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Entomopathogenic nematode- *Heterorhabditis indica* and its compatibility with other biopesticides on the Greater wax moth- *Galleria mellonella* (L.)

M. Sankar[a *], V. Sethuraman[b], M. Palaniyandi[b] and J. S. Prasad[a]
[a] *Directorate of Rice Research, Rajendranagar, Hyderabad, Andhra Pradesh-560001, India*
[b] *Department of Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil Nadu-620024, India*
shankarms10@gmail.com*, sethuramanbio@gmail.com, pmkpalani@gmail.com, jsprasad24@yahoo.com

Abstract: Pathogenic effect of an indigenous entomopathogenic nematode, *Heterorhabditis indica* and commercial biopesticides of three fungal pathogens (*M. anisopliae*, *B. bassiana* and *T. viride*), one antagonistic bacteria (*P. fluorescence*), and two neem based biopesticides (Neem and Nimor) were tested on the Greater wax moth, *Galleria mellonella* larva under laboratory condition. The efficacy of the biopesticides was tested individually or in combination with *H. indica*. Pathogenic interaction on *G. mellonella* larva by *H. indica* and biopesticides was assessed at every twelve hour interval after storage. Significant differences in the percentage of larval mortality were determined among the biopesticide treatments. When tested in isolation, *B. bassiana* imposed greater mortality on host larva (40%) when compared to other biopesticides; while *P. fluorescence* and *H. indica* combination proved to be the most efficient causing 100% mortality on *G. mellonella* after 24 h of storage. Progeny produced by *H. indica* on single *G. mellonella* was found to be more (140108 IJs/larva) in the combination treatment with *T. viride*. Pathogenicity influence of *H. indica* when exposed with other biopesticides on host larva, have proved to be more virulent and compatible. The results on pathogenicity of entomopathogenic nematode- *H. indica* on *G. mellonella* larvae are a novelty in the field of biological control. Understanding the interactions between entomopathogenic nematodes and other soil microorganisms may be the key for success in IPM programme.

Keywords: *Heterorhabditis indica*, *Galleria mellonella*, biopesticides, biological control.

Introduction

Insecticides such as viruses, bacteria, fungi, protozoa, and nematodes can offer effective alternatives to replace chemicals under IPM programme. The greatest strength is their host specificity as most are essentially nontoxic and non-pathogenic to wildlife, humans, and other non target organisms. Several microbial pathogens such as; *Bacillus penetrans, Pseudomonas fluorescence, Bacillus thuringiensis, Trichoderma viride, Beauveria bassiana* and *Metarhizium anisopliae* have been developed and being commercialized to control various economically important crop pests and diseases (Ignacimuthu, 2008). Number of farmers use neem (*Azadirachta indica* A. Juss; Meliaceae) as pesticidal, antifungal, and antifeedant agent. The opportunities for using entomopathogenic nematodes against insect pests in the soil and cryptic habitats in agricultural pest are excellent (Gaugler, 2002). Entomopathogenic nematodes appear to be compatible with many herbicides, fungicides, acaricides, insecticides, nematicides (Georgis & Kaya, 1998; Rovesti & Dese, 1990) azadirachtin (Stark, 1996), *Bacillus thuringiensis* (Kaya *et al.,* 1995). On the other hand, synergistic interaction between entomopathogenic nematodes with various insecticides (Koppenhofer *et al.,* 2000) and pathogens (Thurston *et al.,* 1994; Koppenhofer *et al.,* 1999) has been observed. Our focus in this paper is on an indigenous entomopathogenic nematode- *Heterorhabditis indica* associated with mutualistic bacteria- *Photorhabdus luminescence* complex exposed together with other biopesticides (*M. anisopliae*, *B. bassiana* and *T. viride*, *P. fluorescence,* Neem and Nimor) at various periods, how that works together as a biological control unit to kill an insect larva- *G. mellonella* used as a common host for all the biopesticides.

Material and methods

Insect culture

The laboratory common host, Greater wax moth, *Galleria mellonella* (L.) reared on artificial diet as described by Singh (1994) at a constant temperature of 27 ± 2^{0}C and 65 ± 5% R.H. The final instar larvae (25 days old) were utilized for mass rearing of entomopathogenic nematode, *H. Indica* and the experiment purpose.

