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Isolation and Molecular Characterization of Entomopathogenic Nematode (*Steinernema Siamkayai*)

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Abstract: ***Entomopathogenic nematodes Heterorhabditis and Steinernema together with their symbiotic bacteria Photorhabdus and Xenorhabdus respectively, are obligate and lethal parasites of insect. EPN provide effective biological control of some important pests of commercial crops. A survey was conducted to preserve indigenous Entomopathogenic nematodes in various agricultural fields at Ghaziabad and Meerut district of Uttar Pradesh, India. A total of 50 soil samples were collected, 3 soil samples were detected positive using the G. mellonella baiting technique. Identification and confirmation of species was done with molecular studies using PCR, cloning and sequencing of the ITS region of genomic DNA. In the BLAST analysis all three samples were match with Steirnernema siamkayai. The effect of temperature and moisture on the infection of G. mellonella by Steirnernema siamkayai.were determines. 25 to 30^{0}C temperature were essential for achieving 100% mortality at 48h and 10% moisture level was observed 35% mortality after 48h. The efficacy of both G. mellonella and Maladera insanabillis were observed at 36h. 100% mortality was observed at 100IJs/ G. mellonella and 50% mortality at 150IJs/ Maladera insanabillis.***

Keywords: BLAST, Cloning, Entomopathogenic nematode, Phylogenetic tree *Steirnernema siamkayai*.

1. Introduction

A Survey was conducted to preserve indigenous EPN species or strains because different nematode species and strains exhibit differences in survival, search behavior and infectivity, which makes them more or less suitable for particular insect -pest control programs (Del Pino and Palomo,1996). Entomopathogenic nematode (Rhabditida: Steinernimatidae and Heterorhabdidea) are insect pathogens with great potential for biological control (Kaya, H.K & Gaugler, R 1993, Kaya, H.K. 2006). Chemical treatment against these soil-inhabiting pests have not always been successful in part because of development tolerance or resistance by the host insect and pesticide degradation by soil microorganisms (Ng and Ahamad 1979). They retain many positive aspects, such as their broad host range, safety to non target organism, highly virulent, capable of being culture in vitro and exemption from environmental registration in many countries. Successful use of Entomopathogenic nematodes in soil applications depends on various biotic and abiotic factors. A number of these factors can be controlled and optimized to improve efficacy (Shapiro-Ilan et al. 2006). For example, a biotic factor that can enhance efficacy is the choice of nematode species or strain in relation to the target host (Shapiro- Ilan et al. 2002, 2006a). in terms of biotic factors, application under favorable soil moisture levels is critical (Georgis and Gaugler, 1991, Kaya and Gaugler, 1993). Additionally, efficacy can be enhanced through improved formulation or additional of adjuvants (Wright et al. 2005; Shapiro-Ilan et al. 2006a).

2. Materials and Method

Isolation of Entomopathogenic nematodes

A total of 50 samples were collected from Ghaziabad and Meerut district of Uttar Pradesh, India. Sampling was done by taking soil samples from five random sites at the depth of 8 to 10 cm and the all five subsamples were bulked to compose final samples of about 1kg. The sample were labeled with coding and kept laboratory in BOD at 8-15 ^{o} C for storage purpose. The presence of insect pathogenicity of nematodes was tested by baiting with wax moth larvae *Galleria mellonella* (Bedding and AKHURST 1975).

The soil sample was mixed properly and 4th instars stage *G.mellonella* larvae were placed in the bottom of plastic containers which were then filled with 250 g of soil sample. The containers were covered with muslin cloth for ventilation and kept for incubation in dark place in BOD at (±) 28^{0}C temperature and 85% relative humidity**.** *G.mellonella* larvae were evaluated daily for their viability, if any larvae were found dead, then dead larvae were removed from soil and wash 3 times with distilled water. Cadaver placed in a petriplate on moist tissue paper while live larvae were placed in the bottom same soil container. This test was repeated for 10 days. Collected dead larvae were incubated individually in 'White trap' (White 1927) which consisted of a plastic filled with distilled water to a depth of 1 cm. The bottom of an inverted petri dish was placed in the plastic container. A sheet of filter paper to come in contact with the water. The larvae were placed on the filter paper and incubated at 28^0 C until all nematode progeny had emerged and moved the down into the water of the container. The harvested nematodes further used to *Galleria mellonella* larvae to confirm Koch's postulates for pathogenicity as describes by Kaya and stock (1997). To confirm their pathogenicity to insects, the infective juveniles were transferred onto moist filter paper in petri dishes to living *Galleria mellonella* larvae were added. The new generation IJs were collected in a beaker and rinsed 3 times

