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Research Article

In vitro study on the nematicidal activity of entomopathogenic bacteria against the root knot nematode *Meloidogyne incognita*

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Abstract
National loss of Rs. 21,068.73 million has been estimated due to plant-parasitic nematodes in India. Among plant-parasitic nematodes, one of the major nematodes, root-knot nematodes (RKNs), are well-known diseases causing major losses in vegetable crops. An *in vitro* experiment was conducted to evaluate the nematicidal activities of the cell-free culture filtrate (CFCF) of entomopathogenic bacteria *Photorhabdus spp.* and *Xenorhabdus spp*. isolated from entomopathogenic nematodes *Heterorhabditis indica* (DH3) and *Steinernema abbasi* (CS-39), respectively. The applied doses were 90, 50, 25 and 10% CFCFs. The experiment was performed on the plant-parasitic nematode *Meloidogyne incognita,* and the % mortalities were determined at 6, 12, 24 and 48 hr intervals. The results of the present study revealed that 100% mortality was achieved after 48 hrs with a 10% filtrate of *H. indica* isolate DH3, while no significant result was achieved even after 48 hrs and at 90% CFCF of isolate CS39. Therefore, 10% CFCFs may be recommended for application in root-knot nematode-infected fields for the control.

Keywords: Cell-free culture filtrate (CFCF), *Meloidogyne incognita, Photorhabdus spp.,* Root-knot nematode, *Xenorhabdus spp.*

INTRODUCTION

Eighty percent of the food we consume is available from the plants, while approximately 40% of food crops are lost by agricultural pests, including plant-parasitic nematodes (PPNs) (Food & Agriculture Organization, 2019). Yield losses attributable to PPNs were approximately 14.6% in developing countries compared to 8.8% in developed countries (Gowda *et al.,* 2017). PPNs are considered one of the major pests in the cultivation of vegetables. They are a serious menace to a variety of crop plants worldwide (Ali *et al.,* 2017). PPNs in a subterranean ecosystem play an important role in the food chain (Bernard *et al.,* 2017). PPNs are broadly grouped into three categories based on their parasitic habits: viz. ectoparasites, semiendoparasites, and endoparasites (sedentary and migratory). One of the sedentary endoparasites, commonly called root knot nematodes (RKNs), is the most advanced parasite among all plant-parasitic nematodes and causes heavy economic damage to crops (Khan *et al.* 2019; Mukhtar and Hussain 2019; Asghar *et al.* 2020; Azeem *et al.* 2020). *Meloidogyne incognita* (Kofoid and White, 1919), a severe RKN, is sedentarily endoparasitic in nature and feeds on root contents after entry and establishment in the root system (Das *et al.,* 2015). *M. incognita* induces the development of root galls or knots (Jones and Payne, 1978). The expansion of root cells causes the formation of galls or knots. The secondary symptoms are nutrient deficiency, wilting, yellowing of leaves, and slow or stunted growth (Ralmi *et al.,* 2016). *M. incognita* invades an array of important crops that extensively damage vegetable crops, especially in tropical and subtropical countries (Tariq-Khan *et al.* 2017). Regrettably, chemical methods are considered the most effective for controlling this nematode, but chemicals proved highly toxic, very costly, and have negative impacts on the environment. The utilization of biocontrol agents is an effective alternative option to manage in an eco-friendly manner. EPNs have

been recovered from below-ground soils all over the world. 96 *Steinernema*, 1 *Neosteinernema*, and 21 *Heterorhabditis* species have been described to date (Lewis and Clarke, 2012; Shapiro-Ilan et al., 2017, 2018). They are parasites of insects that transmitted horizontally only and possess an infective juvenile (IJ) stage that energetically invades the insect host. EPNs are always associated with EPBs that play an important role in host infection. Extensive study of the biology of the EPN-EPB complex has been done (Burnell and Stock, 2000; Griffin *et al*., 2005; Lewis and Clarke, 2012; Stock, 2015 and Shapiro-Ilan *et al*., 2017, 2018). Both EPNs have been utilized to treat a vast array of pest insects, PPNs, and plant pathogens (Stock, 2015).

