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The influence of time of storage on performance of the insect parasitic nematode, *Heterorhabditis* sp.

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Summary — The influence of the time of storage on efficacy, persistence and migration rate of the insect parasitic nematode *Heterorhabditis* sp. was investigated. Various batches of the Dutch isolates HFr86 and HL81 stored for different periods (4 to 340 days) at 4-5 °C were compared. Efficacy of HFr86 against the black vine weevil, *Otiorhynchus sulcatus*, ranged from 15 to 93 % effect, the difference in persistence in potting soil ran up to five weeks, migration rate in 9 cm sand columns ranged from 1 to 3 cm in 4 h in the absence of a host and from 1 to 7 cm in the presence of *Galleria mellonella*. An increase of the time of storage caused a decrease of " quality " of the nematodes with respect to persistence and efficacy against black vine weevil. Migration rate in sand columns reflected efficacy on the nematodes for control of the weevil and might be suitable as a sensitive laboratory assay of nematode " quality ", at least for Dutch heterorhabditids.

Résumé — ***Influence de la durée de conservation sur les performances du nématode entomoparasite* Heterorhabditis *sp.*** — L'influence de la durée de conservation sur l'efficacité, la persistance et le taux de migration du nématode entomoparasite *Heterorhabditis* sp. a été étudiée. Plusieurs lots des isolats hollandais HFr86 et HL81 conservés pendant des périodes variables — de 4 à 340 jours — à 4-5 °C sont comparés. L'efficacité de l'isolat HFr86 contre l'otiorrhynque, *Otiorhynchus sulcatus*, varie de 15 à 93 %; la différence en ce qui concerne la persistance dans du terreau croît jusqu'à cinq semaines; la distance de migration dans des colonnes de sable de 9 cm varie de 1 cm à 3 cm en quatre heures en l'absence d'hôte et de 1 à 7 cm en présence de *Galleria mellonella*. Un allongement de la période de conservation a pour résultat une diminution de la « qualité » des nématodes en ce qui concerne la persistance et l'efficacité contre l'otiorhynque. Le taux de migration dans les colonnes de sable démontre l'efficacité des nématodes dans la lutte contre l'otiorhynque et pourrait convenir pour une analyse fine de laboratoire en vue de déterminer la « qualité » du nématode, du moins pour les *Heterorhabditis* hollandais.

Key-words : *Heterorhabditis* sp. (HL81, HFr86), *Otiorhynchus sulcatus*, storage, quality, persistence, efficacy, migration, Nematodes.

Nematodes of the genera *Heterorhabditis* Poinar and *Steinernema* Travassos are obligate parasites of insects and are regarded as a useful alternative to chemical control of many soil-inhabiting insect pests. At the moment, *Heterorhabditis* is mainly used against larvae of the black vine weevil, *Otiorhynchus sulcatus* F. (Simons, 1981; Klinger, 1988), but many more insect species are candidates for control by these nematodes, which have a broad host range. The various species and isolates of the nematodes differ in infectivity and pathogenicity with respect to insect hosts and their developmental stages (e.g. Bedding *et al.*, 1983; Morris *et al.*, 1990). Hence, many species and strains are tested to find the most appropriate nematode for each particular insect pest. There is no reproducible laboratory bioassay that can predict for efficacy in the field and therefore researchers depend so far on time - and labour - consuming field experiments. Variable results are usually attributed to uncontrolled environmental conditions (Kaya, 1985; Gaugler, 1988). It is known that the nematodes are sensitive to soil type and structure, moisture, UV irradiation and temperature (Molyneux, 1986). Biotic factors, like nematophagous mites and collembolans (Epsky *et al.*, 1988), and nematophagous fungi (e.g. Poinar & Jansson, 1986; Timper & Kaya, 1989) also affect persistence and infectivity of insect parasitic nematodes. However, even under controlled conditions in the laboratory, results are often not reproducible. Apart from differences between nematodes isolates and the influences of uncontrolled environmental conditions, there has to be another source of variation. This hinders both the development of bioassays and the effective utilization of nematodes in biological control programs.

Between production and application the nematodes are often exposed to environmental stress, especially during storage and shipment. Although the nematodes generally manage to survive, they suffer from this treatment, resulting in reduced infectivity (Simons, pers. comm.).

In the present study the effect of the time of cold storage (4-5 °C) of some Dutch heterorhabditid popu-

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lations on persistence in potting soil, on migration rates in the presence and absence of *Galleria mellonella* (L.), and on efficacy against *O. sulcatus* larvae in strawberries was investigated under controlled conditions.

