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Linear regression models describing the performance of the insect parasitic nematode, *Heterorhabditis* sp., during storage

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Summary — Performance, defined as migration rates, efficacy and persistence of ten batches of the insect parasitic nematode, *Heterorhabditis* sp. HFr86, stored for different periods of time in a cold-room at 4-5 °C, was described by linear regression models. Input factors were : time of storage, percentage nematodes with ample food reserves, percentage ensheathment and percentage mortality. Migration rate in 9 cm high sand columns in the absence of insects and persistence in non-sterile potting soil was well described by these input factors (R^2 = 95 % and 91 % respectively). Efficacy against *Otiorhynchus sulcatus* in strawberries and migration rate in the presence of *Galleria mellonella*, on the other hand, were not well described (R^2 = 76 % and 68 % respectively). The time of storage was a dominating factor in all models. Percentage mortality of nematodes was the only factor that did not contribute to a better description of migration rates, efficacy or persistence. The equation for efficacy showed that the percentage effect will decrease by 10 % in the first 100 days of storage, followed by more than 30 % in each subsequent period of 100 days. Efficacy and persistence could also be described in terms of migration rates (R^2 = 77 % and 82 % respectively).

Résumé — ***Modèles de régression linéaire permettant de représenter les performances du nématode entomoparasite* Heterorhabditis *sp. pendant sa conservation*** — Les performances — exprimées par le taux de migration, l'efficacité et la persistance — de dix lots du nématode entomoparasite *Heterorhabditis* sp. HFr86 conservés pendant des périodes variables à 4-5 °C sont représentées par des modèles de régression linéaire. Les facteurs pris en compte sont : le temps de conservation, le pourcentage de nématodes avec réserves complètes, le pourcentage de nématodes conservant la cuticule du deuxième stade et le pourcentage de mortalité. La persistance dans du terreau non stérile et le taux de migration dans des colonnes de sable de 9 cm en l'absence d'insectes peuvent être convenablement représentés par les facteurs cités (R^2 = 95 % et 91 %, respectivement). L'efficacité contre *Otiorhynchus sulcatus* dans une culture de fraisiers et le taux de migration en présence de *Galleria mellonella* ne peuvent, par contre, être correctement représentés. Pour tous les modèles, le temps de conservation est un facteur dominant. Le pourcentage de mortalité des nématodes est le seul facteur qui ne contribue pas à une meilleure description du taux de migration, de l'efficacité ou de la persistance. L'équation concernant l'efficacité montre que le pourcentage diminue de 10 % dans les cent premiers jours de conservation, puis de plus de 30 % pour chaque période suivante de cent jours. L'efficacité et la persistance peuvent également être représentées en termes de taux de migration (R^2 = 77 % et 82 %, respectivement).

Key-words : nematodes *Heterorhabditis* sp. efficacy, persistence, migration, storage, food reserves.

Insect parasitic nematodes of the genera *Heterorhabditis* Poinar and *Steinernema* Travassos possess several characteristics that make them very suitable for the biological control of soil-inhabiting insect pests. They are associated with a specific, mutualistic bacterium, *Xenorhabdus*, and this unique combination is responsible for high virulence, broad host range and the relative ease of mass-culturing. In the period between production and application of the nematodes in the field, quality losses occur easily, with a considerable impact on efficacy and persistence (Westerman, 1992). Variable control results can reduce confidence in this biological control method. Besides, comparison of effectiveness of nematode species and isolates is almost impossible, due to unpredictable fluctuations in performance of individual batches.

The presence of the symbiotic bacterium in the intestine of the juvenile is required for maximal virulence after infection. In the case of *Steinernema* the number of bacteria per infective juvenile and the proportion of juveniles that carry the symbiont can vary enormously and may depend on the size of the infective juveniles (Akhurst, 1983, 1986). Very little is known of how the relationship between nematode and bacterium develops during a period of starvation, such as in storage (e.g. Kučera & Mráček, 1989).