Entomopathogenic bacteria (EPB) have been successfully used for nematode management without causing environmental imperilment. *Xenorhabdus spp*. and *Photorhabdus spp*. are the symbiotic bacteria associated with the entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis,* respectively. Studies of the virulence mechanisms and secondary metabolites of *Xenorhabdus* and *Photorhabdus* bacteria have been aimed at the potential of bacterial symbionts to be used for the management of agriculturally important pests (Hinchliffe *et al.* 2010; Zhang *et al.* 2012; Kumari *et al.* 2015; Stock *et al.* 2017), EPB have never been found living freely in the soil; however, they have been commonly detected in the bacterial biota of insect larvae in metagenomic studies (Osimani *et al.* 2018).

Bacterial symbionts of EPNs along with their metabolites, have been used as insecticides, fungicides, antibacterial agents and antitumor agents. Andalo *et al.* (2012) recorded that the nematode-bacteria complex has been commercially developed as a biocontrol agent against insect pests. Symbiotic bacteria contribute to the nematodes' ability to kill the host, establishing suitable conditions for providing nutrients and inhibiting the growth of other microorganisms in the insect host by the release of antimicrobial compounds. Simultaneously, nematodes act as vectors for symbiotic bacteria, and by interacting with the host immune system, they prepare a favorable environment for symbionts. This symbiotic relationship is essential for the efficiency of biocontrol and enables nematodes to exploit a diverse array of insect hosts (Dunphy and Thurston, 1990, Eleftherianos, 2018).

EPB can reduce the chemical efforts used for plant protection and maintain ecological changes (Migunova and Sasanelli, 2021). As expected, entomopathogenic bacterial species of the genus *Photorhabdus* may be a favorable alternative for expanding the biocontrol of many plant pests and pathogens through the secretion of effective bioactive metabolites (da Silva *et al.,* 2020; Abd-Elgawad, 2017; Eroglu *et al.,* 2019; Ffrench-Constant, *et al.,* 2019; Muangpat, *et al.,* 2020 and Ahuja *et al.,* 2021). Therefore, this study aimed to evaluate the nematicidal activity of *Photorhabdus spp.* (DH3) and *Xenorhabdus spp*. (CS-39) isolated from entomopathogenic nematodes *Heterorhabditis indica* and *Steinernema abbasi* at different concentrations on *M. incognita* (J2s) mortality after different exposure times.

MATERIALS AND METHODS

Insect culture

The host insect larvae of *Galleria mellonella* (Linnaeus) were maintained in the Insectary of Department of Zoology, Chaudhary Charan Singh University, Meerut, India (28.9845° N, 77.7064° E) at 28±2°C. *G. mellonella* was reared in the laboratory on an artificial diet based on cereal maize, as David and Kurup (1988) described with some modifications. The late instar stages of *G. mellonella* larvae were collected and used for nematode rearing.

Nematode culture

Cultures of EPNs *S. abbasi* (CS39) (MN527410) and *H. indica* (DH3) (MG914076) were maintained in the Nematology Laboratory, Department of Zoology, Chaudhary Charan Singh University, Meerut, India (28.9845° N, 77.7064° E) at 27±2°C. The greater wax moth *G. mellonella* (Pyralidae: Lepidoptera) was used as a baiting agent. The insect larvae were infected with individual nematode species. Dead larvae from containers were examined for the presence of nematodes and were placed in White (1927) traps to collect emerging infective juveniles (IJs). IJs disinfected in 0.1% sodium hypochlorite solution were transferred to 175 ml culture flasks (75 cm^2 canted neck, polystyrene, nonpyrogenic, DNase/RNase, free sterile flask, Cat. No. 708001) and stored in BOD at 15°C for future use. Seven- to ten-day -old IJs were checked for their pathogenicity against *G. mellonella* larvae.

