeteticontus utilis* (Hymenoptera: Encyrtidae) a parasite of *Carpophilus* spp. (Coleoptera: Nitidulidae) *Annals of Entomological Society of America* **77,** 130–133.

Blumberg, D., Doron, S. & Bitton S. (1985) Effect of triflumuron on two species of nitidulid beetles, *Carpophilus hemipterus* and *Urophorus humeralis*. *Phytoparasitica* **13,** 9–19.

Carpenter, J.B. & Elmer, H.S. (1978) Pests and Diseases of the Date Palm. *Handbook U.S. Department of Agriculture* 527.

Demerec, M. & Kaufman, B.P. (1965) *Drosophila Guide; Introduction to the Genetics and Cytology of Drosophila melanogaster.* Carnegie Institution of Washington, Washington, DC.

Dunphy, G. & Thurston, G. (1990) Insect immunity, in *Entomopathogenic Nematodes in Biological Control* (Gaugler, R. & Kaya, H.K., Eds) CRC Press, Boca Raton, FL, pp. 301–326.

Gaugler, R. (1993) Ecological genetics of entomopathogenic nematodes, in *Nematodes and the Biological Control of Insect Pests* (Bedding, R., Akhurst, R. & Kaya, H., Eds) CSIRO Publications, pp. 89–104.

Georgis, R. & Manweiler, S.A. (1994) Entomopathogenic nematodes: A developing biocontrol technology, in *Agricultural Zoology Reviews* (Evans, K., Ed.) Intersept, Andover, pp. 63–94.

Kaya, H.K. (1990) Soil ecology, in *Entomopathogenic Nematodes in Biological Control* (Gaugler, R. & Kaya, H.K., Eds) CRC Press, Boca Raton, pp. 93–116.

Kaya, H.K. & Gaugler, R. (1993) Entomopathogenic nematodes. *Annual Review of Entomology* **38,** 181–206.

Kaya, H.K. & Stock, S.P. (1997) Techniques in insect nematology, in *Manual of Techniques in Insect Pathology* (Lacey, L., Ed.) Academic Press, San Diego, London, pp. 281–324.

Kehat, M., Swirski, E., Blumberg, D. & Greenberg, S. (1974) Integrated control of date palm pests in Israel. *Phytoparasitica* **2,** 141–149.

Kehat, M., Blumberg, D. & Greenberg, S. (1976) Fruit drop and damage in dates: The role of *Coccotrypes dactyliperda* F. and nitidulid beetles, and prevention by mechanical measures. *Phytoparasitica* **4,** 93–99.

Kehat, M., Blumberg, D. & Williams, R.N. (1983) Seasonal abundance of sap beetles (Coleoptera: Nitidulidae) in date plantations in Israel. *Phytoparasitica* **11,** 109–112.

Lepesme, P. (1947) *Les Insects des Palmiers,* Paris, Paul Lechevalier.

Lindgren, D.L. & Vincent, L.E. (1953) Nitidulid beetles infesting dates. *Hilgardia* **22,** 97–118.

Martin, W.R. (1997) Using entomopathogenic nematodes to control insects during stand establishment. *Hortscience* **32,** 196–200.

Vega, F.E., Dowd, P.F. & Nelsent, T.C. (1994) Susceptibility of driedfruit beetle (*Carpophilus hemipterus* L.: Coleoptera: Nitidulidae) to different *Steinernema* species (Nematoda: Rhabditae: Steinernematidae) *Journal of Invertebrate Pathology* **64,** 276–277.

Williams, R.N., Weiss, M.J., Kehat, M. & Blumberg, D. (1984) The hymenopterous parasites of Nitidulidae. *Phytoparasitica* **12,** 53–64.

