Fundam. appl. Nematol., 1993, **16** (5), 467-472

Identification and biological activity of newly isolated heterorhabditid populations from Israel

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Accepted for publication 18 February 1993.

Summary – The present study describes specific identification and characterization of the biological activity of entomopathogenic nematodes which were isolated from soil samples in a recent survey of the arid " Negev " region of Israel. Morphological examination indicated that the nematode populations designated IS_1, IS_2 and IS_3 represent strains of *Heterorhabditis bacteriophora* Poinar. Whereas IS_4 is considered a strain of *H. megidis* Poinar, Jackson & Klein. The identification for IS_{-3} and IS_4 isolates was confirmed by analysis of total DNA on restriction fragment length differences of repetitive sequences, while the IS_2 and IS_3 isolates showed banding pattern different from that of *H. bacteriophora*. In infectivity assays the IS_4 strain of *H. bacteriophora* was found to be the most pathogenic of the four new population and the HP 88 strain of *H. bacteriophora* against the lepidopteran species *Galleria mellonella, Earias insulana, Spodoptera littoralis*. IS_4 infectivity to the lepidopteran strain was equivalent to that of the steinernematid *Steirnernema carpocapsae* All strain. The superior infectivity of the IS_4 was also demonstrated against the scarab *Maladera matrida*. Viability of the *H. bacteriophora* HP 88 infective juveniles was reduced to 20 % when exposed to 37 °C for 3 h. The level of survival of all other heterorhabditids strains was 3-4.5 fold higher than the HP 88 strain. The viability of *S. carpocapsae* All strain was not affected by the same heat treatment. Exposure to low relative humidity conditions (3 h at 50-60 % RH) resulted in 55-85 % reduction in most of the various nematode populations. Relatively high tolerance to the desiccation conditions was recorded with the IS_1 and IS_3 heterorhabditid isolates.

Résumé – ***Identification et activité biologique de populations d'Heterorhabditides récemment isolées en Israël*** – Cette étude traite de l'identification spécifique et de la caractérisation de l'activité biologique de nématodes entomopathogènes isolés du sol au cours d'une récente prospection de la région aride du Negev, en Israël. Les données morphologiques ont conduit à identifier les populations de nématodes nommées IS_1, IS_2 et IS_3 comme des souches d'*Heterorhabditis bacteriophora* Poinar, alors que la population IS_4 serait une souche de *H. megidis* Poinar, Jackson & Klein. L'identification des isolats IS_1 et IS_4 a été confirmée par analyse de l'ADN total par différences dans la longueur de fragments de restriction de séquences répétitives, tandis que les isolats IS_2 et IS_3 montrent une disposition des bandes différente de celle de *H. bacteriophora*. Lors d'essais d'infectivité envers les Lepidoptères *Galleria mellonella, Earias insulana* et *Spodoptera littoralis*, la souche IS_4 de *H. bacteriophora* s'est montrée plus pathogène que les trois autres souches israéliennes et que la souche HP 88 de *H. bacteriophora*. L'infectivité de la souche IS_4 envers les Lépidoptères équivaut à celle de la souche All du Stenernematide *Steinernema carpocapsae*. L'infectivité plus élevée de la souche IS_4 a été également démontrée envers le scarabée *Maladera matrida*. La viabilité des juvéniles infectants de la souche HP 88 de *H. bacteriophora* est réduite de 20 % après exposition à une température de 37 °C pendant 3 h. Le taux de survie de toutes les autres souches d'Heterorhabditides est 3 à 4,5 fois plus élevé que celui de la souche HP 88. La viabilité de la souche All de *S. carpocapsae* n'est pas affectée par ce même traitement thermique. L'exposition à des taux d'humidité relativement bas (3 h à RH 50-60 %) conduit à une réduction de 55 à 85 % de la plupart des populations des différents nématodes. Une tolérance relativement élevée à la dessication a été observée chez les isolats IS_1 et IS_3.

Key-words : Entomopathogenic nematodes, *Heterorhabditis, Steinernema,* biology, heat resistance, drought resistance, Israel.

The entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae are considered effective biological control agents and serve as alternative measures to chemical control of insect pests (Georgis, 1990). With increasing interest in practical and commercial use of these nematodes as biological control agents, many new populations of steinernematids and heterorhabditids were isolated from soils of natural habitats in surveys conducted in various parts of the world (Akhurst & Brooks, 1984; Akhurst & Bedding, 1986; Blackshaw, 1988; Hara *et al.*, 1990; Hominick & Briscoe, 1990 *a, b;* Griffin *et al.*, 1991). While most steinernematids were isolated from regions characterized by temperate and cooler climates (Hominick & Briscoe, 1990 *a;* Poinar, 1990), heterorhabditid isolates were also recovered from soils in warmer regions (Poinar, 1990). Most surveys were conducted in moist regions.

Recently a survey was conducted at the arid Negev region of Israel (Glazer *et al.*, 1991). Consequently, entomopathogenic nematodes, identified as heterorhabditids, were isolated from soil samples obtained from under the canopy of irrigated fruit trees, planted in

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sandy soils. The new populations were named *Heterorhabditis* sp. IS_{1-4} (Glazer *et al.*, 1991). Characterization of the nematodes' biological activity was initiated by subjecting the newly isolated populations to low relative humidity (75 %) for 3 days after preconditioning for 72 h at 97 % RH. Under these conditions less than 10 % of the infective juveniles (IJs) of the new populations survived for 48 h.

Specific identification of the new isolates as well as further comparative characterization of their biological activity i.e. infectivity to various insect pests and tolerance of extreme environmental conditions, are described in the present paper.

Materials and methods

Nematode cultures

The various nematode strains tested in the present study are listed in Table 1. The nematodes were reared on the last instar of the wax moth *Galleria mellonella* according to Dutky *et al.* (1964). The steinernematid IJs were stored at 4°C and the heterorhabditid IJs at 10 °C.

Table 1. Nematodes used in the present study.

Nematode species	Strain	Origin	References
Heterorhabditis bacteriophora	HP 88	Infected June beetle, Logan, UT, USA	Poinar & Georgis (1990)
Heterorhabditis sp.	IS_1	Soil, apricot orchard, Be'er A'Sluge, Israel	Glazer *et al.* (1991)
Heterorhabditis sp.	IS_2	Soil, pear orchard, Retamim, Israel	Glazer *et al.* (1991)
Heterorhabditis sp.	IS_3	Soil, Citrus orchard, Ze'elim, Israel	Glazer *et al.* (1991)
Heterorhabditis sp.	IS_4	Soil, Citrus orchard, Gevulot, Israel	Glazer *et al.* (1991)
Steinernema carpocapsae	All	Soil, GO, USA	Poinar (1990)

Insect colonies

The spiny ball worm *Earias insulana* was reared in the laboratory on an artificial diet (C-2 MS) according to the method described by Klein *et al.* (1983). The Egyptian cotton worm *Spodoptera littoralis* was grown on leaves of castor beans according to Glazer *et al.* (1990). Grubs of the beetle *Maladera matrida* Argaman were obtained from the laboratory of A. Gol'berg of the Gilat Experimental Station.

Nematode identification

Morphological examination

The nematodes were reared in wax moth larvae *(G. mellonella)* maintained at 21 °C and dissected on day 4 or 5 to recover the first generation hermaphrodites and on days 9 or 10 to recover the second generation adults. Infective stage juveniles were collected as they emerged from the host cadavers approximately 14 days after initial exposure. All stages were killed in hot (55 °C) Ringer's solution or water, fixed in TAF and processed to glycerin for microscope examination.

Restriction fragment length polymorphism (RFLP) analysis of DNA

The procedures for total DNA extraction, digestion and gel electrophoresis were conducted according to Smits *et al.* (1991). For comparison, the total DNA of *H. bacteriophora,* two heterorhabditid isolates from Ireland (HIR-M 145 and HIR-K 122) and one isolate from The Netherlands (HE-87.3), of which their RFLP have been analysed previously (Smits *et al.*, 1991), were run in parallel to the populations from Israel.

