Available Online at EScience Press
Plant Protection
ISSN: 2617-1287 (Online), 2617-1279 (Print)
http://esciencepress.net/journals/PP

RECOVERY OF ENTOMOPATHOGENIC NEMATODES FROM CADAVERS OF *GALLERIA MELLONELLA* STORED UNDER DIFFERENT MOISTURE AND TEMPERATURE REGIMES

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ARTICLE INFO

Article history
Received: 29th October, 2021
Revised: 12th December, 2021
Accepted: 15th December, 2021

Keywords
Entomopathogenic nematodes
Greater wax moth
Mealworm
Emergence
Recovery

ABSTRACT

The terrestrial activities of entomopathogenic nematodes like movement, infection, development, and survival are influenced by a number of abiotic factors and among these soil moisture and temperature are regarded as the most important. Therefore, in the present studies the emergence of *Steinernema feltiae* from infected *Galleria mellonella* cadavers were evaluated in moist and dry conditions at different temperatures and the effects of cool storage on the recovery and duration of recovery of infective juveniles (IJ) from cadavers were also investigated. The results showed highly significant results regarding emergence of *S. feltiae* from infected *G. mellonella* under moist and dry regimes. A total of 154,456 IJ recovered per *Galleria* on moist sand as compared to 11,551 when placed on dry sand. Similarly, greater numbers of IJ recovered from the 10°C treatment than 5 °C. The total IJ recovered from cadavers at 5 °C were 292,314 and at 10°C were 381,135. The relationship between both the temperatures under wet and dry conditions was highly significant ($P<0.001$). The number of IJ emerging from wet at 5 °C was 50,029 and from wet at 10 °C were 81,674. Under dry conditions, 40,892 IJ recovered from 5 °C and 41,260 from 10 °C. There was no significant overall effect of insect host on the numbers of active nematodes recovered from the cadavers. More IJ recovered from *G. mellonella* cadavers than *T. molitor* stored at 5 °C but this trend was reversed at 20 °C.

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INTRODUCTION

The development and use of entomopathogenic nematodes, is one of the important strategies among different biological control agents to manage insect pests ecofriendly. Among all the nematodes tested for the management of insect pests, the members of the families Heterorhabditidae and Steinernematidae have been widely investigated for their efficacy against a number of insect pests as they have many advantages over synthetic detrimental chemicals (Grewal and Peters, 2005; Kaya and Gaugler, 1993; Koppenhöfer and Kaya, 1995; Rahoo et al., 2018a; Rahoo et al.,

2019a; Rahoo et al., 2017).

Application of entomopathogenic nematodes as effective biocontrol agents is well documented as these can be easily mass produced for inundative applications (Griffin et al., 2005; Rahoo et al., 2016a; Rahoo et al., 2019b; Rahoo et al., 2011; Rahoo et al., 2016b; Rahoo et al., 2018b). The mass production of these nematodes using fermentation technology is an industrial based process (Ehlers and Shapiro-Ilan, 2005a, 2005b). Such kinds of industrial tools are lacking in many countries like Pakistan where the use and application of these nematodes is in its early stage. In these countries, the development and use of entomopathogenic nematodes relies mainly on low technology mass production techniques such as the use of host insects for *in vivo* production (Ehlers and Shapiro-Ilan, 2005a). These techniques are laborious but are feasible where labour costs are low. In Pakistan, initial field evaluation of entomopathogenic nematodes is likely to be done with *in vivo* produced nematodes in hosts such as *Galleria mellonella*. Since *G. mellonella* may not always be available, an alternative host could be *Tenebrio molitor*. The conditions for their application need to be evaluated to determine if it is possible to improve the efficacy and persistence of such nematodes.

The terrestrial activities of nematodes like movement, infection, development, and survival are influenced by a number of abiotic factors and among these soil moisture is regarded as the most important (Wallace, 1966). Low moisture contents have been reported to have an adverse effect on nematode activities and survival; however an appreciable number of terrestrial nematode species can survive and tolerate dehydration and desiccation to some extent if there is gradual decrease in the drying process (Womersley, 1987, 1990, 1993). Such drying conditions are commonly found in soils because the relative humidity (RH) in the interstitial spaces stays close to 100% and drops only in extremely dry conditions. Resistance to dehydration has been experienced in the genera *Steinernema* and *Heterorhabditis* to a limited degree. The IJ cannot survive rapid desiccation under laboratory trials under low RH regimes (Kung et al., 1991; Simons and Poinar Jr, 1973; Womersley, 1990), but they can endure substantial lengths of time in dry soils (Kung et al., 1991). The gradual desiccation in soil may provide IJ enough time to adapt physiologically into a partially desiccated and immobilized quiescent state (Womersley, 1990).

