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Genetics of the Nematode *Heterorhabditis bacteriophora* Strain HP88: The Diversity of Beneficial Traits[1]

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Abstract: Genotypic variation among infective juveniles of *Heterorhabditis bacteriophora* (strain HP88) in heat, desiccation, ultraviolet tolerance, and host-finding ability was assessed by comparing the performance of inbred lines of this entomopathogenic nematode in laboratory assays. Each line consisted of highly homozygous offspring originating from one individual obtained from a natural population. Considerable variation in all four traits was detected among the different inbred lines. The heritability values for heat or ultraviolet tolerance and for host-finding ability were high, indicating that selection should be an efficient way for improving these traits in the population. The results for desiccation tolerance varied considerably within each line. Heritability value was low, indicating that the results were influenced mainly by environmental variation and suggesting that selective breeding for higher desiccation tolerance would be inefficient. Improvement through induction of mutations may be a better alternative in this population.

Key words: desiccation, heritability, *Heterorhabditis bacteriophora,* host finding, inbred line, ultraviolet, variation.

Among the alternative measures to chemical control of insect pests, particular attention in recent years has focused on biological control using entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae (15, 18,19). The nematodes are associated with a bacterium from the genus *Xenorhabdus*; together they possess unusual virulence, killing insect hosts in 24–48 hours (1,18,19). They are able to parasitize many economic pests and in some cases provide a level of insect control equivalent to that of chemical insecticides (13,19). Mass production is easily accomplished (3,13). Finally, extensive testing has demonstrated a lack of mammalian pathogenicity and the U.S. Environmental Protection Agency has consequently exempted these nematodes from registration and regulation requirements (15,19).

Despite this impressive list of attributes, the sensitivity of heterorhabditid nematodes to inactivation by extremes of the physical environment (e.g., high temperature, solar radiation, desiccation) prevents them from being used to maximum advantage as bioinsecticides under field conditions (2). Efforts to overcome the poor field persistence of entomopathogenic nematodes have focused on improved formulation (13). In recent years genetic improvement has been suggested as a means of increasing nematode environmental tolerance (8,9,14,15), and the feasibility of improving host finding in *Steinernema carpocapsae* by selective breeding has been demonstrated (11).

Heterorhabditis bacteriophora (strain HP88) is an ideal inundative candidate for genetic improvement. This nematode is commonly used in field experiments against soil-inhabiting insect pests (24, 31). Because *H. bacteriophora* (HP88) has a short generation time (4–6 days), it is especially well suited for genetic studies; large numbers can be screened for mutants and stocks can be stored frozen for prolonged periods (23). As *H. bacteriophora* (HP88) is a self-fertilizing hermaphrodite, new mutations become homozygous automatically and pure lines can be maintained. Moreover, males are produced at a low frequency, allowing the transfer of genetic markers if necessary.

The intolerance of infective stages to

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desiccation (22,25), temperature (17), and solar radiation (10) has caused erratic results in field applications (7,10), reducing field efficacy and restricting nematode application to habitats with favorable microenvironments (i.e., shelter from desiccation and solar radiation). Increasing nematode tolerance to these factors should directly enhance their performance in the field. In addition, an indirect approach could be to enhance nematode host-finding ability and thus reduce the period in which the nematode is exposed to environmental inactivation.

Therefore, we assessed the genetic variance in a natural population of *H. bacteriophora* (HP88) for several of the desirable traits. Ideally this is accomplished by comparing the performance (i.e., phenotype) of many different "pure" lines (presumed homozygous for different alleles) derived from the original population by selfing for several generations. While this approach is impractical in most sexually reproducing organisms, the hermaphroditic nature of Heterorhabditidae should allow a considerable degree of homogeneity to be achieved in the segregating lines. A highly heritable trait responds more readily to selection than a trait of low heritability. The latter would favor an approach based on induction and selection of new mutations affecting that trait. Here we report our results in estimating heritability for several beneficial traits in *H. bacteriophora* (HP88).

