Desiccation Survival of Entomopathogenic Nematodes of the Genus *Heterorhabditis*

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The present study aims at determining the desiccation tolerance of entomopathogenic nematodes from the genus *Heterorhabditis* recently isolated in Israel. We first verified the most suitable desiccation conditions that lead to induction of the anhydrobiotic state using *Heterorhabditis bacteriophora* HP88. After direct exposure of infective juveniles (IJs) to 97% and 93% r.h. for 96 h, the survival rate was > 70%. By contrast, exposing HP88 IJs to 88% and 85% r.h. resulted in poor survival (< 10%) or complete mortality. Following exposure to 97% and 93% r.h. for 24, 48, 72 or 96 h, survival ranged from 68% to 79% with no significant differences between the exposure periods. Stepwise reduction of r.h. conditions (97% > 93% > 88% > 85% r.h.) at a 24-h or 72-h interval resulted in enhanced survival (30% survival) of IJs at the final r.h. level compared with IJs which were either directly exposed to 85% r.h. (0% survival) or were preconditioned at the higher r.h. levels prior to exposure to 85% r.h. (15% survival). *H. bacteriophora* HP88 IJs were able to survive for at least 18 days after preconditioning. At 97% r.h. nematode viability remained stable at 70–85% and at 93% r.h. survival ranged between 37% and 60%. The data indicate that survival is influenced by rate of water removal from the nematode's body and a minimal relative humidity level (>93%). Substantial differences in survival ability were observed among IJs of 12 new heterorhabditid populations, isolated from different climatic regions in Israel, which were preconditioned at 97% r.h. for 72 h following by an additional 72 h at 93% r.h. Maximum survival was recorded with HIS-19 (64%), moderate (40–55%) survival was observed with seven isolates, including *H. bacteriophora* HP88, and five isolates displayed poor (<25%) desiccation tolerance.

KEY WORDS: *Heterorhabditis* species; nematodes; infective juveniles (IJs); relative humidity; anhydrobiosis; dormancy; direct vs stepwise exposure; desiccation.

INTRODUCTION

Certain nematode species can withstand complete desiccation by entering a condition of anhydrobiosis in which their metabolism is lowered (3). Anhydrobiosis is a reversible, physiologically arrested state of dormancy that results from the absence of water. The following traits broadly define the characteristics of anhydrobiotes (1): true anhydrobiotes can lose up to 95–98% of their body water; when induced they have no detectable metabolism, thereby conserving energy reserves; in the anhydrobiotic state, resistance to environmental extremes, vacuum, ionizing radiation and metabolic poisons lethal to

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the active organism, is greatly increased. Most anhydrobiotes require a slow desiccation process to attain the metabolic adaptations necesary for entering the anhydrobiotic state (4,26).

Entomopathogenic nematodes (EPNs) have been used to control many insect pests (12), since the third stage infective juveniles (IJs) can locate and penetrate insect hosts through natural body openings and release symbiotic bacteria within host hemolymph (5). The infective stage is the only stage in the life cycle of these nematodes that can survive outside the nematode-killed insects, but desiccation limits the effectiveness of EPNs as biological control agents in field situations. Survival depends on abiotic and biotic factors in the environment (7,11,12,20).

EPNs are capable only of a low level of dormancy described as quiescent anhydrobiosis (27). Most studies on desiccation survival of IJs are on steinernematid species (2,6,10,14-16,18,21,22,27). Fewer studies were devoted to determine the desiccation tolerance of heterorhabditids (15,17,24). Surrey and Wharton (24) tested the desiccation survival of *Heterorhabditis zealandica*. Their experiments indicated that "survival was poor once water had been lost from the substrate." They concluded that: "the survival would not be improved by adjusting the treatment temperature, the source of the infective larvae, the method of rehydration or the addition of trehalose." Menti *et al.* (15) directly exposed *Steinernema feltiae* and *H. megidis* from Greece and the UK to different relative humidities. They showed that although the survival of *H. megidis* was superior to that of *S. feltiae*, for both species desiccation tolerance was poor (minutes) and there was no evidence of enhanced survival of the populations of either species from Greece compared with those from the UK. Poor survival of *H. megidis* (strain UK211) was also reported by O'Leary *et al.* (17), who exposed the nematodes directly to 57% r.h. for 3 h without preconditioning at higher r.h. levels.