On the other hand, most of the studies related to the effects of drying on nematodes have been carried out on free living nematode species. However, the infective juveniles of entomopathogenic nematodes are either free living in the soil or they can be found within a cadaver. The external environmental conditions may change drastically. Quick decline in soil moisture can occur in the upper soil layers, especially in sandy soils with little water retention capacity; entomopathogenic nematodes are very frequently isolated from such soils (Hara et al., 1991; Stock, 1995; Strong et al., 1996). In addition, the infected host may move to areas with low soil moisture or RH before succumbing to the infection. In such situations, the host cadaver may retain moisture better than the surrounding soil and protect the IJ from dehydration until the moisture conditions become conducive for IJ dispersal and host finding. The rationale for conducting the following series of experiments was to test the principle of applying EPN in cadavers. The hypotheses being that entomopathogenic nematodes' survival and persistence can be prolonged by manipulation of the conditions under which infected larvae are treated. Furthermore it is possible that *Heterorhabditis* (such as *Heterorhabditis bacteriophora*) may not behave in a similar way to *Steinernema feltiae* in which case it would seem appropriate to evaluate both species on different hosts and at different host densities to establish if such considerations need to be addressed when seeking methods of using entomopathogenic nematodes in new locations.

MATERIALS AND METHODS

Entomopathogenic nematodes

The entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis bacteriophora* were obtained from the stock culture supplied by CABI Bioscience, UK. The culture of both the nematodes was maintained in the laboratory at the Department of Agriculture, University of Reading, UK and subcultured on last instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) at 25 °C supplied by Livefoods Direct Ltd. Sheffield, UK following the method described Dutky (Dutky et al., 1964). Only one week old infective juveniles of *S. feltiae and H. bacteriophora* were selected and used in the experiments.

Experiment No. 1: Emergence of *S. feltiae* from infected *G. mellonella* cadavers in moist and dry conditions

Sixty late instar larvae of *G. mellonella* weighing 0.25-

0.35 g were selected and individual weights recorded. Each larva was placed on a filter paper in a 30 mm Petri dish and inoculated with a 0.15 ml suspension of *S. feltiae* containing a mean of 78 infective juveniles. The dishes were stored in an incubator at 20 °C for 4 days in which time all larvae succumbed to nematode infection.

Sixty 30 mm Petri dishes containing 5 g of dry silver sand were prepared. Half of them were left dry and to the remaining 30, 1 ml of tap water was added. An infected larva (cadaver) was added to each dish, supported on a small piece of plastic mesh (Netlon) (www.netlon.co.uk) so that the cadaver could be moved to a fresh Petri dish without it being ruptured. Petri dishes were sealed with Nescofilm to prevent desiccation and kept in an incubator at 20 °C.

One week after inoculation, each cadaver was moved on the supporting Netlon and transferred to a new dish containing either wet or dry sand. These dishes were then sealed and returned to the incubator. Each dish was monitored daily to observe when infective juveniles first emerged from each cadaver. When emergence began, the sand from the original dish was moved to a modified miniature Baermann extraction tray made from a 50 mm Petri dish to recover nematodes that had emerged from the *Galleria* cadavers. This process was repeated every 3 days until no more nematodes emerged from the cadavers.

Experiment No. 2: Emergence of *S. feltiae* from infected *G. mellonella* cadavers in moist and dry conditions at different temperatures

Two hundred and forty late instar larvae of *G. mellonella* were selected for uniformity of size. Each larva was placed on a filter paper in a 30 mm Petri dish and inoculated with 0.1 ml suspension of *S. feltiae* containing an average of 54 infective juveniles. The dishes were stored in an incubator at 20 °C for 3 days in which time all larvae succumbed to nematode infection. All filter papers were changed 4 days after inoculation and the Petri dishes were returned to the 20 °C incubator. On the 10th day, Petri dishes were divided into two groups of 120 for assigning to incubators at 5 or 10 °C. Each group was then divided. Half of them were left with dry filter paper and to the remainder 0.5 ml of tap water was added. An infected larva (cadaver) was added to each dish. Petri dishes were sealed to prevent desiccation. On the 10th day after inoculation and every 5th day up to 40 days, twenty cadavers were moved (10 from moist and 10 from dry) on the supporting Netlon to new Petri dish containing 5 g sand to which 1 ml of tap water was added. An infected larva (cadaver) was added to each dish which was sealed and kept in an incubator at 20 °C.

Each Petri dish was monitored on a daily basis to observe when infective juveniles first emerged from each cadaver and subsequently the infective juveniles emerging were recovered as described above. This process was repeated on every treatment alternating counting days for each treatment. The nematodes were recorded six times until no more nematodes emerged from the cadavers.

