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Productivity of five entomopathogenic nematodes in *Galleria mellonella* L. and their persistence in soil under laboratory conditions

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Abstract The persistence and reproductive ability of entomopathogenic nematodes (EPN) are important factors to consider in insect biological control agents. Therefore, the present study compared the persistence and productivity of five EPN isolates namely, *Steinernema glaseri*, *S. siamkayai*, *S. carpocapsae* All, *Heterorhabditis bacteriophora* and *H. indica* EPNKU80 in *Galleria mellonella* larvae. The productivity among the EPN significantly varied among the nematode species. Generally, *Heterorhabditis* species produced more infective juveniles (IJ) than *Steinernema* species in the cadaver. The IJ production was highest in *H. bacteriophora* but was not statistically different from *H. indica* (EPNKU80). In contrast, the lowest number of IJ was produced by *S. carpocapsae* All. The persistence test showed that *H. bacteriophora* had the highest penetration ability and had the greatest number of EPN produce inside *G. mellonella* larvae at all exposure times than the other EPN species. In addition, the highest efficacy was observed in *H. bacteriophora* (84.40%) at 15 day after application, which was statistically different from *S. glaseri* (63.30%), *S. siamkayai* (59.10%), *H. indica* EPNKU80 (58.50%) and *S. carpocapsae* All (19.30%). It concluded that *Heterorhabditis* species was higher soil persistence and IJ reproduction than *Steinernema* species which used for insect control.

Keywords: Biological control agent, *Heterorhabditis*, Infective juvenile, *Steinernema*, Soil persistence

Introduction

Entomopathogenic nematodes (EPN) has been used as biological control agents because they exhibit wide host range and are native to many regions throughout the world. Several EPN species from the genera *Steinernema* spp. and *Heterorhabditis* spp. and their symbiotic bacterium, *Xenorhabdus* spp. and *Photorhabdus* spp., respectively, are obligate pathogens of insects in nature (Gaugler and Han, 2002). The two genera of EPN species have been used as effective biological control agents (Stock,

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2005; Abd- Elgawad, 2017). These nematodes have been employed with great efficacy to control soil-dwelling and above-ground insects. (Arthurs and Smith, 2002).

The infective juvenile (IJ) stage of both EPN species is found in the soil and persist there to forage for insect hosts (Bedding *et al.*, 1993; Gaugler and Han, 2002). They do not feed but consume stored nutrient reserves, seek out the host and enter it through natural body openings. They then penetrate into the host's hemocoel and release their associated bacteria that kill the host within 24-48 hr. (Kaya and Gaugler, 1993; Boemare *et al.*, 1996). The bacteria transform the host cadaver into a suitable food source for nematode growth, development, and reproduction. Eventually, the nematodes emerge as IJs when the food resources are depleted and migrate into the soil to search for a new host (Poinar, 1990).

The suitability of EPN as biological control agents for specific target insects depends on their persistence in the soil and their productivity (Tanada and Fuxa, 1989; Mannion, 1992). Persistence is simply a measure of the number of live EPN present in the soil and is distinct from infectivity, which is a measure of the ability of these live IJ to invade an insect host (Curran, 1993). The productivity of EPN has also been shown to differ within target insects (Morris *et al.*, 1990; Mannion and Jansson, 1992) and between hosts within specific EPN species or strains (Morris *et al.*, 1990). Most EPN with higher productivity rates within a specific target host may be more effective in controlling a particular insect under field conditions (Morris *et al.*, 1990).

Productivity is also essential to long-term persistence. Most importantly, biotic factors such as virulence, host finding, environmental tolerance and persistence must be considered when selecting an appropriate EPN for field application success. (Shapiro-Ilan *et al.*, 2002a; Shapiro-Ilan *et al.*, 2006). In addition, factors influencing high quality and efficiency of production have been explored, including medium development, optimization of culture parameters, recovery of IJs, formation of IJs, extraction and harvesting of IJs (Han *et al.*, 1995, 1997).

Therefore, the objective of the present study was to compare the persistence in the soil and the productivity in *G. mellonella* larvae of five EPN species used for insect control in Thailand. The results of this study served as a baseline data to indicate the suitability of EPN species for further development as biological control agents for insect pest management.

Materials and methods

Rearing of the greater wax moth (*G. mellonella*)

Adult males and females of *G. mellonella* were reared in a plastic container (15×21×7 cm) and fed with 10 % sucrose syrup. A 4×21 cm piece

of paper was provided under the lid of the container to serve as the oviposition medium. After 24 - 48 hours, the *G. mellonella* eggs were collected and transferred into a new plastic container containing 115 grams of *G. mellonella* artificial diet (Mohamed and Coppel, 1983) and maintained at 25±2 ℃; 31% RH. The diet was added every two days for three weeks until pupation.