Materials and methods

Nematodes

The Dutch heterorhabditid HL81 was used in the first efficacy experiment and HFr86 in all other experiments. The nematodes originated from Limburg (HL81) and Friesland (HFr86) in the Netherlands. They were subcultured routinely every three to four months on larvae of the greater wax moth *(G. mellonella)* at 20 °C and harvested in modified White traps (Poinar, 1975). Culturing was intensified in 1987 in preparation for the large experiment with HFr86 in 1988 (see below). After harvesting, the nematode suspension was cleaned by decanting and adding fresh tap water. The infective juveniles were stored in 500 ml water in 1 l bottles, at 1400-7200 nematodes per ml, in a dark cold room at 4-5 °C under constant aeration with sterile-filtered air, a standard procedure in most laboratories. No attention was paid to other factors, like pH, that might affect the nematodes. The time of storage (tst) is the number of days from the onset of emergence from the host to the start of the experiments. Three to four days before inoculation of the nematodes the bottles were transferred to 20 °C to let the nematodes adjust to the test temperature.

Insect hosts

Larvae of the mealworm (*Tenebrio molitor* L.) were bought from Marba Apeldoorn B.V. and stored at 6 °C until a few weeks before the beginning of the experiments. The larvae were placed at 20 °C to allow pupation and then stored again at 6 °C until use. Black vine weevils were reared on strawberry plants in a greenhouse. The eggs were obtained as described by Simons (1981).

Efficacy of HL81 against black vine weevil

In April 1988 five batches of HL81 (tst 104 to tst 282) were compared. Strawberry plants in potting soil (non-sterile peat soil; organic matter 27 %, pH = 5.1, moisture content 71 %) in 0.7 l pots were infested with ten eggs of the black vine weevil. The plants were kept in the greenhouse at about 20 °C for 7 weeks to allow development of the insects. The plants were then transferred to a climate room (temperature 20 °C ± 1.5; relative humidity 75 % ± 10; 14 h light period) and a week later groups of twenty plants were inoculated; each group with one of the five batches of HL81. The same number of plants remained untreated. A dose of 100 (living) nematodes per cm^2 was applied (approx. 7000 nematodes per plant). Half the pots were sifted for living and dead insect larvae after 3 weeks and the other half was checked after 6 weeks. Data were analysed using generalized linear regression on the number of living black vine weevil larvae (binomial distribution). Percentage effect was also calculated (Abbotts formula; Unterstenhöfer, 1963).

Persistence of HFr86 in potting soil

In October 1988 six batches of HFr86 were compared (tst 25 to tst 337). The presence of infective nematodes in soil was detected by baiting at weekly intervals with mealworm pupae. The ability of the nematodes to parasitize insects in the course of the experiment was used as a working definition of " persistence ". Two hundred and ten pots of 0.7 dm^3 were filled with potting soil and placed in a climate room at 20 °C ± 1.5. A week later groups of 30 pots were inoculated; each group with one of the six batches of HFr86. The same number of pots remained untreated. A dose of 100 (living) nematodes per cm^2 was applied (*ca* 8000 nematodes per pot). Each week five pots of each treatment were baited by placing five mealworm pupae halfway down the pots and a week later the baited pots were sifted for living and parasitized (red) pupae. Occasionally pupae were found that had died from bacterial infections both in the treated and untreated pots. These pupae could easily be distinguished from the parasitized ones, as they turned brown or black. The pots were watered at least twice a week and covered with transparent plastic. Data were analysed using generalized linear regression on the number of parasitized pupae (binomial distribution). Percentage parasitism per week was calculated [red pupae/(total pupae — black pupae) × 100 %].

Migration rates, efficacy and persistence of HFr86

Ten batches of HFr86 were tested. They were grouped on the basis of comparable order of time of storage, i.e. group I = nematodes of the batch tst 4 (time of storage, days); group II = tst 88, tst 93 and tst 114; group III = tst 130, tst 134 and tst 137, and group IV = tst 298, and two batches stored for 343 days.