Experiment No. 3: The effect of cool storage on the recovery and duration of recovery of active infective juveniles from cadavers

Forty uniformly sized late instar larvae of *G. mellonella* and 40 *Tenebrio molitor* were selected. Each larva was placed on a filter paper in a 30 mm Petri dish and inoculated with 0.1 ml suspension of *H. bacteriophora* containing an average of 68 infective juveniles. The dishes were stored in an incubator at 20 °C for 3 days in which time all larvae succumbed to nematode infection. All filter papers were changed 4 days after inoculation and the Petri dishes were returned to the incubator. On the 16th day Petri dishes were divided into two groups containing 40 Petri dishes of *G. mellonella* and *T. molitor* for assigning to incubators at 20 and 5°C. Each group was divided for each temperature; 20 with *G. mellonella* and 20 with *T. molitor.* All of them were changed to a new filter paper and 0.5 ml of tap water was added. An infected larva (cadaver) was added to each dish. Petri dishes were sealed and placed in incubators at different temperatures. Twenty cadavers were moved from each incubator (10 from 20 °C and 10 from 5 °C) (five *G. mellonella* and five *T. molitor* cadavers) were moved to modified miniature Baermann extraction trays made from a Petri dish (7.5 x 4.5 mm) to recover nematodes that had emerged from the cadavers. All the Petri dishes were kept in an incubator at 20 °C. The data were recorded after 1, 2, 4, 8 and 12 weeks.

RESULTS

Experiment No. 1: Emergence of *S. feltiae* from infected *G. mellonella* cadavers in moist and dry conditions

The results showed highly significant results regarding emergence of *S. feltiae* from infected greater wax moth under moist and dry regimes. A total of 154,456 infective juveniles recovered per *Galleria* on moist sand as compared to 11,551 infective juveniles when placed

on dry sand (Figure 1). There was significantly greater emergence of infective juveniles of *S. feltiae* from *Galleria* kept moist than from those kept in dry sand. For example, the number of infective juveniles emerging was 6666 and 89206 on 11 and 14 days, respectively, on moist sand. On the other hand, the numbers were 5568 and 2615 on 11 and 14 days, respectively on dry sand (Figure 2). There was a positive relationship (P> 0.05) between the weight of the *Galleria* larvae at infection and the numbers of infective juveniles recovered in the moist conditions but not for those kept under dry conditions.

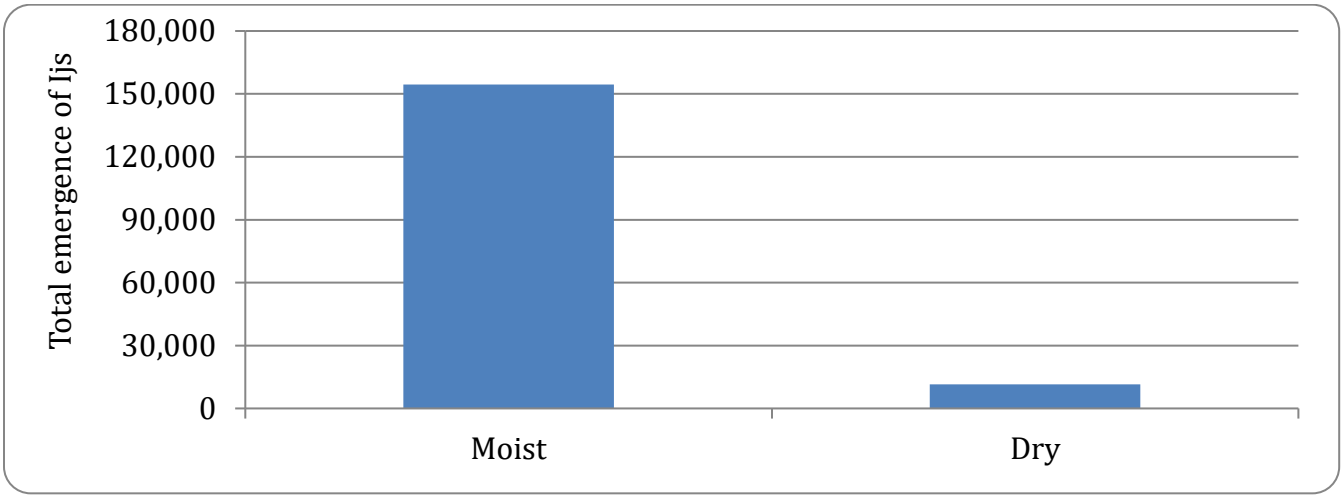

Figure 1: Overall emergence of infective juveniles of *S. feltiae* from *G. mellonella* maintained under moist and dry conditions.