Efficacy of Entomopathogenic Nematode Strains Against Engorged *Boophilus annulatus* Females (Acari: Ixodidae) Under Simulated Field Conditions

MICHAEL SAMISH,[1] EVGENY ALEKSEEV,[1] AND ITAMAR GLAZER[2]

J. Med. Entomol. 36(6): 727–732 (1999)

ABSTRACT The biocidal efficacy of entompathogenic nematodes against engorged females of *Boophilus annulatus* (Say) was evaluated in soil-filled buckets in a greenhouse, where conditions resemble nature. The 9 tested nematode strains differed markedly in their effect upon tick mortality. The Mexican strain of *Steinernema carpocapsae* was most efficient, inducing 100% tick mortality at a concentration of 50 nematodes per square centimeter. An increase in the concentration of the *S. carpocapsae* DT strain to >200 IJ/cm^2 failed to kill more ticks. It appears that entomopathogenic nematodes show promise as tick control agents.

KEY WORDS *Ixodidae, Boophilus, Steinernema, Heterorhabditis,* entomopathogenic nematodes, biological control

BIOCONTROL STUDIES OF ticks lag behind the efforts devoted and success attained in the field of plant pest biocontrol (Samish and Rehacek 1999). This study follows the commercial success of entomopathogenic nematodes in the control of fleas and several plant pests (Henderson et al. 1995, Martin 1997) and is based on the efficient killing of ticks with nematodes in vitro.

Engorged females of *Boophilus annulatus* (Say) were found to be highly susceptible to infection by entomopathogenic nematodes of the genus *Steinernema* and *Heterorhabditis* (Samish and Glazer 1991, 1992). Laboratory studies have shown that the susceptibility to these nematodes differs markedly according to the species and developmental stage of the ticks. Mauleon et al. (1993) found that engorged *B. annulatus* females were most susceptible to entomopathogenic nematode isolates, whereas *B. microplus* (Canestrini) and *Amblyomma variegatum* (F.) were found to be resistant to all tested nematode isolates. Zhioua et al. (1995) and Hill (1998) demonstrated that only engorged *Ixodes scapularis* (Say) females were readily killed by steinernematid nematodes unlike unfed females, males, engorged nymphs, or engorged larvae. Recently, Kocan et al. (1998) reported that steinernematid nematodes kill replete females of *Amblyomma americanum* (L.), *A. cajennense* (F.), *A. maculatum* (Koch), *Dermacenter variabilis* (Say), and *Rhipicephalus sanguineus* (Latreille). In a recent study, we demonstrated substantial differences in the virulence of 5 nematode strains to different developmental stages of 3 tick species (Samish et al. 1996). Engorged females of *B. annulatus* were most susceptible to entomopathogenic nematodes (Samish et al. 1996, Samish et al. 1999).

Previous studies have been conducted only in petri dishes padded with moist filter paper (Kocan et al. 1998). This bioassay provided an initial comparison between the virulence of nematode strains and the susceptibility of tick species. Such an assay however, does not resemble conditions which prevail when ticks, nematodes, and environmental conditions interact in nature. The natural habitat of nematodes is mainly the upper, moist layer of soil, and the engorged *B. annulatus* female ticks also tend to hide in such an ecological niche. With these limitations in mind, the current study attempts near-field experiments that examine the potential use of entomopathogenic nematodes against ticks in soil-filled buckets under greenhouse conditions as a bridging stage between petri dish and field tests. They showed the effect of different dilutions of steinernematid and heterorhabditid nematodes on the death rate of engorged females of *B. annulatus*.

Materials and Methods

Nematodes. The nematodes were reared at 25°C in the last instar of *Calleria mellonella* (L.), according to the method of Kaya and Stock (1997). After storage at 10°C for 7–14 d, they were left to acclimatize (21–23°C) for 24 h. The origin of the nematode species tested in this study is presented in Table 1.

Ticks. *B. annulatus* ticks were collected in 1984 from cattle. They were fed every 2 mo on Friesian calves.

Off-host stages were incubated in the dark at 26°C and 80% RH. Engorged female ticks were tested for nematode susceptibility within 24 h of repletion.

Assays in Buckets. Plastic buckets (10 liter, 6 holes in bottom) were filled with 7 liter of sandy soil (0.05%

[1] Kimron Veterinary Institute, Bet-Dagan, P.O. Box 12, 50250, Israel.
[2] Department of Nematology, ARO, the Volcani Center, Bet-Dagan, 50250, Israel.

