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Significance of metabolites of native *Xenorhabdus*, a bacterial symbiont of *Steinernema*, for suppression of collar rot and root knot diseases of groundnut

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Native isolated *Xenorhabdus* from *Steinernema* spp. manifest wide variety of secretary proteins, mainly in three clusters having mol wt in the range of 20-21, 46-51 and 60-66 kDa. The proteins of high mol wt, 76-90 kDa, apart from regular proteins are produced only in *Xenorhabdus* isolates of *S. riobrave* (*S*rM & A), but not in the isolates *S. carpocapsae* (*Sc*) and *S. thermophilum* (*St*). Under pot culture efficacy, average galls/root of groundnut (root-knot index) was significantly low with higher shoot and root wts in exo- and endo-toxic factor (2% v/w) treatments of different *Xenorhabdus* isolates over control. Effect of *Xenorhabdus* metabolites against collar rot disease of groundnut revealed that soil application of exo- and endo-toxic metabolites (1:10 diluted) in root zone of plants were effective in suppressing fungus *Aspergillus niger*, giving better plant height and survival of groundnut at harvest. PCR amplification of genomic DNA showed multiple amplified products of *Xenorhabdus* isolates (*S*r A & M, *S*t)

Keywords: *Aspergillus*, collar rot, exo- and endo-metabolites, groundnut, *Meloidogyne*, PCR, root-knot nematode, SDS-PAGE, *Steinernema*, toxicity, *Xenorhabdus*

Introduction

Xenorhabdus spp. are facultative Gram-negative symbiotic bacteria, found with beneficial soil nematode *Steinernema*. These bacteria are generally present in nematode gut and entomopathogenic in nature[1-3]. Entomopathogenic nematodes (EPNs) are widely marketed world over and have emerged as key biological control agent of insects, which can easily fit in integrated management system too[4,5]. The EPN, *Steinernema* (Nematoda: Steinernematidae) generally enter in the insect through natural openings and release symbiotic bacteria *Xenorhabdus*, which invade the insect and rapidly kill the host within 48 h causing septicemia[2,6,7]. The bacteria while multiplying in host body produce toxins, antibiotics, bacteriocins, intra- and extra-cellular proteins and many other fermentative products[8,9]. Generally, EPNs are ubiquitous, micro-biovorus and also utilize organic matter of soil food web[7,10]. They can influence non-targeted organisms and soil fauna, besides targeted soil insects, as parasite-host, antagonistic agent or predator-prey, but reported safe to mammals and plants[11].

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In India, during last few decades, research on insect control through exotic and native EPNs has progressed well[6,12], but attention on impact of *Xenorhabdus* complex on plant parasitic nematode-induced root-knot[13] and other plant diseases were few[14]. Recently, antagonistic effect of native *S. riobrave* and other *Steinernema* spp. against root-knot nematodes of okra have been reported from India[13]. Further, bionematicidal and nematostatic activitivities of symbiotic bacteria *Photorhabdus*, isolated from *Heterorhabditis* nematode, were also reported[15].

In view of the positive effect of insect pathogenic nematodes, which are highly preferred potential bioagents for soil inhabiting pests in different parts of the world, present studies were conducted with an objective to establish effect of toxins (toxic metabolites) of native *Xenorhabdus* isolated from *Steinernema* spp. against root-knot and collar rot diseases of groundnut with possible characterization of metabolites and native bacteria.

Materials and Methods

Preparation of Exo- and Endo-toxic Factors/Metabolites

Infective juveniles (Js) of native EPNs[16,17], *viz*. *S. riobrave* Mogar (*Sr* M) and Anand (*Sr* A) isolates, *S. carpocapsae* (*Sc*) and *S. thermophilum*(*St*), that

emerged from dead *Corcyra* larvae were collected in 0.1% formalin prepared in sterile distilled water and surface sterilized by suspending in 70% methyl alcohol for 15 min. Approximately, 500 Js were suspended with 10% sterile sodium hypochlorite in a microfuge tube and incubated at room temperature for 10 min. The tubes were centrifuged twice at 1000 rpm for 5 min with washing of Js with sterile distilled water in between. This process was repeated twice and the final pallets of Js were collected. Treated Js were crushed, streaked on Mac Conkey agar plate (Hi media) under aseptic conditions and incubated at 25±2°C for 48 to 96 h for isolation of symbiont bacteria, *Xenorhabdus* sp. colonies. Isolated bacterial cultures were further streaked on nutrient bromothymol blue tetraethyltetrazolium chloride agar (NBTA) medium for the assessment of purity and to select the primary and secondary phase bacterial variants[18]. Dark blue colonies that still did not have red centers were further plated on nutrient:yeast extract (1:1) agar plates. The plates were incubated at 25°C for 72 h for selection of primary phase colonies of *Xenorhabdus* for the study.

