Combined application of two entomopathogenic nematodes, *Heterorhabditis indica* and *Steinernema asiaticum* to control the rice leaf folder, *Cnaphalocrosis medinalis* (Goen.).

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ABSTRACT

In this study, the efficacy of two entomopathogenic nematode species, *Heterorhabditis indica* and *Steinernema asiaticum* were evaluated against the larvae of the rice leaf folder, *Cnaphalocrosis medinalis* and the greater wax moth, *Galleria mellonella*. Combined inoculation of *H. indica* and *S. asiaticum* each at 75 IJs/larva resulted in faster larval mortality on *C. medinalis* (24.6 h) and *G. mellonella* (31 h). The percent larval mortality caused by *H. indica* alone was resulted significantly more (90% on *G. mellonella* and 60% on *C. medinalis*) than *S. asiaticum* (10% on *G. mellonella* and 40% on *C. medinalis*). Progeny produced by *H indica* and *S. asiaticum* was significantly more (84134 and 80458 IJs/larva respectively) on *G. mellonella* than they produced on *C. medinalis* larva (4843 and 4330 IJs/larva respectively). The combined use of entomopathogenic nematodes may offer an integrated approach to increase the efficacy of control of the rice leaf folder, *Cnaphalocrosis medinalis* and it could be a viable component in the Integrated Pest Management (IPM) where other control measures are ineffective or cannot be imposed.

INTRODUCTION

Rice, *Oryza sativa* (Asian rice), is the world's most important food crop second to wheat, feeding over 2 billion people in Asia alone. The major insect pests that cause significant yield losses are stem borer, leaf folder, plant-hoppers and leafhoppers (Inayatuilah *et at.*, 1986; Mahar and Bhatti, 1986; Rehman *et al.*, 1986). Emphasis on biological alternatives to pesticides has increased in agriculture due to concern about environmental pollution and entomopathogenic nematodes (EPN) play an important role in the regulation of rice pest population (Rao *et al.*, 1971).

Nematodes of the families Steinernematidae and Heterorhabditidae are being utilized as the biological control agents for various foliar and soil pests throughout the world (Padmakumari *et al.*, 2008; Choo *et al.*,1989). Considerable variation in the production and infectivity of entomopathogenic nematodes (EPN) and no single species or strain is suitable for controlling all or even most insect species. They are symbiotically associated with mutualistic bacteria in the genus *Xenorhabdus* for Steinernematidae and *Photorhabdus* for *Heteror habditidae* (Boemare, 2002). Thus, it is a nematode/ bacterium complex that works together as a biological control unit to kill an insect host. The mutualistic bacterium propagates and produces substances that rapidly kill the host and protect the cadaver from colonization by other microorganisms.

Choo *et al.* (1989) reported that *S. carpocapsae* and *Heterorhabditis bacteriophora* are very effective against the rice yellow stem borer, *Scirpophaga incertutas* causing 91 % mortality and proved EPN as a potential biocontrol agent in rice eco system. Srinivas and Prasad (1991) reported natural occurrence of *S. carpocapsae* (DD-136) multiplied fast on 4th and 5 th instar larvae of *C. medinalis* causing upto 98% mortality. Evaluation of the potential entomopathogenic nematodes to use as a biological control agent to the target insect pest usually includes an assessment of effectiveness in relation to all developmental stages of the pests. Keeping an economic importance of rice pest management, laboratory studies were undertaken to investigate the combined pathogenic ability of two indigenous EPN species *H. indica* and *S. asiaticum* were evaluated on rice leaf folder, *C. medinalis* and data were compared with a common laboratory host *G. mellonella*.

MATERIAL AND METHOD

Propagation of entomopathogenic nematode species

Two indigenous entomopathogenic nematodes, Heterorhabditis indica (DRR-1) and *Steinernema asiaticum* (DRR-3) were maintained in vivo on final instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) in the laboratory at Directorate of Rice Research (DRR), Rajendranagar, Hyderabad. Infective juveniles (IJs) of

each EPN species was harvested separately by a modified White trap's method (White, 1927) from the cadavers of *G. mellonella* and they were surface sterilized with formalin (0.1 %) stored in distilled water (250 ml conical flask @ 2000 IJs/ml) at room temperature (27-30 °C) prior to use in the experiment.