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The nematode survives outside the insect host as a third stage juvenile, the so-called dauerlarva. This free-living juvenile is adapted to long term survival and is capable of locating and parasitizing new hosts. The free-living juvenile differs in anatomy, morphology and energy metabolism from normal, parasitic third stage juveniles (Poinar & Leutenegger, 1986; Fodor *et al.*, 1990). Somatic muscles and amphids are well developed, important for locating and finding insects. While in the soil, mouth and anus are closed and the digestive organs are non-functional (Poinar & Leutenegger, 1986). Important to condition and survival of the nematodes are the food reserves in the body, mainly lipids and glycogen. In fact, the quantity of these substances is regarded as one of the major limitations for the live span and activity of the dauerlarvae (e.g. Molyneux, 1985; Fodor *et al.*, 1990). Typical of these nematodes is the retention of the second stage cuticle after moulting to dauerlarva (ensheathment), particularly in the case of *Heterorhabditis*. The extra cuticle makes the juveniles more resistant to environmental stress, like desiccation and it offers extra protection against several nematophagous fungi (Timper & Kaya, 1989; Campbell & Gaugler, 1991). While in the soil or in a storage medium after mass production an increasing number of nematodes exsheaths.

Field testing to assess the quality of nematode inoculum in terms of efficacy or persistence is time consuming, requiring two months or more for completion. The current study was carried out to search for characteristics that could describe performance in a much faster way. For this purpose the data of Westerman (1992) were used. Efficacy, persistence and migration behaviour of nematodes of ten batches of a Dutch *Heterorhabditis*, HFr86, stored for different periods of time at 4-5 °C had been compared. Time of cold storage, percentage mortality and percentage nematodes with plentiful food reserves were taken into account as was percentage ensheathed or exsheathed infectives. In the present study these characteristics were related to the efficacy, persistence and migration data by linear regression, to yield a mathematical description of nematode performance. Migration rate itself already seems a suitable indicator for performance of Dutch heterorhabditid isolates (Westerman, 1992). Therefore migration rates in the absence or presence of an insect were related to the efficacy and persistence data.

Materials and methods

Nematodes

The heterorhabditid HFr86 originated from the province Friesland in the Netherlands. The nematodes were subcultured routinely on larvae of the greater wax moth, *Galleria mellonella* (L.), at 20 °C and harvested in modified White traps (Poinar, 1975). The nematode suspension was cleaned and stored in 500 ml water in 1 l bottles, at 1400-6800 nematodes per ml (Table 2), 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 that might affect the nematodes, like pH. 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 experiments the nematodes were transferred to 20 °C to let them adjust to the test temperature.

Migration rate, efficacy and persistence

For a description of the experiments we refer to Westerman (1992). The nematodes of ten batches of HFr86 were tested. Migration rates in the absence or presence of a greater wax moth larva were calculated as the average distances covered (cm) in 9 cm sand columns in four hours. Efficacy was assessed as percentage effect against black vine weevil larvae (*Otiorhynchus sulcatus* F.) in strawberries, in climate chambers at 20 °C. Persistence was expressed as percentage parasitism of mealworm pupae (*Tenebrio molitor* L.) in non-sterile potting soil, in the course of 6 weeks at 20 °C.

Assessment of percentage ensheathment and " dark " nematodes

One to 2 weeks before the start of the experiment two samples of approx. 200 nematodes were checked under a microscope. Nematodes were recorded dead when they showed a typical straight position, disintegrated body content or when the nematodes showed no movement after prodding with a handling needle. Nematodes with plentiful food reserves were recognized by a dark appearance in contrast with the transparent appearance of nematodes without ample foodreserves. This assessment was subjective to some extent, but was reasonably reproducible when carried out by one person. The second stage cuticle was clearly visible, especially at the anterior end of the nematodes. Assessed were 1) % dead, 2) % dark, ensheathed nematodes (dark/ensh), 3) % dark, exsheathed nematodes (dark/exsh), 4) % transparent, ensheathed nematodes (transp/ensh 5) % transparent, exsheathed nematodes (transp/exsh).

Linear regression analysis

Time of storage (tst), percentage mortality, " dark " nematodes and ensheathment were related to migration rates, efficacy and persistence of the nematodes by multiple linear regression (models $y = a + b_1x_1 + b_2x_2 + \ldots + \alpha$). The explanatory variables (x_i) expressed as percentages were transformed to logratios prior to the regression analysis (Aitchison, 1986). The explanatory variables and their interactions and quadratic terms were successively added to the models. Depending on their t-value they were retained or dropped (5 % significance) by the procedure of forward selection. Interaction or quadratic terms were never included without their corre-

sponding main effects. Where the regression was significant, but the *t*-values of the individual variables were not, the equation with the highest *t*-values was presented.