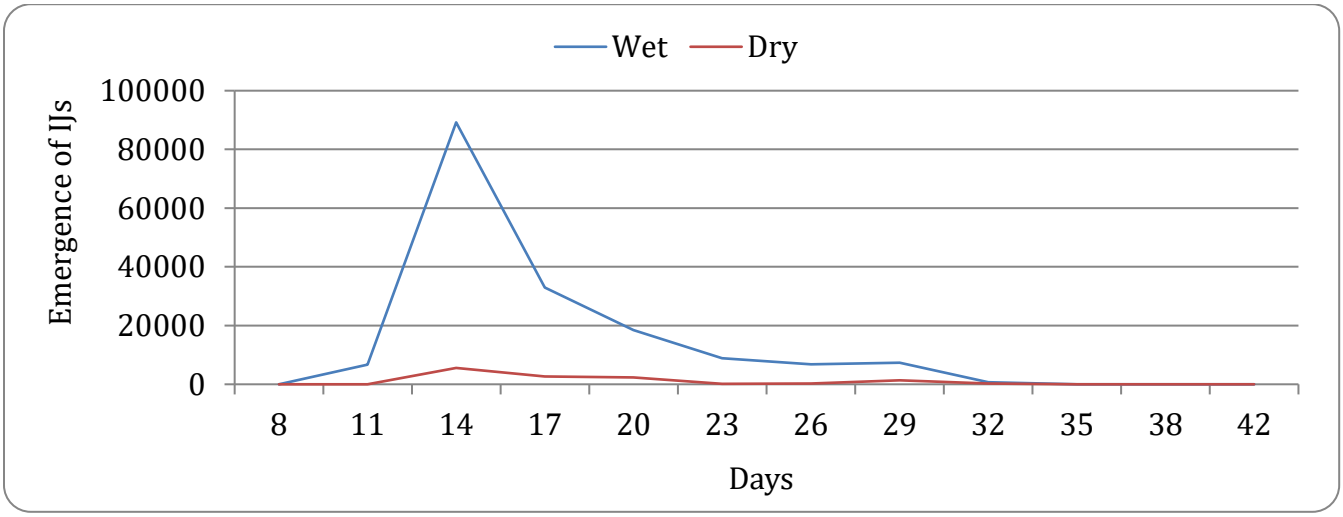

Figure 2: Emergence of infective juveniles of *S. feltiae* from *G. mellonella* maintained under moist and dry conditions over 41 days.

Experiment No. 2: Emergence of *S. feltiae* from infected *G. mellonella* cadavers in moist and dry conditions at different temperatures

The relationship between wet and dry recovery of infective juveniles was highly significant (P<0.001). There was significantly greater emergence of infective juveniles of *S. feltiae* from the *Galleria* kept moist than from those kept in dry (Figure 3). Similarly, greater numbers of infective juveniles were recovered from the 10°C treatment than 5 °C. The total infective juveniles recovered from cadavers at 5 °C from all treatments of wet and dry were 292,314 and at 10 °C 381,135 (Figure 4). The relationship between both the temperatures under wet and dry conditions was highly significant

(P<0.001). For example, the number of IJ emerging was 50,029 from wet 5 °C and 81,674 from wet 10 °C. On the dry, 40,892 recovered from 5 °C and 41,260 from 10 °C (Figure 5). The differences in numbers of IJ recovered from different days between 5 to 30 days were also highly significant (P<0.001) as shown in figure 6.