While experimental studies have shown that heterorhabditid species are very susceptible to severe desiccation, heterorhabditid populations have been isolated from natural environments characterized by low moisture levels, such as arid regions (9) or dunes in coastal regions (23). Furthermore, there is no information about the most suitable desiccation conditions that could induce anhydrobiosis in *Heterorhabditis* and enhance their survival. The objective of the present study was to determine the most suitable desiccation regimes to induce anhydrobiosis in *H. bacteriophora* (strain HP88). We then compared the desiccation survival of 12 isolates of *Heterorhabditis* spp. under those induction conditions.

MATERIALS AND METHODS

The nematode culture *Heterorhabditis bacteriophora* HP88 was obtained from Dr. Randy Gaugler at Rutgers University, NJ, USA. All other *Heterorhabditis* species (listed in Table 1) were isolated from the soil of temperate, subtropical, semiarid or arid regions in Israel (9). The 13 isolates were reared in last-instar wax moth larvae, *Galleria mellonella* L., as described by Kaya and Stock (13). The emerging IJs were stored at 4°C in 250 ml culture flasks in distilled water (2,500–5,000 IJs/ml in 100 ml in each flask). The flasks were stored for 2–4 weeks prior to use in the experiment.

Optimization of the induction regime In this part of the work IJs from *H. bacteriophora* HP88 only were used. Nematode suspension (10 ml) containing approximately 40,000 IJs was concentrated onto a 5-cm-diam filter paper (Whatman No.1) using a vacuum filtration

apparatus. The nematodes on the filter papers were kept in the room for 15 min to let excess water evaporate. Then these filter papers were placed on a cover of a 5-cm-diam petri dish and transferred to a 2.5 *l* desiccator (Nelgen, Rochester, NY, USA). Different r.h. levels were established in the desiccators with different saturated salt solutions at 25±0.3°C as follows: K_2SO_4 for 97% r.h., KNO_3 for 93% r.h., $ZnSO_4.7H_2O$ for 88% r.h. and KCl for 85% r.h. (25) (all salts were obtained from Merck AG, Darmstadt, Germany).

In all treatments, nematode survival was determined by cutting small sections from the filter paper discs with approximately 5,000 IJs on the surface and immersing them in distilled water for 24 h. The number of live and dead nematodes in each sample was counted under a stereomicroscope. The nematode viability was determined by observing motility following a gentle prodding with a needle as described by Glazer (6). Each treatment was replicated three times.

In order to determine the factors affecting the ability of the IJs to enter the quiescent (anhydrobiosis) state, we examined two separate processes during desiccation: *a.* Induction process – during which water is slowly removed from hydrated nematodes and they acclimatized in the low r.h. conditions by reaching a dormant state (4); *b.* Quiescent period in which the dormant nematodes are exposed to lower r.h. to assess long-term survival.

The following factors were tested to determine their effect on the induction process: (i) Optimal r.h. levels – by exposing the IJs to 97%, 93%, 88% and 85% r.h. for 72 and 96 h, followed by further exposure to 85% r.h. for additional periods of 24, 48 and 72 h. (ii) Length of induction process - by exposing the IJs to 97% or 93% r.h. for 24, 48, 72 and 96 h followed by exposure to 85% r.h. for an additional 48 h. (iii) Stepwise desiccation regime – by exposing the IJs to reduced levels of r.h. (97% > 93% > 88% > 85% r.h.) at 24-h or 72-h intervals. Control treatments included direct exposure to 85% r.h.

Long-term survival in a dormant state To determine the most suitable r.h. condition for long-term survival the dormant IJs of strain HP88 were preconditioned at 97% r.h. for 72 h, and then exposed to lower r.h. regimes of 97%, 93%, 88% and 85% r.h. for 96 h. Viability under long-term (up to 18 days) desiccation conditions was determined by exposing the IJs to 97%, 93% and 85% r.h. at 25°C.

Comparison of different heterorhabditid isolates for desiccation tolerance Following the optimization of the desiccation process with the HP88 strain of *H. bacteriophora*, the desiccation tolerance of 12 isolates (see Table 1) was evaluated. The IJs of the various isolates were placed on filter paper disks as described above and subjected to a desiccation regime which consisted of 72 h exposure to 97% r.h. followed by an additional 72 h at 93% r.h. Samples were taken from each isolate for rehydration and viability evaluation as described above. The HP88 strain of *H. bacteriophora* was included in this experiment for comparison. This desiccation regime resulted in 45–50% survival of the HP88 strain, which was a suitable range for comparison.