Materials and Methods

Nematode culture and isolation of inbred lines: The HP88 strain of *H. bacteriophora* was originally isolated in 1982 from a single larva of *Phyllophaga* sp. in Logan, Utah, USA (21). Since then this strain has been reared under nonselective conditions on larvae of the greater wax moth, *Galleria mellonella.* Our population of this strain was obtained from Biosys (Palo Alto, CA, USA). This population was considered as the base population throughout the study. In order to eliminate differences due to variations in culture and storage conditions, we tested three different batches of this base population which were stored for different periods (base population 1, ≤ month; base population 2, 3 months, and base population 3, 6 months).

To obtain homogeneous lines, infective juveniles from the base populations were surface sterilized with 1% Hyamine (18), then seeded in 5-cm-d petri dishes containing 5 ml of sterile dog food–agar medium (DFA): 8% w/w sterile dog food (Gravy Train, Gaines Foods, Kanakakee, IL, USA) mixed with 1.6% w/w agar (Difco-Agar, Difco, Detroit, MI, USA) in distilled water. The DFA plates were pre-inoculated with the bacterium *Xenorhabdus luminescence* subspecies HP88. The bacteria were isolated and propagated (20). Seventy-two hours after nematode seeding, 50 fourth-stage juveniles (J4) and young (virgin) adult females were transferred with the associated bacteria to new DFA plates (1 nematode/plate). As hermaphrodites, they reproduced by self fertilization. One virgin offspring was removed from each plate and seeded again, individually, to separate DFA plates, thus ensuring reproduction only by self-fertilization. This cycle was repeated at least four times. Each line was designated by a specific code number, and its infective juveniles were stored at 10 C in 0.1% formalin suspension. To obtain large numbers of infective juveniles from the stored material for further studies, each line was reared separately, either on DFA or on pig-kidney medium in 250-ml flasks (3). Before use, the nematodes were rinsed twice in distilled water and passed through nylon mesh filters (60 μm) to ensure that the final suspensions contained only viable infective juveniles.

High temperature tolerance assay: Infective juveniles obtained from the three different base populations and 25 inbred lines were each suspended in 15 ml distilled water in 25-ml conical flasks at a concentration of 50,000 nematodes/flask. The flasks were shaken gently at 40 r.p.m. in a water bath (37 C; 4 hours). Then three samples (1 ml each) were withdrawn from each flask and diluted in 9 ml water at room temperature (22–25 C). Nematode viability in each sam-

ple was determined 24 hours later by observing their motility in the water suspension and their response to probing under a dissection microscope.

Ultraviolet tolerance assay: Nematodes were exposed to ultraviolet light by adapting the method of Gaugler and Boush (10). Approximately 300 infective juveniles from each of 13 inbred lines or the three base populations were transferred in a 0.75-ml suspension onto a 5-cm-d filter paper (Whatman No. 1) placed in a 5-cm-d plastic petri dish. The moist paper prevented nematodes from becoming desiccated during irradiation. The ultraviolet lamp emitted medium wavelength radiation (peaking at 301 nm), which is consistent with the wavelength of natural sunlight believed to be principally responsible for nematode inactivation (10). The petri dishes with nematodes were placed 90 cm below the ultraviolet source, a distance that produced an intensity of 57 ± 5 $\mu W/cm^2$ as measured with a digital radiometer. Exposure to the ultraviolet source for 4 minutes was done in a completely darkened room. Immediately after irradiation, while still in darkness, the petri dishes were covered with aluminum foil. One hour later, eight last-instar larvae of the crab moth, *Spectrobates ceratoniae,* were placed in each petri dish, and the dishes were incubated in the dark at 25 C. Insect mortality, as a measure of nematode infectivity, was recorded after 4 days. The ultraviolet treatment of each nematode line was replicated six times. After an additional 3 days, six dead larvae (one from each replicate) were dissected under a stereoscopic microscope for examination of nematode development and reproduction. The number of nematodes in each insect cadaver was recorded according to their developmental stages.

