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Characterization and screening of antifungal activity of bacteria associated with entomopathogenic nematodes from Mizoram, North-Eastern India

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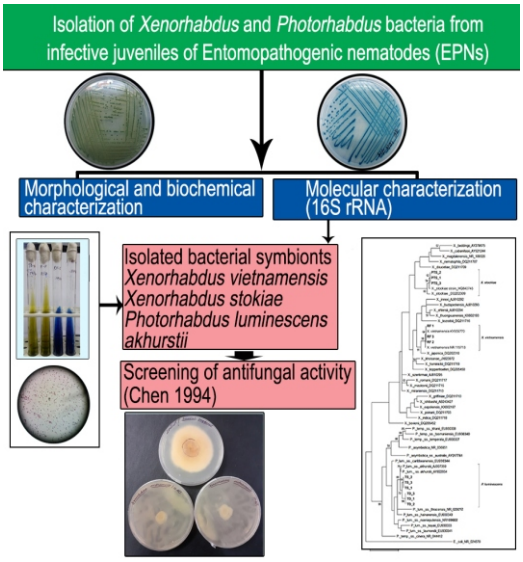
Abstract

Aim: The present study was conducted to isolate and characterize symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus* associated with entomopathogenic nematode isolated from Mizoram, North-Eastern India and screening of their antifungal activity against *Fusarium oxysporum.*

Methodology: Symbiotic bacteria were isolated by macerating infective juveniles of entomopathogenic nematode (EPN) and subsequently plated on nutrient agar supplemented with 0.0025% bromothymol blue and 0.004% triphenyltetrazolium chloride (NBTA). 16S rRNA gene was further sequenced for molecular characterization of isolated bacteria. Screening of antifungal activity was conducted by placing 4 mm of fungal mycelium on petri-plate consisting of spread bacterial suspensions adjusted to 3×10^7 CFU ml^{-1}.



Results: The morphological and molecular characterization (16S rRNA) revealed that three species of symbiotic bacteria were isolated, *Xenorhabdus vietnamensis* from *Steinernema sangi, Xenorhabdus stockiae* from *S. surkhetense* and *Photorhabdus luminescens akhurstii* from both *Heterorhabditis indica* and *H. baujardi.* The maximum likelihood tree showed that *X. vietnamensis* was a sister species of *X. japonica; X. stockiae* formed a sister group with *X. doucetiae, X. nematophila, X. magdalenensis, X. cabanillasii* and *X beddingii* whereas *Phototorhabdus luminescens akhurstii* formed a sister group with *P. luminescens hainanensis* and *P. luminescens thracensis.* The screening of antifungal activity revealed that all the isolated bacterial symbionts successfully inhibited the growth of *Fusarium oxysporum* showing the potential of entomopathogenic bacteria as biological control of pathogenic fungi.

Interpretation: The present study constitutes the first report of symbiotic bacteria, *X. stockiae* and *P. luminescens akhurstii* from Mizoram which contributes to the regional diversity of bacteria. The results emphasized significant toxicity of bacterial isolates against *F. oxysporum* to a great extent, conveying the prospective of isolates as a sustainable biological control agent in future.

Key words: Biological control, Entomopathogenic nematodes, Infective juveniles, Integrated pest management, *Photorhabdus, Xenorhabdus*

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Introduction

Entomopathogenic nematodes (EPNs) belonging to genera *Heterorhabditis* and *Steinernema* are lethal parasites of insects (Poinar, 1979) that have been successfully used for controlling several insect pests (Griffin *et al.,* 2005; Kaya *et al.,* 2006). The nematode enters the host through natural openings like spiracles, oral cavity, anus and also through the cuticle in heterorhabditids (Dowds and Peters, 2002; Bedding and Molyneux, 1982). After gaining entry, the infective juveniles release their symbiotic bacteria into the hemocoel where bacteria multiplies and release a number of virulence factors that kill the insect hosts within 24–48 hr by septicemia. The bacteria proliferate inside the dead insect while the nematodes in turn feeds on the bacteria and remnants of insect tissues for growth and reproduction (Boemare, 2002).

Enterobacteria associated with steinernematids are placed in the genus *Xenorhabdus* (Thomas and Poinar, 1979) and those associated with heterorhabditids are placed in the genus *Photorhabdus* (Boemare *et al.,* 1993). The infective juveniles of EPN harbours the bacterial symbionts in the gut and release them in vascular system of infected insect. The EPN-bacterial associations have been reported as safe potential biocontrol agents (Ehlers and Hokkanen, 1996; Gerdes *et al.,* 2015) capable of infecting wide range of soil dwelling insects (Koppenhöfer and Gaugler, 2009; Stock and Hunt, 2005) which have been successfully used for reduction of several insect pests (Shapiro-Ilan and Gaugler, 2002; Georgis *et al.,* 2006). In addition, the pathogenicity of bacterial symbionts has been tested against various economically important insect pests, including insect vectors of diseases and pathogenic micro-organisms (Benfarhat-Touzri *et al.,* 2014; Ahantarig *et al.,* 2009; Hague *et al.,* 2000; Vagelas *et al.,* 2004). The toxins produced by these symbiotic enterobacteria have been extracted and successfully applied against several agricultural pests in the laboratory (Isaacson and Webster *et al.,* 2002; San-Blas *et al.,* 2013; Ruiu *et al.,* 2013). Therefore, the number of bioactive compounds produced by bacterial symbionts, *Xenorhabdus* and *Photorhabdus* are potential source of future antimicrobial compounds and biopesticides (Webster *et al.,* 2002).

Occurrence of entomopathogenic nematodes has been reported from Mizoram, North–eastern India (Lalramnghaki *et al.,* 2017; Vanlalhlimpuia *et al.,* 2018). However, among the symbiotic bacteria of nematodes, only *X. vietnamensis* has so far been recorded from the region (Lalramnghaki *et al.,* 2017). Identification and characterization of nematodes' bacterial symbionts are crucial importance in an EPN-based biocontrol perspective (Emelianoff *et al.,* 2008). The 16S rRNA gene sequences has proved to be useful for delineating species belonging to the genera *Photorhabdus* and *Xenorhabdus* (Liu *et al.,* 1997; Talliez *et al.,* 2006). Also, the gene region is highly conserved in nature, universal with multiple copies and long gene sequence thereby adding the reliability of 16S rRNA gene for informatics purposes (Woese *et al.,* 1985; Woese, 1987; Janda and Abbott, 2007). Therefore, 16S rRNA still remains to be most frequently target gene for identification of bacteria (Vetrovsky and Baldrian, 2013) despite the insufficiency of the gene for phylogenetic analysis (Tailliez *et al.,* 2010). The present study aimed to isolate, identify and characterize the locally adapted entomopathogenic bacteria and evaluate their potential antifungal activity against one of the most economically important pathogenic fungi, *Fusarium oxysporum.*