Statistical analysis An arcsine of square root transformation was used on percentage survival data. The General Linear Model (GLM) Procedure of SAS (19) was used for analysis of variance. Significance between treatments was determined using Student-Newman-Keuls (SNK) multiple range test at $P<0.001$.

RESULTS

Optimal r.h. levels More than 80% of IJs of *H. bacteriophora* HP88 survived direct exposure to 97% and 93% r.h. for 96 h with no significant difference between these two humidities (Fig. 1). Poor survival (< 10%) or complete mortality was observed among HP88 IJs that were exposed to 88% and 85% r.h., respectively. Further exposure of the IJs to 85% R.H. for 24 h resulted in a three- and seven-fold reduction in viability for nematodes that were preconditioned at 97% and 93% r.h., respectively. The rapid decrease in nematode viability continued until none survived after 72 h exposure to 85% r.h. after preconditioning at 97% and 93% r.h.

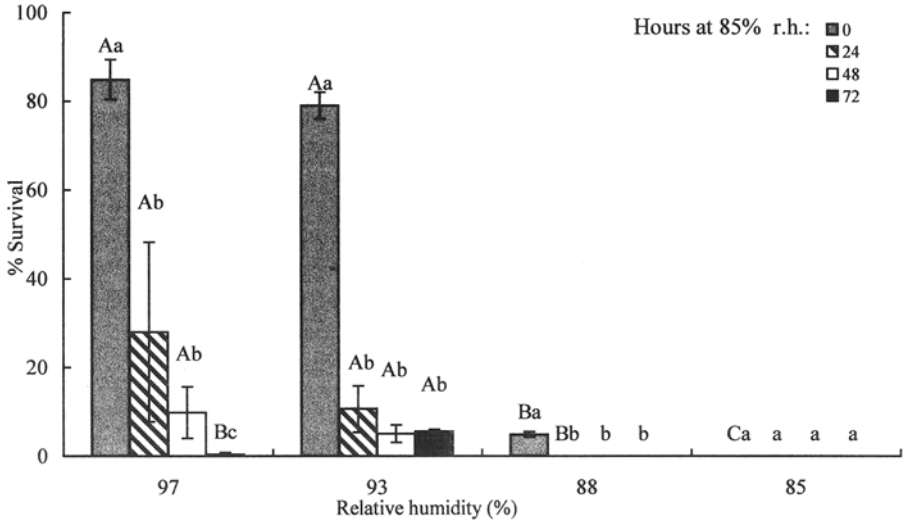

Fig. 1. Effect of different relative humidities on survival of *Heterorhabditis bacteriophora* HP88 strain. The IJs were exposed to various relative humidities at 25±0.3°C for 96 h and then further exposed to 85% r.h. for 0, 24, 48 and 72 h. Viability was determined after rehydration in distilled water for an additional 24 h at room temperature. Bars indicate standard deviation of mean. Columns in different treatments annotated with the same capital letter do not differ significantly (P=0.001) according to the SNK multiple range test. Within treatments, columns annotated with the same lower case letter do not differ significantly (P=0.001), according to the same test.

Length of induction process The percentage survival of HP88 IJs at both 93% and 97% r.h. ranged from 68% to 79% with no significant differences between the exposure periods (P>0.05) (Fig. 2A, B). However, as dehydration intensified by exposure to 85% r.h., survival rate increased. This was especially distinct after 72 and 96 h exposure to 85% (Fig. 2A, B).

Stepwise desiccation A gradual reduction of HP88 survival (from 80% to 20%) was recorded in a stepwise dehydration regime (Fig. 3). However, in control treatments which consisted of direct exposure to 85% r.h. after preconditioning at 97% r.h., survival was less than 10% after the first 24-h exposure to 85% r.h., irrespective of the exposure duration (Fig. 3).