Host-finding assay: Host-finding ability was determined with a procedure described by Gaugler et al. (11). This assay was conducted in rectangular glass plates (30.5 × 14 × 0.2 cm) to which rubber tubing (6 mm o.d.) had been cemented along the plate margins. Each plate contained a 3-mm layer of 2% agar. A Plexiglas top of identical dimensions was modified to hold two pipet tips (101–1,000 μl), each fixed through holes spaced 5 cm apart. Two last-instar larvae of *G. mellonella* were placed in one of the tips 24 hours before testing. Both ends of this experimental tip were loosely packed with steel wool to prevent larvae from escaping, and the top end was sealed with plastic film. The control tip was sealed similarly but did not contain insect larvae. Approximately 1,000 infective juveniles were brushed onto a 1-cm-d inoculation site at the center of the agar plate, equidistant (3.5 cm) from the two pipet tips. The petri dish cover was positioned over the agar, and the dish was placed in an insulated box in a 25-C incubator for a 1-hour test period. Nematode response was monitored by recording the proportion of infective juveniles found on a 1-cm-d agar core, taken directly below the experimental pipette tips containing insect hosts, as a proportion of the total number placed on the agar. The assays were repeated four times for each population.

Desiccation tolerance assay: Infective juveniles from the three different base populations and 22 inbred lines were concentrated by vacuum filtration onto 5-cm-d filter paper disks (Whatman No. 1) at a density of 1,000 nematodes per disk. The disks were transferred to a 4-liter glass desiccator for 24 hours at 25 C in relative humidity (RH) of 93% generated by a saturated solution of potassium nitrate (28). Following this exposure period, the infective juveniles from each population were rehydrated by direct immersion in distilled water for 24 hours. Nematode viability was determined in each population by observing motility and response to probing under a stereomicroscope. Six replicates of each line were counted. Each experiment was repeated three times.

In a second desiccation tolerance assay, the nematodes on the disks were exposed to room conditions (24–26 C, 50–65% RH). After 205 minutes of exposure, samples were taken from each population for rehydration in distilled water. Nematode viability was recorded in each population by