Materials and Methods

Isolation and morphological characterization: Primary forms of bacteria were isolated from freshly emerged infective juveniles of EPNs by maceration (Akhurst, 1980). The infective juveniles were collected in 1.5 ml Eppendorf tube and washed with sterile Ringer solution with 10% sodium hypochloride to avoid external contamination from the tegument. The collected infective juveniles were crushed in 1 ml sterile phosphate-buffered saline (PBS) lacking Mg^{2+} and Ca^{2+} salts (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 1 l H_2O sterile) and 100 µl of the macerated samples were spread on NBTA medium (Nutrient agar supplemented with 0.0025% bromothymol blue and 0.004% triphenyltetrazolium chloride). Growth was observed after 24–48 hr of incubation at 28 °C (Akhurst, 1980; Emelianoff *et al.,* 2008). Single colony of bacteria which absorbs bromothymol blue dye was selected and streaked on nutrient agar medium for storage, biochemical and morphological characterization.

Biochemical characterization of bacterial isolates: Bacterial isolates were sub-cultured on nutrient agar, incubated at temperature of 28 °C. After 24 hr of incubation, gram staining was performed using standard protocol. Motility of isolates were examined by observing the 24 hr nutrient broth culture of bacteria under the microscope. Catalase activity was performed as per Reiner (2010). Citrate utilization, phenylalanine deaminase, indole production, methyl red and oxidase activities were analysed by following the methods of Cowan (2004). Production of acids from mannitol, lactose, maltose, raffinose, L-Arabinose and Rhamnose were conducted using phenol red as pH indicator and nutrient broth as basal medium. All the tests were conducted under aseptic conditions.

DNA extraction, amplification and sequencing: Genomic DNA was extracted following He (2011). For genomic DNA extraction, bacterial cells from 24 hr culture in nutrient broth was harvested in 1.5 ml Eppendorf tube and centrifuged at 13000 rpm at 4 °C. The pellet was collected for extraction of genomic DNA. The 16S rRNA gene was amplified using primers 16SP1 (5'-GAAGAGTTTGATCATGGCTC-3') forward and 16SP2 (5'-AAGGAGGTGATCCAGCCGCA-3') reverse sequence following Tailliez *et al.* (2006). The conditions applied for gene amplification were: 5 min at 94 °C for denaturation followed by 35 cycles for 30 sec at 94 °C, 30 sec at 60 °C for annealing and 1 min at 72 °C for extension followed by 7 min at 72 °C. The PCR products were directly sequenced in forward direction.

Sequence analysis: The generated sequences were edited using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www. geospiza. com) and aligned using MEGA X (Kumar *et al.,* 2018). The sequences were blasted in NCBI (http://www. ncbi. nlm. nih. gov) for the nearest matches and submitted to NCBI GenBank. A total of 28 representative species of *Xenorhabdus* and 14 species of *Photorhabdus* along with one out group species of *E. coli* (NR024570) were retrieved from Genbank NCBI. Based on the lowest BIC (Bayesian Information Criterion) score, maximum likelihood tree (ML) was constructed using the best fit model, T92 (Tamura-3-parameter) with G+I (Gamma distribution + Invariant sites), with a replicate of 1000 bootstraps.

Screening of antifungal activity (DIPG): Antifungal activity of *Photorhabdus* and *Xenorhabdus* was carried out as per Chen *et al.* (1994) with slight modifications. A 10 µl of 48 hr old bacterial culture adjusted to 3×10^7 CFU ml^{-1} was spread using a sterile spreader over 9 cm petriplate containing potato dextrose agar. About 4 mm mycelium of the test fungus was cut from the culture grown on PDA and then subsequently placed at the center of PDA plate containing bacterial symbiont. Control plate consisted of PDA lacking bacterial symbiont. Replicates were made for each plate and incubated at 25 °C. The diameter of fungal growth in each plate was observed and compared with control plate every 48 hr for a period of one week depending on the growth of test fungus. The percent inhibition of the fungus was calculated by the following formula (Balouiri, 2016):

$$\text{Antifungal activity (\%)} = ((Dc - Ds)/Dc) \times 100$$

Where, Dc is the diameter of fungal growth in control plate and Ds is the diameter of fungal growth in the plate containing tested bacterial isolates.

Statistical analysis: Experimental data were statistically analysed and expressed as mean of replicates ± standard error. Student's t-test and one way analysis of variance (ANOVA) were performed by using SPSS 20. The confidence level was set at 95% and $p \leq 0.05$ was considered as statistically significant.

Results and Discussion

Morphological characteristics of the isolates: In this study, two species of *Xenorhabdus* bacteria, *viz. X. vietnamensis* from *Sternernema sangi* and *X. stockiae* from *S. surkhetense* and one species of *Photorhabdus, viz. P. luminescens akhurstii* from *H. indica* and *H. baujardi* were identified (Table 1). Steinernematids are associated with *Xenorhabdus* spp. and heterorhabditids with *Photorhabdus* spp. (Boemare, 2002; Forst and Clarke, 2002). Boemare (2002) reported that a single heterorhabditids can be associated with more than one *Photorhabdus* sp., however, in this study, one species was isolated from two separate species of heterorhabditids. This may be attributed to the ambiguities related to definition of species in bacteriology and nematology. All bacterial isolates grew equally well on dark, NBTA media at 28 °C. *Xenorhabdus* isolates on absorbing bromothymol blue

