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Reproductive potential and Host Searching Ability of Entomopathogenic nematode, *Steinernema feltiae*

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ABSTRACT

Due to non-availability of mass production techniques in Pakistan, the development and application of entomopathogenic nematodes (EPN) depend on the use of host insects for *in vivo* production. As there is little information regarding relationship between nematode dosage and production of infective juveniles (IJ) of *Steinernema feltiae* and ability to locate hosts, therefore, in the present studies investigations were done on these aspects. A significantly greater emergence of IJ of *S. feltiae* from *Galleria mellonella* was observed with White traps than with modified Baermann trays. Maximum juveniles of *S. feltiae* emerged at 50 IJ dose followed by 100 IJ. The emergence of IJ decreased significantly at the dose of 200 IJ and then increased with 400 IJ dose. The minimum number of juveniles emerged at the dose of 200 IJ. The relationship between inoculation dose and emergence of IJ by using both methods was non-significant. Similarly, with an increase in inoculum dose and time, invasion of host was significantly increased. There was little invasion at the 800 IJ dose even after 4 days whereas with the commercial dose (8,000), nematodes did migrate to the larvae and caused some infection after 2, 3 and 4 days. Greatest invasion took place with the highest dose (80,000) with 8 IJ successfully finding and penetrating the larvae. A positive correlation was observed between dose and time and invasion of the host. It is concluded that application of EPN in cadavers may be appropriate in Pakistan because of the non-availability of industrially produced isolates.

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Authors' Contributions
AMR and TM designed and conducted the research and analyzed the data. SRG supervised the research work. RKR and SIA assisted in writing the manuscript

Key words
Entomopathogenic nematode, Galleria mellonella, Steinernema feltiae, Infective juveniles.

INTRODUCTION

Entomopathogenic nematodes (EPN) are now established as potent biological control agents because the ability to mass produce them has allowed the development techniques of inundative application (Griffin *et al*., 2005). The mass production technique based on fermentation technology is an industrial process (Gaugler and Han, 2002; Ehlers and Shapiro-Ilan, 2005a). Such technologies are not yet available in countries like Pakistan where the use of EPN is in its infancy. In these countries, mass production of EPN for their development and use depends on low technology production techniques using host insects for *in vivo* production (Ehlers and Shapiro-Ilan, 2005b). These techniques are labour intensive and are only feasible where labour costs are low. In Pakistan preliminary field evaluation of EPN is done with *in vivo* produced nematodes in hosts such as *Galleria mellonella* (Rahoo *et al*., 2011). As biological control becomes more prevalent in pest management, it will become increasingly important to anticipate interactions between biological control agents (Kaya, 1990a; Kaya *et al*., 1995; Rosenheim *et al*., 1995). Infective juveniles (IJ) are applied as inundative biological control agents of soil-borne insect pests on a variety of crops (Kaya, 1990b, 2002; Kaya and Gauglar, 1993; Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan *et al*., 2002). The efficacy of such IJ applications may be reduced by interactions with other soil organisms that result in IJ mortality (Timper and Kaya, 1992), inhibition of movement, and (or) prevention of IJ from finding target hosts (Kaya and Koppenhöfer, 1996). The IJ responds to various cues including carbon dioxide (Gaugler *et al*., 1980), faeces (Schmidt and All, 1979), and temperature gradients (Byers and Poinar, 1982; Rahoo *et al*., 2016a, b). Infective juveniles aggregating near false stimuli from non-target arthropods would not immediately infect and

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kill the target host. In this case, non-target arthropods would represent a 'sink' for IJ and would reduce mortality of target hosts due to diminished number of IJ. Therefore, the presence of non-target arthropods in the soil could reduce the efficacy of EPN as a biological control agent. Infective juveniles are typically applied to the soil surface and actively foraging IJ migrate in search of a host.

It was hypothesized that if IJ were applied inundatively, will the successful location and invasion of target host be affected by inoculum density of IJ applied and was the reproduction of nematode within the host density dependent? To test the hypotheses, two experiments were conducted. The objective of the first experiment was to investigate the ability of *S. feltiae* to find a host and to determine how many individuals successfully migrate to and invade the host. Similarly, the relationship between nematode dosage and IJ production is well documented for different nematode species (Zervos *et al*., 1991; Selvan *et al*., 1993; Cabanillas *et al*., 1994; Cabanillas and Raulston, 1996). However, no such data exist for *S. feltiae*; therefore, the objective of second experiment of the study was to assess the reproduction potential of *S. feltiae* in relation to the dosage of IJ applied.