For production of exo-toxins, cultures grown in minimal broth medium (Hi media) were centrifuged at 5,000 rpm in microfuge tubes for 10 min and respective supernatants were collected separately. The remaining bacterial pellets were resuspended in 567 µL of TE buffer by repeated pipetting, followed by addition of 30 µL of 10% SDS (sodium dodecyl sulphate) with gentle shaking for cell lyses and incubated for 1 h at 37°C for obtaining the crude endo-toxins from bacterial cells.

Estimation and Characterization of Total Exo-proteins

The supernatant with exo-toxin was filtered using sterile 0.22 µ nylon membrane filter (Millipore). The filtrate was slowly added with 3.3 *M* ammonium acetate at 4°C. The tubes were kept at 8ºC overnight in a refrigerator so that most proteins get precipitated. Next day, 4 mL of filtrate was centrifuged at 13000 rpm so that all precipitated proteins get paletted. The supernatant was discarded and proteins were dissolved in 1× protein loading dye. The tubes were incubated at 95ºC for 15 min so that the proteins get denatured.

The protein samples (20 µL) were prepared in protein sample-buffer containing bromothymol blue and mixed well, then loaded in the wells of freshly prepared acrylamide separating gel (12%) + stacking gel (5%) and electrophoresed over night at 30 V (Biometra power supply) using SDS-PAGE. The gel was carefully removed and silver stained[19] and observed under white light. Standard protein ladder of Bangalore Genei (Medium range protein mol wt marker) was used as protein marker to access mol wt of unknown exo-proteins of native isolated *Xenorhabdus* spp.

Raising of Groundnut Plants

Earthen pots (10 cm diam × 15 cm height) after disinfestation with 4% formalin solution were filled with sandy loam sterilized soil (1.0 kg/pot). Groundnut seeds were sown in sterilized soil and on 5th d germination was observed. Further, all agronomical practices for rising and maintaining good plant growth like time to time watering, fertigation and weeding were carried out during the experimentation.

In *Vitro* Testing of Exo- and Endo-toxins against Root-knot Disease

Testing of exo- and endo-toxins of the entire native *Xenorhabdus* isolates was carried out for the search of more toxic factor against root-knot nematode, *M. javanica* pt. 2 on groundnut cv. GG 2 cultured in pots. Exo- and endo-toxin suspensions of each isolate, i.e., *Xenorhabdus* from *Sr* M and *Sr* A, *Sc*, *St* and OH (a standard *Xenorhabdus* culture obtained from Ohio Univ., USA for comparison) were prepared for application as mentioned above.

Second stage juveniles (J_2) of *M. javanica* pt. 2 were inoculated in the rhizosphere of 1-wk-old groundnut plants as to achieve threshold level of 2-nematode per g of soil. The exo- and endo-toxins of all the test cultures were applied by drenching (2% v/w) the root zone of the plants. The plant roots treated with carbofuran 1.0 kg/ha (Furadan 3G, 6.6 g/m^2) and nutrient broth served as treated check and untreated check, respectively. All the treatments were replicated four times in completely randomized design.

The observations on plant height, root and shoot wt and root-knot index were recorded at 60 d of treatment to see the effect of exo-and endo-toxins against root knot nematodes.

Effect of *Xenorhabdus* Metabolites against Collar Rot Disease (*Aspergillus niger*)

Effect of exo- and endo-toxins of all the *Xenorhabdus* isolates was studied against *Aspergillus niger* on groundnut cv. GG 2. Native isolates cultures of *A. niger* and *Xenorhabdus* maintained and mass produced in the laboratory[20] were used in the study. Exo- and endo-toxins of all native *Xenorhabdus*

isolates as well as Ohio standard were tested at 1:10 dilution @ 2% v/w by soil application in pots. *A. niger* grown on potato dextrose agar (PDA) was inoculated in each pot @ 1.4×10^9 conidia. Three checks, viz., (i) fungal conidia application only, (ii) Carbendazim 2% treatment only, and (iii) untreated pots were maintained for comparison. All the treatments were repeated three times.