Table 1. Investigated entomopathogenic nematode strains, their codes and origins

Nematode species	Strain	Code	Obtained from
Steinernema riobrave (Cabanillas, Poinar and Raulson)	Texas	Sr TX	Dr. Gaugler, Rutgers University, NJ.
Steinernema carpocapsae (Weiser)	DT	Sc DT	Desiccation tolerant strain, selected from "All" strain.
Steinernema carpocapsae (Weiser)	Mexican	Sc MEX	Biosys, MD.
Steinernema feltiae (Filipiev)	IS-6	Sf IS-6	Isolated from a citrus orchard, Gvulot, Negev region, Israel.
Steinernema feltiae (Filipiev)	IS-7	SF IS-7	Isolated from a cattle yard, Tel Goded, Israel.
Steinernema feltiae (Filipiev)	SF	Sf SF	Dr. Ehlers, Christian Albrecht University, Keil, Germany.
Heterorhabditis bacteriophora (Poinar)	HP88	HB HP88	Dr. Gaugler, Rutgers University, NJ.
Heterorhabditis sp.	IS-5	H IS-5	Isolated from soil of an Avocado orchard, Nir-Itzhak, Negev region, Israel.
Heterorhabditis sp.	IS-3	H IS-3	Isolated from a date orchard, Neot Smadar, Arava region, Israel

silt) with a 400-cm^2 upper soil surface. The soil was air-dried and then adjusted to 13% (wt:vol) moisture. Nematodes suspended in 40 ml of water were applied to the soil surface at a concentration ranging from 25 to 400 infective juveniles (IJ) per square centimeter. Control treatment consisted of water application only. After nematode application, 20 engorged females were placed on the soil surface of each bucket and covered with pebbles and dry leaves. The buckets were maintained in a greenhouse at 26 ± 2°C and 80 ± 5% RH, under natural illumination. Tick mortality (no movement of legs when stretching them artificially) was recorded daily for 14 d. Each treatment in an experiment consisted of 4 replicates, and each experiment was repeated 3 times.

Statistical Analysis. Data were analyzed using the SAS LIFETEST procedure (Anonymous 1990). This procedure estimates cumulative tick mortality, according to time of exposure to the nematodes. Estimates of LT_{50} and corresponding 95% confidence intervals (CI) are presented in Table 2. We also calculated the LT_{90} values as follows: (j-1) and j denoted consecutive days that rendered an estimated cumulative daily mortality by day (j-1) < 90%, whereas the cumulative daily mortality was $j \geq 90$. The estimated LT_{90} was given as j days. SAS LIFETEST produces confidence values for LT_{25}, LT_{50}, and LT_{75}, but not for LT_{90}.

Table 2. Effect of various nematode strains at 2 concentrations upon the lethal time of 50 or 90% (LT_{50}, LT_{90}) (in days) of engorged *B. annulatus* females after 14 d of exposure in 10 buckets containing sandy soil, incubated at 26 ± 2°C

Nematode species	Strain	50 IJs/cm^2		200 IJs/cm^2	
		LT_{50} (CI*)	LT_{90}	LT_{50} (CI)	LT_{90}
Steinernema					
S. riobrave	TX	8.1 (7–10)	>14	4.5 (3–6)	8.5
S. carpocapsae	DT	6.5 (5–8)	>14	4.3 (4–7.7)	8.3
S. carpocapsae	MEX	4.8 (3–6)	10	2.8 (3–4)	5
S. feltiae	IS-7	—	—	12.5 (10–14)	>14
Heterorhabditis					
H. bacteriophora	HP88	7.3 (6–9)	11	8 (7–8)	10
H. sp.	IS-5	—	—	6 (5–7)	7.5
H. sp.	IS-3	8 (7–11)	>14	8.3 (6–8)	>14

CI* = Confidence interval for LT_{50}.
— ** = Mortality values below 30% and $LT_{50,90}$ values could not be calculated.