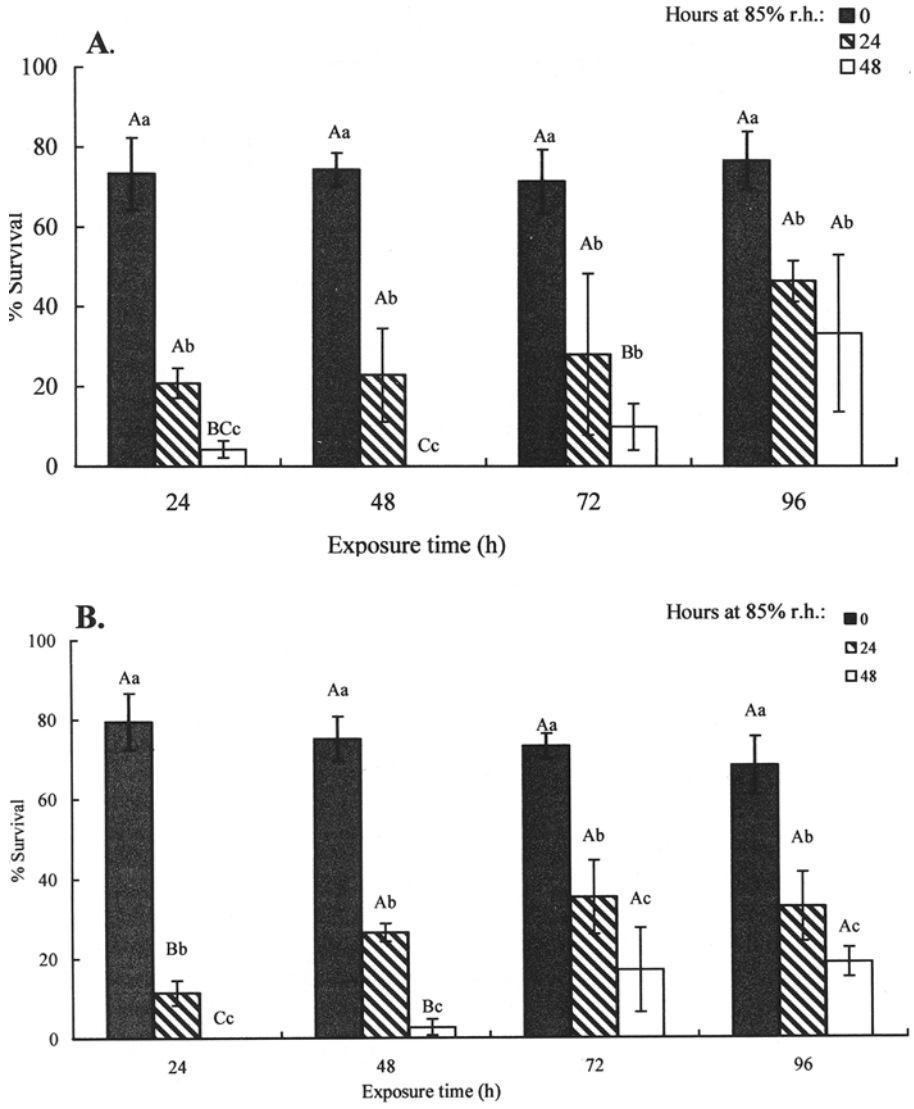

Fig. 2. Effect of different exposure periods on the survival of *Heterorhabditis bacteriophora* HP88 strain at 97% r.h. (A) and 93% r.h. (B) at 25±0.3°C, followed by exposure to 85% R.H. for 0, 24 and 48 h. Viability was determined after rehydration in distilled water at room temperature. Bars indicate standard deviation of means. Columns in different treatments annotated with the same capital letter do not differ significantly (P=0.001) according to the SNK multiple range test. Within treatments, columns annotated with the same lower case letter do not differ significantly (P=0.001), according to the same test.

Long-term survival Following preconditioning for 96 h at 97% r.h., more than 60% of HP88 IJs survived subsequent exposure to 97% and 93% r.h. for periods up to 96 h but at 88% r.h. survival was adversely affected, with only 29% of IJs surviving after 96 h (Fig. 4). No IJ survived for periods of 72 h or longer at 85% r.h.