MATERIALS AND METHODS

Nematode inoculum

The stock culture of entomopathogenic nematode *Steinernema feltiae* obtained from CAB International was maintained in the laboratory at the Department of Agriculture, University of Reading, UK. The nematode was mass cultured on last instar larvae of *Galleria mellonella* (Dukty *et al*., 1964). The infective juveniles (IJ) were harvested in White traps (White, 1927) and stored at 10°C. Only one-week old IJ of *S. feltiae* were used in the experiments.

Effect of S. feltiae *on* G. mellonella *infection and emergence of IJ*

Forty late instar larvae of *G. mellonella* weighing 0.28-0.32 g were selected and individual weights recorded. Each larva was placed on a Whatman No. 1 filter paper in a 30 mm Petri dish. Ten larvae each were inoculated with 50, 100, 200 and 400 IJ of *S. feltiae* contained in 0.1, 0.2, 0.4 and 0.5 ml suspension respectively (and the total volume was made up to 1 ml). The dishes were stored in an incubator at 20°C for 4 days in which time all larvae succumbed to nematode infection. Twenty 30-mm-dia. Petri dishes containing 5 g of dry silver sand were prepared and 1 ml of tap water was added. An infected larva (cadaver) from each treatment cohort was added to each dish. There were five dishes for each treatment. The cadaver was supported on a small piece of Netlon and the Petri dishes were sealed. Each of the five remaining infected larvae from each treatment was placed in each small dish (55 × 20 mm) which was used as a modified White trap. All the Petri dishes and White traps were kept in an incubator at 20°C.

One week after inoculation each cadaver was moved on to the supporting Netlon and transferred to new Petri dish containing 5 g silver sand plus 1 ml water. The Petri dishes were then sealed and returned to the incubator. Nematodes that had migrated into the sand from the original dish were extracted as described previously (Rahoo *et al*., 2011). The procedure was repeated every 3 days until no more nematodes were recovered. Each Petri dish was monitored daily to observe when nematodes first emerged from cadavers. To facilitate counting of nematodes, the two groups were evaluated on different days.

Host searching ability of S. feltiae *in the soil*

To study the ability of *S. feltiae* to search host in the soil, four plastic containers (9.7 cm x 4.5 cm with surface area of 80 cm^2) were connected with 9 x 1.5 cm plastic tubes in a radial arrangement. The tubes and container were filled with loam based compost (John Innes No. 2). Prior to use, the compost was tested to confirm absence of other EPN. Eight *G. mellonella* larvae were placed in each of the four radial containers. Three suspensions of *S. feltiae* were prepared and 20 ml of each suspension containing 800, 8,000 or 80,000 IJ were applied respectively to the central container. A control was treated with 20 ml of tap water. The design was set up with three replicates. One container from each treatment was removed after 24, 48, 72 and 96 h. For the 24 and 48 h treatments larval mortality was recorded and live and dead larvae were taken out from the plastic container. The dead larvae were then put in 30 mm Petri-dishes and placed in a freezer and those still alive were placed in 9 cm Petri-dishes on moist filter paper and observed every 24 h up to 72 h. Those in the 72 h and 96 h treatments were also recorded as dead or alive and then individually put in the freezer. At the completion, the cadavers were taken from the freezer; after thawing they were cut with a scalpel and the body contents contained in 20 ml tap water was homogenised for 30 sec in a universal bottle. The homogenate was carefully examined under a dissecting microscope and the number of IJ that had penetrated the larvae was counted.

Statistical analysis

The data were found normally distributed and did not require transformation. All the data were subjected to Analysis of Variance (ANOVA) using GenStat package 2009, (12^{th} edition) version 12.1.0.3278 (www.vsni.co.uk).

The means were compared by Fisher's Protected Least Significant Difference Test at 5%. Standard errors of means were calculated in Microsoft Excel 2007. Data were also subjected to regression analysis. In the first experiment emergence of IJ from cadavers was regressed as the dependent variable with the inoculation dose of IJ as the independent variable for both extraction methods. In the second experiment penetration of IJ into *G. mellonella* was regressed as the dependent variable with the inoculation dose of IJ and time intervals (days) as the independent variables.