Bacteria isolation and molecular characterization

The symbiotic bacterium was obtained from the hemolymph of *G. mellonella* 2 days after infection with *S. abbasi* (CS39) (MN527410) and *H. indica* (DH3) ((KY311812) by adopting the methods described by Akhurst (1980). The hemolymph was streaked on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue (NBTA medium) and left overnight at 28°C (Akhurst, 1980). Single colonies were transferred with a sterile inoculating loop to YS broth (Akhurst, 1980) and placed in an orbital shaker (180 rpm) at 25°C for 24-36 hours in the dark. Bacterial DNA was extracted from a 2-d-old culture using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

For isolate CS39, the 16S RNA was amplified using primers 10F: 59-AGTTTGATCATGGCT CAGATTG-39

(forward) and 1507R: 59-TACCTTGTTAC GACTTCACCCCAG-39 (reverse) (Sandstrom *et al.,* 2001). PCRs were carried out in a Verti 96 Well Fast Thermal Cycler (AB Applied Biosystems) following the compositions of 30 µl of PCR volume. PCR products were sequenced and deposited in GenBank NCBI (National Centre of Biotechnology Information) with accession number OK413395 for *Xenorhabdus spp.*
Established cultures of isolate DH3 *H. indica* (KY311812) were obtained from the Nematology Laboratory, Chaudhary Charan Singh University, Meerut, India (Kajol *et al.,* 2020) for bacterial isolation. The procedure was followed as described above. The isolate was sequenced and submitted to GenBank NCBI under accession number KY311816 for *Photorhabdus spp.*

Culture maintenance of root-knot Nematode

PPNs were taken from the vegetable fields of CCS University Campus, extraction of infective juveniles was done adopting the method given by Kofoid and White (1919), (Chitwood, 1949) and egg masses of nematodes were picked up using dissecting needle from infected roots of ladyfinger and brinjal plants and placed in a watch glass containing sterile distilled water. RKN-infested roots were washed thoroughly and stained with 0.1% acid fuchsin lactophenol at 85°C for 2-3 minutes (Mc Beth *et al.* 1941). Infested roots were kept in lactophenol for at least 24 hours after washing roots gently under tap water and then examined under a light compound microscope (Magnus, MLX). To obtain freshly hatched juveniles, the egg suspension was poured on a 25 micron sieve mesh covered with filter paper and incubated at 25±2°C. The females were teased out from the roots after staining, and perineal patterns were prepared (Taylor and Netscher, 1974), compared with the “key” by Taylor *et al.* (1955) and finally identified as *M. incognita.* Only juveniles collected within 72 h were used for experimentation.

Preparation of cell-free culture filtrate (CFCF)

Loopful single colonies of each bacterium were removed from NBTA indicator plates and transferred to TSB medium (15 g Tryptic Soya Broth (TSB) [Himedia], 500 ml of distilled water [pH 6.8]) as an inoculum for 100 ml culture. The cultures were shaken for maximum aeration at room temperature in a shaker incubator kept overnight. Then, the cells were transferred to 1000 ml Erlenmeyer culture flasks containing 250 ml of TSB media and shaken (200 rpm, 4 days). Centrifugation of the bacterial suspension was performed at 13,000 rpm for 15 minutes in 50 ml centrifuge tubes. The filtrate obtained was filtered through a Millipore filter with a 0.22 micrometer (µm) pore size to remove unwanted bacterial cells. These CFCFs were diluted with sterile distilled water to provide concentrations of 90%, 50%, 25%, and 10%.

Nematicidal activity test

To prepare the concentration at 90%, approximately 120 newly hatched second-stage juveniles (J2s) of *M. incognita* were collected. Afterwards, solutions prepared in 90 µl sterilized distilled water were poured into each cavity block over 900 µl of the tested bacterial filtrates and 10 µl streptomycin sulfate to adjust the concentrations up to 1 ml. The cavity blocks were then covered with glass lids and kept in an incubator at 25 ± 2°C. A 1.0 ml aliquot of TSB medium or sterilized distilled water containing nematode larvae served as a control. The experiment had three replicates and was repeated once. The numbers of dead nematodes were recorded after 6, 12, 24 and 48 hrs. Indicatively, infective juveniles with no movement were touched with a fine needle and kept in water for an hour to confirm mortality. Nematodes that appeared with no real movement were considered dead. The percentage mortality of the second-stage juveniles was calculated and listed. Statistically, the obtained data were subjected to analysis of variance (ANOVA) (Gomez and Gomez, 1984) followed by Tukey's multiple range test ($p<0.05$) to compare means.