Infectivity bioassays

Infectivity against lepidopteran larvae

Infective juveniles of the various strains were suspended in distilled water at a concentrations of 200, 400 and 800 nematodes per 0.5 ml. Half a milliliter of the nematode suspension was applied to a 5-cm-diam. Petri dish lined with a filter paper (Whatman No. 1). In the control, only water was applied to the filter paper. After 1 h, a single *S. littoralis* fourth instar, three *E. insulana* 3rd stage larvae or five *G. mellonella* 5th stage larvae were transferred from the insect colonies into each of the dishes and exposed to the nematodes. The dishes were placed at 25 °C in the dark. Insect mortality was recorded 48 h after inoculation. Each treatment consisted of 40 insects. The experiments were repeated three times.

Nematode activity in soil

Plastic pots (150 cm^3, 7 cm-diam.) were filled with moist (10 % w/w) sandy soil (sand 75 %, silt 13 %, clay 12 %). One ml of distilled water containing nematode suspensions of the various strains was applied to the surface of each pot at a concentration of 800 IJs/cm^2 (approx. 29 000 IJs/pot). The pots were incubated for 24 h at 25 °C in the dark and then four last instar *G. mellonella* were placed in each pot. In order to prevent escape, each larvae was confined to a 3-cm-diam. plastic Petri dish, the lid of the dish being replaced by a metal screen (150 mesh). The Petri dish containing the larvae was completely buried in the nematode-infested soil of the pot, perpendicular to the surface. Each pot was considered one replicate. The pots were placed in humidity chambers which consisted of rectangular plastic boxes (35 cm long, 30 cm wide and 20 cm deep) sealed with

covers. Water (250 ml) was added to provide 100 % RH. The pots were held above the water by a 1.5 cm thick plastic screen. The boxes were incubated at 25 °C for 96 h. At the end of the incubation period insect mortality was scored. The number of nematodes within the body of the dead larvae was determined by dissecting each larva separately in distilled water and counting under a stereoscopic microscope. Each nematode treatment consisted of four replicates. The experiment was repeated twice.

The activity of the various strains against 3-week-old grubs of *M. matrida* was determined using the same assay. However this time only three insects were placed in each pot. To prevent mutual predation the grubs were placed in separate netted Petri dishes. In this assay insect mortality was the sole criteria for comparison of nematode activity. Mortality was recorded following 5 days (120 h) incubation at 25 °C.

Assays for comparison of tolerance to environmental extremes

Heat tolerance assay

Infective juveniles obtained from the various nematode strains were each suspended in 15 ml distilled water, in 25 ml conical flasks at a concentration of 5 000 nematodes per flask. The flasks were shaken gently at 40 RPM in a water-bath shaker at 37 °C for 3.5 h. Then three 1 ml samples were withdrawn from each flask and diluted with distilled water to 10 ml final volume at room temperature (22-25 °C). Nematode viability in each sample was determined 24 h later by observing their motility in the water suspension and response to probing under a dissection microscope.

Desiccation tolerance assay

Infective juveniles of the various strains were concentrated by vacuum filtration onto 5 cm-diam. filter paper disks (Whatman No. 1) at a density of 1000 nematodes per disk. The disks were then exposed to ambient conditions (24-26 °C, 50-65 % RH). After 3 h of exposure, samples were taken from each population for rehydration in distilled water. Nematode viability was recorded in each population by observing motility and response to probing under a stereomicroscope 24 h after rehydration. Three replicates were counted and each strain was tested three times.

Statistical analysis

The data presented in percentage values were normalized using arcsine transformation. The significance of the main effects was determined by analysis of variance. The significance of the various treatments was evaluated by *t*-test (P = 0.05).

Results and discussion

Nematode identification

Morphological examination reveals that the cultures of IS_1, IS_2, IS_3 represent strains of *H. bacteriophora* Poinar. IS_4 is considered a strain of *H. megidis* Poinar, Jackson & Klein, although some of quantitative values do differ somewhat from the type specimens which were collected in Ohio, USA (Poinar, 1990).