Nematode culture

Infective juveniles (IJs) of entomopathogenic nematode, *Heterorhabditis indica* Poinar, Poinar *et al*., (1992) isolated from naturally infected larvae of Greater wax moth, *G. mellonella* collected from deserted honey comb in research farm at Directorate of Rice Research (DRR), Hyderabad and, the multiplied IJs were harvested through modified White trap (White, 1927) after incubating 7 to 9 days at room temperature (27 ± 2^{0}C). Freshly harvested IJs were surface sterilised in formalin (0.1%) solution and stored in distilled water with a drop of Triton X-100 (0.5%). Nematode concentration was maintained at 2000 IJs/ml in tissue culture flasks (100 ml/flask) and the

stock suspension was regularly replaced by changing fresh water at 2 weeks interval. The dose of nematodes was prepared either by direct count or dilution count as described by Woodring and Kaya (1988). The stock suspension was concentrated by centrifugation at 500 rpm for 10 min. to settle the IJs and the supernatant was decanted. Further, required dilutions of the suspension were prepared using distilled water.

Biopesticides

The most commonly used biopesticides in the experiments such as; entomopathogenic fungi, *Beauveria bassiana* (Baba) and *Metarhizium anisopliae* (Metarhizium), antagonistic fungi, *Trichoderma viridae* (Nisarga), antagonistic bacteria, *Pseudomonas fluorescence* (Sparsha) and the botanical biopesticides, Neem-3000 ppm (Multineem) and Nimore-1500 ppm (Nimbicidine) (manufactured by M/s Multiplex Agricare Pvt. Ltd., Bangalore, India) was diluted separately in 100 ml nematode (*H. indica* at 200 IJs/ml) suspension to prepare a storage flask and five such kind of replicates were made it to all the biopesticides individually followed by the field recommended concentrations *viz*., *B. bassiana*, *M. anisopliae*, *T. viridae*, (1 X 10^9 spores/ml), *P. fluorescence* (1 X 10^9 CFU /ml) and Neem (3000 ppm) and Nimore (1500 ppm) as procedure given in the label by the company. Each biopesticide was considered as one treatment and each treatment has five replicates. All the flasks were stored for 60 h duration to find out the interaction with nematodes.

Bioassays

Bioefficacy of each biopesticide was evaluated at an every 12 h intervals by inoculating on a final instar larva of *G. mellonella* at 1 ml suspension obtaining from each flask as topical application to the larva released on Whatman's No-1 filter paper lined in Petri dish (9 cm dia.). Each treatment was replicated 20 times and the experiment was repeated twice to confirm the results. Care was taken for accurate number of IJs and other biopesticides while inoculation to the larva. The mean percent mortality of *G. mellonella* larva killed by each biopesticides was recorded. Microscopic observations and the growth of biopesticides on larva was confirmed the result of infection and death. After death, each cadaver was individually transferred to White trap and allowed them to multiply (nematodes or fungi or bacteria if any) for 8-10 days with suitable moisture. The percent mortality of larva caused either by fungi / bacteria / neem or *H. indica* was identified under microscopic observation (Nikon SMZ 800, Japan). The progeny produced by nematodes (if any) was harvested by White trap method and the total recovery of nematodes/larva was worked out. Data were analysed and subjected to ANOVA or factorial analysis of variance (FANOVA) and statistical significance was judged at the level ($P < 0.05$).

Results

Pathogenicity

The level of larval mortality on *G. mellonella* varied and depended on the type of treatment. All treatments gave an excellent lethal effect against *G. mellonella*. Among various biopesticides treatments (12 h of exposure) in isolation, the mortality rate of *G. mellonella* infected by *P. fluorescence* recorded higher (49.42%) when compared to that of *T. viride* (47%) and Nimor (42.28%). When assessed for the combined lethal effect on *G. mellonella* larva caused by *H. indica* and other biopesticides, the synergistic effect of *H. indica* and *P. fluorescent* brought 100% complete mortality in 24 h of exposure. But the IJs of *H. indica* combined with Neem, Nimor and *T. viride* also resulted in 100% mortality but after 48 h of interaction. However, the IJs combination with *M. anisopilae* and, *B. bassiana* required more time (60 h) of interaction to cause the same level of insect mortality (Fig.1).