with sterile distilled water and stored at 11^{0}C in a tissue culture bottle.

DNA Extraction of entomopatthogenic nematodes

Genomic DNA was extracted from IJs using a modification of the method of Joyce et al. (1994). Centrifuge the nematode suspension and take in an eppendorf tube. 50µl of lysis buffer (0.5M KCl,0.5M Tris Cl, 0.5M $MgCl_2$, 1M DTT, 4.5% Tween 20, 2% Gelatin) and 10-20 grains of sterile sand were added and crush with the help of grinder glass rode. After that 20µl of proteinase K were added and then tubes were incubated at 65^{0}C for 1h, 95^{0}C for 10min. and snap cool for 10 min. in ice. Centrifugation the tubes for 13,000 rpm for 7 minutes. Take 5ul of the DNA suspension for PCR reaction.

PCR Amplification, Cloning, sequencing and phylogenetic analysis

The reaction was set up on ice and 5µl of DNA sample were used in PCR. In PCR reaction 5µl of 10X buffer with $MgCl_2$, 1µl of dNTPs (10mM),1µl (10mM) of each forward and reverse primer , 0.5µl of Taq-polymerage, and make a final volume 50µl with added of 36.5µl of nuclease free water. The forward (5'ATATGCTTAAGTTCAGCGGGT 3') and reverse (5'GTTTCCGTAGGTGAACCTGC 3') primers were used in the PCR reaction for amplification of the complete ITS region. The cyclic conditions were as following 4 min. at 94^{0}C for denaturation, 35 cycles of 60 sec. at 94^{0}C, annealing 90 sec at 54^{0}C, extension at 72^{0}C for 2 min. and incubate 10 min.at 72^{0}C for final extension then store for 4^{0}C. PCR product was separated by 1% agarose gel electrophoresis and images were obtained in U.V-transilluminator. The PCR products were cloned into P$^{GEM®-T}$ Easy Vector System (Promega catalog no. A1360) and transformed into the E.coli DH5∝ competent cell according to the instruction of the manufacture. After selection of transformed colonies, plasmid DNA isolation was performed using plasmid DNA minispin kit (chromous Biotech (P) Ltd) and digested by restriction enzyme (EcoRI) to confirm whether the gene was successfully cloned into the vector or not. Positive clones were send to the sequencing. The obtained sequence of *Steinernema* isolates were compared with sequence of *Steinernema* species available in GenBank (NCBI). The DNA Sequences were edited using BioEdit (Hall.1999) with sequences of related species and new isolates. The evolutionary relationship of the isolated with 21 species/srains *Steinernema* were evaluated (Swofford, 2002**)**. Phylogenetic (Neighbor joining) analysis the ITS sequence data were done using MEGA fig.1 (Tamura et.al. 2007).

Temperature effect

To study the effect of temperature on the infective juveniles in *Galleria mellonella* larvae, 60 fifth instar larvae were used in replications. The infective juveniles were cleaned three times with distilled water and kept at 15 – 45^{0}C for 6 h prior to testing at each temperature. Nematode suspension was then shaken and 250 µl was pipette and put in each one of the multiwall plate onto the double layer of Whatman No.1 filter paper, following the methods of Kaya and Stock (1997) in concentrations of 100 IJs per larva. A single larva was transferred into each well and plate was covered with lid and sealed paraffin film. The plates of each concentration was incubated at 15,20, 25,30, 35, 40 and 45^{0}C. The mortalities were checked after 24, 48 and 72h.