Migration rate

Migration rate of the nematodes in the ten batches was assessed in 9 cm high PVC cylinders (diam. : 4.5 cm), made of six separate rings of 1.5 cm high, filled with fine sterile sand (particle size 93 % between 180-300 µm; approx. 220 g/cylinder), and moistened with demineralized water (8 % w/w; pF *ca* 1.4) (Wester man & Godthelp, 1990). Four cylinders with a last instar larva of the greater wax moth on the bottom, and four cylinders without a host were inoculated with approximately 2000 nematodes in 0.5 ml water. After 4 h at 20 °C the rings were separated and the sand of each ring was rinsed in 50 ml water. The rinse water for rings 2 to 5 was combined to reduce the amount of work with little effect on the final results. The number and percentage

nematodes in ring 1, rings 2 to 5 and ring 6 were estimated by counting four samples of 3 ml of the rinse water. Nematodes found in a ring were considered to have moved the average height of the ring, i.e. 1.5 cm/2 = 0.75 cm. The migration rate, the average distance covered by the nematodes, was calculated [(no. ring 1 × 0.75 cm + no. ring 2-5 × 4.5 cm + no. ring 6 × 8.25 cm)/total no. nematodes]. Experimental data were analysed statistically using analysis of variance (ANOVA; l.s.d., $\alpha = 0.05$).

Efficacy

In February and March 1989, a week after the migration experiment, an efficacy experiment was carried out with 198 plants. They were potted and inoculated as described in the efficacy experiment with HL81. Each of ten groups of eighteen plants were inoculated with one of the ten batches of HFr86. The same number of plants remained untreated. A dose of 100 (living) nematodes per cm^2 was used (approx. 8000 nematodes per pot). Half the pots were sifted for living and dead insect larvae after three weeks. During this first assessment, some black vine weevil pupae were found. Therefore the experiment was terminated and a second assessment after six weeks was omitted. Data from the three week assessment were analysed as described in the previous efficacy experiment.

Persistence

This experiment was also carried out in February and March 1989 in a climate room at 20 °C ± 1.5 with 264 pots filled with potting soil similar to that used in the previous persistence experiment. Groups of 24 pots were inoculated with nematodes of one of the ten batches of HFr86. The same number of pots remained untreated. A dose 100 (living) nematodes per cm^2 was used (approx. 9500 nematodes per pot). Each week four pots per treatment were baited with five mealworm pupae, during a period of six weeks. During the first week four pupae were used per pot for baiting due to a shortage of mealworm pupae at that time. One week after baiting the pots were sifted for living, red and black pupae. Data were analysed as in the persistence experiment described above.

Results

Efficacy of HL81 against black vine weevil

The batch of HL81 stored for the shortest period (tst 104) tended to give highest control of black vine weevil larvae both after three (78 % effect) and six weeks (100 % effect). However, differences between batches were not significant, except that nematodes of the oldest batch tst 282 gave a significantly lower result than the other nematodes after three weeks (27 % effect). Efficacy after six weeks, as the average over all batches, was better than after three weeks (87 % and 61 % effect respectively).

Persistence of HFr86 in potting soil

Table 1 shows the results of the first persistence experiment with HFr86. The nematodes of batch tst 25 (stored for only 25 days) were most persistent in this test; after five weeks they still killed more than 50 % of the pupae. The nematodes of tst 337, the longest time of storage, caused an initial mortality of only 30 %, and after four weeks these nematodes had reached a non-infective level. The nematodes of the other four batches that had all been stored for a similar period of time hardly differed in persistence, with 50 % mortality reached within three weeks. The rate of decline of infectivity was almost the same for all nematodes, but the onset of decline was delayed for the freshest nematodes.

Table 1. The influence of the time of storage at 4-5 °C (tst [days]) on persistence of the heterorhabditid HFr86 in potting soil (percentage parasitism of *T. molitor* pupae in the course of time), after application of the nematodes in week 0. (Percentages in each column followed by the same letter are not significantly different, l.s.d., n = 5, $\alpha \leq 0.05$).

	Number of weeks after application of HFr86					
tst	*1*	*2*	*3*	*4*	*5*	*6*
control	0 *a*	0 *a*	0 *a*	0 *a*	0 *a*	0 *a*
25	100 *c*	100 *c*	80 *d*	84 *c*	56 *c*	33 *b*
189	100 *c*	76 *b*	56 *cd*	38 *b*	13 *b*	8 *a*
189	100 *c*	64 *b*	36 *bc*	32 *b*	4 *ab*	8 *a*
234	100 *c*	72 *b*	36 *bc*	33 *b*	9 *ab*	4 *a*
234	100 *c*	65 *b*	48 *c*	36 *b*	4 *ab*	0 *a*
337	29 *b*	0 *a*	12 *ab*	0 *a*	0 *a*	0 *a*