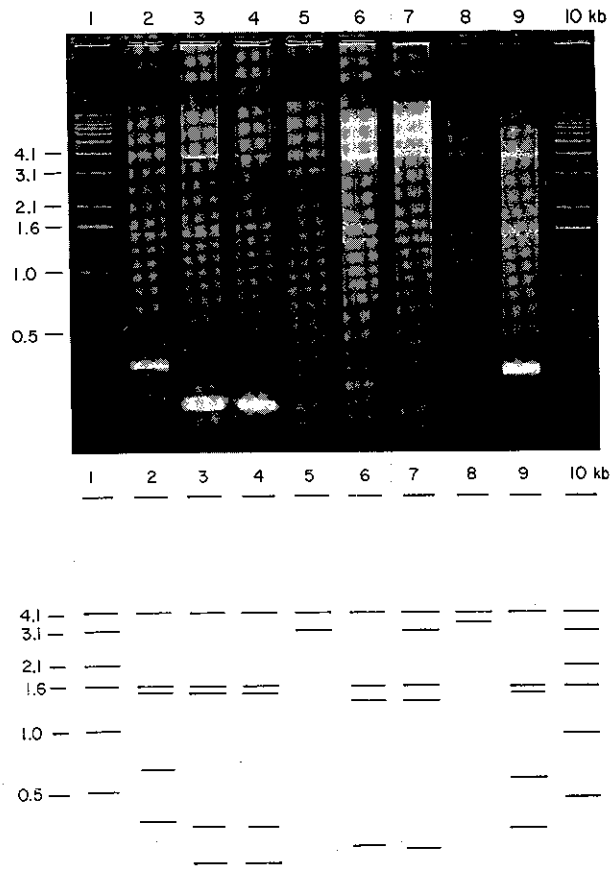

Fig. 1. EcoR 1 digestion patterns of total DNA showing repetitive DNA fragments of the listed *Heterorhabditis* isolates from Israel, USA, Ireland and the Netherlands. Photograph of the ethidiumbromide stained 1.1 % agarose gel (above). Line drawing showing only repetitive DNA bands (below). (1 : 1 kb DNA-maker; 2 : HE-87.3, The Netherlands; 3 : HIR-K 122. Ireland; 4 : HIR-M 145, Ireland; 5 : *Heterorhabditis bacteriophora*, USA; 6 : IS_2, Israel; 7 : IS_3, Israel; 8 : IS_1, Israel; 9 : IS_4, Israel.)

In the analysis of DNA RFLP the IS_1 isolate shows identical pattern to that of *H. bacteriophora* (Fig. 1), confirming its identification by morphological examination. The size of fragments for these nematode species fol-

lowing digestion with EcoR 1 is 4.1 and 3.2 kilobase-pairs (kb). Similarly, the IS_4 presents identical banding pattern to that of the Dutch isolate HE-87.3, which has also been identified as *H. megidis* (Smits *et al.*, 1991). Their fragments size on the EcoR 1 gel is 4.1, 1.6, 1.5, 0.6 and the characteristic intense band at 0.35 kb (Smits *et al.*, 1991). These findings also support the identification achieved by " classical " means. Whereas, the IS_2 and IS_3 isolates which were identified morphologicaly as *H. bacteriophora* show resembling pattern which is different from all other populations. The size of fragments for the IS_2 and IS_3 following digestion with EcoR 1 is 4.1, 3.2, 1.6, 1.4, 0.5 and 0.3 kb. The fact that the IS_2 and IS_3 isolates share two common bands at 4.1 and 3.2 kb, with *H. bacteriophora* might suggest that these isolates are indeed subspecies which are morphologically undistinguishable. Further morphological studies are needed to verify this discrepancy.