Groundnut plant stand in each pot was assessed after 40 d of inoculation for plant growth parameters, such as plant height and fresh shoot and root wt. The data were analyzed following unequal completely randomized design because of plant mortality induced by collar rot disease.

Xenorhabdus Strain Characterization

Isolation of Genomic DNA from Xenorhabdus

Grown (24 h) cultures of *Xenorhabdus* isolates (1.5 mL) were used to isolate genomic DNA using Ultra Pure Genomic DNA kit of Genei, Bangalore, India. High purity of DNA was ensured using column purification procedure.

The genomic samples ready for augmentation were PCR-amplified using following oligonucleotide primers of 16S rRNA of *Xenorhabdus* sp.[21,22] custom synthesized at Genei, Bangalore: GCAGAGTTAGAT CTTGGCTC and AAGGAGGTGATCCAGCCGCA. Samples were amplified following a 50 µL reaction as 100 pico moles of each primers, 0.2 µg of DNA template, 25 µL 2X PCR-master mix and distilled water[23].

DNA amplification was performed in Eppendorf personal thermocycler under a program of one cycle at 95ºC for 10 min, followed by 35 cycles of denaturation at 95ºC for 1 min, primer annealing at 60ºC for 1 min and DNA extension at 72ºC for 2 min with last extension cycle of 5 min. 10 µL of the PCR product was electrophoresed using 2% agarose gel supplemented with ethidium bromide at 150 V for 75 min using TAE buffer. λ DNA Hind III digest and 100 bp DNA ladder from Genei, Bangalore were used as standard. The Gel was observed under Spectronics UV transilluminator and photographed through Olympus 4 mega pixel digital camera.

Results and Discussion

The results on total exo-protein analysis showed that all four native *Xenorhabdus* isolates from different *Steinernema* spp. produced a wide variety of different proteins. The equation derived from migration of medium range protein mol wt marker, i.e., $y = 12224e^{0.3327x}$, where $R^2 = 0.986$, was used to calculate the approx mol wt of unknown proteins from *Xenorhabdus* isolates (Table 1).

As evident from Fig. 1, mainly three clusters of secretary proteins having mol wt in the range of 20-21, 46-51 and 60-66 kDa were observed. A single band was observed in 20-21 and 46-51 kDa range in all the four species (Fig. 2A). This can be the protein of similar characteristics[24]. Thus, 20-21 kDa protein may be Lecithinase[25]. Our plate assays showed secretary lecithinase presence in all the four species. The 46-51 kDa protein had similar characteristics as A24 tox secretary protein from *X. nematophilia*[24]. Similarly, US Patent Nos 20020147148 2002 and 6,841,165 B1 (January 2005) have also claimed toxic proteins in the range of 20-350 kDa having entomotoxic property of *X. bovienii* (*http://www.usptogov/; http://ep.espacenet.com*). Likewise, *Xenorhabdus* also had proteins in the 20-100 kDa

Table 1—Proteins of *Xenorhabdus* isolates (Found on gel, read by $y = 12224e^{0.3327x}$ $R^2 = 0.986$)

Particulars	Displacement (cm)	Mol wt (kDa)
Standard (Medium range protein mol wt marker)	0.7	14.3
	1.1	20.1
	2.65	29.0
	4	43.0
	5.2	66.0
	6	97.4
Xenorhabdus Sc	1.6	20.8
	2.2	25.4
	4.2	49.4
	5.1	66.6
Xenorhabdus St	1.6	20.8
	2.5	28.0
	3.4	37.8
	4.8	60.3
Xenorhabdus Sr M	1.6	20.8
	2.4	27.1
	3.3	36.6
	4	46.2
	4.3	51.1
	4.8	60.3
	4.9	62.4
	5.1	66.6
	5.8	84.1
	5.9	87.0
Xenorhabdus Sr A	1.6	20.8
	2.4	27.1
	3.3	36.6
	4.3	51.1
	4.8	60.3
	5	64.5
	5.5	76.1
	5.7	81.4
	6	89.9