Migration rates, efficacy and persistence of HFr86

Migration rates

Migration rate of HFr86 was affected by the time of storage in cylinders both with and without a caterpillar of the wax moth (correlation coefficients $r^2 = 0.58$ and 0.67 respectively) (Fig. 1). In the cylinders without an insect host the nematodes from the batches tst 4 and tst 88 migrated 3 cm in 4 h, while the nematodes stored for longer periods such as tst 298, tst 343a and tst 343b migrated at most 1 cm from the point of application. The latter nematodes showed no increase in migration in cylinders with wax moth larvae. For comparison, migration rates of tst 4 and tst 114 were 7 cm in 4 h in the cylinders with a wax moth larva. Analysis of variance showed a significant effect of batch ($P < 0.001$), response to wax moth larvae ($P < 0.001$), and a significant

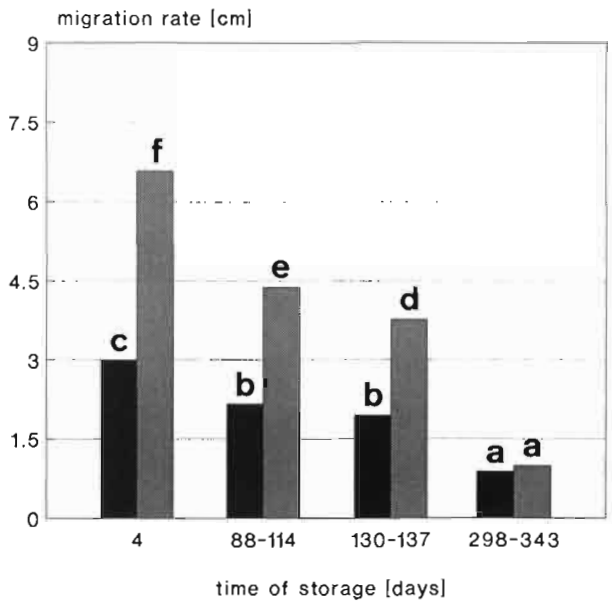

Fig. 1. The influence of the time of storage at 4-5 °C [tst (days)] on migration rates (average distances covered [cm] in 4 h) of the heterorhabditid HFr86 in 9 cm sand cylinders without (black bars) and with (grey bars) a *G. mellonella* larva. *(Bars with the same letter are not significantly different, I.s.d., n = 4, α ⩽ 0.05).*

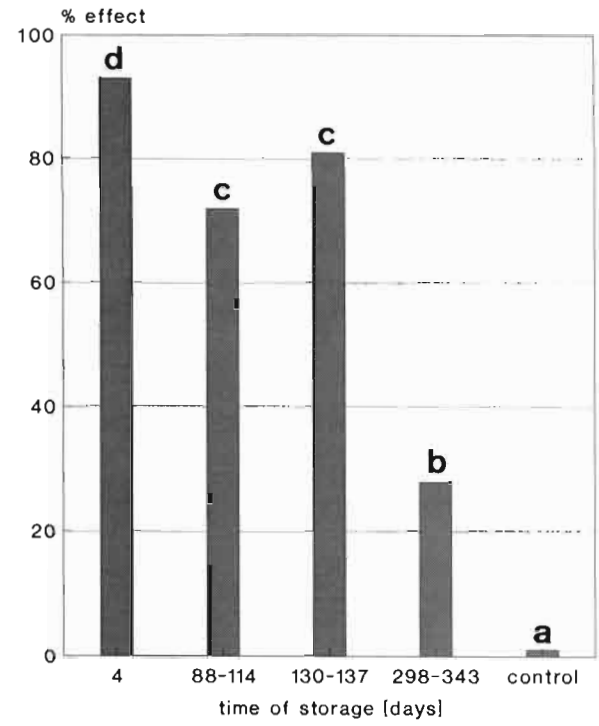

Fig. 2. The influence of the time of storage at 4-5 °C [tst (days)] on efficacy (percentage effect) of the heterorhabditid HFr86 against the black vine weevil *O. sulcatus* after 3 weeks in climate chambers at 20 °C. *(Bars with the same letter are not significantly different, I.s.d., n = 9, α ⩽ 0.05).*

interaction effect ($P < 0.001$), indicating that the nematodes of some batches responded much better to the presence of a wax moth larva than others.