Fifth instar larvae of *Galleria mellonella* were exposed to 100 IJs concentration of EPN species at 15, 20,25,30, 35, 40 and 45^{0}C in multi well dishes. The dead larvae were removed from dishes and rinsed three time deionized water and transferred individually on White trap. The emergence of IJs were collected daily up to a period of 20 days, till the emergence of IJs was stopped from insect cadavers. The total number of IJs produced per larvae was determine. Nematode concentration there were 6 replicates and the experiment was repeated thrice. Untreated controls were identical to the treatment except that no IJs were added.

Soil moisture effect

To optimized the soil moisture in a laboratory bioassay on the *Galleria mellonella* larvae. The native EPN species was used in the bioassay. Fifth instar larvae of *Galleria mellonella* were exposed to 100 IJs concentration of EPN species. Experiment was conduct in multi well dishes. One hundred IJs in 1000 µl of water were placed on the bottom of each well and filled with oven dried 250gm soil which was premoistured. Final soil moisture 2,4,6,8,10,15,20 %. After the soil was compacted by tapping the plate, one *Galleria mellonella* larvae was placed on the surface of the soil in each well and plate were covered with lid. Multi well dishes put on the BOD at 28±2^{0}C and maintain the moisture. Larval mortality was recorded after 48 h.

***G.mellonella* Larval mortality and reproduction bioassay**

Larval mortality bioassays were carried out in multi-well dishes lined with one layer of Whatman No. 1 filter paper, following the methods of Kaya and Stock (1997). Nematodes in 250 µl of concentrations of 10, 20, 40, 60, 80, 100 IJs per larva. After 30 min, a single larva of *Galleria mellonella* was placed in each of multi well dishes. For each concentration there were 6 replicates and the experiment was repeated thrice. Untreated controls were identical to the treatment except that no IJs were added (Arun 2012). The multi well dishes were incubate in BOD at 27 ± 2^{0}C. Larval mortality was checked after 36 h. Fifth instar larvae of *Galleria mellonella* were exposed to 100 IJs concentration of EPN strains in multi well dishes. The dead larvae were removed from dishes and rinsed three time deionized water and transferred individually on White trap. The emergence of IJs was collected daily up to a period of 20 days, till the emergence of IJs was stopped from insect cadavers. The total number of IJs produced per larvae was the determine. For each nematode strains and concentration there were 6 replicates and the experiment was repeated thrice. Untreated controls were identical to the treatment except that no IJs were added.

***Maladera insanabillis* larval mortality**

Larval mortality bioassays were carried out in multi-well dishes lined with one layer of Whatman No. 1 filter paper, using Kaya and Stock (1997) method. For EPN species 20,40,60,80,100,150 doses were applied on the larva of *Maladera insanabillis*. The experiment was repeated thrice and three replicates were done. Untreated controls were identical to the treatment except that no IJs were added (Arun 2012). The petri-dishes were incubating in BOD at 27 ± 2^{0}C. Larval mortality was checked after 36 h.

3. Statistical Analyses

Statistical analyses were performed SPSS 22.0 programme. The percentage of *Galleria mellonella* mortality was arcsine transformed before statistical analysis. One-way ANOVA was used for the analysis of soil moisture. Two-way ANOVA was used for analysis of temperatures effect with various times (24, 48, 72hrs). The values of lethal dose and lethal time were determined by probit analysis with SPSS.

4. Resul.Ts

Isolation and molecular characterization

The sample collected from different cultivated lands showed positive for the presence of the Entomopathogenic nematode of the genus *Steinernema.* A total of 50 collected soil samples, EPN were recovered from three samples. Suitable quantity of purified total DNA template was extracted from the lysate of crushed nematode isolates and DNA template was yielded from each extraction. The ITS region of genomic DNA from the three unidentified EPNs using the forward and reverse primers were identified the PCR products were 804 base pairs. The purified PCR- amplified products were cloned and sequenced. The sequences from multiple clones were successfully obtained from PCR-amplicon of different samples. All three samples in the BLAST search indicate similarity between *Steirnernema siamkayai.* The evolutionary relationship of the isolates and other closely related species/strains were evaluated. The phylogenetic analysis of the ITS genomic DNA sequence data placed *Steinernema* isolates in a clade with other isolates of *Steinernema* species/strains.