TABLE 1. List of isolates from surveys in Israel

Isolates of *Heterorhabditis* spp.	Isolation site	Region (Climatic characteristic*)	Habitat
HIS-2	Gvulot	Negev (SAr)	Pear orchard
HIS-3	Ze'elim	Negev (SAr)	Citrus orchard
HIS-5	Nir Yizhaq	Negev (SAr)	Avocado orchard
HIS-13	Magen	Negev (SAr)	Orange orchard
HIS-16	Yahel	Arava (Ar)	Pomelo orchard
HIS-19	Almog	Jordan Valley (Ar)	Palm trees
HIS-20	Argaman	Jordan Valley (Ar)	Palm trees
HIS-26	Retamim	Negev (SAr)	Pear orchard
HIS-28	Kefar Hess	Coastal plain (STr)	Citrus orchard
HIS-31	Yahel	Arava (Ar)	Palm trees
HIS-33	Hedera	Coastal plain (STr)	Citrus orchard
HIS-34	Gush Halav	Upper Galilee (Tm)	Apple orchard

*Ar = Arid, SAr = Semiarid, STr = Subtropical, Tm = Temperate.

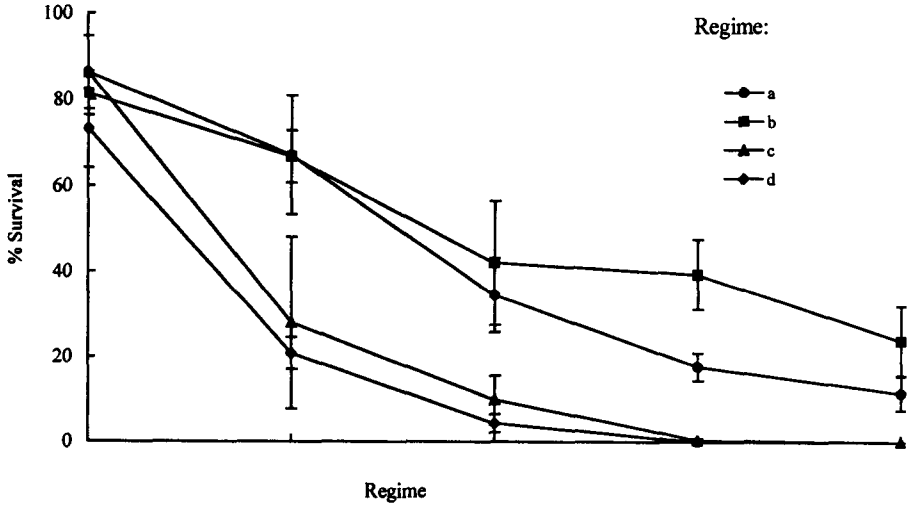

Fig. 3. Survival of *Heterorhabditis bacteriophora* HP88 strain at 85% r.h. following different dehydration regimes: **a.** Stepwise dehydration by exposure to 97%, 93%, 88% and 85% r.h. at 72-h intervals. **b.** Stepwise dehydration as in Fig. 3a at 24-h intervals. **c.** Direct exposure to 85% r.h. after 72 h at 97% r.h. **d.** Direct exposure to 85% r.h. after 24 h at 97% r.h. Viability was determined after rehydration in distilled water for 24 h, at room temperature. Bars indicate standard deviation of mean.

Longer-term survival at higher relative humidities (>93%) is presented in Figure 5. Infective juveniles of *H. bacteriophora* HP88 could survive at least 18 days after preconditioning. The survival varied from 85% to 37%. No significant difference ($P<0.05$) in survival was observed between IJs exposed to 97% and 93% r.h. within the first 8 days. At 97% r.h. nematode viability remained stable at average levels between 85% and 70% and, at 93% r.h., the average survival after the 8th day ranged between 60% and 37% (Fig. 5).