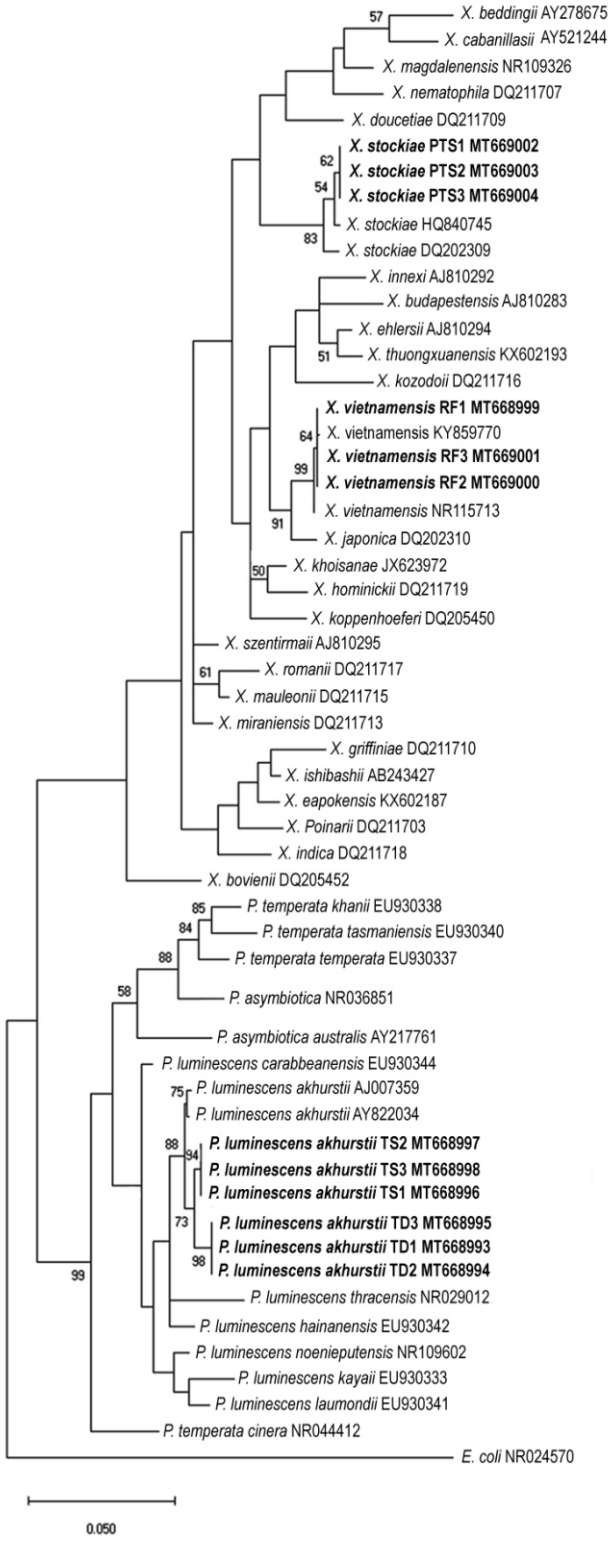

Fig. 1: Maximum likelihood tree of *Xenorhabdus* and *Photorhabdus* inferred from 16S rRNA gene. (The numbers specified at the nodes represents bootstrap proportion values (50% or more, 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers).

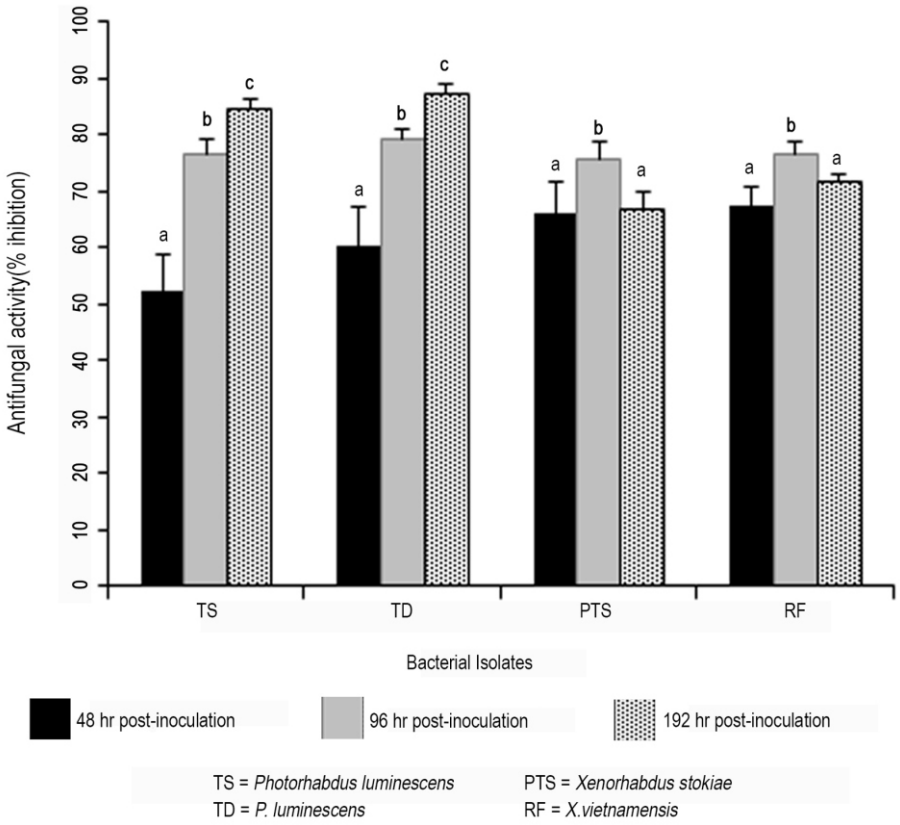

Fig. 2: Antifungal activity of bacterial isolates. The results are presented as growth inhibition (%) and mean ± S.M. For all the isolates, bars with different lowercase letter stand for significant differences ($P \leq 0.05$) of inhibition at different incubation time (hr).

produced blue colored colonies, while *Photorhabdus* isolates produced blue green colored colonies which are in compliance with Akhurst (1980) and Sáenz-Aponte *et al.* (2014), respectively. The absorbtion of dye on NBTA was used as preliminary identification of the bacterial isolates (Akhurst, 1980). Moreover, a single colony from NBTA plate was further sub cultured on nutrient agar media which showed distinct colouration between the genera. Both the *Photorhabdus* isolates (TD and TS) showed yellow pigment whereas both the *Xenorhabdus* isolates (RF and PTS) showed off-white color on nutrient agar which collaborates the reports of Akhurst (1980) and Shahina *et al.* (2004).

Biochemical analysis: The overall result of the biochemical analysis is shown in Table 2. The microscopic examination showed that all the isolated symbionts were motile, Gram negative and rod-shaped. One of the remarkable characters to distinguish the two genus is the presence or absence of catalase activity (Forst *et al.,* 1997) and in accordance to this, *Photorhabdus* isolates showed a strong catalase activity, while *Xenorhabdus* isolates did not show any sign of catalase activity. Furthermore, all the isolated bacteria showed negative results for phenylalanine deaminase, oxidase, inositol, including carbohydrate acidification tests (Lactose, Raffinose, Arabinose, Rhamnose) while acidification of maltose and mannitol were observed for all the isolates which were in consistent with the biochemical characteristics of *Photorhabdus* and *Xenorhabdus*

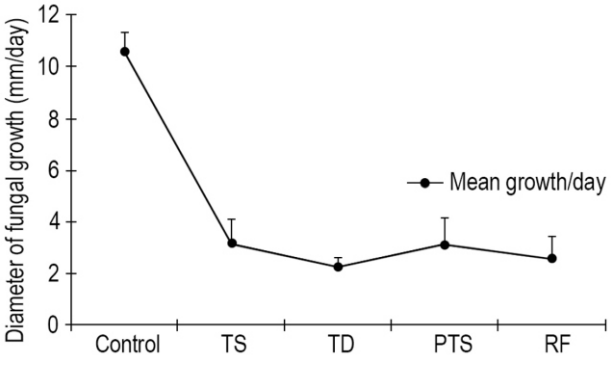

Fig. 3: Effects of bacterial isolates on the growth of *F. oxysporum* in comparision to the control plate. The growth rate of fungus per day is presented as mean ± S.M.