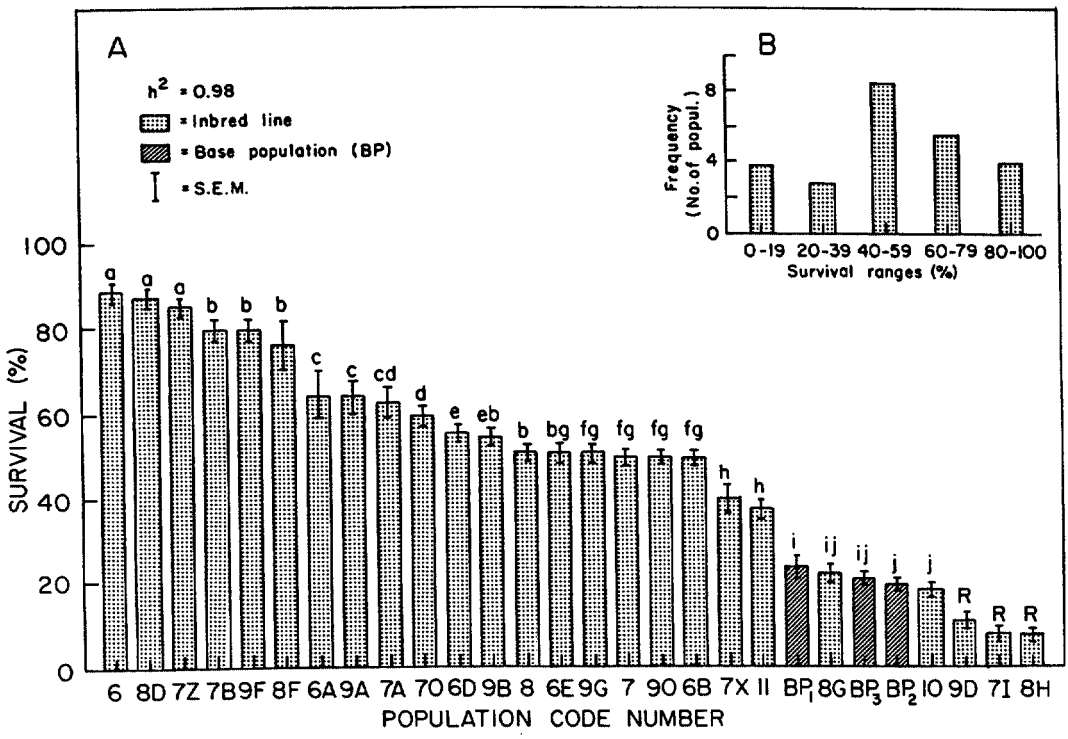

FIG. 1. *Heterorhabditis bacteriophora* (strain HP88) infective juveniles from three base populations (BP) and 25 inbred lines. A) Survival after suspension in water at 37 C for 4 hours. Bars bearing a common letter do not differ significantly ($P = 0.05$; Duncan's multiple-range test). B) Distribution of BP and inbred lines among various survival ranges following suspension in water at 37 C for 4 hours.

observing motility and response to probing under a stereomicroscope 24 hours after rehydration. Six replicates were counted, and each line was tested three times.

Data analysis: The data presented in percentage values were normalized using arcsine transformation. Significance of main effects was determined by analysis of variance (ANOVA). The significance of the score of individual homogeneous lines was evaluated by Duncan's multiple-range test ($a = 0.05$). In order to determine the influence of the genetic variation between the populations, the value of heritability (h^2) was calculated. This value represents the ratio between the genetic variation and the total variation, i.e., genetic + environmental (27). The values of h^2 were calculated according to the following equations:

$$h^2 = Vg/(Ve + Vg)$$

where Ve = environmental variation and Vg = genetic variation.

$$Ve = SS/DF$$

where SS = sum of squares and DF = degrees of freedom.

$$Vg = (Ve - MSE)/r$$

where MSE = mean square error and r = number of replicates per treatment.

Results and Discussion

Temperature tolerance: High variability was observed between inbred lines in their ability to withstand high temperature (37 C) for 4 hours. Survival ranged from 7.6 to 88.6% (Fig. 1A). The distribution analysis (Fig. 1B) indicates that the survival of the majority of the populations lies between 40 and 60%. The survival values of the three base populations were around 20%. The heritability value ($h^2 = 0.98$) suggests that the results were influenced almost entirely by the genetic variation between the populations and only slightly by environmental variation.

There was high genetic variability for high temperature tolerance within the original base population of *H. bacteriophora* (HP88) (Fig. 1), suggesting that selection should be an efficient means of improving this trait in the population. Successful selection for adaptation to high temperatures has been achieved in the free-living nematodes *Panagrellus redivivus* and *Caenorhabditis briggsae* (16). However, temperature adaptation was not displayed by *S. carpocapsae* and *H. heliothidis* when cultured for several generations at either 15, 20, or 25 C (5). This may have resulted from the relatively mild, hence less selective, temperature regime to which these nematodes were exposed. Moreover, because an apparently heterozygous population was examined, rather than inbred lines, the possible positive response to selection by a small proportion of the population may have been undetected. The insecticidal activity of nematodes has been reported to be most effective at temperatures in the range of 18–28 C (2), with the exception of several heterorhabditids like the HL81 strain (26) which were effective at lower temperatures (12–18 C). Increasing the ability of nematodes to withstand high temperatures (35–40 C) could improve their efficacy under such conditions in the field and also allow for extending the use of the nematodes as biological control agents in warmer climatic regions. Moreover, such heat tolerance would improve the shelf-life of the nematode.

