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Inoculum Doses and Exposure Periods Affect Recovery of *Steinernema feltiae* and *Heterorhabditis bacteriophora* from *Tenebrio molitor*

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Authors' Contribution
AMR and TM designed the study, executed experimental work, recorded and analyzed the data. AMJ and RKR assisted in analyzing the data and writing the manuscript.

Key words
Steinernematid, Heterorhabditid, Emergence, Yellow mealworm, Infective juveniles.

ABSTRACT

In Pakistan, techniques for mass production of entomopathogenic nematodes (EPN) are not yet available and the development and use of EPN mainly depends on the use of host insects such as greater wax moth (*Galleria mellonella*) for *in vivo* production. Since *G. mellonella* may not always be available, therefore, yellow mealworm (*Tenebrio molitor*) could be an alternative host. Therefore, in the present studies recovery of two EPN (*Steinernema feltiae* and *Heterorhabditis bacteriophora*) from *T. molitor* cadavers was compared in relation to the dosage of infective juveniles (IJ) applied and exposure periods. Significantly (P< 0.001) greater numbers of nematodes (predominantly IJ) were recovered from the cadavers of *T. molitor* that had been inoculated with *S. feltiae* than *H. bacteriophora*. The inoculum dose also had an influence on the numbers of nematodes recovered. There were significantly greater numbers of *S. feltiae* in the 50 and 500 IJ treatments than the 10 IJ dose. On the other hand, the lowest dose of *H. bacteriophora* did not yield any IJ. Similarly, days did not affect the recovery of nematodes from *T. molitor* cadavers. However, this effect was consistent over all the exposure periods but in the case of *S. feltiae* there was a significantly greater recovery from cadavers of larvae that had been exposed to nematodes for longer periods.

INTRODUCTION

In Pakistan, the techniques for mass production of entomopathogenic nematodes (EPN) are not yet available for inundative application and the development and use of EPN mainly depends on low technology mass production techniques such as use of host insects for *in vivo* production (Ehlers and Shapiro-Ilan, 2005; Rahoo *et al*., 2011, 2017). Such approaches are labour intensive but are feasible where labour costs are low. Initial field evaluation of EPN in Pakistan can be done with *in vivo* produced nematodes in hosts such as greater wax moth (*Galleria mellonella*). Since *G. mellonella* may not always be available, therefore, yellow mealworm (*Tenebrio molitor*) could be an alternative host. One of the advantages of production of EPN in *T. molitor* is that it does not produce cocoons and retains structural integrity while infected by nematodes and is being commercially produced on large scale in many countries of the world. Use of *T. molitor* as a host for *in vivo* production of EPN in biological control has been reported by Shapiro-Ilan *et al*. (2002).

The use of EPN against soil-dwelling insects attacking citrus, cranberries, turf and ornamentals is well established (Georgis, 1990) and has the potential to be used against root-knot nematodes and other insect pests (Hussain *et al*., 2016; Fateh *et al*., 2017; Javed *et al*., 2017a, b; Kayani *et al*., 2017; Khan *et al*., 2017; Mukhtar *et al*., 2017a, b; Tariq-Khan *et al*., 2017; Kassi *et al*., 2018; Nabeel *et al*., 2018). Many companies are currently engaged in producing and selling nematodes in the USA, Australia, Japan and Europe but these companies are not yet producing EPN for use in the warmer countries of the tropics and sub-tropics. Despite the increasing commercial and scientific interest in steinernematids and heterorhabditids, a universal standard infectivity assay has not been established. The need to evaluate nematode insecticidal activity in the laboratory has resulted in the development of a variety of assays that measure nematode infectivity by recording host mortality.

There are possibilities that *Heterorhabditis bacteriophora* may not behave in a similar way to *Steinernema feltiae* in which case it would seem appropriate to evaluate both species in *T. molitor* at different inoculum

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densities to seek methods of using EPN in new locations such as Pakistan. Results on the relationship between nematode dosage and infective juveniles (IJ) production are available for different nematode species in *G. mellonella* (Selvan *et al*., 1993; Cabanillas and Raulston, 1996) and no such data exist for *T. molitor*. Therefore, in the present studies recovery of two EPN (*Steinernema feltiae* and *Heterorhabditis bacteriophora*) from *T. molitor* cadavers was compared in relation to the dosage of IJ applied and exposure periods.