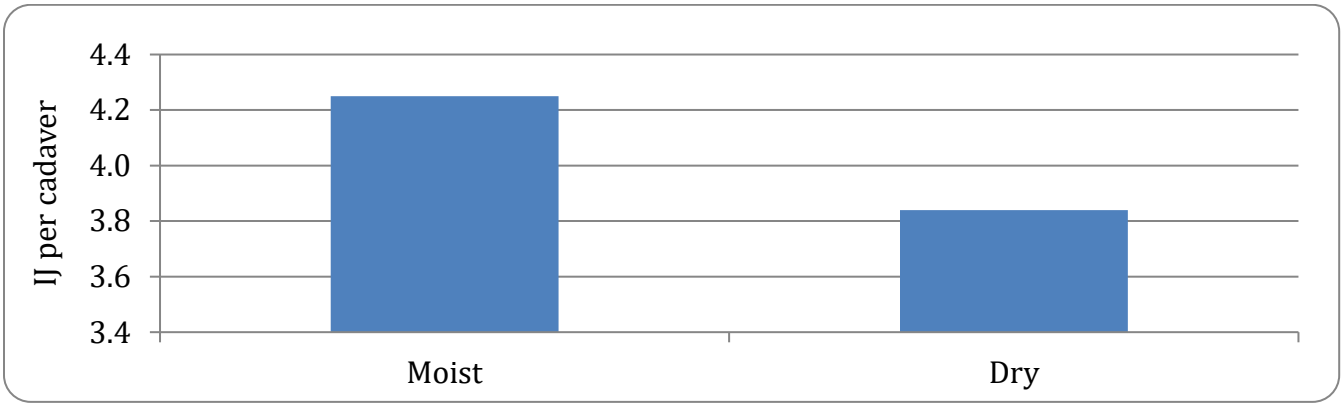

Figure 3: Recovery of *S. feltiae* infective juveniles from cadavers of *G. mellonella* following storage under moist and dry conditions. (Means of 10 cadaver data transformed log 10)

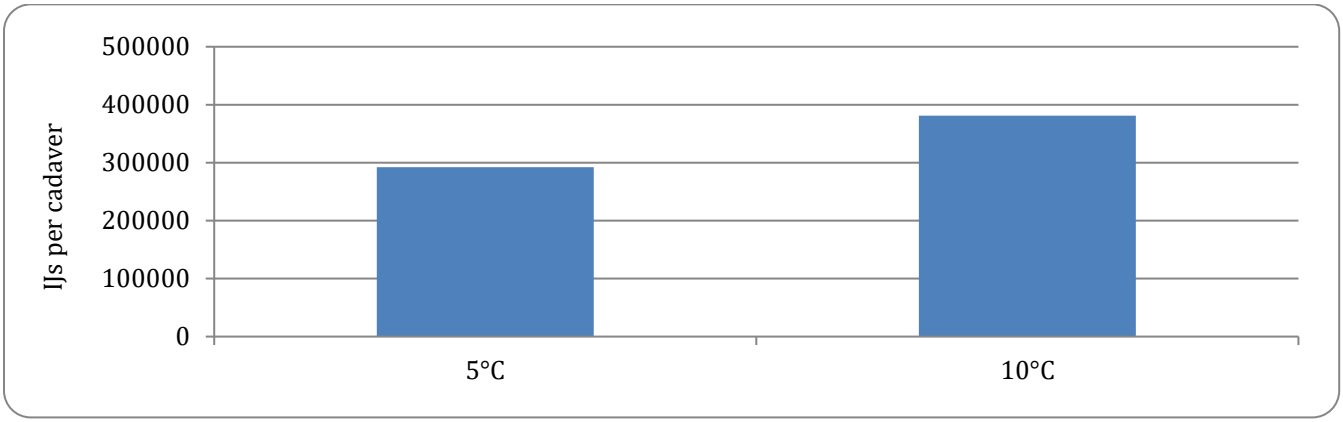

Figure 4: Recovery of *S. feltiae* infective juveniles from cadavers of *G. mellonella* following storage under 5 and 10 °C. (Means of 10 cadaver data transformed log 10)

Figure 5: Effect of different storage conditions of *G. mellonella* cadavers infected with *S. feltiae* on the numbers of infective juveniles recovered. (Means of 10 cadaver data transformed log 10).

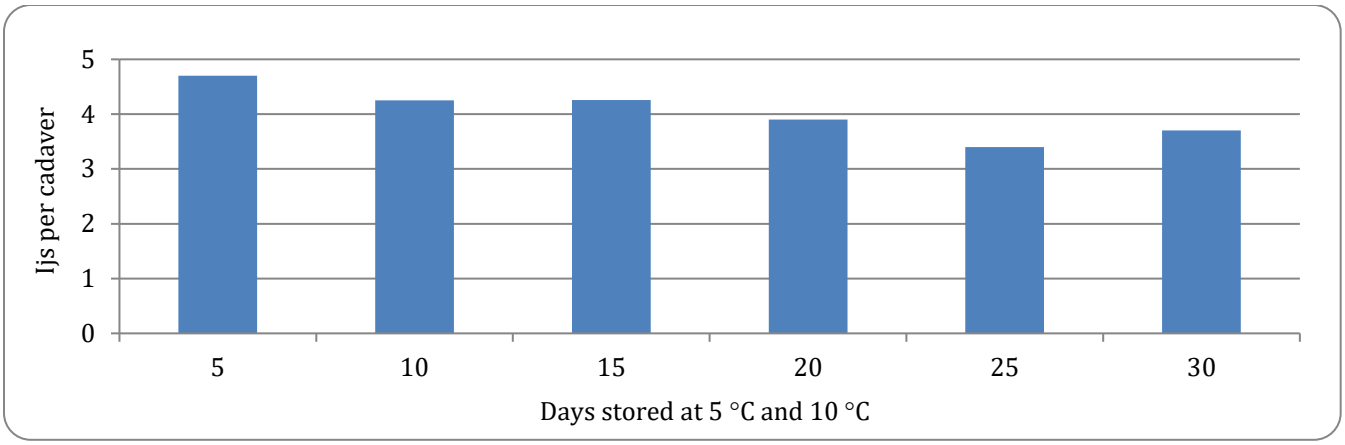

Figure 6: Recovery *of S. feltiae* from cadavers of *G. mellonella* following different periods of storage at 5 and 10 °C. (Means of 10 cadaver data transformed log 10).