Results

When engorged female *B. annulatus* were exposed in near-field experiments to 9 different entomopathogenic nematodes strains, mortality of the ticks increased in the course of 2 wk, with up to 90% mortality after 5 d (200 IJ/cm^2; Table 2). In most cases the mortality rate was relatively high during the first 10 d of exposure but slow later on (Figs. 1 and 2).

Higher concentrations of nematodes resulted in a higher tick mortality rate (Fig. 1; Table 2). A small, but significant ($P > 0.05$), increase in tick mortality was recorded with 50 and 100 *S. carpocapsae* DT IJ/cm^2, compared with 25 IJ/cm^2, but the percentage of tick mortality was not proportional to the increase in nematode concentration. Concentrations higher than 100 IJ/cm^2 had no significant additive effect on tick mortality (Fig. 1). Therefore, we compared the biocidal efficacy of various nematode strains against engorged *B. annulatus* females at a concentration of 50 IJ/cm^2.

Substantial differences in the mortality rate of engorged females of *B. annulatus* were recorded after exposure to the various nematode strains (Fig. 2). At 50 IJ/cm^2 the highest mortality level was recorded for the *H. bacteriophora* Mexican strain of *S. carpocapsae* (96.3 ± 3.4%), followed by *H. bacteriophora* HP88 (82 ± 5.7%) and the Sp SF, as well as Sc DT strains of *S. carpocapsae* nematodes. *Heterorhabdilis* sp. IS-5, *S. feltiae* IS-6, and IS-7 killed <40% of the ticks within 14 d (Fig. 2).

The LT_{50} values differed considerably among the 7 nematode strains. The LT_{50} seems to be specific to each strain rather than to the genus of the nematode (Table 2). At 50 IJ/cm^2, the LT_{50} ranged from 4.8 to 8.1, and at 200 IJ/cm^2 from 2.8 to 12.5. The most rapid tick mortality rate was recorded after exposure to the Mexican strain of *S. carpocapsae* (Table 2).

When the concentration of nematodes was increased from 50 to 200 IJ/cm^2, the LT_{50} increased by 1.5–1.8 for the steinernematid strains, and by <1 for the measurable heterorhabditid strains (Table 2).

Discussion

Our previous studies were carried out in petri dish assays (Samish and Glazer 1992, Samish et al. 1996). In the current study, sand-filled buckets were used to