Mass-rearing of EPN

Four EPN species, *S. carpocapsae* All, *S. glaseri*, *H. bacteriophora* and *H. indica* EPNKU80 used in the study were obtained from the stock cultures of the Department of Entomology, Faculty of Agriculture, Kasetsart University, Thailand. The commercial EPN, *S. siamkayai* was obtained from the Department of Agriculture, Ministry of Agriculture, Thailand. All EPN species were mass-reared in last instar larvae of *G. mellonella* (Kaya and Stock, 1997). Two thousand IJ of each EPN were applied as 700 µL droplet into 5 cm diameter Petri dishes lined with double layers of filter paper (Whatman® No. 1). Then, ten *G. mellonela* larvae were placed in the Petri dish and kept in the dark. Three days later, the cadavers were transferred to a modified White trap (White, 1927). The emerging IJs were stored in tap water at 15±1 ℃ for subsequent use in the experiments within two weeks.

Productivity of EPN

The study of reproductive ability was performed in *G. mellonela* larvae. For this purpose, a drop containing 700 µL of each EPN species containing 2,800 IJs was applied into 5 cm diameter Petri dishes lined with double layers of filter paper. Ten last instar larvae of *G. mellonela* were placed in the Petri dish and kept in the dark. Three days after application (DAA), the cadavers were individually transferred in a small aluminum cage (1 cm x 1 cm), and the cages were then placed in plastic cups with 3 cm diameter and 1.5 cm high. Then 0.2 ml of distilled water was added to the cups. Ten replicates (cups) were used for each EPN. After inoculation, the number of emerging IJs moving into the distilled water was daily monitored.

Persistence of EPN in the soil

The persistence experiment was conducted in plastic rearing cups (7 × 9.5 × 5 cm) filled with 50 grams autoclaved soil (no indigenous EPN population was present). Sterile water was added to the soil to achieve a water content of 15.5% (w/v). Each EPN isolate was applied into a cup at a rate of 2,000 IJs/500 µL. The test cup was kept opened and incubated at

25±2 ℃ and 31% RH. Ten last instar larvae of *G. mellonella* were added to the cups as bait for the EPN. Baiting was performed four times at 15-day intervals (1, 15, 30 and 45 DAA). The infected larvae were dissected and the number of IJs per insect was counted under the microscope. The experiment was repeated twice.

Statistical analysis

The experiments were all designed in complete randomized design (CRD). The significance of differences between means was determined using analysis of variance (ANOVA). Comparisons were made using the least significant test (LSD) using the SPSS program version 16.0.

Results

Productivity of EPN

The total productivity of EPN, as indicated by the number of emerging IJ from the insect cadaver (last instar larvae of *G. mellonella*), was significantly affected by the EPN species ($F=2.62$; $df=2, 29$; $P<0.05$). Compared to the other EPN species, the highest number of IJ was produced by *H. bacteriophora* (396,056±3.57), followed by *H. indica* EPNKU80 (300,852±1.67), *S. siamkayai* (257,860±2.00), *S. glaseri* (150,420±2.04), and *S. carpocapsae* All (146,590±1.07), respectively (Figure 1).

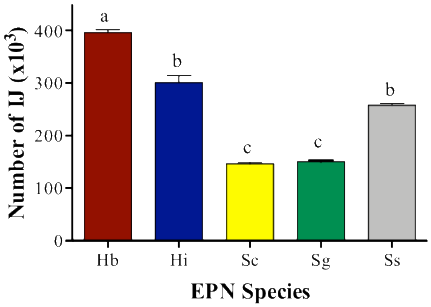

Figure 1. Mean number (±SE) of infective juveniles (IJ) of five EPN species namely, *Heterorhabditis bacteriophora* (Hb), *H. indica* EPNKU80 (Hi), *Steinernema carpocapsae* All (Sc), *S. glaseri* (Sg), and *S. siamkayai* (Ss), recovered from *Galleria mellonella* cavaders. Bars with the same letter are not significantly different according to Least-Significant Difference test (LSD test, $P \leq 0.05$)

The emergence of IJ from the two *Heterorhabditis* spp., *S. siamkayai*, *S. glaseri* and *S. carpocapsae* All began on the 7th, 7th, 10th and 14th day after exposure, respectively. The maximum emergence of *H. bacteriophora* (147,724±3.10), *H. indica* EPNKU80 (105,202±3.37), *S.*

siamkayai (116,108±1.39) and *S. carpocapsae* (99,620±1.31) was recorded on the 14th day; while *S. glaseri* (10,040±1.12) showed maximum emergence on the 10th day (Figures 2A-2B).