Temperature effect

After 24h mortality of *Galleria mellonella* of caused by *stainernima saimkayai* was grater at 30 and 35^{0}C than 20, 25, 35, 40^{0}C ($F_{4,30}$=11025.556,$F_{2,30}$=11326.667,$F_{8,30}$=1357.222, df = 8, 30, P=.000). After 48h, however mortality increased to 100% at 25, 30^{0}C and 70% at 35^{0}C, 10% at 20 and 40^{0}C. after 72h. 90% mortality at 35^{0}C, 50% at 20^{0}C and 20 % at 40^{0}C. Emergence from *Galleria mellonella* earlier at the intermediate temperature (25, 30, 35^{0}C) than at 20, 45^{0}C. The no. of IJs that emerged per host was grater at 25,30,35^{0}C than 20, 40^{0}C (F= 516.623, df=5,10, P= .000). No mortality present and IJ emerged at 15 and 45^{0}C.

Moisture effect

The moisture effect detected in the test of *Galleria mellonella* larvae. The mortality was significantly increased as moisture levels increased. Lower survival was observed at 10% moisture level after 24 hrs. than 2, 4, 6, 8, 15 and 20%. The higher survival was detected in 2% moisture level.

Efficacy of *Galleria mellonella* and *Maladera insanabillis*

The larvae of *Galleria mellonella* and *Maladera insanabillis* both found susceptible to the test EPN. The degree of susceptibility of insect larvae to nematode infection varied according to the dose of their infective juvenile as well as the exposure period. At 36h 100% mortality were observed at 100 IJs/*Galleria mellonella* and 50% mortality were found at 150 IJs/*Maladera insanabillis.*The higher progeny production of *Galleria mellonella* at 100IJs.

5. Discussion

EPN were recovered 3 out of 50 of all samples taken, the recovery of three *Sternernima* species clearly shows the incredible richness of EPN species in this state. Based on these results we think it likely that more indigenous new species will be discovered in future surveys. These native isolates may prove more effective in controlling important agriculture pests then exotic EPN isolate. Pests of economically important crops in this region may be more susceptible to these native EPN, providing the opportunity to develop and implement these entomopathogens in biological control or integrated pest management programmes in this country.