Infectivity bioassays

Infectivity against lepidopteran larvae

The effect of the various nematode strains, applied at different concentrations, on the mortality of *S. littoralis* and *E. insulana* larvae is presented in Fig. 2. The IS_4 isolate of *Heterorhabditis* sp. was found to be the most infective among the various heterorhabditid strains. At all concentrations, exposure of the two lepidopteran species to the IS_4 strain resulted in complete mortality (Fig. 2), whereas with all other heterorhabditid strains the mortality of *S. littoralis* and *E. insulana* larvae was proportional to the nematode concentration. The poorest infectivity was recorded with the HP 88 strain of *H. bacteriophora,* where the level of insects mortality exceeded only 70 % even at the highest nematode concentration (Fig. 2). Exposure of *G. mellonella* larvae to the various nematode strains, resulted in 100 % mortality at all concentrations.

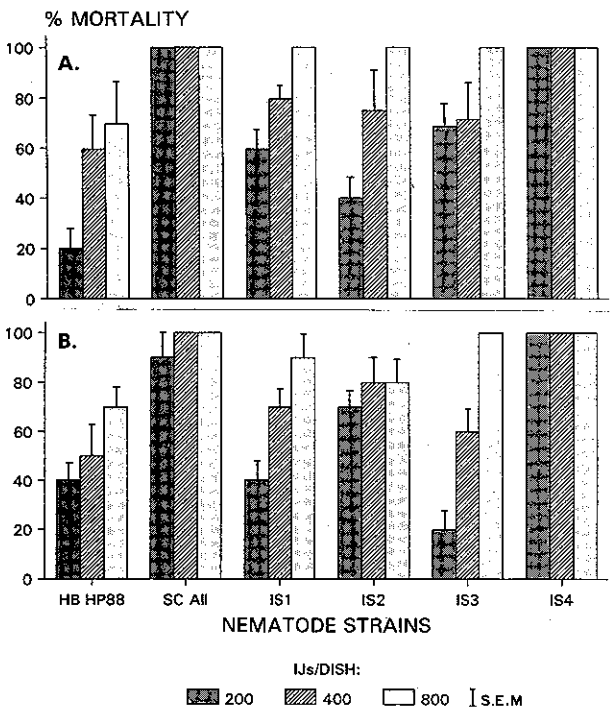

Fig. 2. Effect of different nematodes strains at various concentrations on the mortality of larvae of *Spodoptera littoralis* (A) and *Earias insulana* (B), following 48 h exposure at 25 °C in 5-cm diameter Petri dishes lined with moist filter paper (HB = *Heterorhabditis bacteriophora;* SC = *Steinernema carpocapsae;* IS_{1-4} = newly isolated heterorhabditid populations).

The data obtained in the infectivity assay against the lepidopteran species indicate that the steinernematid *S. carpocapsae* " All " is more pathogenic than the heterorhabditid *H. bacteriophora* " HP 88 " or the new isolates IS_{1-3} (Fig. 2).

These findings confirm previous observations shown that steinernematids are in general more pathogenic against lepidopteran larvae than heterorhabditids (Glazer *et al.*, 1990). However, the new *H. bacteriophora* IS_4 isolate was found to be equally pathogenic to insects as the steinernematid strain (Fig. 2).

Nematode activity in the soil

Exposure of *G. mellonella* larvae to the various nematode strains in the soil assay resulted in 100 % mortality (Fig. 3). Therefore, insect mortality was not suitable for distinguishing the infectivity of the various nematode strains. The average number of nematodes in the cadavers of the larvae was used as an alternative measure to determine differences in infectivity of nematode strains against a highly sensitive insect such as *G. mellonella,* as suggested by Fan and Hominick (1991). The highest number was recorded with the IS_4 strain (Fig. 3). This criterion indicates the superior ability of the IS_4 strain to seek the target insect, to penetrate and establish in the host.

Under the same conditions the effect of the various nematodes on *M. matrida* mortality varied considerably (Fig. 4). The highest mortality level of grubs was also recorded with the IS_4 strain (100 %, Fig. 4). All other heterorhaditids resulted in 60-70 % mortality with no significant difference from each other ($P < 0.05$). The lowest mortality of grubs was recorded with the *S. carpocapsae* " All " (33 %, Fig. 4).