Insects' culture

The larvae of *G. mellonella* reared on wheat bran and com flour based artificial diet as described by Singh (1994) in the laboratory. The moths of rice leaf folder, *Cnaphal ocrosis medinalis* (Guen.) (Lepidoptera: Pyralidae) were collected in the rice crop (*cv*. TN (1)) grown in DRR rice research form. The adults of male and female were identified based on the wing markings (Khan *et al.*, 1988) and they were sexed, released into Mylar tube covered potted rice plants for egg laying. The neonate larvae hatched from the eggs were cultured on rice *cv.* TN (1) potted plants and the larvae were periodically changed onto another plants at week interval under greenhouse condition. The late instar larvae of both insects were used in the experiments.

Bioassay

Bioassays were carried out to study the pathogenic ability and reproduction potential of two native EPN isolates of *H indica* and *S. asiaticum* onto a final instar larva of *C. medinalis* and *G. mellonella* separately. To determine the bioefficacy, the test insects were separately exposed to nematode species together at different concentration (25, 50, 75 and 100 IJs of each species/larva). The experiment was arranged according to a completely randomized block design (RBD) with fifteen replicated larva of each insects of all the treatments separately. Late instar larvae of each test insect were separately released on Petri dish (4.5 mm. dia.) lined with moist Whatman's No.1 filter paper.

Inoculation of 25, 50, 75 and 100 IJs of each species/larva was counted individually and applied to the larval body as topical application. EPNs exposed larva was then incubated at 28 DC and allowed them to infect for 24 h. All the dead cadavers (if any) and nematodes treated larva was washed separately and transferred to another fresh Petri dish having respective food (artificial diet for *G. mellonella* and two rice leaf cut pieces for *C. medinalis*) and renewed till they survive. Each concentration was considered as one treatment inoculating fifteen final instar larvae of each test insects separately. The experiment was repeated twice to confirm the results with maintaining appropriate untreated controls separately for both the test insects.

Pathogenicity and determination of nematode infection

Larval death was confirmed by touching the head with needlelbrush and assessing for reflexive movement. The nematode infected cadavers of both the tests insects were determined by the following symptoms (i) color of cadaver produced by symbiotic bacteria associated with EPN (*H. indica* associates with Photorhabdus /uminescens which produces red, brick red, yellowish and green and *S. asiaticum* associates with *Xenorhabdus nematophilus* which produces grey or black colour (ii) date of infective juveniles (Us) emergence from the cadaver (*H. indica* emergence mostly on day 8th or 9th after infection and *S. asiaticum* emergence on day 5th or 6th after infection) (iii) size and length of infective juveniles could be evolved by the expertise people (iv) re-inoculation of IJs to host larva to confirm the nematode species. The infective juveniles were harvested through modified White trap's method (White, 1927) and the collected IJs were counted by dilution count method under stereo zoom microscope. Observations were recorded on time (h) taken for larval mortality at 12 h interval, reproductive potential of each nematode species on both the test larva and percent larval mortality caused by each nematodes on test insects were worked out separately. The data on multiplication rate of IJs counts were normalized by square root transformation and percent larval mortality was normalized by arcsine transformation. The significance of differences in strains was determined by analysis of variance (ANOVA).

RESULTS

Identification of nematode infection

Preliminary identifications were determined based on the color of larval cadavers induced by the growth of symbiotic bacteria of nematodes. Color of the cadavers infected with symbiotic bacteria, *Photorhabdus luminescens* associated with *H indica* showed red, brick red, yellowish and green and *S. asiaticum* associates with *Xenorhabdus nematophilus* showed grey or black (ii) date of infective juveniles (Us) emergence from the cadaver infected with *H.indica* took more than 8 days after infection whereas *S. asiaticum* emergence was started on day 5th or 6th after infection (iii) size and length of infective juveniles was evolved by the microscopic observations (iv) re-inoculated both the nematodes species individually on host larva and confirmed the nematode species in compared with control treatment (v) IJs of *H. indica* was identified with presence of dorsal tooth and position of excretory pore under research microscope.