Experiment No. 3: The effect of cool storage on the recovery and duration of recovery of active infective juveniles from cadavers

There was no significant overall effect of insect host on the numbers of active nematodes recovered from the cadavers. More infective juveniles were recovered from *G. mellonella* cadavers than *T. molitor* stored at 5 °C but this trend was reversed at 20 °C (Figure 7).

Likewise, there was no overall effect of storage regime on yield of active infective juveniles. The majority of infective juveniles were outside the cadavers after the first 2 weeks but live infective juveniles were recovered for up to 12 weeks. Although numbers were low, there were more infective juveniles from the 5°C treatment at 8 and 12 weeks. The differences in emergence of infective juveniles of *H. bacteriophora* from the *G. mellonella* and *T. molitor* were non-significant. The relationship between host and temperature was highly significant (P< 0.001) as shown in figure 8.

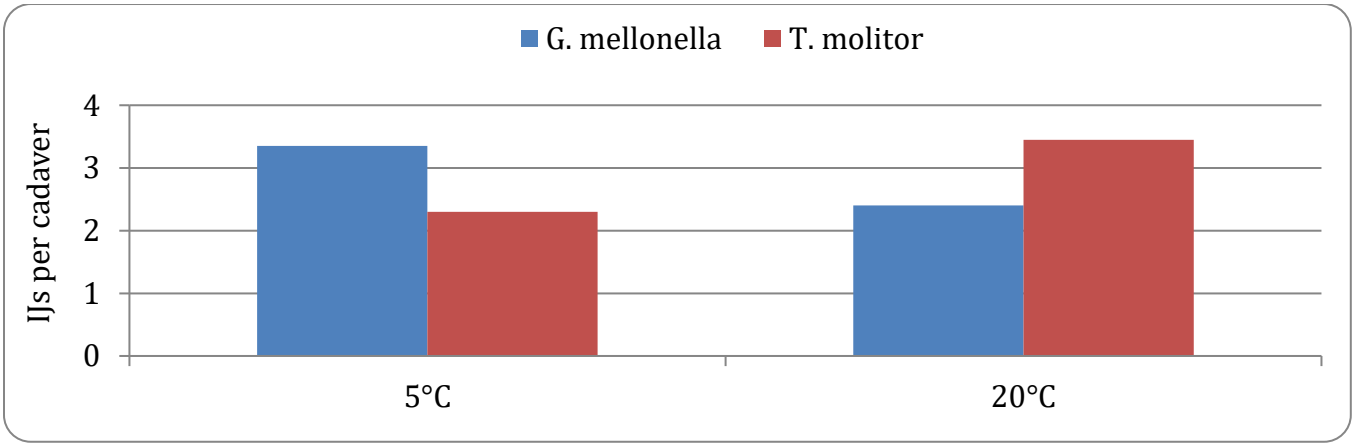

Figure 7: Effect of different storage temperatures on survival of *H. bacteriophora* from *G. mellonella* and *T. molitor* cadavers. (Means of 5 cadavers data transformed log 10).

DISCUSSION

The success of nematode application for insect control in soil and the survival of naturally occurring nematode populations depends on the ability of the IJ to disperse and persist until it can locate a host. Many entomopathogenic nematodes have been shown to be good parasites for soil dwelling insects. Under natural conditions insects that become infected with entomopathogenic nematodes will remain in soil and eventually IJ will emerge from the cadavers.

If infected insect cadavers are to be used as a means of deployment of entomopathogenic nematodes under field

conditions, information on how the cadavers are to be prepared and treated needs to be collected. The recovery and survival of IJ in cadavers left in moist and dry conditions was compared. The population level was higher in cadavers placed on the moist sand (Figure 1). This showed that the soil conditions in which an infected insect occurs can have an effect on the subsequent emergence of IJ from the cadaver.