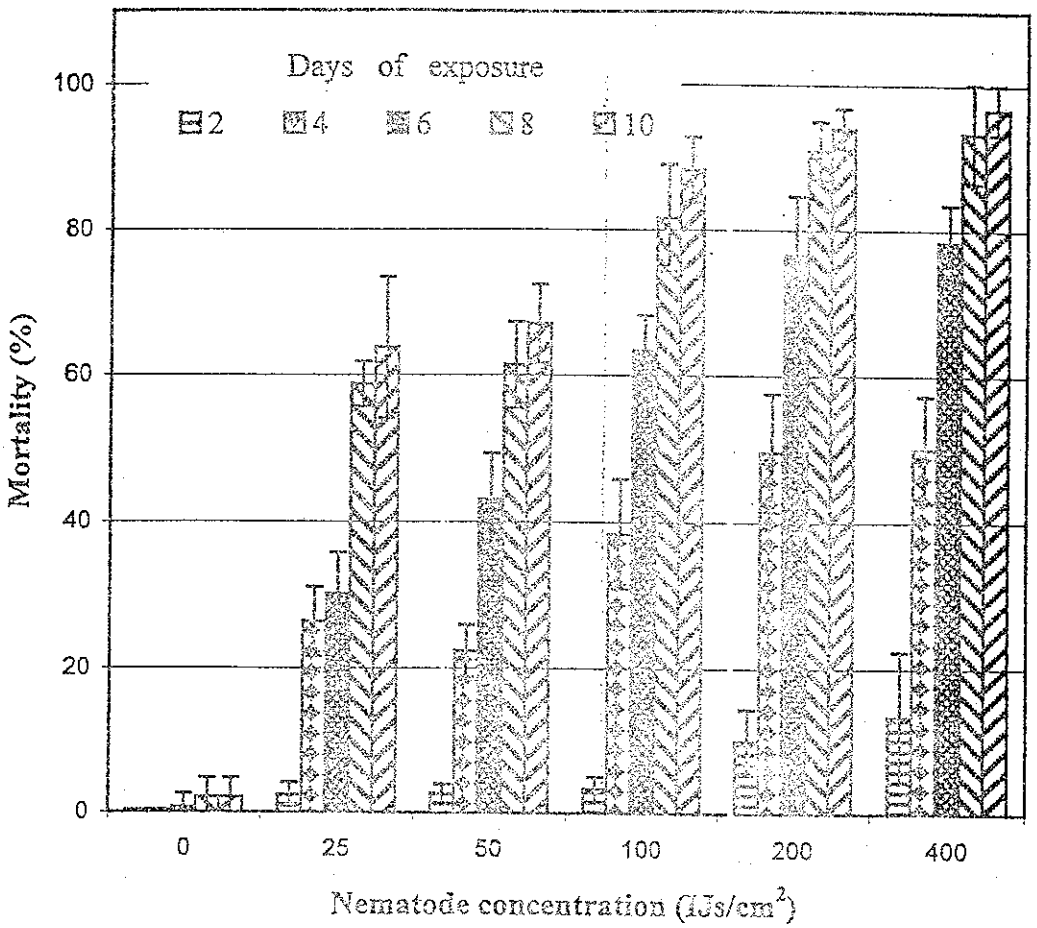


Fig. 1. Effect of concentration of the nematode *S. carpocapsae* DT on the mortality of engorged *B. annulatus* female ticks. Tested in 10-liter buckets containing sandy soil, incubated at 26 ± 2°C.

simulate near-field condition. Pebbles and dry leaves were placed on the moist sand in an attempt to mimic a natural environment favorable to both engorged ticks and nematodes. Under these conditions, some strains of entomopathogenic nematodes were found to kill engorged *B. annulatus* females very efficiently.

The rate of mortality of ticks in buckets varied greatly according to nematode species–strain, concentration, and time of exposure. Tick mortality differed markedly with different concentrations of infective juveniles of *S. carpocapsae* (Fig. 1). It seems that at a concentration of >100 IJ/cm^2, the ticks' surroundings are saturated with entomopathogenic nematodes. Some additional factors appear to govern the overall effect on tick mortality.

In the commercial application of entomopathogenic nematodes against insect pests, a concentration of 100–150 IJ/cm^2 is economically feasible (Georgis and Manweiler 1994, Martin, 1997) and should be cost-effective for the application of nematodes against ticks.

The current study indicates that, under favorable conditions, the most important factor affecting tick mortality level is the nematode strain. When the nematodes were applied at a suboptimal concentration of 50 IJ/cm^2, the Mexican strain of *S. carpocapsae* was found the most effective (Fig. 2). At a higher concentration (200 IJ/cm^2), this strain also had the lowest LT_{50} value, indicating its high potency. The heterorhabditid strains tested here were less effective (Fig. 2). These findings are not parallel with those obtained in previous laboratory assays, where the H IS-3 and H IS-5 strains of *Heterorhabditis* sp. were the most virulent among the steinernematid and heterorhabditid nematodes tested (Samish et al. 1999). Such relative differences in virulence of nematode strains in petri dish assays may be attributed to different host–parasite interactions (e.g., the ability of the infective juveniles to invade the host, to penetrate its hemocoel, or to overcome the host immune response), or perhaps they can be attributed to differences in the multiplication rate and pathogenic effect of the specific symbiotic bacterium associated with each nematode strain (Forst et al. 1997). In the current bucket tests, nematodes' survival and their ability to locate their host were apparently dominant factors, as well as the