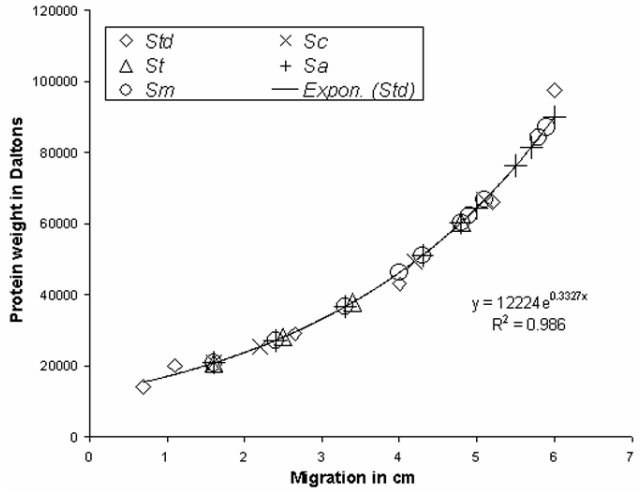

Fig. 1—Migration of standard and unknown proteins of *Xenorhabdus* isolates

range but these proteins and their encoding genes may have some difference with respect to their weight and activity, which needs further investigations.

The native strains also produced some higher mol wt proteins in the range of 76-90 kDa apart from regular proteins. These proteins were not secreted in isolates from *Sc* and *St*. Thus, *Xenorhabdus* isolates from native *Sr* M and *Sr* A were distinct from each other by comparing different proteins of high mol wt, even though they were isolated from *Steinernema* spp. of close geographical area. There are possibilities of novel proteins with native strains, which have shown good efficacy against root-knot nematode, *M. javanica* pt2 and *A. niger* in pot testing. These proteins of high mol wt of *Xenorhabdus* isolates from *Sr* M and *Sr* A may have some additional benefits too.

Effect of Exo- and Endo-toxins of *Xenorhabdus* Isolates against Root-knot Disease of groundnut

The average root-knot index (0-5 scale) on groundnut plants was found to be the lowest (1.52) in each of the treatments of exo-toxins of *Xenorhabdus* isolates from *Sc* and *St* as compared to control (2.08), which was comparable to chemical nematicide carbofuran treatment (1.38; Table 2). Besides *Sc* and *St* treatment, the next effective treatment was *Sr* M (1.63). These exo-toxin treatments were unable to influence the plant height, but they increased the fresh wt of roots and shoots. Similarly, the endo-toxins treatments of different isolates against *M. javanica* pt. 2 revealed that root-knot index was minimum in OH treatment (1.38), followed by *Sr* M (1.52), *Sc* (1.63) and *St* (1.72) treatments. This clearly indicates that both exo- and endo-toxins of *Xenorhabdus*