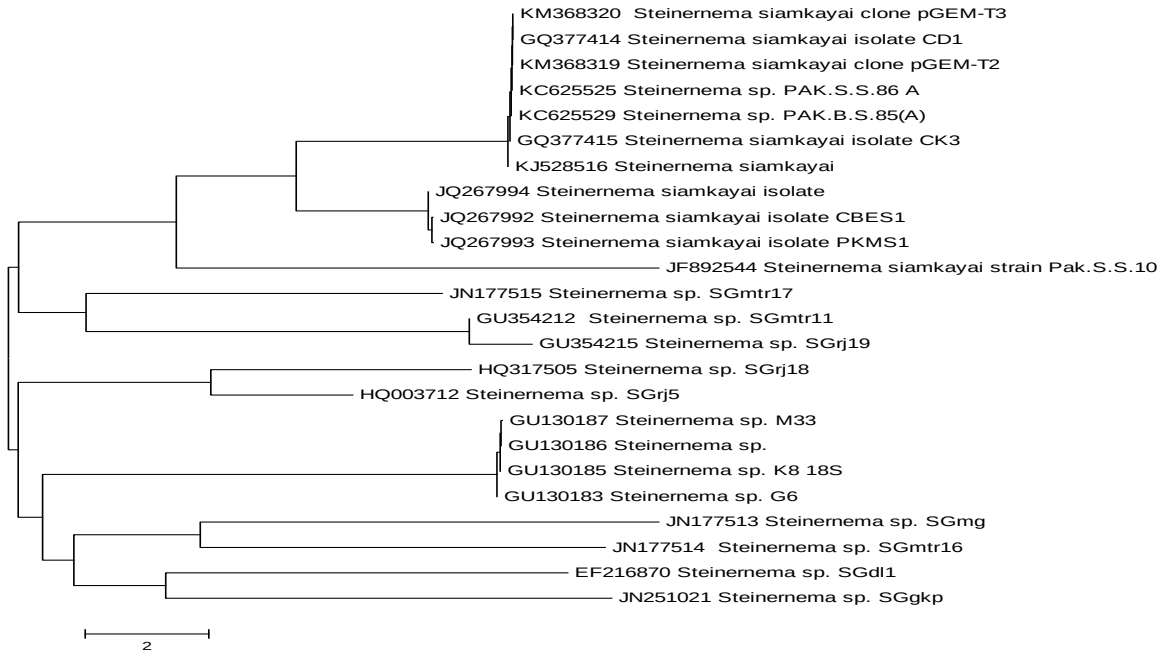

Figure 1: Phylogenetic relationship of *Steinernema* species based on ITS region by Neighbour-joining method

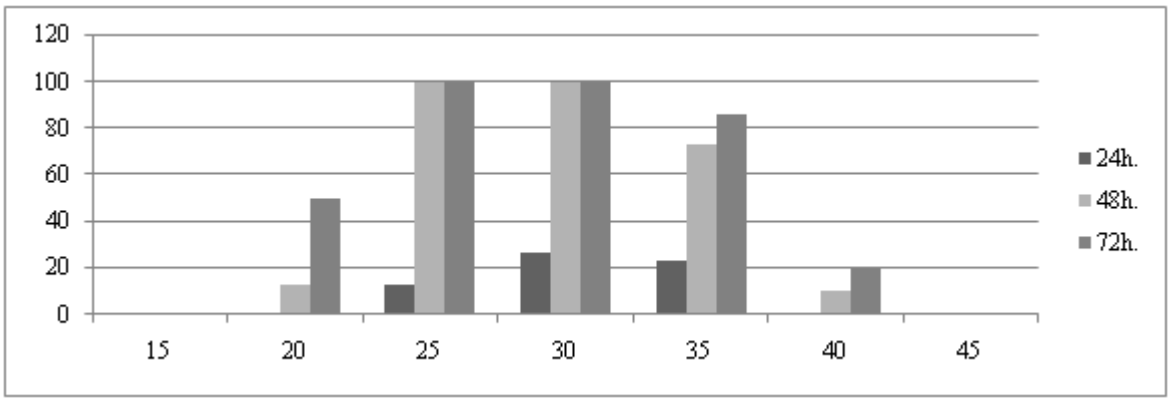

Figure 2: Mortality percentage of *Galleria mellonella* larvae following exposure time interval to different temperature of infective juveniles (IJs) of *Steinernema siamkayai*

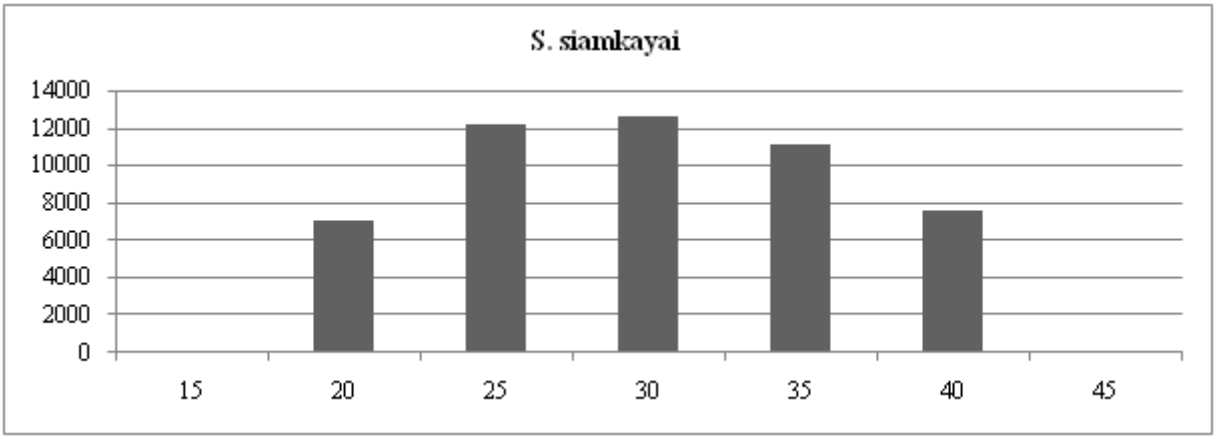

Figure 3: Progeny production by *Galleria mellonella* larvae at different temperature of infective juveniles (IJs) of *Steinernema siamkayai*.