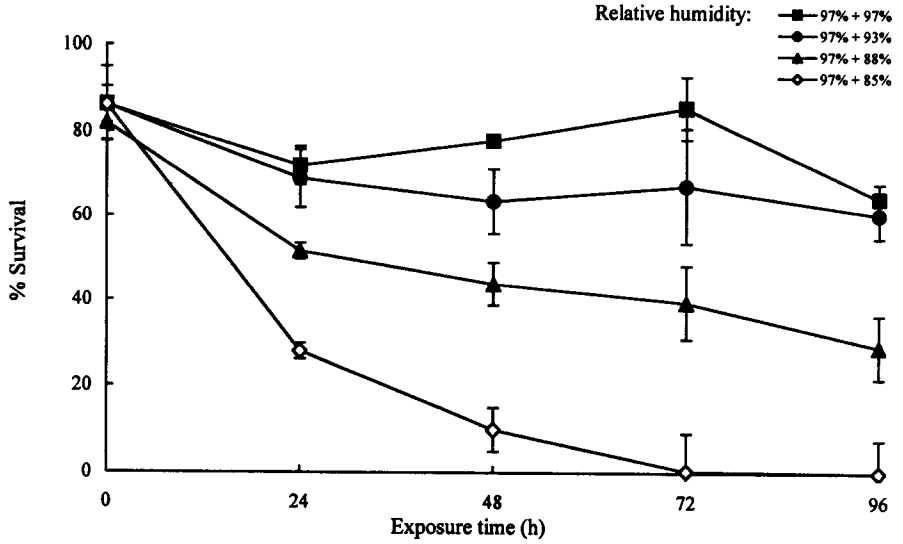

Fig. 4. Effect of different relative humidities on survival of *Heterorhabditis bacteriophora* HP88 strain during a 96-h period, after preconditioning at 97% r.h. for 96 h at 25±0.3°C. Viability was determined after rehydration in distilled water for 24 h at room temperature. Bars indicate standard deviation of mean.

Comparison of different heterorhabditid isolates for desiccation tolerance Substantial differences in survival ability were observed between IJs of 12 new heterorhabditid populations, isolated from different climatic regions in Israel (Fig. 6). The greatest survival was recorded with HIS-19 (64%), moderate survival (40–55%) was observed with seven isolates, including *H. bacteriophora* HP88, and five isolates displayed poor desiccation tolerance (< 25% survival). When the desiccated nematodes were further exposed to 85% r.h. for 24 h (data not shown), the isolates HIS-19 and HIS-3 retained a relatively high level of viability (25% and 19%, respectively), whereas the viability of all other Israeli isolates ranged between zero and 7%.

DISCUSSION

Heterorhabditid nematodes are known to be very poor anhydrobiotes (27). Under various desiccation regimes IJs survived for minutes (17) or, at the most, hours (24). In the present study we demonstrated, for the first time, that the IJs of *H. bacteriophora* are capable of surviving at r.h. ≥93% for extended periods (up to 18 days). Under these conditions the IJs were no longer motile, decreased in size, and lost *ca* 25% of their water content (Liu & Glazer, unpublished data). Unlike steinernematids, which tend to aggregate (22), the heterorhabditid IJs tested here moved over the filter paper before motility ceased.

The data presented here indicate that the high survival rate is attributable to two main factors:

1. Slow water removal from the nematode's body – A slow desiccation process is required by most anhydrobiotes to enable the metabolic changes necessary to enter the anhydrobiotic state (3,26). Good anhydrobiotes require a short period of preconditioning at high r.h. before they can be exposed to extremely low r.h. (0–30%) (1). Steinernematids were able

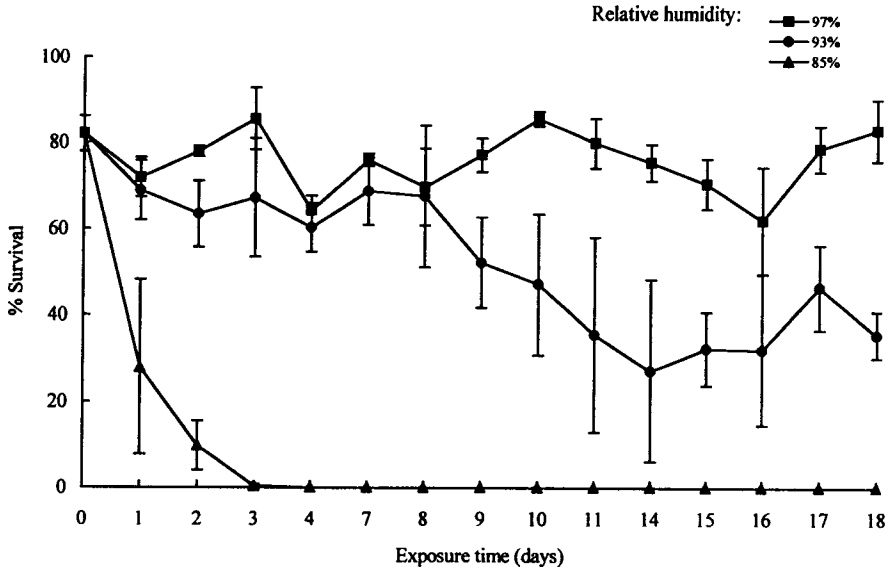

Fig. 5. Effect of different relative humidities on survival of *Heterorhabditis bacteriophora* HP88 strain during an 18-day period, after preconditioning at 97% r.h. for 96 h at 25±0.3°C. Viability was determined after rehydration in distilled water for 24 h at room temperature. Bars indicate standard deviation of mean.