On the other hand, the number of emerging IJs of two *Heterorhabditis* spp., *S. siamkayai* and *S. glaseri* decreased on 14th day while *S. carpocapsa* showed a decreasing on 10th day. After 50 days no new IJs were observed on the cadavers in all EPN treatments.

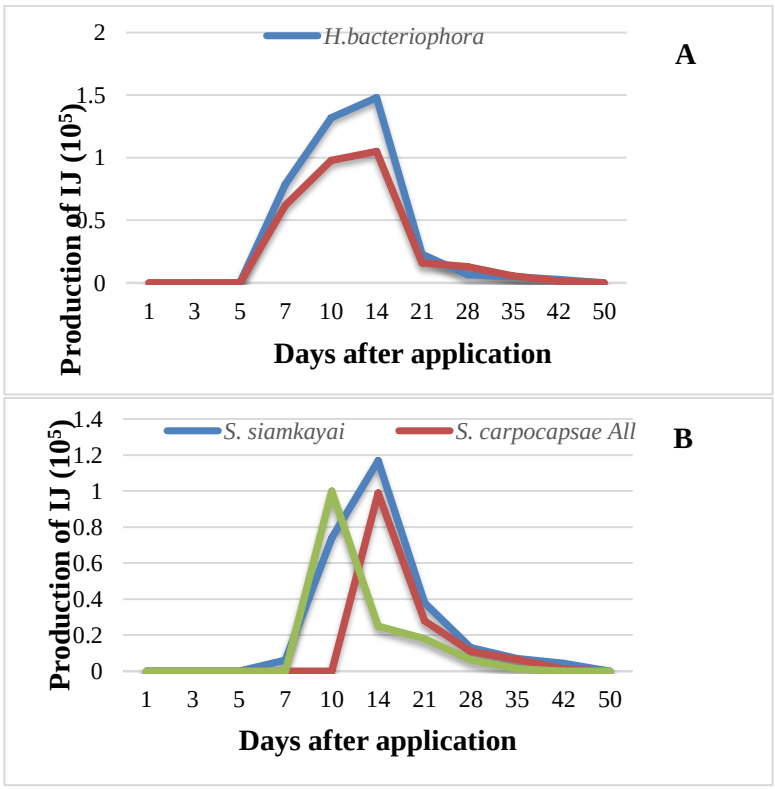

Figure 2. Productivity of *Heterorhabditis* spp. (A) and *Steinernema* spp. (B) recovered from *Galleria mellonella* cadavers using the baiting technique at different time points. (*Heterorhabditis bacteriophora*, *H. indica* EPNKU80, *Steinernema siamkayai*, *S. carpocapsae* All, *S. glaseri*)

Persistence of EPN in soil

The persistence of IJs in the soil was evaluated by the number of adult EPN found inside *G. mellonella* cadavers and the incidence of infected *G. mellonella* larvae. The number of adult EPN found inside *G. mellonella* cadavers on different days after application was recorded by dissecting the insect cadavers and counting the adult EPN under the microscope. EPN infection was observed in all EPN species up to 45 days after application. Significant differences in the number of adult EPN were observed in different EPN species and on different days. For all five EPN species,

significantly (df=4, 16; $P<0.01$) more adult EPN (22-69 adults/insect) were found on the first day after application than at other times (1-11 adults/insect).

On the first day after application, the number of adult EPN was significantly higher (F=470.83; df=4, 16; $P<0.01$) in the two *Heterorhabditis* spp. (55-69 adults/insect) than in the three *Steinernema* spp. (22-45 adults/insect). However, *H. bacteriophora* showed the highest penetration ability and had a greater number of adult EPN inside *G. mellonella* larvae than the other EPN species. However, there were no significant differences between EPN species at 30 and 45 DAA. The number of adult EPN decreased with increasing days after (Table 1).