RESULTS

The bacterial CFCFs of DH3 and CS39 concentrations of 90%, 50%, 25%, and 10% compared to NBTA media on the mortality percentage of newly hatched juveniles of *M. incognita* after 6, 12, 24 and 48 hrs are illustrated in Table 1. In general, larval mortality percentages increased with increased bacterial filtrate concentrations and exposure durations tested. Among the bacterial strain tested, DH3 (*Photorhabdus spp.*) showed the highest mortality % at concentrations of 90%, 50%, and 10%, except at 25%. In isolate CS39 (*Steinernema spp.*), mortality % at 25% concentration were found higher at 6hr (43.3%) and 24hr (83.3%) as compared with isolate DH3 (*Photorhabdus spp.*) at 6hr (40%) and 24hr (80%) respectively. All concentrations observed from the DH3 strain gave the highest mortality (100%) after 48 hrs of exposure time. However, the mortality percentage of the CS39 strain varied at different concentrations (90%, 50%, 25% and 10%) at 48 hr 96.6%, 93.3%, 96.6%, 86.6%, respectively. It is evident from the data that mortality at 90% and 25% concentration was found same, i.e., 96.6%. In contrast, a difference was observed at a 50% concentration (93.3%), followed by a 10% concentration (86.6%). The lowest mortality was observed in isolate CS39 at a 10% concentration (20%), followed by DH3 with 30% mortality. The larval mortality percentage was found to be similar for both strains CS39 and DH3 at 25% (i.e., 70%) after 12 hr of exposure. With application of 90% concentration of IJs the larval mortality percentages were found 53.3%, 73.3%, 90.0%, 96.6% and 60.0%, 80.0%,

80.0%, 10.0% of isolate CS39 and DH3, respectively concluded that the DH3 was more pathogenic as compared to isolate CS39. When *M. incognita* J2s were exposed for 6 and 12 hrs to bacterial filtrates of both strains at 50%, DH3 ranked first, followed by CS39, with mortality percentages averaged to 60.0% and 80.0% and 36.6% and 40.0%, respectively. On the other hand, DH3 strain at 50% concentration after 12 hrs resulted in significantly (P<0.01) higher larval mortality percentage than that of isolate CS39, double with the value of 80.0%, followed by CS39 (40.0%).

Effect of entomopathogenic bacteria on *M. incognita* (infective juvenile) mortality

According to two-way ANOVA, isolate CS39 possesses two dependent factors, time and concentration, dependent on each other, in nematicidal activity concentration of CFCF was not significant [F (3, 6) =4.594; P=0.054] means conc. was not a dependent factor, whereas % mortality was dependent on time [F (3, 6) =11.24; P=0.007], which was significant at P<0.01.

In isolate CS-39, at 90% concentration of CFCF, the mortality % was observed less significant for 6- 24 hr (P<0.05) than 6- 48 hr (P<0.01) and whereas at 50% concentration of CFCF, the % mortality was observed less significant at 6-24 hr (P<0.05) and 12-24 hr (P<0.05) as compared to 6 hr to 48 hr (P<0.001) and 12 hr to 48 hr (P<0.001). At a CFCFCF concentration of 25%, the mortality at 6-24 hr (P<0.01) was less significant than that at 6-48 hr (P<0.001). At a 10% concentration of CFCFs, the % mortality at 6-48 hr (P<0.0001) was highly significant compared to the % mortality at 6-24 hr (P<0.01) and 12-48 hr (P<0.01).

In isolate DH3 (*Photorhabdus spp.*), the concentration of IJs per larva [F (3, 6) =6; P=0.03] and exposure duration [F (3, 6) =5; P=0.05] were dependent on each other for the very cause of % mortality, depicted by the concentration and time both being significantly different (P<0.05). The result of Tukey's multiple comparisons test indicated that at 90% concentration of CFCF at 6 - 48 h, the mortality rate of the *M. incognita* IJs was significant (P<0.01), whereas at the concentration of 50%, the % mortality observed at 6 - 24 hr was less significant than at 6-48 hr (P<0.01). At a CFCFCF concentration of 25%, the % mortality at 12-24 hr (P<0.05) was much less significant than that at 6-24 hr (P<0.01), whereas at 6-48 hr (P<0.0001), the mortality rate was moderately significant. At a 10% concentration of CFCFs, % mortality at 6-48 hr (P<0.0001) was highly significant compared to the mortality rates at 6-12 hr (P<0.01) and 12-48 hr (P<0.001).