Fig 1. Percent mortality of G. mellonella larva exposed to various biopesticides

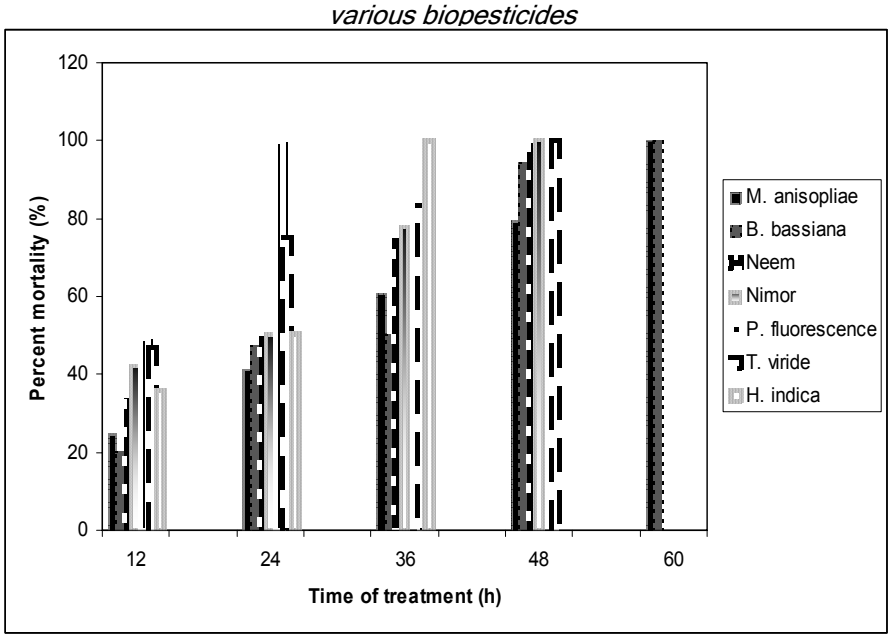
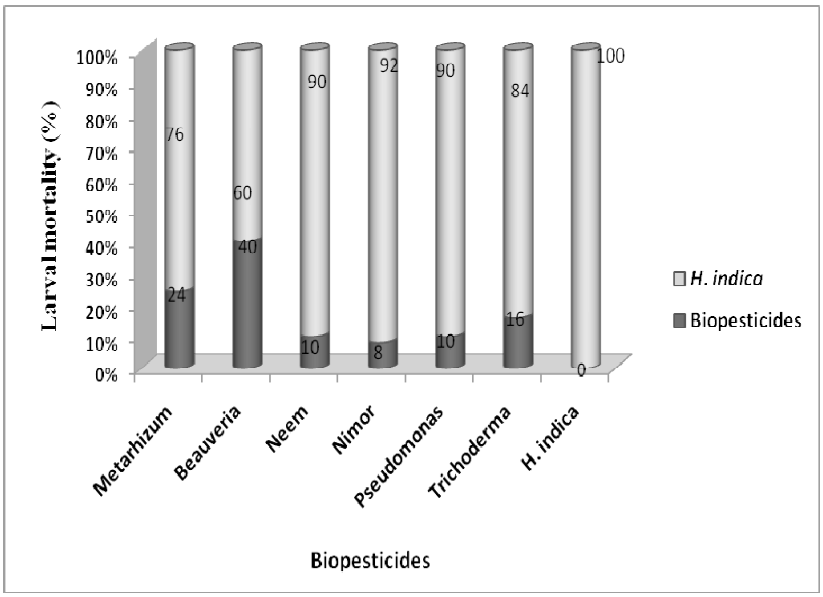


Fig. 2. Percent larval mortality influenced by individual biopesticides treated in combination with H. indica on Galleria mellonella.



The investigation on pathogenicity of entomopathogenic nematode- *H. indica* on *G. mellonella* larvae is a novelty in the field of biological control. Maximum mortality of insects (100%) was also achieved when they were treated with *H. indica* alone in a control treatment. By the individual statistical analysis of all competition bioassay treatments with *H. indica*, a statistically significant influence on larval mortality caused by *H. indica* was found to be more with the combination treatment of Neem (90%), Nimore (92%) and *P. fluorescence* (90%) on *G. mellonella*. No such influence was determined with the other three biopesticides such as; *T. viride* (84*%), M. anisopliae* (76%), and *B. bassiana* (60%) (Fig.2).