to survive at low r.h. (75–85% r.h.) following exposure to 97% r.h. for 72 h (22,27). Our data indicate that *H. bacteriophora* requires a more gradual and longer period of adaptation than that of steinernematids. The greater survival level was obtained after longer exposure time (96 h) at 93% or 97% r.h. or stepwise reduction of relative humidities.

2. Minimal relative humidity level – In the present study it was demonstrated that *H. bacteriophora* can survive extended periods at relative humidities ≥93%. Womersley (27) had referred to steinernematids and heterorhabditids as one group "predominantly associated with the upper soil profile." However, later studies had demonstrated the differences in foraging behavior between members of the two genera (12). Heterorhabditids tend to search for sedentary insects that occupy the deeper soil layer (5,12). Examination of the spatial and temporal distribution of natural heterorhabditid populations showed that they were more abundant in deeper soil layers (15–30 cm) than in the upper ones (5–10 cm) throughout the year (7). Thus, heterorhabditids may have adapted to an environment in which a certain minimum level of humidity is maintained all year round. The results presented here suggest that *H. bacteriophora* is capable of limited anhydrobiosis that can be induced by slow dehydration.

We also demonstrated that under controlled desiccation regimes there is a considerable variation in desiccation tolerance among different heterorhabditid isolates (Fig. 6). The fact that other isolates from a desert environment did not show enhanced desiccation tolerance may be attributed to the characteristics of the micro-habitat which these populations inhabit. Most isolates were obtained from soil samples that were taken from under the tree canopy in the orchards that were sampled (9). The water evaporation rate from the soil in this habitat is slower than that of the soil in exposed areas of the orchard. It may allow the nematodes

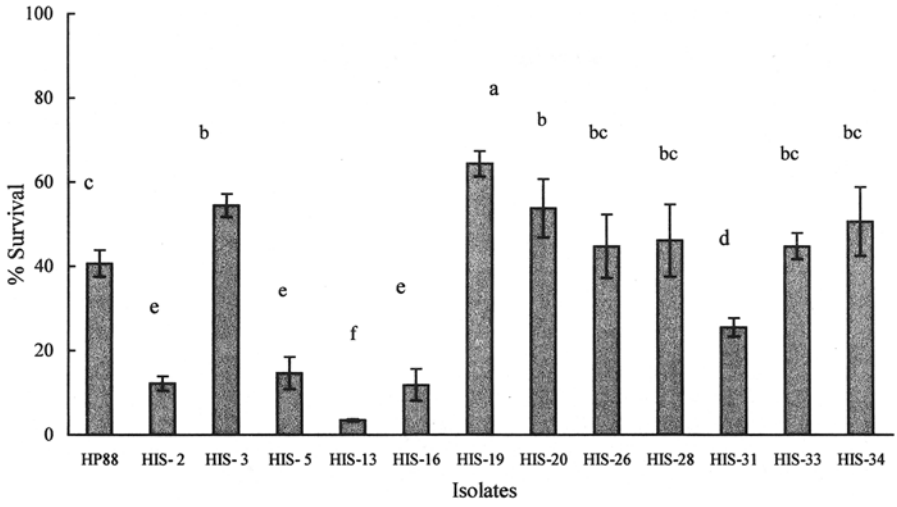

Fig. 6. Effect of exposure of different heterorhabditid isolates to 97% r.h. for 72 h followed by further exposure to 93% r.h. for an additional 72 h, at 25±0.3°C. Viability was determined after rehydration in distilled water for 24 h at room temperature. Bars indicate standard deviation of mean. Within treatments, columns annotated with the same lower case letter do not differ significantly (P=0.01), according to the SNK multiple range test.

to reach deeper, moist soil layers (8). Knowledge about the natural environments in which these organisms persist is needed to indicate the optimal induction and maintenance conditions. It is possible that other heterorhabditids, isolated from dry environments, can survive under more extreme dry conditions.

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