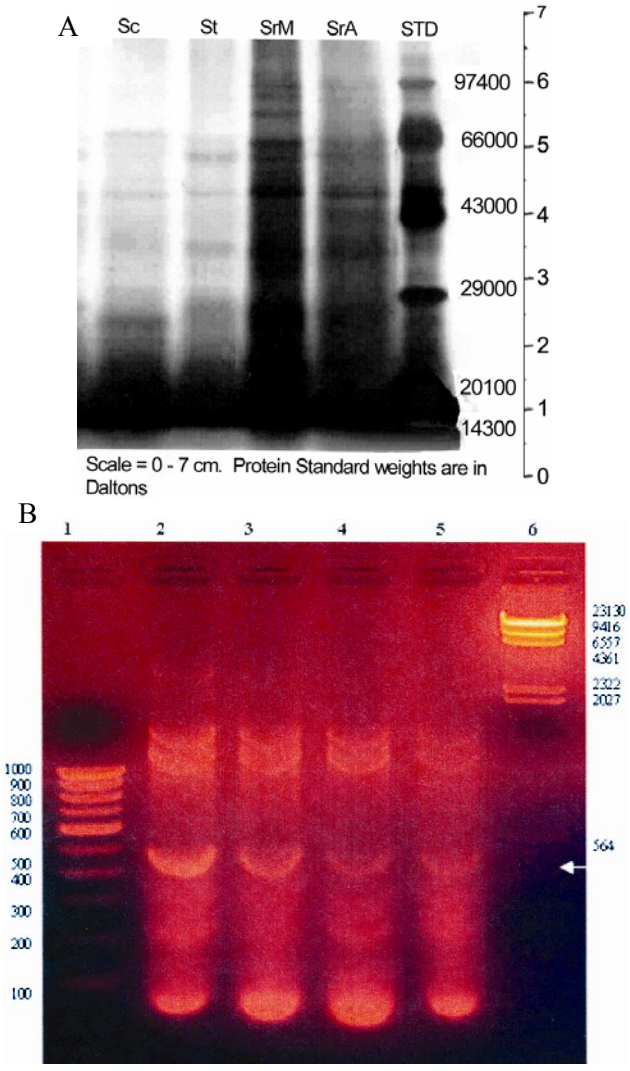

Fig. 2 (A-B)—A. SDS-PAGE of exo-proteins of *Xenorhabdus* isolates; B. Agarose gel electrophoresis of 16S rDNA amplified products of *Xenorhabdus* isolates.

isolates have suppressive effects against *M. javanica* pt. 2 on groundnut. The antagonism of plant parasitic nematodes by EPNs has been documented since late 1980s from Japan[26,27] and subsequently from other parts of the world [28-30]. The present results are similar to observation of Grewal *et al*[31], who have confirmed the role of *Xenorhabdus* metabolites (allelopathic effect) for suppression of plant parasitic nematodes.

Effect of Exo- and Endo-toxins of *Xenorhabdus* Isolates against Collar Rot Disease of Groundnut

The soil application of both exo- and endo-toxins of all *Xenorhabdus* isolates tested, except *St*, in the present study gave protection against *A. niger* in terms of plant survival, which was comparable to the fungicide Carbendazim (Table 3). However, they

differ in their response in terms of plant height and root and shoot fresh wt. The exo-toxin treatment of OH gave the maximum plant height, followed by the toxins of *Sc*, *Sr* M and *Sr* A isolates; whereas the same trend was not followed in case of root and shoot fresh wt. On the other hand, the endo-toxin treatment of *Sr* M isolate gave the maximum plant height followed by *Sc*, OH and *Sr* A isolates. These

Table 2 —Interaction of exo- and endo-toxins of *Xenorhabdus* isolates against *M. javanica* pt. 2 on groundnut plants

Treatment	Exo-toxin				Endo-toxin			
	Plant height (cm*)	Root knot index (0-5)*	Weight* (g)		Plant height (cm*)	Root knot index (0-5)*	Weight* (g)	
			Root	Shoot			Root	Shoot
Xenorhabdus Sr M	5.60^{ab} (31.33)**	1.63^{bc} (2.67)	1.93^{e} (3.75)	3.10^{ab} (9.64)	5.47^{ab} (30.00)	1.52^{b} (2.33)	1.93^{ab} (3.76)	3.26^{ab} (10.60)
*Xenorhabdus Sr*A	5.42^{b} (29.33)	1.72^{abc} (3.00)	1.76^{cd} (3.10)	2.90^{b} (8.42)	5.57^{a} (31.00)	1.72^{ab} (3.00)	1.78^{bc} (3.19)	3.33^{ab} (11.08)
Xenorhabdus OH	5.66^{ab} (32.00)	1.82^{ab} (3.33)	1.94^{bc} (3.81)	3.16^{ab} (10.01)	5.38^{ab} (29.00)	1.38^{b} (2.00)	1.98^{ab} (3.92)	3.13^{b} (9.86)
Xenorhabdus Sc	5.71^{a} (32.67)	1.52^{bc} (2.33)	1.86^{cd} (3.46)	3.24^{a} (10.52)	5.35^{ab} (28.67)	1.63^{b} (2.67)	1.88^{ab} (3.54)	2.83^{c} (8.01)
Xenorhabdus St	5.71^{a} (32.67)	1.52^{bc} (2.33)	2.13^{ab} (4.52)	2.93^{b} (8.58)	5.26^{b} (27.67)	1.72^{ab} (3.00)	2.06^{ab} (4.27)	3.22^{ab} (10.36)
Carbofuran	5.57^{ab} (31.00)	1.38^{c} (2.00)	2.25^{a} (5.06)	3.25^{a} (10.55)	5.26^{b} (27.67)	1.28^{b} (1.67)	2.23^{a} (4.98)	3.45^{a} (11.88)
Control (Nutrient broth)	5.68^{ab} (32.33)	2.08^{a} (4.33)	1.63^{d} (2.66)	2.49^{c} (6.21)	5.29^{b} (28.00)	2.16^{a} (4.67)	1.48^{c} (2.26)	2.30^{d} (5.31)
± S.E.	0.08	0.13	0.08	0.08	0.08	0.15	0.11	0.09
CV %	2.36	13.69	7.59	4.91	2.52	15.58	10.20	5.29

*Square root $\sqrt{X + 0.5}$ transformation

**Figures in parentheses indicate retransformed values

Figures indicating common letters do not differ significantly from each other at 5% level of significance according to DNMRT.