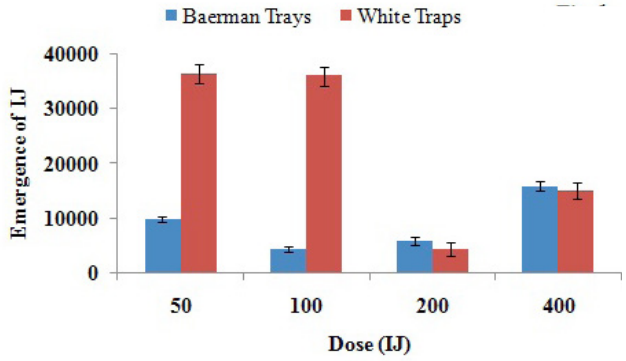

Fig. 1. Effect of inoculum doses on the emergence of infective juveniles of *Steinernema feltiae* from *Galleria mellonella* cadavers and comparison between Baermann extraction trays and modified White traps. Bars are the standard errors of means.

RESULTS

Emergence of IJ from G. mellonella *cadavers as affected by dose of* S. feltiae

There was significantly greater emergence of IJ of *S. feltiae* from the *Galleria mellonella* extracted by White traps than those kept on sand and extracted with modified Baermann extraction trays. For example, at 50 IJ inoculation treatment, 9,975 IJ emerged in the Baermann extraction as compared to 30,200 IJ from the White trap. A similar trend was observed for other inoculum doses (Fig. 1). The relationship between inoculation dose and emergence of IJ by using both methods was non-significant. Maximum juveniles of *S. feltiae* emerged at 50 IJ dose followed by the dose of 100 IJ. The emergence of juveniles decreased significantly at the dose of 200 IJ and then increased with 400 IJ dose. The minimum number of juveniles emerged at the dose of 200 IJ. The relationships between doses and emergence have been shown by regression equations given in Figure 2.

Ability of S. feltiae *to search for host targets in the soil*

The analysis of variance regarding the effects of dosage and time (days) on the searching ability of *S. feltiae* was highly significant (P<.001). At the dose of 800 IJ, few individuals migrated towards the larvae and successfully penetrated. On the other hand, with a dose of 8000 IJ some IJ succeeded in locating and penetrating *G. mellonella* larvae. With an increase in inoculum dose, significantly more IJ moved towards the host and caused infection and was found significantly correlated with the latter (Fig. 3). Similarly, significantly more IJ were successful in locating and invading the host after 3 and 4 days than after 1 or 2 days. At the dose of 80,000 greater numbers of IJ successfully invaded the host and by the 4th day up to 8 IJ had penetrated a larva. The relationship between time interval and host invasion was significantly positive which meant that with an increase in time significantly more IJ migrated to and entered the host as shown in Figure 4.

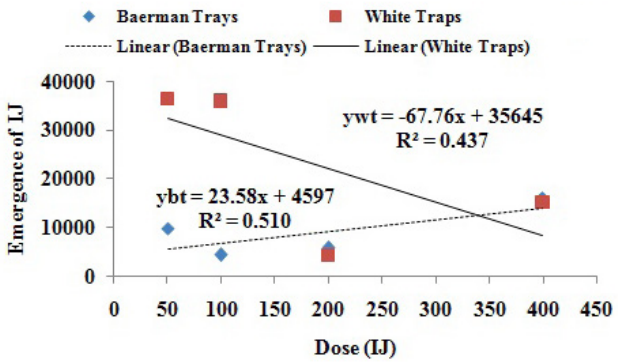

Fig. 2. Relationship between inoculum doses and emergence of infective juveniles of *Steinernema feltiae* from *Galleria mellonella* cadavers using Baermann extraction trays and modified White traps. ywt and ybt represent regression equations regarding emergence of infective juveniles of *Steinernema feltiae* from *Galleria mellonella* with modified White traps and Baermann extraction trays, respectively.