Ultraviolet tolerance: Exposing the base populations of *H. bacteriophora* (HP88) to ultraviolet radiation for only 4 minutes reduced their pathogenicity by 50% in comparison with the untreated control (cf. BP and BPT in Fig. 2A). The inbred lines varied greatly in their ability to infect crab moth larvae after ultraviolet treatment, with insect mortality ranging from 33 to 100%. Most of the populations killed 75–100% of the tested insects (Fig. 2B). Nematode development within the insect's cadaver was also influenced by ultraviolet radiation. In populations with high ultraviolet tolerance (killing at least 90% of the insects; i.e., populations 9F, 9C, 7B, 7O in Table 1), the proportion of infective juvenile offspring that developed was similar to the proportion that developed in insects infected with untreated base populations (Table 1). On the other hand, in populations where pathogenicity was reduced by the ultraviolet treatment (populations 7I, 6, 8G, 8D in Table 1), larger proportions of juveniles (1st–4th stages) were recovered from the insects' cadavers. In some cases (populations 6A, 7 in Table 1), nematode development was almost completely inhibited. Only the original infective juveniles that infected the insect larvae or a small number of poorly developed progeny were found in the cadaver. Nevertheless, the total number of nematodes found in each cadaver was not proportional to their virulence as expressed by the percentage of insect mortality (Table 1). Nematode pathogenicity and reproductivity are therefore differently affected by the ultraviolet radiation. The heritability value ($h^2 = 0.66$) suggests that the different results obtained for the various populations were influenced mainly by the genetic variation between them, but environmental effects may also play an important role.

Previous studies have demonstrated that *S. carpocapsae* is highly sensitive to the ultraviolet component of solar radiation (10). In a more recent study, limited genetic variation for tolerance to ultraviolet radiation was detected among different strains of *S. carpocapsae* (12). Like the steinernematids (12), *H. bacteriophora* (strain HP88) is also inactivated by brief exposure to ultraviolet light. Unlike the steinernematids, however, there is a substantial amount of genetic variation in the HP88 strain of *H. bacteriophora* and, therefore, this trait should respond well to genetic selection. Such improvement would facilitate the utilization of entomoparasitic nematodes under suboptimal field conditions. Enhancement of tolerance to solar radiation would improve activity of the nematodes on the foliage, allowing their use against foliage pests and thus widening the range of their