Table 1: List of sequences (n=55) analysed in this study

Species	Nematode Host	NCBI Accession No.	Country	Reference
Photorhabdus luminescens akhurstii TS1	*Heterorhabditis indica*	MT668996	Mizoram, India	This study
Photorhabdus luminescens akhurstii TS2	*Heterorhabditis indica*	MT668997	Mizoram, India	This study
Photorhabdus luminescens akhurstii TS3	*Heterorhabditis indica*	MT668998	Mizoram, India	This study
Photorhabdus luminescens akhurstii TD1	*Heterorhabditis baujardi*	MT668993	Mizoram, India	This study
Photorhabdus luminescens akhurstii TD2	*Heterorhabditis baujardi*	MT668994	Mizoram, India	This study
Photorhabdus luminescens akhurstii TD3	*Heterorhabditis baujardi*	MT668995	Mizoram, India	This study
Xenorhabdus vietnamensis RF1	*Steinernema sangi*	MT668999	Mizoram, India	This study
Xenorhabdus vietnamensis RF2	*Steinernema sangi*	MT669000	Mizoram, India	This study
Xenorhabdus vietnamensis RF3	*Steinernema sangi*	MT669001	Mizoram, India	This study
Xenorhabdus stockiae PTS1	*Steinernema surkhetense*	MT669002	Mizoram, India	This study
Xenorhabdus stockiae PTS2	*Steinernema surkhetense*	MT669003	Mizoram, India	This study
Xenorhabdus stockiae PTS3	*Steinernema surkhetense*	MT669004	Mizoram, India	This study
Photorhabdus luminescens akhursti	Unknown	AY822034	Taiwan	GenBank
Photorhabdus luminescens akhursti	*Heterorhabditis indica*	AJ007359	Guadeloupe	Fischer- Le Saux *et al.* (1999)
Photorhabdus luminescens caribbeanensis	*Heterorhabditis* sp.	EU930344	St. Martin, France	Tailliez *et al.* (2010)
Photorhabdus luminescens thracensis	*Heterorhabditis bacteriophora*	NR029012	Turkey	Hazir *et al.* (2004)
Photorhabdus luminescens hainanesis	*Heterorhabditis* sp.	EU930342	China	Tailliez *et al.* (2010)
Photorhabdus luminescens noenieputensis	*Heterorhabditis noenieputensis*	NR109602	South Africa	Ferriera *et al.* (2013)
Photorhabdus luminescens kayaii	Unknown	EU930333	France	Tailliez *et al.* (2010)
Photorhabdus luminescens laumondii	*Heterorhabditis bacteriophora*	EU930341	Australia	Tailliez *et al.* (2010)
Photorhabdus temperata cinerea	*Heterorhabditis downesi*	NR044412	Hungary	Toth and Lakatos (2008)
Photorhabdus temperata khanii	*Heterorhabditis megidis*	EU930338	Cuba	Tailliez *et al.* (2010)
Photorhabdus temperata tasmaniensis	*Heterorhabditis marelatus*	EU930340	USA	Tailliez *et al.* (2010)
Photorhabdus temperata temperata	*Heterorhabditis megidis*	EU930337	Belgium	Tailliez *et al.* (2010)
Photorhabdus asymbiotica	Clinical specimen	NR036851	Texas	Fischer- Le Saux *et al.* (1999)
Photorhabdus asymbiotica australis	Clinical specimen	AY217761	Australia	Akhurst *et al.* (2004)
Xenorhabdus vietnamensis	*Steinernema sangi*	KY859770	India	Lalramnghaki *et al.* (2017)
Xenorhabdus vietnamensis	*Steinernema sangi*	NR115713	Vietnam	Tailliez *et al.* (2006)
Xenorhabdus japonica	*Steinernema kushidai*	DQ202310	Japan	Tailliez *et al.* (2006)
Xenorhabdus khoisanae	*Steinernema khoisanae*	JX623972	South Africa	Ferreira *et al.* (2013)
Xenorhabdus hominickii	*Steinernema karii*	DQ211719	Kenya	Tailliez *et al.* (2006)
Xenorhabdus koppenhoeferi	*Steinernema scarabaei*	DQ205450	USA	Tailliez *et al.* (2006)
Xenorhabdus szentirmaii	*Steinernema rarum*	AJ810295	Argentina	Lengyel *et al.* (2005)
Xenorhabdus romanii	*Steinernema puertoricense*	DQ211717	Puerto Rico	Tailliez *et al.* (2006)
Xenorhabdus mauleonii	*Steinernema* sp.	DQ211715	St. Vincent	Tailliez *et al.* (2006)
Xenorhabdus miraniensis	*Steinernema* sp.	DQ211713	Australia	Tailliez *et al.* (2006)
Xenorhabdus griffiniae	*Steinernema hermaphroditum*	DQ211710	Indonesia	Tailliez *et al.* (2006)
Xenorhabdus ishibashii	*Steinernema aciari*	AB243427	Japan	Kuwata *et al.* (2006, 2013)
Xenorhabdus eapokensis	*Steinernema eapokense*	KX602187	Vietnam	Kampfer *et al.* (2017)
Xenorhabdus poinarii	*Steinernema glaseri*	DQ211703	Portugal	Tailliez *et al.* (2006)
Xenorhabdus indica	*Steinernema abbasi*	DQ211718	Oman	Tailliez *et al.* (2006)
Xenorhabdus bovienii	*Steinernema weiseri*	DQ205452	Czech Republic	Tailliez *et al.* (2006)
Xenorhabdus stockiae	Unknown	HQ840745	China	GenBank
Xenorhabdus stockiae	*Steinernema siamkayai*	DQ202309	Thailand	Tailliez *et al.* (2006)
Xenorhabdus innexi	*Steinernema scapterisci*	AJ810292	Uruguay	Lengyel *et al.* (2005)
Xenorhabdus budapestensis	*Steinernema bicornutum*	AJ810293	Serbia	Lengyel *et al.* (2005)
Xenorhabdus ehlersii	*Steinernema serratum*	AJ810294	China	Lengyel *et al.*. (2005)
Xenorhabdus thuongxuanensis	*Steinernema sangi*	KX602193	Vietnam	Kampfer *et al.* (2017)
Xenorhabdus kozodoii	*Steinernema arenarium*	DQ211716	Russia	Tailliez *et al.*. (2006)
Xenorhabdus beddingii	*Steinernema* sp.	AY278675	Australia	Marokhazi et al. (2003)
Xenorhabdus caballinasii	*Steinernema riobrave*	AY521244	USA	Tailliez *et al.* (2006)
Xenorhabdus magdalenensis	*Steinernema australe*	NR109326	Chile	Tailliez *et al.* (2012)
Xenorhabdus nematophila	*Steinernema carpocapsae*	DQ211707	Spain	Tailliez *et al.*. (2006)
Xenorhabdus doucetiae	*Steinernema diaprepesi*	DQ211709	Martinique	Tailliez *et al.* (2006)
E. coli ATCC 1175T	Clinical specimen	NR024570	CIP	Cilia *et al.* (1996)