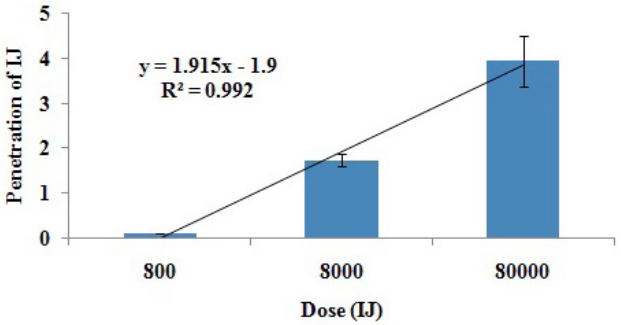

Fig. 3. Effect of inoculum doses on overall penetration of infective juveniles of *Steinernema feltiae* into *Galleria mellonella* and relationship between penetration of IJ and inoculum doses. Bars are the standard errors of means.

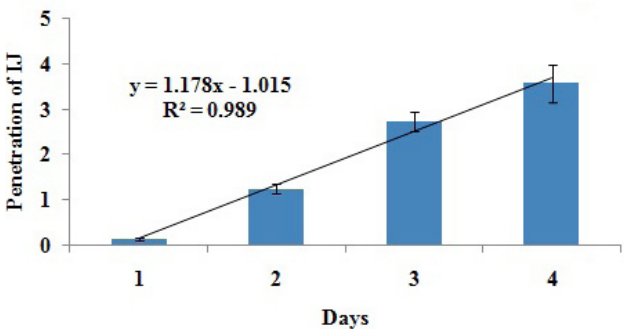

Fig. 4. Effect of time intervals (days) on overall penetration of infective juveniles of *Steinernema feltiae* into *Galleria mellonella* and relationship between penetration of IJ and days. Bars are the standard errors of means.

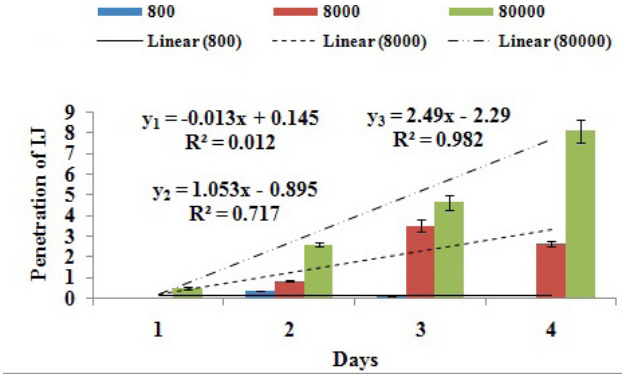

Fig. 5. Effect of each inoculum dose on the penetration of infective juveniles of *Steinernema feltiae* into *Galleria mellonella* at each time interval (day). Bars are the standard errors of means. y_1, y_2 and y_3 represent regression equations regarding penetration of infective juveniles of *Steinernema feltiae* into *Galleria mellonella* at 800, 8000 and 80000 IJ dose.

The interaction between dose and time was also highly significant (P <0 .001). With an increase in dose and time, invasion of host was significantly increased. There was little invasion at the 800 IJ dose even after 4 days whereas with the commercial dose (8,000) nematodes did migrate to the larvae and some infection occurred after 2, 3 and 4 days. Greatest invasion took place with the highest dose with 8 IJ successfully finding and penetrating the larvae (Fig. 5). The relationships between time and invasion at each inoculum dose have been shown by trend lines and regression equations given in Figure 5.

DISCUSSION

For *S. feltiae*, the relationship between nematode dosage and IJ production was non-significant. Similar to the results by Zervos *et al*. (1991), the variability in the number of juvenile production per host was high. *In vivo* production of IJ depends on the nematode dosage applied (Zervos *et al*., 1991; Selvan *et al*., 1993; Cabanillas *et al*., 1994). Entomopathogenic nematode species have different optima for the nematode dosage in relation to IJ production. *Steinernema riobravae* showed highest reproduction rates in pre-pupae and pupae of *Helicoverpa zea* at low application rates of 5 to 25 nematodes whereas *S. glaseri* and *S. carpocapsae* reached optimum reproduction rate in *G. mellonella* after exposure to 100 to 500 and 800 IJs respectively (Zervos *et al*., 1991; Selvan *et al*., 1993).