Pathogenicity test

The time taken for 100 % larval mortality by both EPN species varied among the treatments. The larval mortality and differences among the treatment were most noticeable after 31.2 h on *G. mellonella* and 24.6 h on *C. medinalis*

Table 1. Progeny produced by *Heterorhabditis indica* and *Steinernema asiaticum* on *Galleria mellonella* and *Cnaphalocrocis medinalis* larva

Dose IJs of *H.indica* + *S.asiaticum* / larva	*Recovery of EPNs/larva (nos.)			
	G. mellonella		*C. medinalis*	
	H.indica	*S.asiaticum*	*H.indica*	*S.asiaticum*
25 + 25 = 50	83618 (287.9)^{b}	80458 (282.9)^{a}	4017 (63.1)^{c}	2864 (53.9)^{d}
50 + 50 = 100	73143 (272.6)^{d}	66370 (258.2)^{b}	3682 (60.0)d	4330 (65.5)a
75 + 75 = 150	75485 (274.3)^{c}	0 (0.707)^{a}	4843 (69.3)^{a}	4260 (65.3)^{a}
100 + 100 = 200	84143 (288.0)^{a}	0 (0.707)^{a}	4519 (67.3)^{b}	4270 (64.7)^{b}
SEM	3.0224	4.0162	1.403	1.454

* Each value is the mean of fifteen replications
Figures in Parentheses are square root transformed values.
In a column, means followed by a common letter(s) are not significantly different by DMRT (P=0.05)

which received concentration of 200 Us/larva of both EPN species and it was varied from 31.2 to 43.2 h on *G. mellonella* and 24.6 to 48.8 h on *C. medinalis*. Differences in larval mortality caused by both EPN species were considerably non significant among the test insects. The time taken for 100% mortality with 100 and 75 IJs of each species / larva was 24.6 h and 27.6 h respectively for C. *medinalis* and 31.2 hand 40 h respectively for *G. mellonella* larva. However, both EPN species had slow infectivity taking more time treated at 25 and 50 IJs of each species / larva was recorded at 43.2 h and 34.4 h on *C. medinalis* and 48.8 h and 42 h on *G. mellonella* respectively (Fig. 1).

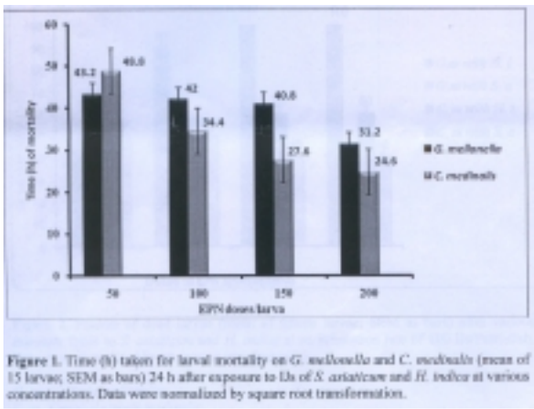

Figure 1. Time (h) taken for larval mortality on *G. mellonella* and *C. medinalis* (mean of 15 larvae; SEM as bars) 24 h after exposure to IJs of *S. asiaticum* and *H. indica* at various concentrations. Data were normalized by square root transformation.

Percent larval mortality

In combined bioassay, the mean percent mortality caused by *H. indica* and *S. asiaticum* at 25 IJs of each species / larva (50 IJs/larva) resulted 50:50 equal percent larval mortality on both the host larva. However, the larval mortality caused with 50 IJs of each species (100 IJs/larva) were found to be significantly more with *H. indica* infection (90%) than *S. asiaticum* infection (10%) on *G. mellonella* and however, it was equal on *C. medinalis* (50:50). The larva received at 75 IJs of each species / (150 IJs/larva), *H. indica* showed significantly highest larval mortality (100%) on *G. mellonella* and (60%) on *C. medinalis* than those infected with *S. asiaticum* on *G. mellonella* (0%) and *C. medinalis* (40%) and similar trend of percent larval mortality was recorded with the higher dose at 100 IJs of each species larva (200 Us/larva) against both the test insects (Fig. 2).