MATERIALS AND METHODS

Nematode cultures

EPNs *Steinernema feltiae* and *Heterorhabditis bacteriophora* used in the studies were taken from stock cultures supplied by CABI Bioscience and were maintained in the laboratory at the Department of Agriculture, University of Reading, United Kingdom. The nematodes were cultured in the last instar larvae of greater wax moth, *G. mellonella* (Lepidoptera: Pyralidae) (Livefoods Direct Ltd. Sheffield, UK) at 25°C. Ten *G. mellonella* larvae were placed on each 9 cm Petri dishes lined with a Whatman® No. 1 filter paper. The larvae in dishes were individually inoculated with approximately 2000 IJ of *S. feltiae* and *H. bacteriophora* contained in 1 ml of tap water. The Petri dishes were sealed with Nescofilm® sealing film (Azwell Inc., Osaka, Japan) and placed in an incubator at 20°C (Dutky *et al*., 1964).

After incubation at 20°C for 10 days, the infected *G. mellonella* larvae were taken from Petri dishes and placed on modified White traps (White, 1927). After some days, nematodes moved from the *G. mellonella* cadavers to the water. The water containing IJ was transferred to a clean beaker filled with fresh tap water and the IJ were allowed to settle for 30 min. The supernatant was decanted, the beaker was refilled with fresh tap water and the process was repeated three times until a clean suspension was obtained. Excess water was discarded and nematodes were kept at 10°C and used within two weeks (Kaya and Stock, 1997). IJ of both the nematode species were acclimatized at room temperature (~21-23) for an hour and their viability was tested under a stereomicroscope before use.

Effect of inoculum doses and exposure periods on recovery of S. feltiae *and* H. bacteriophora *from* T. molitor

Seventy two larvae of *Tenebrio molitor* weighing 0.11-0.20 were selected. Each larva was placed on a filter paper in a 30 mm Petri dish and inoculated with a 0.1 ml suspension of *S. feltiae* or *H. bacteriophora.* The different treatments were doses of 10, 50 or 500 IJ. The dishes were sealed and kept in an incubator at 20°C for 12 days. After 24 h Petri-dishes from each treatment dosage were taken from the incubator and filter paper was changed, moistened by adding 0.1 ml of tap water and placed in the same Petri-dish and were labelled. This practice was repeated after 2, 3, 4, 8, and 12 days. The mortality was recorded every day in the morning and deveining. When IJ emergence from the meal worms (which were in the 20°C incubator) began, the cadavers were placed in a modified White trap for up to two weeks and IJ recovery was recorded.

Statistical analysis

The data were not found normally distributed and transformed to log 10 prior to statistical analysis. All the data were subjected to Analysis of Variance (ANOVA) using GenStat package 2009, (12th edition) version 12.1.0.3278 (www.vsni.co.uk). Means were compared by Fisher's Protected Least Significant Difference Test at 5%.

Table I.- Recovery of all nematode stages from cadavers of *Tenebrio molitor* following exposure to different dosages of *S. feltiae* and *H. bacteriophora* (Data transformed to log 10).

Dose (No. of IJ)	Nematode species	
	H. bacteriophora	*S. feltiae*
10	0.00 a	1.83 b
50	3.52 c	4.70 d
100	4.72 d	4.72 d

Means sharing common letters do not differ significantly.

RESULTS

The analysis of variance showed highly significant results regarding effect of dose, days and species on the recovery of IJ from *T. molitor*. Similarly, the interaction between dose and days, dose and species and among dose, days and species were also significant. However, the interaction between days and species was non-significant (Supplementary Table I).

Significantly ($P< 0.001$) greater numbers of nematodes (predominantly IJ) were recovered from the cadavers of *T. molitor* that had been inoculated with *S. feltiae* than *H. bacteriophora*. The inoculum dose also had an effect on the recovery of nematodes. There were significantly greater numbers of *S. feltiae* in the 50 and 500 IJ treatments than the inoculum treatment of 10 IJ. On the other hand, the lowest dose of *H. bacteriophora* did not yield any IJ (Table I). Similarly, days did not affect the recovery of nematodes from *T. molitor* cadavers. However, this effect was consistent over all the exposure periods but in the case of *S. feltiae* there was a significantly greater recovery from cadavers of larvae that had been exposed

to nematodes for longer periods (Table II). The individual recovery of both the nematode species at three doses and six exposure periods is shown in Table III.