Table 2: Biochemical profiles of isolated *Photorhabdus* and *Xenorhabdus* bacteria

Name of the test	*Xenorhabdus* sp.		*Photorhabdus* sp.	
	RF	**PTS**	**TD**	**TS**
Gram stain	–	–	–	–
Shape	Rod	Rod	Rod	Rod
Motility	Motile	Motile	Motile	Motile
Catalase	–	–	+	+
m-inositol	–	–	–	–
Citrate utilization	–	–	+	+
Phenylalanine deaminase	–	–	–	–
Indole production	–	–	–	–
Methyl Red	–	–	+	+
Oxidase	–	–	–	–
Mannitol	+	+	+	+
Lactose	–	–	–	–
Maltose	+	+	+	+
Raffinose	–	–	–	–
L-Arabinose	–	–	–	–
Rhamnose	–	–	–	–
Lysine decarboxylase	–	–	–	–
Pigmentation	Off-white	Off-white	Yellow	Yellow

+ : positive; –: negative

reported by Salvadori *et al.* (2012). However, negative reaction of mannitol in *Xenorhabdus* has been earlier reported by Akhurst (1983). Negative results for lysine decarboxylase test observed in all the isolates confirms the previous studies of Sáenz-Aponte *et al.* (2014) and Nashimura *et al.* (1994) who also reported negative test for lysine decarboxylase in *Photorhabdus* and *Xenorhabdus,* respectively. However, the above studies contradict the reports of Salvadori *et al.* (2012) who showed positive test in both the genera. Similar to the report of Pervez *et al.* (2015), in this study, *Photorhabdus* isolates showed positive test for methyl red and negative for indole production whereas *Xenorhabdus* isolates showed negative tests which is in accordance with the report of Yamanaka *et al.* (1992) and Akhurst (1983). *Photorhabdus* isolates showed positive test for citrate utilization which is in confirmation with the reports of Sáenz-Aponte *et al*. (2014) and Peel *et al.* (1999). In contrast, *Xenorhabdus* isolates showed negative test for citrate utilization. However, Akhurst (1983) reported positive results for citrate utilization test in *Xenorhabdus.* It is thus clear that some variations may occur in the biochemical characters of the bacterial symbionts leading to complications in defining the precise biochemical characteristics. This may be due to several variable characters. The reasons may also be attributed to dimorphism as described by Akhurst (1980), choice of pH indicator, basal media used for the test and phase variation (Boemare and Akhurst, 1988).

Molecular characterization: The 16S rRNA gene of 1192 bp long sequence was used for molecular identification of isolated bacterial symbionts, *Xenorhabdus* and *Photorhabdus.* The BLAST search result of NCBI GenBank revealed that isolate, *Xenorhabdus vietnamensis*, RF (MT668999–MT669001) showed 99.8–100% similarity with *X. vietnamensis* (KY859770 and NR115713) indicating that they belonged to the same species. The developed sequences, RF (MT668999–MT669001) and the database sequences, *X. japonica* (DQ202310) and *X. khoisanae* (JX623972) showed interspecific distance of 1–3%. *Xenorhabdus stockiae,* PTS (MT669002–MT669004) exhibited high similarity (99.4–99.7%) with the database sequences of *X. stockiae* (HQ840745 and DQ202309) with a zero gap (0.00%) between them. Comparing with the other closely related species, the developed sequence of *X. stockiae* (MT669002–MT669004) exhibited a distance of 3.7% and 4.3% with *X. doucetiae* (DQ211709) and *X. nematophila* (DQ211707), respectively.

The two *Photorhabdus* isolates, TD (MT668993–MT668995) and TS (MT668996–MT668998) along with *P. luminescens akhurstii* (AY822034 and AJ007359) showed similarity ranging from 99.4–99.8% with genetic distance of 0.00–1.00% indicating that they are conspecific. However, the interspecific distance of developed sequences with *P. luminescens thracensis* (NR029092) and *P. luminescens hainanensis* (EU930342) ranged from 1.3–3.8%. The relationships of the isolated symbiotic bacteria and database sequences retrieved from NCBI GenBank are shown in Figure 1. The maximum likelihood tree revealed that *X. vietnamensis* is a sister species of *X. japonica; X. stockiae* is clustered cohesively and formed a sister group with *X. doucetiae, X. nematophila, X. magdalenensis, X. cabanillasii* and *X beddingii* whereas *Phototorhabdus luminescens akhurstii* formed a sister group with *P. luminescens hainanensis* and *P. luminescens thracensis* respectively. All the sequences analysed in this study are shown in Table 1.

Screening of antifungal activity of *Xenorhabdus* and *Photorhabdus* against *Fusarium oxysporum*: The inhibition percentage of bacterial isolates over *F. oxysporum* is depicted in (Fig. 2). The growth inhibition of fungus was observed 48 hr post–incubation. Among the isolates, *Photorhabdus luminescens akhurstii* (TD and TS) caused 50–60% inhibition after 48 hr that increased to 76–79% inhibition after 96 hr of incubation. A further rise in the antifungal activity showed 84–87% inhibition after 192 hr of incubation. In the case of *Xenorhabdus* isolates, the percent inhibition of growth increased 48 hr to 96 hr post-incubation, followed by decrease in inhibition of the fungal growth after 192 hr of incubation. After 48 hr post-inoculation, *X. surkhetense* inhibited *F. oxysporum* upto 66% with a further rise of 75% after 96 hr of incubation. *X. vietnamensis* inhibited 67 % and 76% fungal growth after 48 and 96 hr of incubation, respectively. The study showed that all the bacterial isolates were highly effective against *F. oxysporum* with significant growth inhibition at different time ($p \leq 0.05$). However, no significant difference in antifungal activity was observed among the isolates ($p \geq 0.05$).