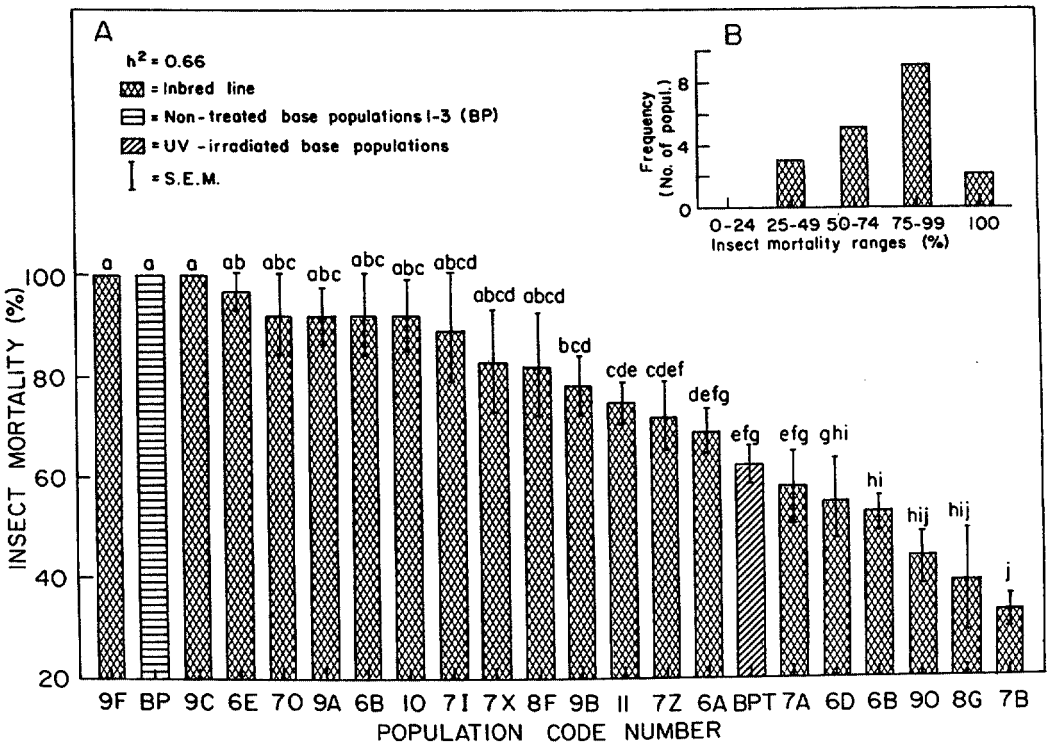

FIG. 2. A) Mortality of the last larval instar of the crab moth, *Spectrobates ceratoniae,* caused by infective juveniles from three base populations (BP) and 20 inbred lines of *Heterorhabditis bacteriophora* (strain HP88). Nematodes were exposed to UV irradiation for 4 minutes before inoculation, except for BP (strain HP88 not exposed to UV light). BPT = strain HP88 exposed to UV light. Bars bearing a common letter do not differ significantly ($P = 0.05$; Duncan's multiple-range test). B) Distribution of the BP and the inbred lines among various insect mortality ranges.

TABLE 1. Pathogenicity and development of base population (BP) and inbred lines of the nematode *Heterorhabditis bacteriophora* (strain HP88) infecting the larval crab moth, *Spectrobates ceratoniae.*

Population code no.	Insect mortality (%)	Nematodes/cadaver (n)†				
		Adults	J4‡	J1-3	IJ	Total
BP 1-3 (untreated)	100	112	31	52	221	416
9F	100	85	26	78	197	386
9C	100	156	20	65	313	554
7O	92	22	58	76	247	403
7B	92	40	19	43	126	228
7I	88	8	24	281	0	313
7X	83	21	173	80	0	274
6A	69	2	2	7	4	15
7A	58	3	82	41	0	126
7	55	0	0	0	3	3
6B	52	22	94	48	0	164
BP 1-3 (UV treated)	50	55	112	208	17	392
6	44	34	209	164	0	407
8G	39	49	188	286	0	523
8D	33	29	39	201	3	272

† All cadavers were examined 7 days after infection.
‡ J = nematode juvenile; IJ = infective juvenile.

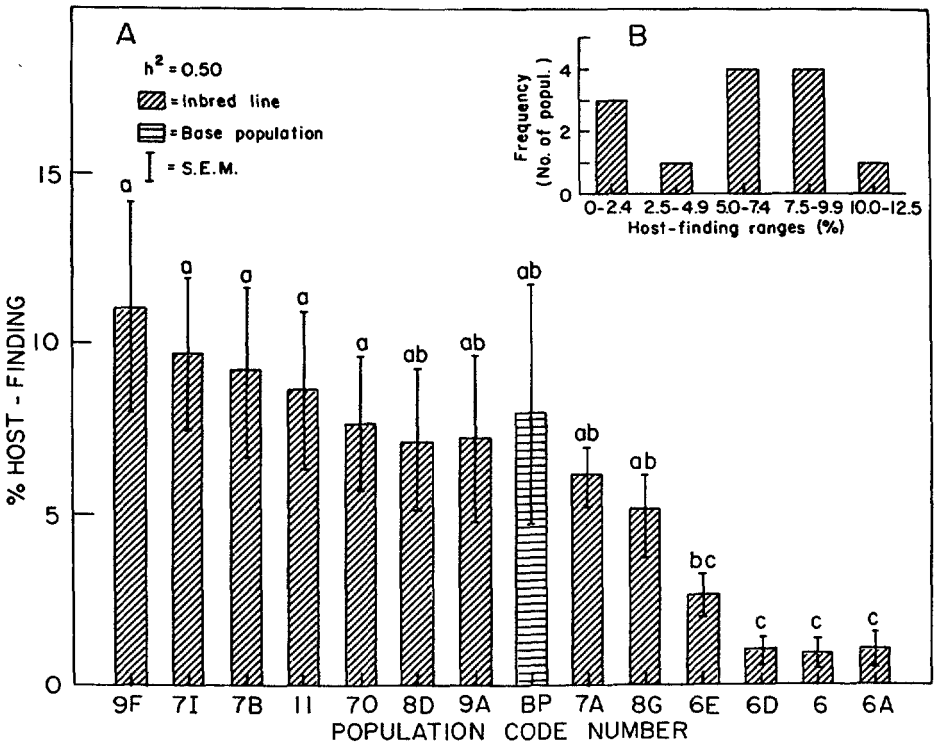

FIG. 3. A) Host-finding abilities of *Heterorhabditis bacteriophora* (strain HP88) infective juveniles obtained from three base populations (BP) and 13 inbred lines. Bars bearing a common letter do not differ significantly ($P = 0.05$; Duncan's multiple-range test). B) Distribution of the BP and the inbred lines among various host-finding ranges.

targets to include many important agricultural pests.