Previous studies had shown that heterorhabditid species are more effective against scarabs in general (Klein, 1990) and particularly grubs of *M. matrida* (Glazer & Gol'berg, 1989) than steinernematids. The superior activity of the IS_4 strain in both soil assays indicates that this nematode may be a good candidate for use against *M. matrida* as well as lepidopteran pests.

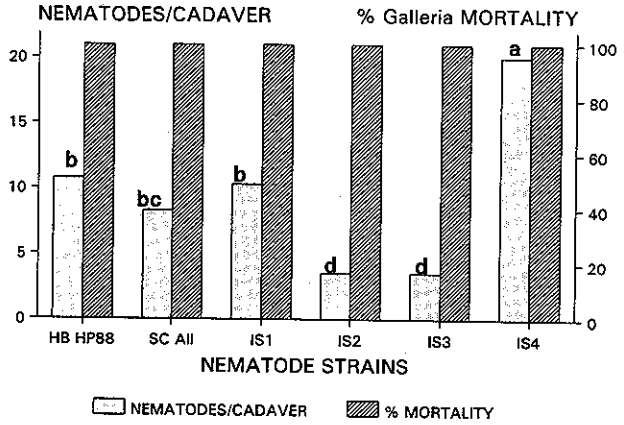

Fig. 3. Mortality of *Galleria mellonella* larvae and the number of nematodes recovered from the insect cadavers following 96 h exposure to different nematode strains in 150 cm³ plastic pots filled with moist sand. The various strains were applied to the surface of each pot at a concentration of 800 infective juveniles/cm². The pots were incubated at 25 °C (HB = *Heterorhabditis bacteriophora;* SC = *Steinernema carpocapsae;* IS_{1-4} = newly isolated heterorhabditid populations). Columns with different letters are significantly ($P \leq 0.05$) different according to *t* test.

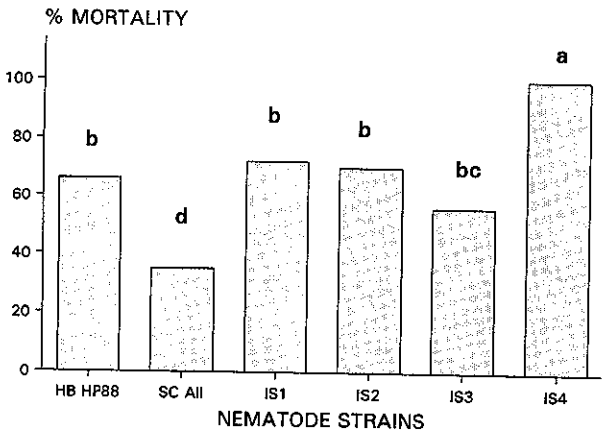

Fig. 4. Mortality of *Maladera matrida* grubs following 96 h exposure to different nematode strains in 150 cm³ plastic pots filled with moist sand. The various strains were applied to the surface of each pot at a concentration of 800 infective juveniles/cm². The pots were incubated at 25 °C (HB = *Heterorhabditis bacteriophora;* SC = *Steinernema carpocapsae;* IS_{1-4} = newly isolated hererorhabditids populations). Columns with different letters are significantly ($P \leq 0.05$) different according to *t* test.

Nematode tolerance to environmental extremes

Heat tolerance

The viability of the steinernematid representative (*S. carpocapsae* All) was not affected by the heat treatment (3 h exposure to 37 °C). However the viability of the *H. bacteriophora* HP 88 IJs was reduced to 20 % (Fig. 5). The level of survival of all other heterorhabditid strains was 3-4.5 fold higher then the HP 88 strain. This enhanced ability to withstand high temperature is attributed to the conditions in their natural habitat, the north-western part of the Negev desert, which is characterized by high temperatures during the summer season (Glazer *et al.,* 1991).