Table II.- Recovery of all nematode stages from cadavers of *Tenebrio molitor* following different periods of exposure to IJ of *S. feltiae* and *H. bacteriophora* (Data transformed to log[10]).

Days	Nematode species	
	H. bacteriophora	*S. feltiae*
1	2.61	3.13
2	2.64	3.13
3	2.13	3.17
4	2.71	4.23
8	3.26	4.71
12	3.14	4.14

Table III.- The individual recovery of both the nematode species at three doses and six intervals (Data transformed to log[10]).

Dose (No. of IJ)	Days	Nematode Species	
		H. bacteriophora	*S. feltiae*
10	1	0.00 a	0.00 a
	2	0.00 a	0.00 a
	3	0.00 a	0.00 a
	4	0.00 a	3.08 bc
	8	0.00	4.76 cd
	12	0.00 a	3.15 bcd
50	1	3.09 bc	4.63 cd
	2	3.16 bcd	4.64 cd
	3	1.65 ab	4.78 cd
	4	3.28 bcd	4.76 cd
	8	4.99 d	4.57 cd
	12	4.97 d	4.82 cd
100	1	4.75 cd	4.75 cd
	2	4.76 cd	4.76 cd
	3	4.74 cd	4.74 cd
	4	4.84 cd	4.84 cd
	8	4.80 cd	4.80 cd
	12	4.45 cd	4.45 cd

Means sharing common letters do not differ significantly.

DISCUSSION

At the lowest dose of 10 IJ, there was no infection of *T. molitor* by *H. bacteriophora* and slight infection in the case of *S. feltiae*. The recovery of nematodes after inoculation with 50 and 500 IJ was similar in the case of *S. feltiae* but different for *H. bacteriophora*. This suggests that a minimum of 50 IJ of *S. feltiae* and *H. bacteriophora* are required for obtaining nematode populations. It is also suggested that *T. molitor* may not be a good host for the infection by EPN. Mealworms are not natural hosts of EPN since they live in very different habitats and it is unlikely that they come in contact in the environment. However, they can be readily mass-produced on flour of bran meal and therefore would be a useful host for use with *in vivo* production systems. Mealworms have a hard, smooth cuticle with shallow segments (relative to some soil dwelling insect larvae) which could be a barrier to infection impeding penetration by both nematode species. Secondly, mealworms are comparatively more active than *G. mellonella* and thus could avoid infection by EPN.

The life cycles of steinernematid and heterorhabditid nematodes are different. The mode of reproduction of the first generation adults is bisexual for *Steinernema* spp. (Wouts, 1984; Kondo and Ishibashi, 1987), while it is hermaphroditic for *Heterorhabditis* spp. which begins sexual reproduction from the second generation (Zioni *et al*., 1992; Glazer *et al*., 1994). In most of the previous studies, attention has been placed mainly on the production and/or pathogenicity of IJ (Selvan *et al*., 1993; Glazer *et al*., 1994). Contrarily, not so much attention has been placed on the origin of juveniles via endotokia matricida which is generally considered as the failure of normally oviparous nematodes to deposit their eggs which may then accumulate and continue development within the female body. In the comparison between *H. bacteriophora* and *S. feltiae*, the former differed from the latter in the occurrence rate of endotokia matricida and the production of IJ. Generally the heterorhabditid produced more IJ than the steinernematid. Irrespective of the nematode species tested in the present experiment, endotokia matricida occurred even in actively moving adults of the first generation, although it occurred more frequently in the aged ones. In conclusion, the findings of the present studies have suggested that great numbers of *H. bacteriophora* and *S. feltiae* infective juveniles could be produced in *T. molitor* in the absence of *G. mellonella* for the management of insect pests.

Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2018.50.3.983.987

Statement of conflict of interest

Authors have declared no conflict of interest.

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