Host finding: In this assay only one base population and 13 homogeneous populations were tested. In general only a small proportion of the nematodes in all the populations reached the target insect under these assay conditions (Fig. 3). However, a tenfold difference was recorded between the highest (population #9F; 11 ± 3%) and the lowest (populations #6D, 6, 6A; 1 ± 0.5%) rates of host-finding ability, as expressed by a percentage of the nematodes reaching the target insect. Variation analysis gave a heritability value of 0.5. The data suggest that there is sufficient genetic variation in host-finding ability in these populations to warrant an attempt at improvement by selection. In most of the populations, 5–10% of the infective juveniles were able to locate the insect larvae (Fig. 3B).

Enhancing nematode host-finding ability is important because highly motile nematodes, able to locate hosts rapidly from a greater distance, would be less vulnerable to environmental inactivation. The host-finding ability by *S. carpocapsae* also was found to be low (ranging between 1.8 and 8%), even though highly significant phenotypic differences were found between different strains (12). Furthermore, Gaugler et al. (12) demonstrated that host-finding ability of *S. carpocapsae* can be enhanced 20–27-fold by selection.

Desiccation tolerance: In the first assay (24-hour exposure at 25 C, 93% RH), infective juvenile viability in the three base populations was 57% (Fig. 4A), whereas viability of infective juveniles obtained from 18 inbred lines ranged from 0 to 85%. The results varied considerably within each line (including the base population). Although significant variation was observed between different lines (e.g., #9C as compared with #8-11, $P > 0.05$, Fig. 4A), the heritability