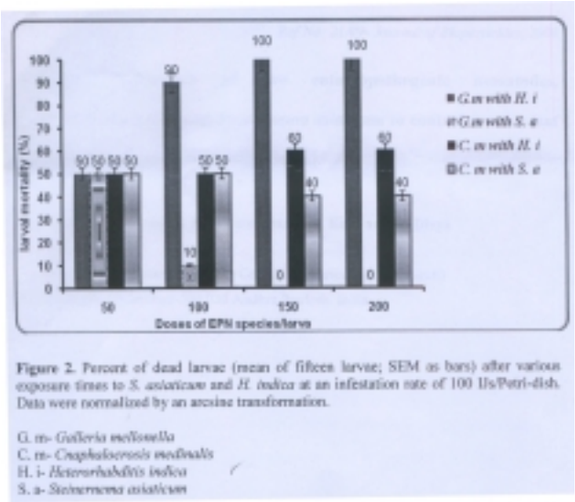

Figure 2. Percent of dead larvae (mean of fifteen larvae; SEM as bars) after various exposure times to *S. asiaticum* and *H. indica* at an infestation rate of 100 IJs/Petri-dish. Data were normalized by an arcsine transformation.

G. m- *Galleria mellonella*
C. m- *Cnaphalocrocis medinalis*
H. i- *Heterorhabditis indica*
S. a- *Steinernema asiaticum*

Progeny production

The mean recovery of IJs of *H. indica* and *S. asiaticum* infected cadavers of *G. mellonella* and *C. medinalis* varied significantly among the test insects. Data presented in the experiment there was no significant differences found among the treatments on both the test insects (Table 1). The maximum number of IJs (84134 IJs/larva) was produced by *H. indica* in *G. mellonella* larva which received inoculum at 100 IJs of each species / larva however; it varied from 73143 to 84134 IJs/larva among the treatments however, on *C. medinalis* it was recorded maximum of 4843 IJs/larva and varied from 3682 to 4843 IJs/larva. Similarly, the highest recovery (80458 IJs/larva) of *S. asiaticum* recorded on *G. mellonella* and varied from 66370 to 80458 IJs/larva and it was less in number on *C. medinalis* (4330 IJs/larva) varied from 2864 to 4330 IJs/

larva (Table 1). Results in the study revealed that highest recovery of nematodes were recorded on *G. mellonella* than *C. medinalis* larva by both the EPN species.

DISCUSSION

The results of this study confirmed that *H. indica* was significantly dominant over *S. asiaticum* when both species were applied together at various dosages. In combined bioassay, *H. indica* caused 100% mortality on *G. mellonella* larvae and it was 60% on *C. medinalis* but no infectivity was found by *S. asiaticum* on *G. mellonella* (0%) and it was equal or on par on *C. medinalis* (40%) larva when they exposed at more than 100 IJs/larva. Results of this study proved that rice leaf folder, *C. medinalis* more susceptible to both nematode species causing equal percentage of larval mortality. Both nematode species showed increasing activity with increasing dose of inoculums. In a similar study, Alatorre-Rosas and Kaya (1990) demonstrated that when two nematode species were placed together in a sand column, *S. feltiae* in the presence of *H. heliothidis* infected more insect hosts only when the hosts were in close proximity to nematode placement. It may be the reason that *S. feltiae* exhibits more virulence than *H. indica* in a sand bioassay study. In the study, combined application of IJs of *H. indica* and *S. asiaticum* at 25 IJs of each species caused equal percentage of larval mortality on both the insects (Fig. 2). It appears that the observed differences in nematode infection among the two species are due to either their mutualistic bacteria *Xenorhabdus* sp. (for *S. asiaticum*) and *Photorhabdus* sp. (for *H. indica*) or their response to environmental conditions such as temperature or moisture. Boemare *et al*. (1993) supports to our results that bacterial infection on larva that without the presence of the symbiotic bacteria in the insect cadaver, the nematodes are unable to kill the insect or to reproduce.