Table 1. Number of adult EPN inside *G. mellonella* cadavers (mean ±SE) on different days after application under laboratory conditions

EPN Species	Number of adult EPN inside *G. mellonella* (IJs/larvae)			
	1 day	15 days	30 days	45 days
H. bacteriophora	69.20 ±2.92Aa[1/]	11.40 ±0.68Ab	1.40 ±0.24Ac	1.00 ±0.00Ac
H. indica EPNKU80	55.00 ±2.02Ba	5.20 ±0.37Bb	1.00 ±0.32Ac	0.80 ±0.20Ac
S. carpocapsae All	22.20 ±3.17Da	5.40 ±0.50Bb	1.40 ±2.45Ac	0.20 ±0.20Ac
S. glaseri	45.80 ±0.97Ca	9.00 ±0.71Ab	1.40 ±0.24Ac	1.00 ±0.00Ac
S. siamkayai	23.60 ±0.93Da	1.80 ±0.37Cb	1.20 ±0.20Ab	0.60 ±0.24Ab

[1/]Means number (±SE) of adult EPN inside *G. mellonella* in rows and columns followed by the same letter are not significantly different according to Least-Significant Difference test (LSD test, $P \leq 0.05$). Capital letters compare means in columns; small letters compare means in rows.

To demonstrate EPN persistence, the incidence of infected *G. mellonella* larvae was also evaluated. The results at 1 DAA showed that all EPN species had an average efficacy of 80-97% against *G. mellonella* larvae. At 15 DAA, the efficacy of *H. bacteriophora* was found to be still high in suppressing insects, close to the efficacy after one day exposure (84%) and different from other EPN species (F=598.26; df=3, 36; $P<0.01$). For some of *S. siamkayai*, *S. glaseri* and *H. indica* EPNKU80, the efficacy against *G. mellonella* larvae was still above 50%, while *S. carpocapsae* All had the lowest efficacy. However, the efficacy of all EPN species decreased to below 29 % after 30 days of application (Table 2).

Table 2. Persistence of five EPN species in soil detected from infected *G. mellonella* larvae by date of evaluation

EPN Species	Persistence (%)			
	1 day	15 days	30 days	45 days
H. bacteriophora	97.30±0.30Aa[1/]	84.40±1.63Aa	28.70±2.49Ab	9.40±1.79Ac
H. indica EPNKU80	92.40±0.42Aa	58.50±2.00Bb	23.20±2.13Ac	6.60±1.63Bd
S. carpocapsae All	80.40±1.22Ba	19.30±3.48Cb	12.60±2.90Bb	3.20±1.52Cc
S. glaseri	92.90±0.50Aa	63.30±1.52Bb	29.50±1.79Ac	13.20±1.52Ad
S. siamkayai	85.50±0.45Ba	59.10±2.33Bb	27.90±2.13Ac	11.00±1.00Ad

[1/]Means persistence (±SE) in rows and columns followed by the same letter are not significantly different according to Least-Significant Difference test (LSD test, $P \leq 0.05$). Capital letters compare means in columns; small letters compare means in rows.

All five EPN species continue to live in the soil and infect the last instar larvae of *G. mellonella* EPN species until 45 days after application, however the efficacy was very low. Nonetheless, *H. bacteriophora* showed higher efficacy and survivability in soil than other EPN species when observed on 15 days after application. In addition, the EPN species should be re-applied 15-20 days after EPN spraying to further increase the optimum efficacy.

Discussion

The use of EPN for insect control seems to be increasing in Thailand (Somsook *et al.*, 1986). For effective biological control, the selection of the appropriate EPN species in a particular formulation against a specific pest in a specific ecosystem is crucial (Shapiro-Ilan *et al.*, 2002b). In this study, we investigated the persistence in soil and IJ productivity in *G. mellonella* larvae of five EPN species. The results of the study showed that of *H. bacteriophora* exhibited the highest productivity among the EPN species compared, while the least number of IJ was found in *S. carpocapsae.* Similarly, Mannion (1992) found that *Heterorhabditis* spp. produced higher productivity per cadaver infected more new hosts in the soil than *Steinernema* spp. In addition, *Steinernema* spp. and *Heterorhabditis* spp. have distinct life cycles. In *Steinernema* spp., bisexual reproduction is the mode of reproduction for the first generation of adults (Kondo and Ishibashi, 1987; Wouts, 1984), while *Heterorhabditis* spp. reproduce hermaphroditically in the first and sexually in the second generation (Glazer *et al.*, 1994; Zioni *et al.*, 1992). In the present study, *Heterorhabditis* spp. produced more IJ than the *Steinernema* spp., and hence can be used to

control insect pests. (Fateh *et al.,* 2017; Hussian *et al.,* 2016; Kassi *et al.,* 2018).

The results also showed a difference in the time at which the IJ emerge from the host cadavers, as some *Steinernema* spp. emerged earlier from the insect cadavers than *Heterorhabditis* spp. These results were similar to Stock *et al.* (1998) who reported that the IJ of *S. siamkayai* inoculated in blood drops of *G. mellonella* emerged 8-10 days after exposure. This could be due to the larger size of IJ of *Steinernema* spp. than those of *Heterorhabditis* spp. The larger IJ of *Steinernema* spp. would occupy more space inside the cadavers and require more nutrient resources, thus producing fewer offspring. It is known that the emergence of IJ is related to the depletion of food reserves and crowding in the host cadavers (Kaya, 1985, 1987) and possibly build-up of ammonia (San-Blas *et al.,* 2008).