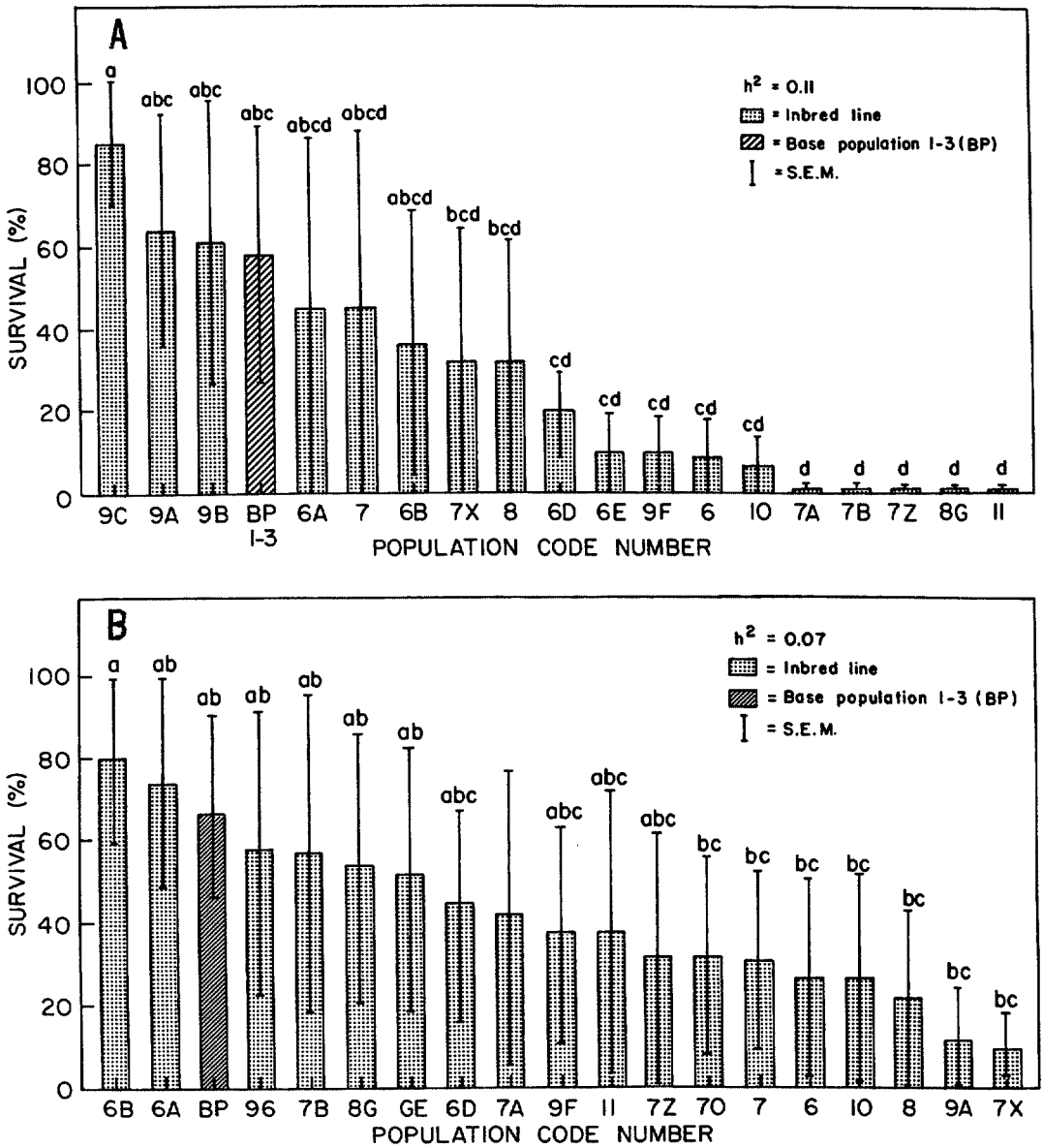

FIG. 4. Survival of *Heterorhabditis bacteriophora* (strain HP88) infective juveniles from three base populations (BP) and 18 inbred lines at low relative humidity. A) After exposure to 93% RH for a period of 24 hours at 25 C. B) After exposure to room conditions (24–26 C, 50–65% RH) for 205 minutes. Bars bearing a common letter do not differ significantly ($P = 0.05$; Duncan's multiple-range test).

value was low ($h^2 = 0.11$), indicating that the results were influenced mainly by environmental variation and only slightly by genetic variation among populations. A similar phenomenon was observed in the second assay, under room conditions (Fig. 4B). Viability was highly variable, ranging from 10 to 80%. As in the previous assay, a low heritability value ($h^2 = 0.07$) was found, indicating a minor genetic component. The data obtained from the two different assays suggest that because of the high environmental variation, the response of this population to selection for higher desiccation tolerance is expected to be slow at best and will most likely make this approach impractical.

Improving the ability of *H. bacteriophora*

(HP88) to withstand low RH is an essential goal, as nematode performance and persistence in the field is limited by low RH tolerance. Improvement through induction of mutations for desiccation resistance may be the best alternative in this case. Desiccation survival, by means of an anhydrobiosis mechanism, is a well-known phenomenon in soil-inhabiting, free-living, and plant-parasitic nematodes during the dry season (6,30). For most anhydrobionts, slow desiccation is required for metabolic adaptation to the onset of the anhydrobiosis state (4,6,29). However, good anhydrobiont nematodes will survive prolonged periods (weeks) even when they are introduced directly into a low RH environment (30). *Steinernema carpocapsae* was able to survive several weeks at RH above 90%, but this was followed by a subsequent dramatic drop in survival (22,25).

We found significant phenotypic differences among inbred lines derived from the HP88 strain of *H. bacteriophora* for several phenotypes: heat, ultraviolet or desiccation tolerance, and host-finding ability. These differences result mainly from genetic variation, with the exception of desiccation tolerance. This information enables us to decide on the most efficient genetic improvement strategy for this population (i.e., selection for heat tolerance or induction of mutations for desiccation tolerance).

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