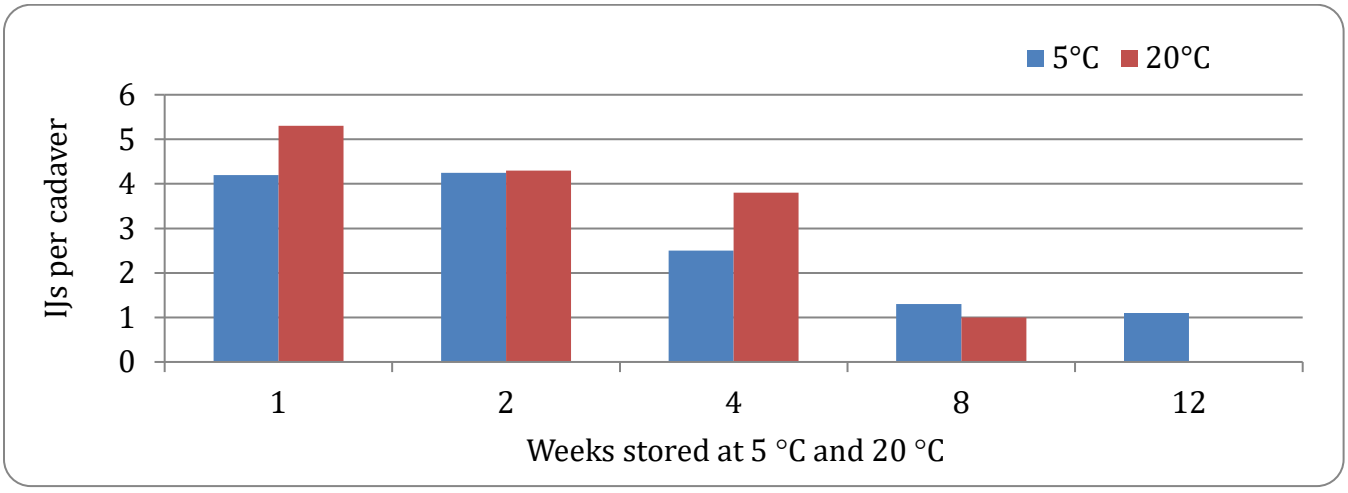

Figure 8: Overall recovery of active infective juveniles of *H. bacteriophora* from cadavers of *G. mellonella* and *T. molotor* following different periods of storage at 5 and 20 °C. (Means of 5 cadaver data transformed log 10).

The emergence of entomopathogenic nematode IJ from host cadavers is influenced by soil moisture, a question that is asked can IJ survive for considerable lengths of time within desiccating host cadavers in dry soil. Only very limited IJ emergence was observed from cadavers in dry soil and the IJ emerged readily from moist soil (Figure 3). It is not clear whether IJ persistence within the cadavers is an adaption to low soil moisture conditions or whether the nematodes are simply trapped in the cadaver. There appears to be some correlation between persistence within cadavers and the ecology of the different nematode species (Kamionek, 1977; Kung et al., 1991; Schmiege, 1963). However, if the IJ complete their development inside a host cadaver located in unfavourably dry soil, they probably have no choice but to endure within the cadaver for two reasons. First, if they manage to exit the cadaver, they are instantly exposed to the low moisture without time to time to adapt physiologically into a quiescent state. Under such rapid desiccation regimes, IJ may survive for only a few days or even hours. Secondly, the cuticle of the host dries out and hardens at a rate and to a degree that probably restricts the escape of the IJ from the cadaver until it is rehydrated. By retaining moisture and functioning as a buffer, the host cadaver may serve as a means for nematodes populations to persist through dehydration conditions. It is not known how commonly this occurs under field conditions which are more variable and different insect species serve as hosts. For short periods with insufficient moisture, remaining inside the host cadaver could be an efficient mechanism for entomopathogenic nematode survival (Koppenhöfer and Kaya, 1995).

The period over which IJ emerge could be quite important as it may influence how long they stay viable. It was hypothesised that if cadavers are kept relatively dry, the period of emergence will be extended relative to those that were kept moist. It is known that when cadavers are placed in very humid conditions such as a White trap as described above, IJ will readily emerge. Under the treatment imposed in experiment no.1 maintaining the cadavers dry did not extend the period of emergence and in fact it seems that the nematodes died as many fewer emerged from the dry treatment. A mistake was not to have investigated the dry cadavers to see if there were dead IJ inside. Future experiments should be planned to vary the duration of “dry” periods. The fact that in some dishes, there were 26,000-119,000 IJ emerging from the dry cadavers (and in one case after 29 days) suggests that the original hypothesis needs further testing.