The five EPN species exhibited different patterns of complete nematode reproduction. *Heterorhabditis* species consistently produced more productivity than *Steinernema* species. Patterns of emergence from cadavers of *G. mellonella* were consistent. As mentioned earlier, the emergence of IJs is related to depletion of food reserves and crowding (Kaya, 1985, 1987). These factors may have been less evident in the emergence of IJs of all species from *G. mellonella* larvae. In addition, differences in reproductive potential of EPN species may also be related to isolates, species, and host susceptibility, number of bacteria per infective stage, invasion rate, temperature and humidity (Rahoo *et al.,* 2016a, 2016b, 2017b; Nabeel *et al.,* 2018). Nematode productivity has also been found to vary across nematodes inside target insects (Morris *et al.,* 1990; Mannion and Jansson, 1992) and among hosts within specific nematode species or strains (Morris *et al.,* 1990).

EPN with higher productivity within a specific target host may be more effective in controlling a particular insect under field conditions. Morris *et al.* (1990) found that a high infection rate of soil insects followed by a high reproductive rate is critical to ensure re-infestation of the habitat by EPN progeny. On the other hand, the production of IJ through *endotokia matricida,* commonly describe as the failure of normally oviparous nematodes to deposit their eggs, which may then accumulate and develop in the body of females has received less attention (Morris *et al.,* 1990). It was observed that *H. bacteriophora* and *S. feltiae* occurrence rate of *endotokia matricida* differed in these EPN resulting to difference in the number of IJ produced.

Persistence refers to the quantity of live nematodes in the soil as opposed to infectivity, which refers to the ability of these live nematodes to infect an insect host (Curran, 1993). In general, *Steinernema* spp. appear to survive better than *Heterorhabditis* spp. under laboratory and field conditions (Baur and Kaya, 2001). Surprisingly, in this study, *H.*

bacteriophora showed the highest penetration ability and had a greater number of nematodes inside *G. mellonella* larvae than the other EPN species, whereas the lowest number of EPN inside insect larvae was observed in *S. carpocapsae* All. According to Stuart and Gaugler (1994), the natural populations of *Steinemema* spp. have patchy distribution and the number of nematodes is often very low. These results also showed that the number of EPN species decreased as the time of observation progressed. Molyneux (1985) reported that most data showed a rapid decline in viable nematodes in the first few days after application but after that period the population rate declined rapidly in many laboratory tests under various conditions in sterile soil. The data generally indicate a survival period of weeks, rather than months and a gradual decline in the number of live nematodes recovered (Womersly, 1990).

Other previous studies investigating the persistence of *Heterorhabditis* spp. in soil showed long-term survival. Jansson *et al.* (1991) showed that *H. bacteriophora* (HP88) survived in gravelly loam for 253 days after treatment. This could be due to nematode-specific foraging strategies, the effects of different soil types, or a combination of factors (Kaya and Gaugler, 1993). For example, IJ of *S. carpocapsae* stay near the soil surface due to their sit and wait strategy (Kaya *et al.,* 1993) therefore, cannot escape the warmer and potentially detrimental soil temperatures in the upper 5 cm of soil (Molyneux, 1985; Kung *et al.,* 1991). Laboratory studies have demonstrated *Steinernema* spp. may not be able to escape detrimental soil temperatures in some types due to limited pore diameter (Kung *et al.,* 1991). *H. bacteriophora* dispersed to greater depths in all soil types, in part due to their more active host-search strategies which possibly allow these nematodes to escape warm temperatures in the upper 10 cm of soil. Thus, difference in behavior may be more important factors determining the differences in persistence among EPN species.

Several previous studies indicated that EPN persistence and productivity are important factors for the success of EPN application in field trials and long-term viability also depends on EPN production (Womersly, 1990). It is generally accepted that a laboratory bioassay that predicts success of EPNs in the field is needed to simplify EPNs selection in biological control programs. (Hominick, 1990; Mannion, 1992).

We conclude that the persistence of *H. bacteriophora* and *S. glaseri* in soil is longer than that of the other nematode species and that *Heterorhabditis* spp. produced more IJ than *Steinernema* spp. in *G. mellonella* larvae. Based on the overall results, we recommend the use of these EPN species as biological control agents with 15 days re-introduction. Moreover, future studies should focus on field conditions for these nematode species against other insect pests.

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