DISCUSSION

EPNs have been recovered from soils of all continents worldwide except Antarctica (Abate *et al.,* 2017). In a few EPN species-insect species combinations, host defenses and immune reactions in response to EPN infection have been studied extensively, and 96 *Steinernema*, 1 *Neosteinernema*, and 21 *Heterorhabditis* species have been described to date (Lewis and Clarke, 2012; Shapiro-Ilan *et al.,* 2017, 2018). EPN-EPB symbiotic associations have been studied for a long time, and the latest results have been excellently reviewed by Murfin *et al.* (2012). Toxic secretions of large molecular diversity play a significant role in the mechanism of mortality. The symbiotic complexes of *Xenorhabdus stockiae* and *Steinernema surkhetense*, *X. stockiae* and *Steinernema siamkayai, Xenorhabdus indica* and *Steinernema pakistanense* were discovered and analysed earlier by Bhat *et al*. (2017). In *X. nematophila*, the leucine responsive protein (lrp) plays a vital role in regulating symbiosis with nematodes and pathogenicity to insects (Cowles *et al.,* 2007; Hussa *et al.,*

Table 1. Mean mortality percentage of *M. incognita* (infective juveniles) tested with culture filtrate of EPB isolates CS39 and DH3. All values are in the form of means.

Treatments	Conc. (µg/ml) %	% Mortality of *M. incognita*			
		6Hr	12Hr	24Hr	48Hr
CS-39 *(Xenorhabdus spp.)*	90	53.3	73.3	90.0	96.6
	50	36.6	40.0	70.0	93.3
	25	43.3	70.0	83.3	96.6
	10	20.0	40.0	60.0	86.6
DH3 (*Photorhabdus spp.*)	90	60.0	80.0	80.0	100
	50	60.0	80.0	90.0	100
	25	40.0	70.0	80.0	100
	10	30.0	60.0	80.0	100

#Each figure represents the mean of three replicates; N= 30 *M. incognita* infective juveniles (IJs)

2015). Structurally diverse secondary metabolites with a broad spectrum of bioactivity, including antifungal, antibacterial, insecticidal, nematicidal, and cytotoxic effects, have been isolated from different *Xenorhabdus strains* (Brachmann and Bode, 2013). These include depsipeptides such as xenematides, xenocoumacins, fabclavines, pristinamycin, xenortides, rhabdopeptides, benzylideneacetone, bicornitun, PAX peptides, cabanillasin, nemaucin, dithiolopyrrolone derivatives, indole-containing compounds, rhabduscin, bacteriocins, etc. In the case of *Photorhabdus luminescens*, lrp led to the overproduction of desmethylphurealipid A. Injection of desmethylphurealipid A into *G. mellonella* and *Manduca sexta* larvae reduced the mRNA levels of antimicrobial peptide-encoding genes, suggesting that these molecules may play a role in insect pathogenicity (Nollmann *et al.,* 2015). lrp regulates genes encoding the biosynthesis of xenematides, xenortides, rhabdopeptides, xenocoumacins and peptide-antimicrobial-*Xenorhabdus* (PAX) - peptides, concluded relying on results obtained from microarray analyses (Engel *et al.,* 2017).