In accordance with our result, Chen *et al.* (1994) reported that bacterial isolates, *Xenorhabdus* and *Photorhabdus* showed antimycotic activity against *F. oxysporum*. They have shown that a clear inhibition after several days was later colonised by *F. oxysporum.* However, in this study, no further growth of *F. oxysporum* was observed after 48 hr of incubation with bacterial isolates, *Photorhabdus luminescens akhursti* (TS and TD) while *Xenorhabdus* isolates shows lower inhibition after 48 hr of incubation which illustrated the diverse antagonistic effect of different isolates of entomopathogenic bacteria. Thus, this study highlights the antagonistic effect by direct competition which collaborates the report of Sing and Faull (1988). Similarly, Vanitha *et al.* (2010) reported collective mean percent growth inhibition of *F. oxysporum* (31.03 ± 1.76) by supernatants of bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) after 10 days of inoculation, depicting lower inhibition compared to this study, 77.78 ± 4.28 after 6 days of inoculation. Variations of antifungal activity on *F. oxysporum* may be attributed to the inconsistency of fungal response towards bioactive compounds or different culture media which may impact the general metabolic profile of the bacteria (Sing and Faull,1988; Yonghong *et al.,* 2011).

The present study showed that all the isolated bacteria, *X. vietnamensis, X. stockiae* and *P. luminescens* showed high antifungal activity against *F. oxysporum.* The antifungal activity of the isolates given in Fig. 2 and 3 indicates a clear inhibition of growth with respect to the growth of the fungus in control plate. Therefore, the inhibitory effects that the isolates exhibited on *F. oxysporum,* one of the most economically important fungal pathogens of agricultural crops (Nelson *et al.,* 1981; Beckman *et al.,* 1987) revealed the potential of entomopathogenic bacteria as biological control agent against pathogenic fungi. Also, *Fusarium* spp. have been isolated from Mizoram and reported to be one of the major causative agent of ginger soft root diseases resulting in a huge loss of rhizome yield (Rosangkima *et al.,* 2018). In addition, the fungus is known to emerged as human pathogenic, reported to develop resistance against antifungal drugs and responsible for causing mycological infections in immunocompromised patients (Boutati and Anaissie, 1997; Nucci and Anaissie, 2002; Ponton *et al.,* 2000). Therefore, the results of this study highlights the potential antifungal activity of the isolates to a certain degree and its application in the field conditions needs further scientific investigation either through formulations with other fungicides or the secondary products of the isolates. Thus, more species of EPN-bacterial complex could be isolated from Mizoram and further research work is required to enhance knowledge on the bacterial isolates for their successful applications.

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References

Ahantarig, A., N. Chantawat, N.R. Waterfield, R. Ffrench-Constant and P. Kittayapong: PirAB toxin from *Photorhabdus asymbiotica* as a larvicide against dengue vectors. *J. Appl. Environ. Microbiol.,* **75**, 4627–4629 (2009).

Akhurst, R.J.: Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis. Microbiology,* **121**, 303–309 (1980).

Akhurst, R.J.: Taxonomic study of *Xenorhabdus,* a genus of bacteria symbiotically associated with insect pathogenic nematodes. *Int. J. Syst. Evol. Microbiol.,* **33**, 38–45 (1983).

Akhurst, R.J., N.E. Boemare, P.H. Janssen, M.M. Peel, D.A. Alfredson and C.E Beard: Taxonomy of Australian clinical isolates of the genus Photorhabdus and proposal of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. and *P. asymbiotica* subsp. australis subsp. nov. *Int. J. Syst. Evol. Microbiol*., **54**, 1301–1310 (2004).

Balouiri, M., M. Sadiki and S.K. Ibnsouda: Methods for *in-vitro* evaluating antimicrobial activity: A review. *J. Pharm. Anal.,* **6**, 71–79 (2016).

Beckman, C.H.: The nature of wilt diseases of plants. APH, St. Paul, Minnesota (1987).

Bedding, R.A. and A.S. Molyneux: Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (Heterorhabditidae: Nematoda). *Nematologica,* **28**, 354–359 (1982).

Benfarhat–Touzri, D., A.B. Amira, S.B. Khedher, A. Givaudan, S. Jaoua and S. Tounsi: Combinatorial effect of *Bacillus thuringiensis* kurstaki and *Photorhabdus luminescens* against *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Basic Microbiol.,* **54**, 1160–1165 (2014).

Boemare, N.E. and R.J. Akhurst: Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp.