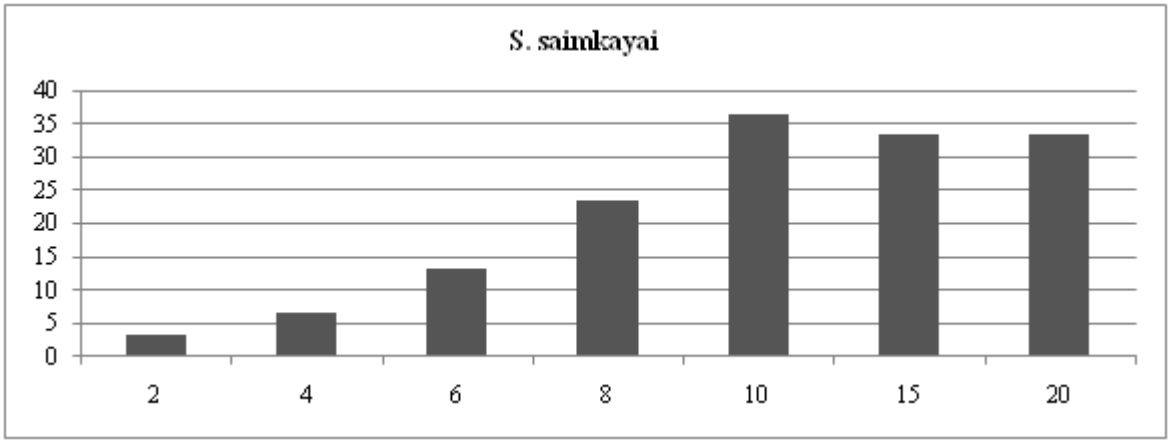

Figure 4: Mortality percentage of *Galleria mellonella* larvae following soil moisture of infective juveniles (IJs) of *Steinernema siamkayai*

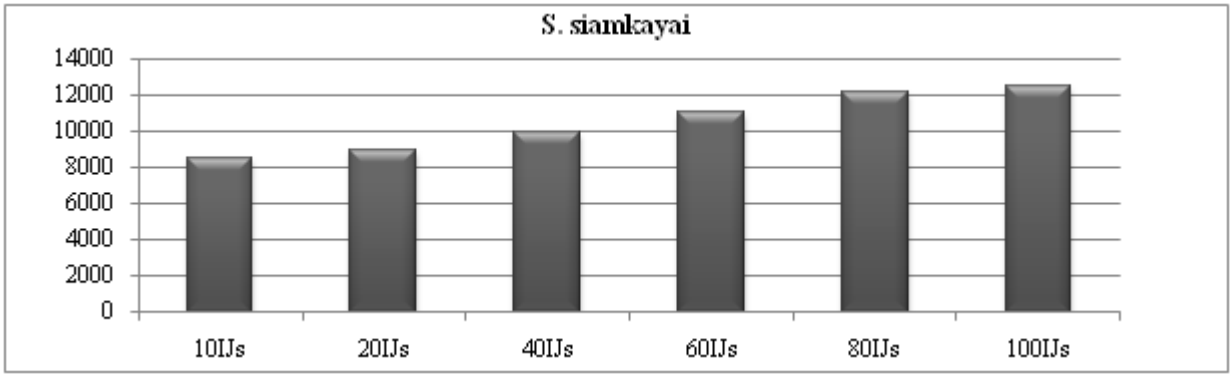

Figure 5: Progeny production by *Galleria mellonella* larvae at different dosages of infective juveniles (IJs) of *Steinernema siamkayai*.