It is also suggested from these results that the numbers

of IJ that form in the body of an insect might be dependent on the size of that insect. The results of experiment no. 2 indicated that IJ can survive adverse environmental conditions by remaining in the host cadaver for up to 40 days after inoculation. Survival is dependent upon the environmental conditions to which the cadaver is exposed. Observations indicate desiccation causes the cadaver to shrink in volume, possibly hardens the cuticle, and impedes nematode emergence. This suggests that the cadavers can provide protection from desiccation, but only for a limited period and eventually the nematodes become trapped and die inside the cadaver. *S. feltiae* showed high levels of emergence at 10°C on wet conditions as compared to dry condition and at 5°C showed a decrease in emergence on dry condition as compared to wet condition although the temperature effect was not statistically significant. Infective juvenile mortality in the cadaver may result primarily from water loss. Water loss may change the cadaver environment by further concentrating the solutes in the cadaver thereby increasing osmotic stress and restricting movement. Drying of the cuticle may restrict oxygen diffusion into the cadaver resulting in increased physiological stress, e.g. anoxia was shown to reduce thermo tolerance and cold hardening abilities in the flesh fly *Sarcophaga crassipalpis* (Yocum and Denlinger, 1994). In addition, the surface area available for diffusion becomes greatly diminished due to cadaver shrinkage. In contrast to the sudden decline at 5°C, survival and nematode numbers at 10°C remained almost constant over time, although variation between cadavers was considerable. Some cadavers did not produce infective juveniles and in others no nematodes survived. Lower nematode numbers may also contribute to the increased survival at 5°C. Exposure to low temperatures generally prolonged the time to emergence for both steinernematids and heterorhabditids (Grewal et al., 1994a). For the one steinernematid species tested, exposure to 5 and 10°C temporarily inhibited emergence from most cadavers. Low temperature and relative humidity combinations may intensify the effects of one or both factors on nematode survival (Wharton, 1995). Prolonged exposure to desiccation caused greater mortality than long-term exposure to low temperature for the free-living nematode *Panagrolaimus davidi* (Brown, 1993; Brown and Gaugler, 1996, 1997). Low temperature could slow or prevent emergence and increase the chance of the nematodes becoming trapped in the desiccating cadaver. However, preventing emergence of infective juveniles may prolong their survival by reducing their metabolic and oxygen consumption rates at low temperature. Desiccation may also reduce the likelihood of nematode freezing at low temperatures by increasing the osmolality (Wharton, 1995). In conclusion, low temperatures and relative humidity prevent infective juvenile emergence from the host cadavers. Remaining in the cadaver for extended periods affords infective juveniles limited protection from desiccation and low temperatures; eventually the nematodes become trapped and die within the cadaver. The degree of protection offered is determined by the temperature and relative humidity regime to which the cadaver is exposed. The effects of temperature and relative humidity may also be exacerbated by their combined effect.

Brown and Gaugler (1997) showed that IJ will survive adverse environmental conditions by remaining in the cadaver for up to 50 days but commented that survival of IJ was dependent on the conditions and that different species responded differently. Similarly, Grewal et al. (1994a) had reported that exposure of heterorhabditids and steinernematids to low temperatures prolonged the time to emergence. This work suggested that more work could be done to determine if the duration of IJ survival in a cadaver can be extended by storing cadavers in a cool environment.

The results of Brown and Gaugler (1996) with *H. bacteriophora* do not show that there is a clear prolongation of IJ emergence if cadavers of *G. mellonella* or *T. molitor* are kept at 5°C. Although there were active IJ after 8 and 12 weeks at 5°C numbers were not sufficiently great to justify this treatment with this species of nematode. Conceivably, more nematodes might survive cool storage if a species more adapted to colder conditions had been used. Brown and Gaugler (1997) also showed that the importance of humidity in the emergence of IJ. In experiment no. 3, cadavers stored in sealed dishes to which moisture had been added and should not have been limiting. In addition, IJ survival is related to their metabolic rates and their initial reserve levels (Glazer, 1996). Nematode metabolism is reduced at low temperature; hence, IJ do not use their lipid reserve (Georgis et al., 2006; Georgis and Manweiler, 1994), which is rapidly used at higher temperatures due to their great mobility. However, for *H. bacteriophora*,

the proportion of mobile IJ started to noticeably decrease after 7 days at both storage temperatures, which allow them not to use their lipid reserve and maintain their infectivity. Results presented here agree with those obtained by (Jung, 1996) for different isolates of *Heterorhabditis* in which the percentage of living IJ decreased noticeably after 7 weeks of storage at 5 ± 2°C. This work provides data which could optimize storage conditions under which *H. bacteriophora* is maintained in laboratory. The limited room temperature shelf life is one of the obstacles for using entomopathogenic nematodes (Georgis et al., 2006).

AUTHORS' CONTRIBUTION

AMR designed the study, executed experimental work, collected data, AMR and RKR analyzed the data, AMR wrote the manuscript, RKR assisted in writing the manuscript, SRG supervised the work and proofread the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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