(Enterobacteriaceae). *Microbiology,* **134**, 751–761 (1988).
Boemare, N.E., R.J. Akhurst and R.G. Mourant: DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *Int. J. Syst. Bacteriol.,* **43**, 249–255 (1993).
Boemare, N.: Interactions between the partners of the entomopathogenic bacterium nematode complexes, *Steinernema-Xenorhabdus* and *Heterorhabditis-Photorhabdus. Nematology,* **4**, 601–603 (2002).
Boutati, E.I. and E.J. Anaissie: *Fusarium,* a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer centre and implications for management. *Blood,* **90**, 999–1008 (1997).
Chen, G., G.B. Dunphy and J.M. Webster: Antifungal activity of two *Xenorhabdus* species and *Photorhabdus luminescens,* bacteria associated with the nematodes *Steinernema* species and *Heterorhabditis megidis. Biol. Control,* **4**,157–162 (1994).
Cilia, V., B. Lafay and R. Christen: Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. *Mol. Biol. Evol.,* **13**, 451–461 (1996).
Cowan, S. T.: Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, Cambridge, UK (2004).
Dowds, B.C.A. and A. Peters: Virulence mechanisms. In: Entomopathogenic Nematology (Ed.: R. Gaugler). CABI Publishing, Wallingford, UK., pp. 79–98 (2002).
Dreyer, J., A.P. Malan and L.M. Dicks: Bacteria of the genus *Xenorhabdus,* a novel source of bioactive compounds. *Front. Microbiol.,* **9**, 3177 (2018).
Ehlers, R.U. and H.M.T. Hokkanen: Insect biocontrol with non-endemic entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp): conclusions and recommendations of a combined OECD and COST workshop on scientific and regulatory policy issues. *Biocontrol Sci. Technol.,* **6**, 291– 293 (1996).
Emelianoff, V., N. Le Brun, S. Pagès, S.P. Stock, P.Tailliez, C. Moulia and M. Sicard: Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Hérault and Gard (Southern France). *J. Invertebr. Pathol.,* **98**, 211–217 (2008).
Ferreira, T., C.A. Van Reenen, A. Endo, C. Spröer, A.P. Malan and L.M. Dicks: Description of *Xenorhabdus khoisanae* sp. nov., the symbiont of the entomopathogenic nematode *Steinernema khoisanae. Int. J. Syst. Evol. Microbiol.,* **63**, 3220–3224 (2013).
Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand and N.E. Boemare: Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. *Int. J. Syst. Evol. Microbiol.,* **49**, 1645–1656 (1999).
Forst, S., B. Dowds, N. Boemare and E. Stackebrandt: *Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs. *Annu. Rev. Microbiol.,* **51**, 47–72 (1997).
Forst, S. and D. Clarke: Bacteria-nematode symbiosis. In: Entomopathogenic nematology (Ed.: R. Gaugler). CABI Publishing, Wallingford, U.K., pp. 57–77 (2002).
Georgis, R., A.M. Koppenhöfer, L.A. Lacey, G. Bélair, L.W. Duncan, P.S. Grewal, M. Samish, L. Tan, P. Torr and R.W.H.M. van Tol: Successes and failures in the use of parasitic nematodes for pest control. *Biol. Control,* **38**, 103–123 (2006).
Gerdes, E., D. Upadhyay, S. Mandjiny, R. Bullard-Dillard, M. Storms, M. Menefee and L.D. Holmes: *Photorhabdus luminescens*: Virulent properties and agricultural applications. *AJAF,* **3**, 171–177 (2015).
Griffin, C., N. Boemare and E. Lewis: Biology and Behaviour. In: Nematodes as Biocontrol Agents. (Eds.: P.S. Grewal, R.U. Ehlers and D.I. Shapiro-Ilan). CABI Publishing, Wallingford, UK., pp. 47–64 (2005).
Hague, N., S. Gowen, F. Andreoglou, S. Elawad and H. Samaliev: The nematicidal effects of the bacteria *Pseudomonas oryzihabitans* and *Xenorhabdus nematophilus* on the root-knot nematode *Meloidogyne javanica. Nematology,* **2**, 507–514 (2000).
Hazir, S., E. Stackebrandt, E. Lang, P. Schumann, R.U. Ehlers and N. Keskin: Two new Subspecies of *Photorhabdus luminescens,* Isolated from *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae): *Photorhabdus luminescens* subsp. *kayaii* subsp. nov. and *Photorhabdus luminescens* subsp. *thracensis* subsp. nov. *Syst. Appl. Microbiol.,* **27**, 36–42 (2004).
He, F.: *E. coli* genomic DNA extraction. *Bio. Protoc.,* **1**, 11–13 (2011).
Isaacson, P.J. and J.M. Webster: Antimicrobial activity of *Xenorhabdus* sp. RIO (Enterobacteriaceae), symbiont of the entomopathogenic nematode, *Steinernema riobrave* (Rhabditida: Steinernematidae). *J. Invertebr. Pathol.,* **79**,146–153 (2002).
Janda, J.M. and S.L. Abbott: 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.,* **45**, 2761– 2764 (2007).
Kämpfer, P., N.J. Tobias, L.P. Ke, H.B. Bode and S.P. Glaeser: *Xenorhabdus thuongxuanensis* sp. nov. and *Xenorhabdus eapokensis* sp. nov., isolated from *Steinernema* species. *Int. J.* Syst. *Evol. Microbiol.,* **67**, 1107–1114 (2017).
Kaya, H.K., M.M. Aguillera, A. Alumai, H.Y. Choo, M. dela Torre, A. Fodor, S. Ganguly, S. Hazir, T. Lakatos, A. Pye, M., M. Wilson, S. Yamanaka, H. Yang and R.U. Ehlers: Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. *Biol. Control,* **38**,134–155 (2006).
Koppenhöfer, H.S. and R. Gaugler: Entomopathogenic nematode and bacteria mutualism. In: Defensive Mutualism in Microbial Symbiosis (Eds.: J.F. White and M.S. Torres). CRC Press, Boca Raton, FL., pp. 99–116 (2009).
Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.,* **35**,1547–1549 (2018).
Kuwata, R., L.H. Qiu, W. Wang, Y. Harada, M. Yoshida, E. Kondo and T. Yoshiga: *Xenorhabdus ishibashii* sp. nov., isolated from the entomopathogenic nematode *Steinernema aciari. Int. J. Syst. Evol. Microbiol.,* **63**, 1690–1695 (2013).
Lalramnghaki, H.C., Vanlalhlimpuia, Vanramliana and Lalramliana: Characterization of a new isolate of entomopathogenic nematode, *Steinernema sangi* (Rhabditida, Steinernematidae), and its symbiotic bacteria *Xenorhabdus vietnamensis* (γ-Proteobacteria) from Mizoram, northeastern India. *J. Parasitic. Dis.,* **41**,1–9 (2017).
Lengyel, K., E. Lang, A. Fodor, E. Szállás, P. Schumann and E. Stackebrandt: Description of four novel species of *Xenorhabdus,* family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov. and *Xenorhabdus szentirmaii* sp. nov. *Syst. Appl. Microbiol.,* **28**, 115–122 (2005).
Liu, J., R. Berry, G. Poinar and A. Moldenke: Phylogeny of *Photorhabdus* and *Xenorhabdus* species and strains as determined by comparison of partial 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.,* **47**, 948–959 (1997).
Marokhazi, J., N. Waterfield, G. LeGoff, E. Feil, R. Stabler, J. Hinds and A. Fodor and R.H. ffrench-Constant: Using a DNA microarray to investigate the distribution of insect virulence factors in strains of *Photorhabdus* bacteria. *J. Bacteriol.,* **185**, 4648–4656 (2003).
Nelson, P.E., T.A. Toussoun and R.J. Cook: *Fusarium*: Diseases, Biology,

and Taxonomy. Pennsylvania State University Press, University Park, PA, USA (1981).

Nishimura, Y., A. Hagiwara, T. Suzuki and S. Yamanaka: *Xenorhabdus japonicus* sp. nov. associated with the nematode *Steinernema kushidai*. *World J. Microbiol. Biotechnol.,* **10**, 207–210 (1994).

Nucci, M. and E. Anaissie: Cutaneous infection by *Fusarium* species in healthy and immunocompromised hosts: implications for diagnosis and management. *Clin. Infect. Dis.*, **35**, 909–920 (2002).

Peel, M.M., D.A. Alfredson, J.G. Gerrard, J.M. Davis, J.M. Robson, R.J. McDougall and R.J. Akhurst: Isolation, identification, and molecular characterization of strains of *Photorhabdus luminescens* from infected humans in Australia. *J. Clin. Microbiol.,* **37**, 3647–3653 (1999).