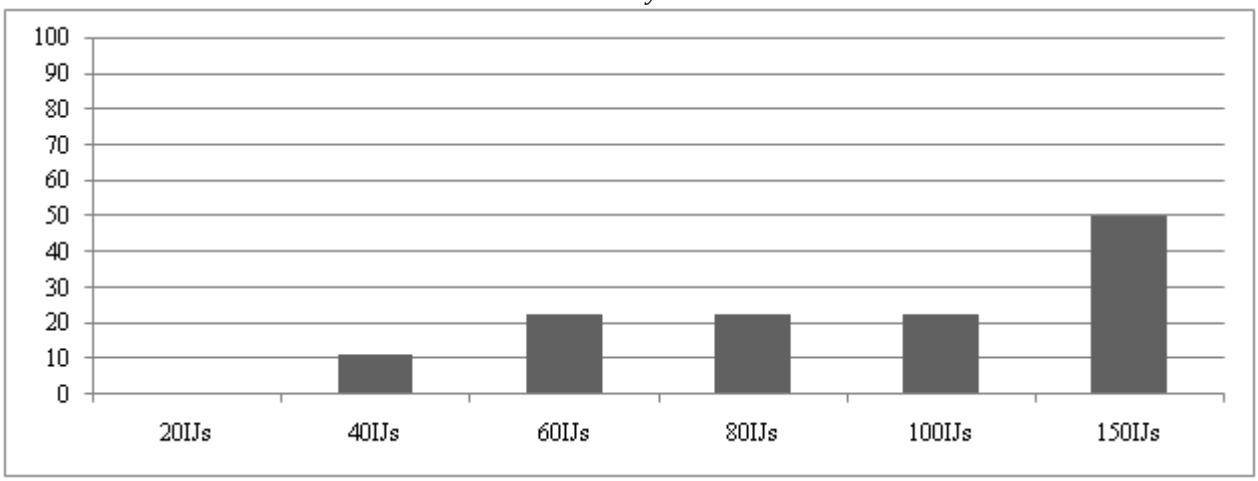

Figure 6: Mortality percentage of *Maladera insanabillis* larvae following exposure time interval to different concentrations of infective juveniles (IJs) of *Steinernema siamkayai*.

Table 1: LT_{50} values calculated from dosage response *G.mellonella* assays conducted with *Steinernema siamkayai*.

Dose	**LT_{50}*G.mellonella***	**LT_{90}*G.mellonella***
10IJs	58.063 (52.362-70405)	79.978 (67.356-142.529)
20IJs	50.566 (48.543-57.789)	72.559 (64.197-93.343)
40IJs	50.004 (46.197-55.373)	68.284 (60.295-88.274)
60IJs	41.811(34.362-51.288)	72.842 (57.918-138.237)
80IJs	33.649 (28.495-38.806)	44.672 (38.748-62.803)
100IJs	24.275 (23.564-24.965)	29.109 (28.076-30.528)

Table 2: LT_{50} values calculated from dosage response *Maladera insanabillis* assays conducted with *Steinernema siamkayai*.

Dose	LT_{50}*Maladera insanabillis*	LT_{90}*Maladera insanabillis*
20IJs	57.223 (51.943-66.931)	78.743 (67.207-125.400)
40IJs	53.070 (49.450-57.688)	72.664 (64.971-90.002)
60IJs	49.664 (43.728-57.417)	67.692 (58.257-103.779)
80IJs	47.318 (42.802-52.416)	64.438 (57.031-83.266)
100IJs	43.431 (38.864-48.264)	62.755 (55.033-81.019)
150IJs	37.418 (33.982-40.651)	51.555 (46.700-60.700)

Table 3: LC_{50} and LC_{90} values calculated at 36h.

Selected incubation hour	LC_{50} (Confidence limit 95%)		LC_{90} (Confidence limit 95%)	
36 hr.	*G.mellonella*	*M. insanabillis*	*G.mellonella*	*M. insanabillis*
	69.350 (-)	164.886 (116.300-446.694)	177.659	615.051 (286.840-8297.163)

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