The migration rates in the presence or absence of a wax moth larva in sand cylinders were used as dependent variables *(y)*, together with the data from the efficacy and persistence experiments. These data, the number of living (efficacy) or parasitized insects (persistence) summarized per batch, were corrected for natural mortality and logit transformed. The same equation as was selected for the summarized data of the persistence experiment was used for each of the 6 weekly assessments of the persistence experiment separately.

Linear regression analysis was also used to relate migration rates in cylinders with and without wax moth larvae to the efficacy and persistence data, to investigate the descriptive value of migration rates. This time migration rates were used as explanatory variables *(x)*, and logit transformed data of the efficacy and persistence experiments as dependent variables (model $y = a + bx + \alpha$).

Results

Migration rate, efficacy and persistence

The results of the experiments are summarized in Table 1. For detailed information we refer to Westerman (1992).

Table 1. Migration rates (average distances covered [cm] in 9 cm sand columns in 4 h) in the absence (—) or presence (+) of a *G. mellonella* larva, efficacy against *O. sulcatus* (% effect after 3 weeks) and overall persistence in non-sterile potting soil (% parasitism of *T. molitor* pupae over a 6 week period) in climate chambers at 20 °C, of ten batches of the heterorhabditid HFr86.

Batch	Migration		Effect (%)	Parasitism (%)
	—	+		
1	3.0	6.6	93	88
2	2.9	4.8	83	67
3	1.1	1.4	56	39
4	2.5	7.0	76	66
5	1.8	3.4	80	60
6	2.1	4.7	88	42
7	2.0	3.2	75	60
8	0.8	0.8	17	23
9	0.9	1.1	51	25
10	0.9	1.0	15	22

Percentage ensheathment and "dark" nematodes

Up to 137 days of storage at 4-5 °C mortality of the nematodes was low (1-12 %) and most nematodes remained dark (88-99 %), except for batch 3, which had only 39 % dark nematodes (Table 2). The main decline in survival occurred between 137 and 298 days (> 50 %).

Table 2. Time of storage (tst) [days] at 4-5 °C, the number of nematodes per ml at the end of the storage period, and percentage of nematodes that were dead, dark and ensheathed (dark/ensh), dark and exsheathed (dark/exsh), transparent and ensheathed (transp/ensh) and transparent and exsheathed (transp/exsh) in ten batches of the heterorhabditid HFr86.

Batch	tst	Nem. ml × 100	Dead	Dark/ ensh	Dark/ exsh	Transp/ ensh	Transp/ exsh
1	4	13.9	3.6	95.5	0.9	0.5	0.5
2	88	46.1	6.0	20.9	73.2	0.1	0.1
3	93	22.4	1.7	1.1	37.4	4.5	55.3
4	114	29.4	0.9	49.4	49.4	0.4	0.2
5	130	28.6	2.2	63.8	34.1	0.2	0.2
6	134	67.8	8.7	26.2	63.8	0.6	0.7
7	137	19.1	11.8	74.5	13.7	0.3	0.3
8	298	52.9	55.1	8.7	6.6	21.0	8.5
9	343	46.6	53.6	20.4	2.9	20.4	2.7
10	343	37.0	64.2	12.5	10.1	8.8	4.4

Linear regression analysis

The regression coefficients of the best fitting equations are presented in Table 3. Descriptions of both efficacy against black vine weevil and migration rate in the presence of a wax moth larva by the best fitting equations were poor; the factors in the equations accounted for only 76 % and 68 % of the observed variation respectively, and apart from the regression coefficient for tst none of the coefficients was significant. The factors selected for persistence and migration rate in the absence of a host, on the other hand, gave good descriptions of these dependent variables (R^2 = 95 % and 91 % respectively). The equation for persistence contained a significant regression coefficient for percentage ensheathed nematodes in addition to tst, and the equation for migration in the absence of a host contained significant coefficients for tst, percentage dark nematodes and the interaction between these two.

The fitted equations reveal that for each storage period of 50 days the migration rate in cylinders with and without a wax moth larva will decrease by approximately 8 and 6 mm respectively, persistence (parasitism of mealworm pupae) will decrease by 8 % every 50 days, but the decline will be less rapid after 200 days. Efficacy in particular appeared to be affected by time of storage. The equation indicated that efficacy (percentage effect) will decrease by 10 % in the first 100 days, followed by more than 30 % in the next 100 days. After 300 days of storage 80 % of efficacy will be lost.