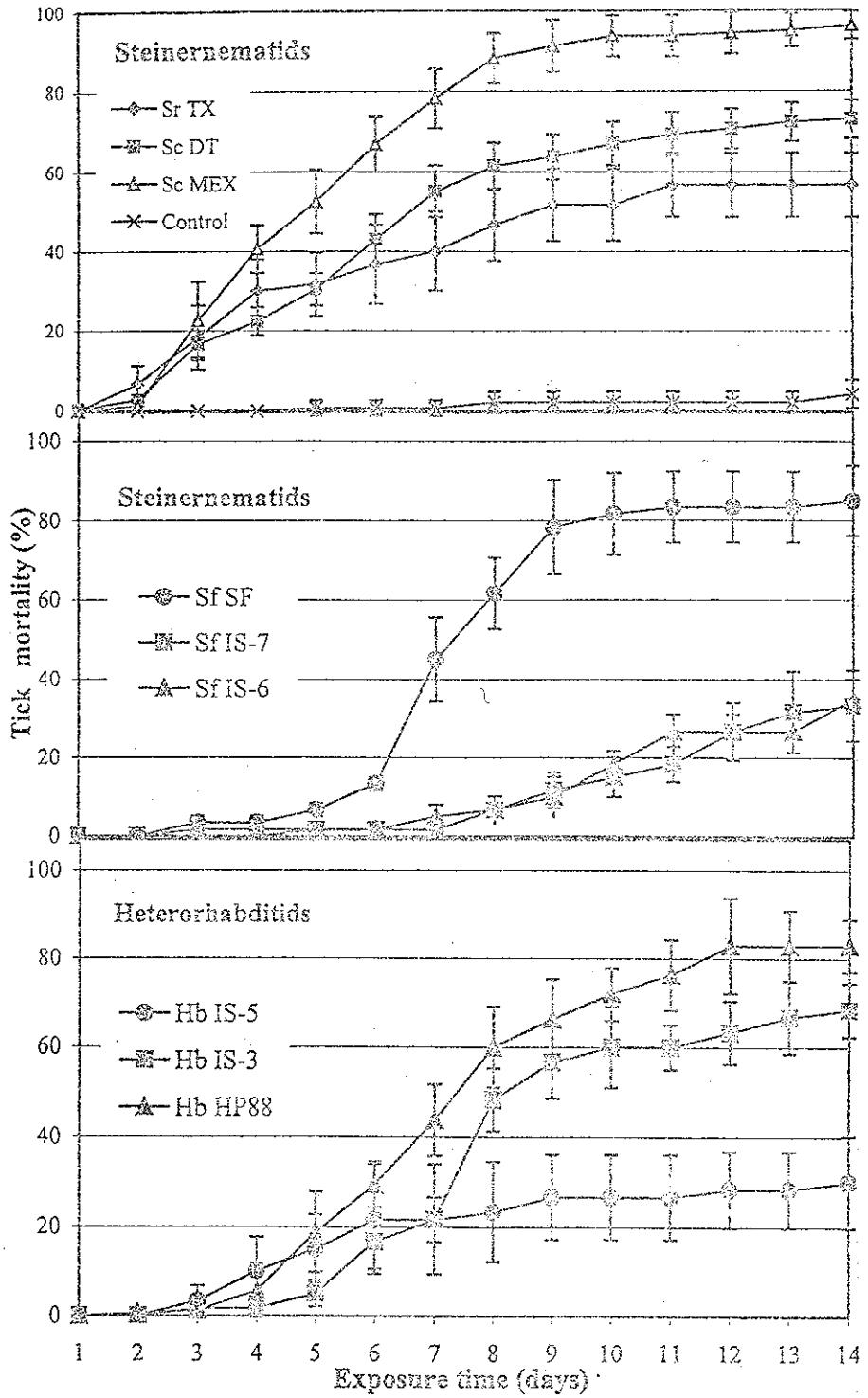


Fig. 2. The mortality of engorged *B. annulatus* female ticks exposed for 14 d to 9 nematode strains. For nematode strain codes, refer to Table 1. Tested in 10-liter buckets containing sandy soil, incubated at 26 ± 2°C (50 IJ/cm^2). Calves serving as food for ticks were kept according to a protocol approved by the committee for animal welfare of the institute. The approved protocol is on file in the laboratory in Israel.

moisture content of the upper soil layer and the differences in depths of soil penetration of the various nematode strains.