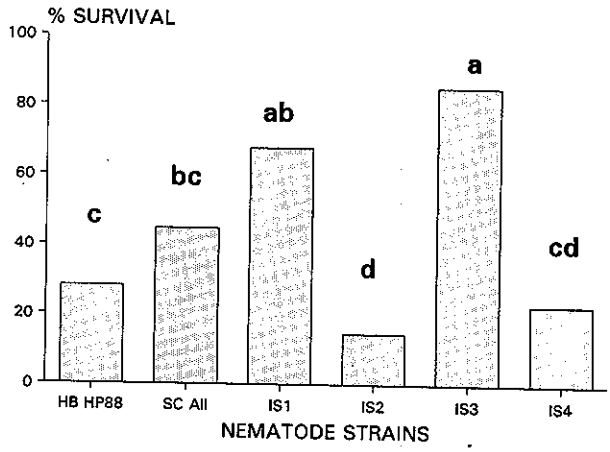

Fig. 5. Survival of various nematode strains following exposure to 37 °C for 3.5 h (HB = *Heterorhabditis bacteriophora;* SC = *Steinernema carpocapsae;* IS_{1-4} = newly isolated heterorhabditid populations). Columns with different letters are significantly ($P \leq 0.05$) different according to *t* test.

Desiccation tolerance

Exposure to low relative humidity conditions (3 h at 50-60 % RH) resulted in 55-85 % reduction in most of the various nematode populations (Fig. 6). Relatively high tolerance to the desiccation conditions was recorded with the IS_1 and IS_3 strains of *H. bacteriophora.* Survival of these strains was 70 and 90 % respectively. This adaptation is also attributed to the natural conditions characteristic to the arid region of their origin.

Unlike the present findings, poor desiccation tolerance was recorded with all the heterorhabditid strains when subjected to 75 % RH for 3 days after preconditionning for 72 h at 97 % RH (Glazer *et al.,* 1991). The discrepancy between the data suggest that nematode ability to withstand rapid desiccation following direct exposure to low RH, as conducted in the present study, might differ from their ability to survive slow desiccation regime as tested previouly (Glazer *et al.,* 1991).

The data presented in the present study demonstrated that the newly isolated strains performed better in the various infectivity and tolerance assays than the common heterorhabditid strain. The fact that those strains were recovered from an arid environment should encourage the survey of areas characterized by extreme environmental conditions.

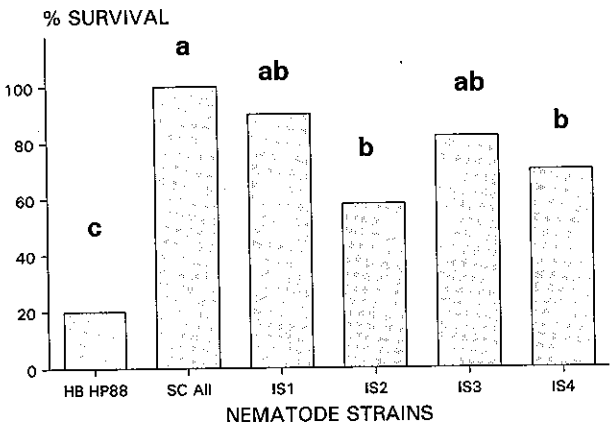

Fig. 6. Survival of various nematode strains following exposure to low relative humidity conditions (50-65 % RH) at 24-26 °C (HB = *Heterorhabditis bacteriophora;* SC = *Steinernema carpocapsae;* IS_{1-4} = newly isolated heterorhabditid populations). Columns with different letters are significantly ($P \leq 0.05$) different according to *t* test.

However it should be noted that the vigour expressed by some of the new isolates may be attributed, in part, to the fact that they were only recently obtained from their natural habitat and reared in the lab for only 3-5 generations, whereas the steinernematid *S. carpocapsae* " All " and the heterorhabditid *H. bacteriophora* " HP 88 " have been reared under laboratory conditions for many generations.

With the enhanced interest in genetic improvement of entomopathogenic rhabditids as biological control agents new populations from natural environments with superior characteristics could serve as a source to breed genetically-improved heterorhabditid lines.

Acknowledgments

We thank Alla Gol'berg, Department of Entomology, Gilat Experimental Station, for the supply of *M. matrida* grubs and Meir Klein for the supply of *S. littoralis* and *E. insulana* larvae. Thanks to Liora Salame, Department of Nematology, ARO, The Volcani Center, for technical assistance.

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