Hu *et al*. (1999) demonstrated that these bacteria produce metabolites that participate actively in nematicidal activity towards a large number of nematodes. The filtrate obtained from DH3 at all concentrations achieved the highest percentage of *M. incognita* mortality (100%) after 48 hrs of exposure. These results are on par with Samaliev *et al.* (2000), who demonstrated that *X. nematophila* completely inhibited the hatching of *M. javanica* and paralyzed the emergence of J2. On the other hand, Fallon *et al.* (2004) observed that the *Steinernema feltiae-Xenorhabdus bovienii complex* could not suppress the development of *M. javanica* and penetration of the parasite into the roots of the host. In the present investigation, *M. incognita* J2s mortality percentages increased as the concentrations of bacterial filtrates and tested exposure times increased compared to TSB or C alone controls. Moreover, bacterial filtrates of DH3 surpassed the strain CS39 of entomopathogenic bacteria tested in increasing larval mortality percentages, especially with low concentrations (10% culture filtrate) and few times for exposure with values of 30.0, 60.0, 80.0 and 100% of culture filtrate after 6, 12, 24 and 48 hrs of exposure time, respectively (Table 1).

Toxic secondary metabolites have shown nematicidal properties *in vitro* against plant parasitic nematodes (Hu *et al.,* 1999). The performance of root insect herbivores is negatively affected by cues emitted by IJs (Helms et al., 2019), adding the dual benefits of using EPNs in pest management strategies. The biocontrol method is the safest way to control pests and pathogens (Sikandar *et al.,* 2020a). They have tremendous consideration in biological control (Davari and Parker, 2018; Trdan *et al.,* 2020). Orozco *et al.* (2016) reported that the inhibition of *M. incognita* J2 and that the mortality of nematodes was concentration-dependent. The nematicidal activity of the culture filtrate (*Pseudomonas aeruginosa*) tested against *M. javanica* has also been reported by Afzal *et al.* (2013).

The present findings are in accordance with those reported by Samaliev *et al.* (2000), who found that symbiotic bacteria *P. oryzihabitans* from *S. abbasi* and *Xenorhabdus nematophilus* from *S. carpocapsae* are responsible for plant-parasitic nematode, *M. javanica* suppression because of the production of defensive compounds. The present investigation indicated that the possible use of DH3 at 10% after 48 hrs of exposure time achieved reliable larval mortality percentages of 100%. In contrast, the use of CS-39 at 10% after 48 hrs of exposure time achieved 86.6% larval mortality (Table 1). In addition to the nematicidal activity observed in bacterial symbionts *Xenorhabdus* spp.and *Photorhabdus* spp., they have been the target of research including discovery of novel antibiotics to extend the medical applications (Tobias et al., 2018; Xue et al., 2018), together with feeding-deterrents as well as repellents for mosquito control (Yooyangket et al., 2018; Kajla et al., 2019). The mechanism of action behind the above said bioactivities depends on presence of secondary metabolites in bacteria. In the present study, the presence of natural nematicidal activity of either *Xenorhabdus* or *Photorhabdus* was confirmed with the mean larval mortality percentages of *M. incognita* J2s, a situation that can justify the reasons for applying such components against *M. incognita* J2s in both conditions *in vitro and in vivo* in the future. These findings agree with Kaya and Gaugler (1993), who mentioned that *Xenorhabdus* and *Photorhabdus* produce several agents with nematicide and antimicrobial activity. Bacteria produce a number of substances such as thiolutin, dithiolopyrrolone, indole etc., and many metabolites such as photobactin, rhabduscin, pristinamycin, Xentrivalpeptides, nematophin, gliobactin, xenorhabdins, xenofuranone etc. among compounds may produce nematicide effects.

Conclusion

The study indicated that the highest percentages of *M. incognita* J2 mortality were obtained from a lower concentration of *Photorhabdus* after 48 hrs of exposure time, which amounted to 100% mortality. It may be concluded that such entomopathogenic bacteria (*Xenorhabdus spp.* (OK413395) *and Photorhabdus spp*. (KY311816)) could be effectively used to suppress plant-parasitic nematodes, including RKNs. Chemical control is expensive, and crop rotation does not work against species with wide host ranges. Hence, sustainable agriculture will rely increasingly on biocontrol

agents for pest management that are environmentally friendly and minimize the contact of humans with hazardous pesticides. These extracts of secondary metabolites (culture filtrate) have been and continue to be a productive and natural source of new biologically active molecules for the suppression of plant-parasitic nematodes, especially *M. incognita.*

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Conflict of interest

The authors declare that they have no conflict of interest.

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