The Sc Mex strain of *S. carpocapsae,* which was previously shown to be more desiccation-tolerant than other steinernematid strains (Glazer and Navon 1990), was the most effective strain in the current study. The lower LT_{50} values obtained by most of the steinernematid strains, compared with the heterorhabditids, are probably also related to their enhanced capability to withstand low relative humidities (Glazer and Navon 1990). Although the soil was kept moist throughout all treatments, nematode activity at the surface and in the upper layer of the soil is probably influenced by the ambient relative humidity.

Furthermore, the higher efficacy of the steinernematid strains may be attributed to a better host-finding capability. Some entomopathogenic nematodes, among them *S. carpocapsae,* appear to prefer to wait for hosts at, or near, the soil surface (Kaya and Gaugler 1993), whereas others are adapted to searching deeper in the soil (e.g., *H. bacteriophora*) (Choo et al. 1989). Nematode species adapted to the 1st strategy of reaching their host are known as ambushers and those adapted to the 2nd as cruisers (Gaugler et al. 1989, Lewis et al. 1992). Ambusher types, like *S. carpocapsae,* remain nearly sedentary at the soil surface, whereas cruisers approach their hosts. According to Campbell and Gaugler (1993), nictation behavior, in which the nematode raises all but ≈5% of its body from the substrate for extended periods, is essential for ambush foraging. Engorged females of *B. annulatus* live on the soil surface. Thus, they are probably more suitable hosts for ambushing nematodes. This ambusher foraging strategy of the *S. carpocapsae* Mexican strain may help to explain its superiority in the current study, as opposed to its lesser success in petri dish tests (Samish et al. 1999).

The difference in foraging strategies of steinernematid and heterorhabditid nematodes also may explain the drastic reduction in LT_{50} values when the steinernematid nematode concentration was increased from 50 to 200 IJ/cm^2, but did not change with a similar increase in heterorhabditids. Most ambushing nematodes will remain mainly on the soil surface ready to approach passing ticks, and a higher concentration of nematodes will be available to attack the ticks, whereas cruising nematodes tend to move to lower layers of soil away from the target host.

How fast the ticks are killed is of major practical importance because they should be destroyed before they can lay eggs or attach and transfer tick-born pathogenic agents to a new host. Engorged female ticks require several preoviposition days after they drop off the host. Other engorged larvae and nymphal ticks also need several days after dropping off before molting and completing their prefeeding period and becoming active host searchers. The Sc Mex strain (200 IJ/cm^2) killed 90% of the females on day 5 of exposure (Table 2). In our bucket tests, the *B. annulatus* females started to lay eggs only on days 1–7. Most other tick species have a far longer preoviposition period (Hadani et al. 1969).

Some of the tested nematode strains may be promising as potential killing agents for commercial use in the field. But a search for superior strains is highly important, requiring the screening of numerous, known nematode strains. In our limited study of the few nematode strains, a correlation between the nematode genera and their anti-tick virulence could not be demonstrated. Such screening for a superior strain is thus expected to be a tedious job. The petri dish test could be used first to exclude strains which do not kill ticks, whereas simulated field tests could serve as a second step.

Acknowledgments

We thank Liora Salame for her technical help and Miriam Zarchi and Ruth Marcus for their valuable assistance in statistical analysis. This research was supported by BARD (The United States–Israel Binational Agricultural Research and Development Fund) Research Grant No. IS-2156-92 and by the U.S. Agency for International Development CDR Grant No. TA-MOU-97-C17-032.

References Cited

Campbell, J. F., and R. Gaugler. 1993. Nictation behaviour and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). Behaviour 126: 155–169.

Choo, H. Y., H. K. Kaya, T. M. Burlando, and R. Gaugler. 1989. Entomopathogenic nematodes: host-finding ability in the presence of plant roots. Environ. Entomol. 18: 1136–1140.

Forst, S., B. Dowds, N. Caninum Boemare, and E. Stackebrandt. 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annu. Rev. Microbiol. 51: 47–72.