The equation selected for persistence was also used to describe parasitism in each week of the persistence

Table 3. Regression coefficients (regr. c.; a, b_i), and standard errors (s.e.) of time of storage at 4-5 °C (tst) [days], percentage dark and percentage ensheathed nematodes (logratio of fractions) and interaction terms, and percentage accountable variation (R^2) of models ($y = a + b_1x_1 + b_2x_2 + ... + \alpha$) for migration rates (average distances covered [cm] in 9 cm sand columns in 4 h) in the I) absence or II) presence of *G. mellonella*, III) efficacy against *O. sulcatus* (logit of the number of living insect larvae), and IV) persistence in potting soil (logit of the number of parasitized *T. molitor* pupae), of the heterorhabditid HFr86. Regression coefficients followed by one, two or three asteriks are significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively.

Model		a	b_i					R^2_{adj}
			tst ($\times 10^{-3}$)	% dark	% ensh	% dark × % ensh	% dark × tst ($\times 10^{-3}$)	
I migration (—)	regr. c.	1.30**	— 1.24	0.382**	—	—	1.44*	
	s.e.	0.31	1.12	0.080	—	—	0.50	91.2 %
II migration (+)	regr. c.	5.61	— 1.66	0.198	0.765	— 0.176	—	
	s.e.	2.66	9.86	0.331	0.617	0.171	—	68.3 %
III efficacy	regr. c.	3.48	— 16.29 *	— 0.078	0.812	— 0.202	—	
	s.e.	1.46	5.41	0.182	0.338	0.094	—	76.0 %
IV persistence	regr. c.	0.71*	— 6.49 ***	0.091	0.188*	—	—	
	s.e.	0.27	0.96	0.042	0.04	—	—	94.7 %

experiment separately. The *t*-values for the regression coefficients of percentage ensheathment and percentage dark nematodes in each week were used to give an indication of the relative importance of these factors in the course of time. At first the percentage dark nematodes contributed significantly to the model. *T*-values were 0.76, 7.30***, 0.45, 2.48*, 0.75, 0.76 for the respective weeks (numbers followed by one, two or three asteriks are significant at $P = 0.05$, 0.01 and 0.001 respectively). Gradually the percentage ensheathment became relatively more important. *T*-values for percentage ensheathment were — 0.22, 0.67, 1.41, 1.46, 4.53**, 2.16, for the respective weeks.

Migration rate in the absence of a host was highly correlated both with efficacy and persistence ($r = 0.89$ and 0.92 respectively). Linear regression with migration rate as the explanatory variable accounted for 77 % (efficacy) and 82 % (persistence) of the variation. The setting of the models are presented in Table 4. The migration rate in the presence of a wax moth larva was slightly less well correlated with efficacy or persistence ($r = 0.88$ and 0.87 respectively), and the equation based on migration rate accounted for 74 % (efficacy) and 72 % (persistence) of the variation.

The corresponding equations indicate that, starting from 9 % effect and 12 % parasitism without measurable migration, efficacy and persistence will increase by approximately 14 % and 12 % respectively for every 0.5 cm the nematodes migrate into the cylinders in the absence of a wax moth larva. After 3 cm of migration the increases in efficacy and persistence will be less rapid. For every centimeter they migrate in cylinders with a wax moth larva, efficacy will increase by 11 % effect and persistence by 9 % parasitism, starting from an initial level of 20 and 21 % respectively. After 6 cm of migration the increases in efficacy and persistence will be less rapid.

Table 4. Regression coefficients (regr. c.; a, b), and standard errors (s.e.) of migration rates (average distances covered [cm] in 9 cm sand columns in 4 h) in the absence (—) or presence (+) of *G. mellonella*, of models ($y = a + bx + \alpha$) for I) efficacy against *O. sulcatus* (logit of the number of living insect larvae) and II) persistence in potting soil (logit of the number of parasitized *T. molitor* pupae of nematodes from the heterorhabditid HFr86. Regression coefficients followed by one, two or three asteriks are significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively.

Model		Migration (—)		Migration (+)	
		a	b	a	b
I efficacy	regr. c.	— 2.335**	1.552 ***	— 1.417**	0.553***
	s.e.	0.540	0.276	0.432	0.108
II persistence	regr. c.	— 2.084***	1.148***	— 1.352**	0.393***
	s.e.	0.343	0.175	0.322	0.080

Discussion

The present study demonstrated that migration rates in the presence or absence of a wax moth larva, efficacy against black vine weevil and persistence in potting soil of the Dutch heterorhabditid HFr86 can be described in terms of time of storage at 4-5 °C, percentage dark

nematodes and percentage ensheathment (Table 3). The time of storage at 4-5 °C (tst) was the governing factor in all equations (Table 3). This factor accounted for the highest percentage of variation (R^2 = 53-75 %).