Table 3—Antagonistic effect of *Xenorhabdus* metabolites against *A. niger* on groundnut plants

Treatment	Soil application (2% v/w)									
	Exo-toxin					Endotoxin				
	Initial plant stand	Plant height (cm*)	Weight* (g)		Final plant stand	Initial plant stand	Plant height (cm*)	Weight* (g)		Final plant stand
			Root	Shoot				Root	Shoot	
Xenorhabdus Sr M	3	33.00	1.10	6.78	3	3	39.50	4.74	10.38	3
Xenorhabdus Sr A	3	29.00	2.89	4.07	3	3	28.67	2.94	12.79	3
Xenorhabdus OH	3	37.00	1.50	6.96	3	3	30.67	2.17	12.90	3
Xenorhabdus Sc	3	36.00	0.78	6.67	3	3	34.33	1.68	7.81	3
Xenorhabdus St	3	32.67	2.58	9.73	2	3	38.00	2.80	6.99	2
Carbendazim	3	41.00	1.47	13.41	3	3	34.33	3.76	17.58	3
A. niger only	3	33.50	1.14	7.50	1	3	38.00	0.94	10.59	1
Control (Nutrient broth)	3	42.00	1.74	12.69	3	3	40.50	3.02	11.08	3
± S.E.	--	2.46	0.23	0.65	--	--	1.77	0.57	0.94	--
CD at 5%	--	7.66	0.73	2.00	--	--	5.57	1.83	2.99	--
CV %	--	8.54	22.74	10.89	--	--	6.97	27.09	10.06	--

treatments also had higher root and shoot fresh wt, except *Sc* isolate. The endo-toxin treatments had better response in terms of plant height and fresh wt of root and shoot as compared to exo-toxin treatments (Table 3). These results clearly indicate that both exo- and endo-toxin of native *Xenorhabdus* isolates were useful in protecting the groundnut plants against collar rot disease induced by *A. niger* by their antagonistic effect[32]. The results are akin to earlier observations on suppression of plant pathogenic fungi, *Pythium*[33,34], *Rhizoctonia*[34], *Mucor*[33], *Phoma betae*[35] by EPN symbiotic bacterial (*X. nematophilus*/ *X. bovinie*) toxins and metabolites *in vitro*.

Characterization of *Xenorhabdus* Isolate

In the present study on *Xenorhabdus* 16S rRNA gene amplification, a region of approximately 1.5-1.6 kb was identified, which PCR amplifiied in all the native *Xenorhabdus* isolates (*Sr* A & M, *St*) of Indian subcontinent (Fig. 2B). Similar results were reported earlier by Brunel *et al*[23] and Boemare *et al*[35]. In addition to this, approximately 1 kb and 400 bp fragments were also amplified, indicating some new regions. However, RFLP analysis could not be done due to the additional lighter amplified products[36], which would interfere during restriction digestion of the 1.5 kb product.

Conclusion

Under pot culture conditions, soil application of both endo-and exo-toxins (*Xenorhabdus* metabolites) were found effective against root knot nematode and *A. niger*. The native *Xenorhabdus* isolates produced some higher mol wt proteins in range of 76-90 kDa, apart from the regular proteins. These proteins were not secreted by Indian *Xenorhabdus* bacteria isolated from *Sc* and *St*[37]. Toxic properties and genome analysis of *Xenorhabdus* isolates showed that our native species are similar to the exotic *Xenorhabdus* because in PCR reaction they amplified 1.5 kb product, a specific characteristics of *Xenorhabdus* genus. However, native bacteria also showed amplification of two more bands of 1.0 kb and 400 bp size, which suggest variations suspecting different patho-types of Indian *Xenorhabdus*.

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