Gaugler, R., T. McGuire, and J. F. Campbell. 1989. Genetic variability among strains of the entomopathogenic nematode *Steinernema feltiae.* J. Nematol. 21: 247–253.

Georgis, R., and S. A. Manweiler. 1994. Entomopathogenic nematodes: a developing biological control technology, pp. 62–95. *In* K. Evans [ed.], Agricultural Zoology Reviews. Agricultural Zoology Reviews, Andover, Hampshire.

Glazer, I., and A. Navon. 1990. Effects and persistence of entomogenous nematodes used against *Heliothis armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 83: 1795–1800.

Hadani, A., R. Cwilch, Y. Rechav, and Y. Dinur. 1969. Some methods for the breeding of ticks in the laboratory. Refuah Vet. 26: 87–100.

Henderson, G., S. A. Manweiler, W. J. Lawrence, R. J. Templeman, and L. D. Foil. 1995. The effects of *Steinernema carpocapsae* (Weiser) application to different life stages on adult emergence of the cat flea *Ctenocephalides felis* (Bouche). Vet. Dermatol. 6: 159–163.

Hill, D. E. 1998. Entompathogenic nematodes as control agents of developmental stages of the blacked-legged tick, *Ixodes scapularis.* J. Parasitol. 84: 1124–1127.

Kaya, H. K., and R. Gaugler. 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38: 181–206.

Kaya, H. K. and S. P. Stock. 1997. Techniques in insect nematology, pp. 281–324. *In* L. Larcy [ed.], Manual of techniques in insect pathology. Academic, San Diego, CA.

Kocan, K. M., M. S. Pidherney, E. F. Blouin, P. L. Claypool, M. Samish, and I. Glazer. 1998. Interaction of entomopathogenic nematodes (Steinernematidae) with selected species of ixodid ticks (Acari: Ixodidae), J. Med. Entomol. 35: 514–520.

Lewis, E., R. Gaugler, and R. Harrison. 1992. Entomopathogenic nematode host finding: response of host contact cues by cruise and ambush foragers. Parasitology 105: 309–315.

Martin, W. 1997. Using entomopathogenic nematodes to control insects during stand establishment. Hortic. Sci. 32: 196–200.

Mauleon, H., N. Caninum Barre, and S. Panoma. 1993. Pathogenicity of 17 isolates of entomophagous nematodes (Steinernematidae and Heterorhabditidae) for the ticks *Amblyomma variegatum* (Fabricius), *Boophilus microplus* (Canestrini) and *Boophilus annulatus* (Say). Exp. Appl. Acarol. 17: 831–838.

Samish, M., and I. Glazer. 1991. Killing ticks with parasitic nematodes of insects. J. Invertebr. Pathol. 58: 281–282.

Samish, M., and I. Glazer. 1992. Infectivity of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to female ticks of *Boophilus annulatus* (Acari: Ixodidae). J. Med. Entomol. 29: 614–618.

Samish, M., and J. Rehacek. 1999. The natural enemies of ticks and biological approaches to tick control. Annu. Rev. Entomol. 44: 159–182.

Samish, M., I. Glazer, and E. A. Alekseev. 1996. The susceptibility of the development stages of ticks (Ixodidae) to entomopathogenic nematodes, pp. 121–123. *In* M. Rodger, D. J. Horn, G. R. Needham, and W. C. Welborn [eds.], Acarology IX. Ohio Biological Survey, Columbus, OH.

Samish, M., E. A. Alekseev, and I. Glazer. 1999. Mortality rate of adult ticks due to infection by entompathogenic nematodes. J. Parasitol. (in press).

SAS Institute. 1990. SAS lifetest. SAS/STAT user's guide. SAS Institute, Cary, NC.

Zhioua, E., R. A. Lebrun, H. S. Ginsberg, and A. Aeschlimann. 1995. Pathogenicity of *Steinernema carpocapsae* and *S. glaseri* (Nematoda: Steinernematidae) to *Ixodes scapularis* (Acari: Ixodidae). J. Med. Entomol. 32: 900–905.

Received for publication 28 July 1998; accepted 11 June 1999.