Pervez, R., J. Revathi, J.S. Eapen, S. Devasahayam and T.K. Jacob: Isolation and identification of symbiotic bacterium associated with the entomopathogenic nematode, *Heterorhabditis* sp. (IISR-EPN 01) from India. Res. *J. Pharm. Biol. Chem. Sci.,* **6**, 339-343 (2015).

Poinar, G.O. Jr.: Nematodes for biological control of insects. CRC Press, Boca Raton, FL, USA (1979).

Ponton, J., R. Ruchel, K.V. Clemons, D.C. Coleman, R. Grillot, J. Guarro, D. Aldebert, P. Ambroise-Thomas, J. Cano, A. J. Carrillo-Munoz, J. Gene, C. Pinel, D.A. Stevens and D.J. Sullivan: Emerging pathogens. *Med. Mycol.,* **38**, 225–236 (2000).

Reiner, K.: Catalase test protocol. ASM Microbe Library. (2010).

Rosangkima, G., Vanramliana, H. Lalthanzara, L. Ringngheti and H.C. Lalramnghaki: Isolation and molecular characterization of ginger soft rot pathogenic fungi in Aizawl district of Mizoram, India. *Sci. Vis.,* **18**, 111–119 (2018).

Ruiu, L., A. Satta and I. Floris: Emerging entomopathogenic bacteria for insect pest management. *Bull. Insectol.,* **2**,181–186 (2013).

Sáenz-Aponte, A., O.F. Pulido and C. Jaramillo: Isolation and characterization of bacterial symbiont SL0708 (Enterobacteriales: Enterobacteriaceae). *Afr. J. Microbiol Res.,* **8**, 3123–3130 (2014).

Salvadori, J.D.M., M.S. Defferrari, R. Ligabue-Braun, E.Y. Lau, J.R. Salvadori and C.R. Carlini: Characterization of entomopathogenic nematodes and symbiotic bacteria active against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and contribution of bacterial urease to the insecticidal effect. *Biol. Control,* **63**, 253–263 (2012).

San-Blas, E.: Progress on entomopathogenic nematology research: A bibliometric study of the last three decades: 1980–2010. *Biol. Contr.,* **66**, 102–124 (2013).

Shahina, F., H. Manzar and K.A Tabassum: Symbiotic bacteria *Xenorhabdus* and *Photorhabdus* associated with entomopathogenic nematodes in Pakistan. *Pak. J. Nematol.,* **22**, 117 (2004).

Shapiro-Ilan, D.I and R. Gaugler: Production technology for entomopathogenic nematodes and their bacterial symbionts. *J. Ind. Microbiol. Biotechnol.,* **28**, 137–146 (2002).

Singh, J and J.L. Faull: Antagonism and biological control. In: Bio Control of Plant Disease (Eds.: K.G. Mukerji and K.L. Garg). CRC Press, Boca Raton, FL., pp. 167–177 (1988).

Stock, S. P. and D.J. Hunt: Morphology and Systematics of Nematodes Used in Biocontrol. In: Nematodes as Biocontrol Agents (Eds.: P. S. Grewal, R. U. Ehlers and D.I. Shapiro-Ilan). CABI Publishing, Wallingford, U.K., pp. 3–43 (2005).

Tailliez, P., S. Pages, N. Ginibre and N. Boemare: New insight into diversity in the genus *Xenorhabdus,* including the description of ten novel species. *Int. J. Syst. Evol. Microbiol.,* **56**, 2805–2818 (2006).

Tailliez, P., C. Laroui, N. Ginibre, A. Paule, S. Pages and N. Boemare: Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp. *thracensis* as *P. temperata* subsp. *thracensis* comb. nov. *Int. J. Syst. Evol. Microbiol.,* **60**, 1921–1937 (2010).

Tailliez, P., S. Pagès, S. Edgington, L.M., Tymo and A.G. Buddie: Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. *Int. J. Syst. Evol. Microbiol.,* **62**, 1761–1765 (2012).

Thomas, G.M. and G.O. Poinar Jr.: *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. *Int. J. Syst. Evol. Microbiol.,* **29**, 352–360 (1979).

Tóth, T. and T. Lakatos: *Photorhabdus temperata* subsp. *cinerea* subsp. nov., isolated from *Heterorhabditis* nematodes. *Int. J. Syst. Evol. Microbiol.,* **58**, 2579–2581 (2008).

Vagelas, I.K., F.T. Gravanis and S.R. Gowen: Soilborne fungi and bacteria symbiotically associated with *Steinernema* spp. acting as biological agents against *Fusarium* wilt of tomato. *IOBC Bulletin,* **27**, 279–284 (2004).

Vanitha, L.S., K. Manju, J. Jayappa and S.C. Chandrashekar: Symbiotic bacteria, *Xenorhabdus* spp. of entomopathogenic nematodes: Source of antifungal compounds against four plant pathogens. *Asian J. Biol. Sci.,* **5**, 174–177. (2010).

Vanlalhlimpuia, Lalramliana, H.C. Lalramnghaki and Vanramliana: Morphological and molecular characterization of entomopathogenic nematode, *Heterorhabditis baujardi* (Rhabditida, Heterorhabditidae) from Mizoram, northeastern India. *J. Parasit. Dis.,* **42**, 341–349 (2018).

Větrovský, T. and P. Baldrian: The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS ONE,* **8**, e57923 (2013).

Wang, Y., X. Fang, F. An, G. Wang and X. Zhang: Improvement of antibiotic activity of *Xenorhabdus bovienii* by medium optimization using response surface methodology. *Microb. Cell fact.,* **10**, 98 (2011).

Webster, J.M., G. Chen, K. Hu and J. Li: Bacterial Metabolites. In: Entomopathogenic Nematology (Ed.: R. Gaugler). CABI Publishing, Wallingford, UK., pp. 99–114 (2002).

Woese, C.R., E. Stackebrandt, T.J. Macke and G.E. Fox.: A phylogenetic definition of the major eubacterial taxa. *Syst. Appl. Microbiol.,* **6**, 143–15 (1985).

Woese, C.R.: Bacterial evolution. *Microbiol. Rev.,* **51**, 221–271 (1987).

Yamanaka, S., A. Hagiwara, Y. Nishimura, H. Tanabe and N. Ishibashi: Biochemical and physiological characteristics of *Xenorhabdus* species, symbiotically associated with entomopathogenic nematodes including *Steinernema kushidai* and their pathogenicity against *Spodoptera litura* (Lepidoptera: Noctuidae). *Arch. Microbiol.,* **158**, 387–393 (1992).