Results for *Heterorhabditis bacteriophora* are variable and may be influenced by the nematode isolate. Zervos *et al*. (1991) observed highest reproduction for *H. bacteriophora* in *G. mellonella* larvae after exposure to 5 and 25 nematodes whereas Selvan *et al*. (1993) reached optimum IJ production with 800 IJs applied per *G. mellonella* larva.

Differences between nematode species in response to nematode inoculum on production rate might be due to different metabolism rates (Selvan *et al*., 1993) and differences in processing of host tissue by symbiotic bacteria (Akhurst and Smith, 2002). Zervos *et al*. (1991) suggested that decrease in production rate at high inoculum level is due to a crowding effect which affects nematode species differently. At very low application rates production of *Steinernema* species might be negatively affected by their need to find a mate in the first generation. Low nematode density within the host may cause difficulties for nematode to find a mate or possibly only one sex is present within host (Zervos *et al*., 1991). This might be the reason that in the present experiment the numbers of IJ per *G. mellonella* did not exceed those in the Baermann extraction (Fig. 1).

IJ production was not significantly affected by the number of nematodes applied (Fig. 2). Inoculum levels might have been too low to result in a crowding effect within host and consequently lower production levels. Results have proved that any nematode dosage of up to 400 IJ per host can be applied without a detrimental effect resulting in reduced IJ production.

The results regarding ability to find host (Fig. 3) suggest that *S. feltiae* will migrate towards a host however; the chance of a host becoming infected was influenced by the numbers of nematodes applied. The objective was to study how far IJ covered in 24 h to locate and penetrate a target host body. Nematodes may have different behaviour strategies and EPN species often are classified accordingly as cruisers, ambushers or intermediates (Csontos, 2002). On the basis of this definition, Campbell and Gaugler (1993, 1997) designated *S. glaseri, S. feltiae* and *H. bacteriophora*

as cruisers. Predators and parasites use chemical and physical cues when they forage on their hosts. For example by using those cues, *S. carpocapsae* prefers foraging on the soil surface, whereas *H. bacteriophora* would rather forage at the bottom (Gaugler and Kaya, 1990). The lateral migration of *S. feltiae* IJ was also studied by Csontos (2002) who showed that the presence of *G. mellonella* pupae stimulates the movement of *S. feltiae* IJ. Parallel to their results, we also observed that the movement of *S. feltiae* was positively affected by the presence of *G. mellonella* larvae. Furthermore, according to Grewal *et al*. (1994a) the IJ of *S. feltiae*, *S. anomali, S. glaseri, H. bacteriophora* and *H. megidis* showed a positive directional response to host cues. These results confirm that *S. feltiae* have cruiser foraging tactics and showed a greater tendency to move towards the host. According to Grewal *et al*. (1994b), *H. bacteriophora, H. megidis, S. anomali, S. glaseri* and *S. feltiae* are adept at finding distant hosts in sandy soil columns, thus representing the cruising extreme. In order to support the above statement, more studies should be performed with different nematode species. Considering the studies of various researchers and our results the existence of a host and its chemical cues appear to affect a nematode's foraging behaviour (Jagdale *et al*., 2002). In addition, when IJ are placed in the middle of the soil column or container, different species move into different directions. For instance, most of the *H. bacteriophora* and *S. carpocapsae* move upwards rather than downwards, whereas, most of the *S. glaseri* move downwards rather than upwards (Gaugler and Kaya, 1990). It is generally accepted that soil texture and presence of a host affect the nematode's direction of movement (Gaugler and Kaya, 1993). The significance of the relationship between behavioural ecology and biological control potential has been recognised for many years (Lewis, 2002). In order to apply more effective insect pest controls by using EPN, investigation into the searching and foraging strategies of EPN should be performed.

CONCLUSIONS

As EPN have been recovered from soils in Pakistan (Shahina *et al*., 1998), so there is likelihood to exploit local EPN populations that might be adapted to the prevailing environmental conditions. The application of EPN in cadavers (Shapiro-Ilan *et al*., 2003) may be appropriate in Pakistan because of the non-availability of industrially produced isolates. *Steinernema feltiae* was used in these experiments because of its worldwide distribution (San-Blas, 2007). In the surveys of the Sindh Province, Shahina *et al*. (1998) found *Steinernema* spp. frequently suggesting that the genus may be better adapted to Pakistani conditions.

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Conflict of interest statement

We declare that we have no conflict of interest.

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