Percentage mortality was the only factor in this study that did not contribute to a better description of efficacy, persistence or migration ability. Apparently, mortality is not a good indicator for the performance of the remaining living nematodes. Nevertheless mortality of nematodes is being used as a criterion for assessing storage conditions or for testing the effects of pesticides on the nematodes (e.g. Heungens & Buysse, 1987). Some conditions may affect infectivity and pathogenicity rather than causing the death of the nematodes. It is possible that the outcome of the regression analysis and thus the importance of the factor mortality would have been different if data were obtained between 137 and 298 days of storage, the period in which survival decreased most rapidly (Table 2). Unfortunately no batches of HFr86, stored for a period between 137 and 298 days, were available at the time of the experiments.

The presence of ample food reserves proved very important in describing the migration rate of HFr86 (Table 3). Migration rates in the absence of a host decreased with declining percentages of dark nematodes. The significant interaction between time of storage and percentage dark nematodes indicates the importance of ample food reserves to migration ability with increasing time of storage. Apart from its descriptive value, the model may reflect a causal relationship between mobility of the nematodes and the demand for energy, needed for mobility. Migration is the most energy consuming part in the life of, for instance, free-living stages of plant parasitic nematodes (Van Gundy *et al.*, 1967). This concept has already led to the development of new storage methods for insect parasitic nematodes based on inactivation so that endogenous food reserves are not called on, for instance by immobilizing nematodes in gels or clay, partial desiccation or storing the nematodes under anaerobic conditions (Kaya & Nelsen, 1985; Yukawa & Pitt, 1985; CSIRO Patent, 1988). Increasing the initial fat content of the nematodes during (mass) production by improving production conditions may further contribute to persistence and life span of the nematodes. In the present study percentage dark nematodes was only moderately correlated with time of storage (r = — 0.69), indicating that, although the percentage of nematodes with ample food reserves did decrease with time, time of storage only partially described the observed decline in stored food reserves.

The visual assessment of food reserves was a qualitative and subjective method, as it was difficult to split a continuum of shades of grey into two categories. Moreover, within the groups of dark or transparent nematodes the actual lipid and glycogen contents will vary between individuals. Therefore the visual assessment will not fully correspond with the actual level of food reserves present in the nematodes. More quantitative and objective methods will be needed in future research to refine the assessments of stored food.

The fact that the percentage ensheathed nematodes was important to the equation describing persistence of the nematodes (Table 3) is not surprising. Persistence of the nematodes will to an extent depend on their ability to tolerate stresses and to withstand pathogens and predators in the soil for as long as possible. The second stage cuticle protects against nematophagous fungi and it may reduce desiccation (Timper & Kaya, 1989; Campbell & Gaugler; 1991). Towards the end of the experiment percentage ensheathment became more significant (see t-values for separate weeks). We observed that the nematodes generally retained the second stage cuticle for some time after they became transparent. It is imaginable that when the nematodes lose food reserves, they become more vulnerable to infections and stress factors. At that stage the presence of a second stage cuticle may be decisive for survival and persistence.

Migration rate in the absence of a wax moth larva also seems a suitable criterion for describing and predicting efficacy and persistence of *Heterorhabditis*. However, Dutch heterorhabditids seem to belong to the most active migrators among insect parasitic nematodes known so far (e.g. Georgis & Poinar, 1983; Westerman & Godthelp, 1990). A test based on migration rates could therefore be less discriminating for other species and isolates of *Heterorhabditis* (Westerman, 1992).

The question is whether the obtained relations apply to both genera of insect parasitic nematodes, *Steinernema* and *Heterorhabditis*, as they seem to use different strategies for survival and infection. Molyneux (1985) suggested that *Heterorhabditis* has a behaviour similar to many plant parasitic nematodes, i.e. of continuous movement and rapid utilization of food reserves until a host has been found, while *Steinernema* tends to conserve lipid reserves, at the expense of host searching activity. Furthermore Timper and Kaya (1989) found that, after moving through sand, *Heterorhabditis* retained its second stage cuticle whereas infective juveniles of *Steinernema* readily lost theirs. Consequently, in describing the performance of nematodes of different genera and species different characteristics might have to be used.

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