Among the biopesticides exposed with *H. indica* and treated to *G. mellonella* larva, the mortality rate influenced by *B. bassiana* was significantly more (40%) than the other treatments (*M. anisopliae*, *T. viride*, and *P. fluorescence*) with increasing the growth of spores and mycelia (24%, 16% and 10% respectively). The percent larval mortality of *G. mellonella* was significantly low by Neem (10%) and Nimor (8%) treated with *H. indica* combination and those cadavers did not support for nematode multiplication thus the death was consider due to neem activities (Fig. 2).

Nematodes recovery

The combined effect of biopesticides- fungi, bacteria and neem treatments with *H. indica* adversely affected the multiplication of nematodes on *G. mellonella* larva. The emergence of infective nematode stages from the nematode-killed wax moth larvae infected by *H. indica* began on day 9th after the host's death and continued for approximately 12-15 days. In control treatment, the recovery from *G. mellonella* larva exposed to *H. indica* alone yielded better results. The recovery of nematodes and the mean number of nematodes emerging from each host larva was ranging from 161559 to 134374 IJs/larva (CD (0.05) =62.85) than they applied in combination with other biopesticides. Among the treatments, *H. indica* and *T. viride* combination resulted in producing the highest number of infective juveniles and ranged from 140108 to 123961 IJs/larva. However, the larvae produced nematodes with *P. fluorescence* combination treatments did not influence the recovery and it was statistically on par ranging from 126080 to 112690 IJs/larva. The results obtained in this study reveal that the total mean recovery of nematodes was significantly less in number and was varied from 91152 to 72638 and from 87598 to 67703 IJs/larva by the combination treatments of *H. indica* exposed in Nimor and *B. bassiana* respectively (Table 1).

Discussion

Pathogenicity

The study provides evidence that all treatments had significant effect on the incidence of the disease ($p \leq 0.05$) and the efficacy of control. The results obtained in the study on percent larval mortality influenced by *H. indica* exposed with *P. fluorescence, T. viride* and Nimor recorded significantly higher efficacy i.e., 49.42%, 47.00% and 42.28% respectively after 12 h of interaction. The pathogenic effect caused on *G. mellonella* larva was completed within 24 h with an interaction of *P. fluorescence* and *H. indica,* whereas in the control treatment (*H. indica* alone) took 36 h to cause the same level of mortality. It is in close agreement with Hara and Kaya (1982) reported that the nematodes are symbiotically associated with a mutualistic bacterium, which allows them to kill their hosts quickly (36 to 48 h) and thus gives them an advantage over other predators, parasitoids and pathogens. The IJs of *H. indica* treated together with neem based pesticides, Neem and Nimor combination took 48 h for 100 % larval mortality. Gaffney *et al*., (2005) investigated that treatment with azadirachtin completely stopped the physiological development of larvae of *Otiorhynchus sulcatus* within 48 h of treatment which showed the growth-disruptive properties of

Table 1. Progeny produced by H. indica on G. mellonella larva exposed in biopesticides.

Time (h) of treatment	*Multiplication of *H. indica* (numbers)						
	M. anisopliae	*B. bassiana*	Neem	Nimor	*P. florescence*	*T. viride*	*H. indica*
12	104534 (305)	87598 (297)	120634 (345)	91152 (287)	126080 (357)	140108 (372)	161559 (400)
24	98580 (298)	79831 (274)	105318 (312)	89328 (280)	122845 (347)	131114 (360)	151093 (385)
36	96682 (310)	74537 (271)	104429 (319)	86605 (290)	118164 (348)	125322 (354)	157967 (397)
48	95234 (312)	71430 (264)	95918 (308)	77747 (276)	115448 (323)	124778 (354)	157450 (397)
60	81477 (286)	67703 (259)	89382 (284)	72638 (266)	112690 (337)	123961 (353)	134374 (347)
CD (0.05) =	78.78	23.16	79.38	70.12	62.18	18.92	62.85

*Figures in the parentheses represent Sqrt transformed. *original values

neem formulations. Zimmerman (1996) and Tkaczuk *et al.,* (2005) reported that all the stages of *Otiorhynchus sulcatus* are susceptible to the entomopathogenic fungi *Beauveria brongniartii* treated along with neem formulations, but the total mortality after different treatments was not statistically different. Among the treatments, in the combined effect between *H. indica* exposed with *M. anisopliae* and *B. bassiana* took maximum of 60 h to extend their complete mortality on *G. mellonella.* Such could be the reason that the interaction between the entomopathogenic nematodes and fungi by tough competitions stress, may loose their infectivity and the larvae may acquire resistant against them.