Efficacy

The nematodes differed significantly in efficacy against black vine weevil larvae (Fig. 2). The time of storage and efficacy against black vine weevil in strawberries were correlated ($r^2 = 0.71$). Occasionally a large variation within groups of comparable time of storage was observed, for instance, the nematodes of tst 93 only caused 56 % effect, while the average in group II was 72 %. Also the nematodes of tst 343a and 343b, stored for the same period of time, differed in efficacy (50 % and 15 % effect, respectively).

Persistence

Large differences existed between nematodes stored for different periods in their ability to infect insects over a six week period (Table 2). As with efficacy, the time of storage had an adverse effect on persistence ($r^2 = 0.77$). Again variation was observed within groups, for instance the nematodes of tst 93 and tst 134 were less persistent than the nematodes of the other batches in either group II or group III. As in the first persistence experiment the rate of decline of infectivity was almost the same for all batches, but the onset of decline was retarded for batches with the shortest time of storage.

Table 2. The influence of the time of storage at 4-5 °C (tst [days]) on persistence of the heterorhabditid HFr86 in potting soil (percentage parasitism of *T. molitor* pupae in the course of time), after initial application of the nematodes in week 0. (Percentages in each column followed by the same letter are not significantly different, I.s.d., n = 4, 4, 12, 12, 12, α ⩽ 0.05).

		Number of weeks after application of HFr86						
group	*tst*	*1*	*2*	*3*	*4*	*5*	*6*	*overall*
	control	0 *a*	0 *a*	0 *a*	0 *a*	0 *a*	0 *a*	
I	4	100 *c*	100 *d*	100 *c*	80 *c*	90 *c*	60 *b*	88
II	88-114	100 *c*	93 *c*	81 *c*	47 *b*	24 *b*	12 *a*	57
III	130-137	100 *c*	98 *d*	64 *b*	45 *b*	20 *b*	5 *a*	54
IV	298-343	81 *b*	51 *b*	12 *a*	12 *a*	0 *a*	0 *a*	23
average		94	84	57	39	22	11	

Discussion

The effects of the length of storage and of conditions during storage on insect parasitic nematodes are usually

expressed as a decrease of percentage parasitism of certain insects in laboratory tests or as a loss of viability of the nematodes (e.g. Molyneux, 1985; Molyneux & Bedding, 1984; Westerman & Simons, 1988). This study clearly demonstrated an effect of the time of storage on efficacy and persistence of the nematodes (Fig. 2; Tables 1 & 2). The observed variation in results is within the range found in most studies on infectivity of nematode species and isolates for certain insect pests (e.g. Bedding & Miller, 1981).

The time of storage alone cannot account for all observed variation, especially not for the variation within the groups of nematodes stored for roughly the same period. It is possible that factors such as oxygen supply, nematode concentration, pH, accumulation of waste products and presence of contaminants, like bacteria and fungi in the storage medium affected the nematodes during storage (e.g. Dutky *et al.*, 1964; Howell, 1979; Molyneux, 1985; Westerman & Simons, 1988). Differences might even have originated from factors during culturing on wax moth larvae.

The observed problem is comparable to that of entomophagous insects being mass reared for use in biological control programs. Genetic drift and behavioural changes in these insects, due to continuous rearing of small populations under artificial conditions and on artificial hosts, are the main concerns in this field of research (Bigler, 1989; Van Lenteren, 1986). These mechanisms can also affect insect parasitic nematodes as current populations are generally based on small numbers of nematodes and it is likely that genetic drift and behavioural changes after continuous mass rearing on artificial medium will occur with time. At present however, these effects are overshadowed by the effects of storage and storage conditions.

It is impossible to do efficacy and persistence testing as in this study, on a routine basis. During the period required to perform efficacy or persistence experiments the quality of the batches will have further declined. Nematode migration was clearly affected by storage. It appeared to be a sensitive parameter, and ranking of the batches according to increase in migration corresponded with ranking both in efficacy and persistence (Fig. 1). Migration testing can be done in the laboratory, quickly and easily. Therefore, migration rate might be a suitable criterion for assessing the influence of storage on nematode performance. Of all hererorhabditid and steinernematid species and isolates tested so far, the Dutch *Heterorhabditis* exhibited the highest dispersion and host searching activity (Georgis & Poinar, 1983*a, b, c;* Westerman & Godthelp, 1990). Thus, a test based on migration might be suitable for the Dutch isolates tested so far, but might be less discriminating for other species and isolates. In those cases a test based on penetration could be useful, as for some plant parasitic nematodes the ability to penetrate decreases more rapidly then mobility (Van Gundy *et al.*, 1967).

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