The time taken for larval mortality on *G. mellonella* and *C. medinalis* by EPN species were negatively correlated with increased the dose of inoculum. However, *C. medinalis* larvae were more susceptible than *G. mellonella* in all the treatments except the lower dose (25 IJs /larva). It is in close agreements with Padmakumari *et al*. (2008) reported that lethal time of 36 h was recorded by *H. indica* and 29 h by *S. asiaticum* on *G. mellonella* whereas on *C. medinalis* it was recorded 19.8 h by *H. indica* and 37.8 h by *S. asiaticum* in a separate bioassay study. It is proved that the larvae of *C. medinalis* highly susceptible to EPNs than the other insect pests.

Combined application of *H. indica* and *S. asiaticum* exposed at 50 IJs of each species / larva (100 IJs/larva) was resulted highest percent larval mortality (90 %) only by *H. indica* on *G. mellonella* whereas on *C. medinalis* both EPNs was showed equal percent larval mortality (50 % of each). It was observed that *H. indica* exhibited the highest out competed than *S. asiaticum* on *G. mellonella* and both the EPNs are equally expressed their pathogenicity on *C. medinalis* and it is also evident proved by Alatorre-Rosas and Kaya (1990) and Choo *et al* (1989) in soil bioassay, *Heterorhabditis* sp. (*H. bacteriophora*) searches for hosts and generally infects deeper in the soil profile than S*teinernema* sp. (*S. carpocapsae*) on *G. mellonella* larva in the laboratory test.

The multiplication rate of both EPN species was found to be similar and it was significantly more on *G. mellonella* larva (varied from 73143 to 84143 IJs/larva by *H. indica* and from 66370 to 80458 IJs/larva by *S. asiaticum*) than they produced on *C. medinalis* (from 3682 to 4843 IJs/larva by *H. indica* and from 2864 to 4330 IJs/larva by *S. asiaticum*). Unlu and Ozer (2003) also harvested more recovery on *G. mellonella* (average from 50905 to 271593 IJs/larva) only by *Heterorhabditis* sp. infected cadaver. Later, Sankar *et al.* (2009) reported that highest recovery of nematode, *H. indica* (varied from 140108 to 123961 IJs/larva) was harvested on final instar *G. mellonella* larva. The low recovery was recorded on *C. medina/is* larva may attribute due to the larval body size and body mass. Differences between the reproduction potential of EPN may be related to the isolates, species, and host susceptibility, number of bacteria per infective stage, invasion rate, temperature and humidity. It is possible that differences in virulence between species and isolates might be greater for a less susceptible host. According to Alatorre-Rosas Kaya (1991) in competition trials between *S. carpocapsae* and *H. bacteriophora*, *S. carpocapsae* was the most successful species in every competitive condition they tested. It is observed that *S. asiaticum* does not seem to have as much competitive advantage as *H. indica* on both the hosts when they used two species .simultaneously. 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 Kaya and Gaugler (1993) reported the combination of two nematode species with different search strategies to control one or two 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

Awareness of hazards caused by the usage of chemicals in agriculture is increasing the demand for pesticide-free

produce in India, particularly in northern areas where quality rice and basmati varieties are grown mainly for export purposes. In these areas, rice leaf folder and stem borers are the major insect pests and the farmers are being encouraged to resort to non-pesticidal and organic inputs for their management. With identifying potential of EPN species and the added advantages of organic rice attracting premium prices, development of EPNs as a component in rice pest management will be economically beneficial to the farmers. Further their efficacy in infecting all the larval stages, pupae and adults of leaf folder have proved the potential of deploying these EPNs under laboratory condition against leaf folder. Results of this investigations suggested that the indigenous isolates of EPNs *H. indica* and *S. asiaticum* could be used effectively as combined application to control rice leaf folder, *C. medinalis*.

The microclimate of rice culture with high humidity and moderate temperature is also conductive for the survival, movement, tracking and invasion of the host by EPN and their establishment as a bio-control agent. However, there is a need for development of suitable delivery mechanisms including formulation technology for field application of this EPN. Studies are required to evaluate their bioefficacy against other rice pests as well. Use of entomopathogenic nematodes could be a viable component in the Integrated Pest Management (IPM) where other control measures are ineffective or cannot be imposed.

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