All groups of pathogens (bacteria, fungi, neem, and nematodes) have shown at least some pathogenicity to *G. mellonella* larva. Investigations found in this study demonstrate the antimycotic substances produced by *H. indica* on associated bacteria, *Xenorhabdus luminescence* may inhibit growth of fungi and bacteria. Gottwald and Tedders (1983) observed < 30 and 6% larval mortality from *B. bassiana* and *M. anisopliae* applications, respectively using relatively high rates (up to 10^7 conidia/g soil) against pecan weevil. It is in support of our investigations that the level of mortality (%) caused on *G. mellonella* by *B. bassiana* was more (40%) than the other treatments (*M. anisopliae (*24%), *T. viride (*16%), and *P. fluorescence (*10%). The larval infection was confirmed with increased growth of fungi/ bacteria after 3 days of infection. Inam-Ul-Haq *et al*., (1997) reported that the antimycotic substances produced by *Xenorhabdus* spp., inhibit the growth of *F. oxysporum* and *F. lycopersici* and the toxins are their most likely mode of action in suppressing the pathogen. The toxic action of most microbial insecticides is specific to a single group or species of insects and this specificity means that most microbial insecticides do not directly affect the other beneficial organisms in treated areas. Data presented in Fig. 1 shows that the entomopathogenic nematode, *H. indica* proved maximum level of larval mortality on *G. mellonella* and proved to be a potential control agent among the biopesticides. In competition bioassay, Shapiro-Ilan *et al.,* (2002) observed >90% suppression of *C. nenuphar* larvae with *S. riobrave* when they treated along with pathogens.

Multiplication

Understanding the interactions between entomopathogenic nematodes and other soil microorganisms may be the key for success in IPM programme. Differences between the reproduction potential of entomopathogenic nematodes may be related to the isolates, species, host susceptibility, number of bacteria proliferating in the host, invasion rate, and environment. It is possible that differences in virulence between species and other biotic factors might be greater for a less susceptible host. The effectiveness of *H. indica* exposed with *T. viride* increased through a synergistic relationship and sub lethal doses of the insecticide causing > 84% of larval mortality and well supported to produce maximum number of yield 129056 IJs/larva varied from 123961 to 140108 IJs/larva on *G. mellonella.* However, the total mean recovery of nematodes was not affected when the IJs exposed upto 60 h in antagonistic bacteria, *P. fluorescent* recording 119045 IJs/larva and varied from 126080 to 112690 IJs/larva. Results of this study is in close agreement aaccording to Poinar, entomopathogenic

nematodes can be reared by *in vivo* methods, with yields of 100,000-200,000 infective stage juveniles per *G. mellonella* larva. However, an average production is much less, from the cadaver infected by IJs exposed in Neem and *M. anisopliae* yielding with an average of 103136 and 95301 infectives per insect respectively and we could not attain such great numbers of nematodes when the IJs exposed in Nimore and *B. bassiana* suspensions (83494 and 76219 IJs/larva respectively). It is in clear understood that biopesticides such as *B. bassiana*, *M. anisopliae* and neem are not supported the host definitely affecting the total number of IJs developing inside the cadaver. In biological-control campaigns, it is vital to know whether releasing one natural enemy against a pest is likely to be more effective than the release of many, especially where competition between enemies might reduce their overall effectiveness (Selvan *et al*., 1993). As Gaugler and Kaya (1990) reported, the combination of 2 nematode species with different search strategies to control 1 or 2 susceptible insect pest species in a soil habitat appears feasible. Indeed, combinations of different nematode species and other biological control agents may increase their overall efficacy against an insect pest (Stiling, 1992).

Conclusion

The benefits of utilizing entomopathogenic nematodes combined with other microbial pesticides can offer better control management IPM programme compared to that of broad spectrum insecticides. Apart from the reduction of non-target impacts, potential benefit as tools for resistance management, safety for applicators, and no re-entry or pre-harvest interval growth are important when a sustainable IPM system is considered.

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