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Characterization of Antinematicidal and Antifungal Bacterial Microbes by 16s Rrna Gene Sequence

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A B S T R A C T

The culture filtrates of endophytic bacterial isolates *viz*., EB16, EB18, EB19and EB3 significantly inhibited the egg hatching (93.36, 93.72, 91.08 and 85.80 per cent respectively), causing the juvenile mortality (95.67, 89.0, 82.67 and 77.33 per cent respectively) of root knot nematode, *Meloidogyne incognita* at 100% concentrations with 60h of exposure. All the four isolates significantly inhibited the mycelial growth of fungal pathogens *viz.*, *Fusarium oxysporum* f.sp. *lycopersici* and *Rhizoctonia solani in vitro*. The four promising endophytic bacterial isolates *viz*., EB16, EB18, EB1 and 9 EB3 were identified as *Bacillus cereus* (Accession no. GU 321330), *Bacillus pumilus* (Accession no. GU 321331), *Methylobacterium radiotolerans* and *Brevundimonas diminuta* (Accession no. GU 321330), respectively by 16S rRNA gene sequence and phylogenetic tree construction.

Introduction

Endophytes are live inside the plant tissue for all or part of their life cycle by penetrating host plants through natural openings, wounds induced by biotic factors such as plant parasitic nematodes (Hallmann *et al.*, 1998) or actively using hydrolytic cellulose. The endophytes colonized root tissues, able to manage sedentary endoparasitic nematodes due to the fact that both of them occupy the same ecological niche and protected from nematode attack and host plant in turn provides shelter and nutrition to the endophytes. The identification of endophytic bacteria has been performed mainly with morphological and physiological studies required skillful techniques and is very complex and time consuming. Over the years, a sizeable database of 16S rRNA gene has been built and successfully applied in identifying bacteria or determining the phylogenetic relationships. Moreover, it has been reported that a partial region of 16S

rRNA is effective for the classification and identification of bacteria (Yamada *et al.*, 1997). The present investigation was taken up to isolate and characterize endophytic bacteria and tested their antimicrobial activity against root knot nematode and soil borne pathogens.

Materials and Methods

Nematicidal efficacy of endophytic bacterial isolates

Bacterial cell free filtrates of the isolates at different concentrations were tested for their effect on hatching of eggs and juvenile mortality of *M. incognita*. One egg mass and 100 J_2/ dish of *M. incognita* was placed in a Syracuse dish with bacterial filtrate different concentrations *viz*.,100, 75, 50 and 25 per cent and incubated at 28 ± 2^{o}C. The broth without bacteria and tap water were used as control. Observations were recorded on the numbers of hatched and immobilized juveniles after 24, 36, 48, and 60 h of incubation in inhibition of egg hatching and juvenile mortality experiments under *in vitro* studies.

Antifungal activity of endophytic bacterial isolates

In vitro screening of endophytic bacterial isolates against two fungal pathogens *viz*., *F.oxysporum* f.sp. *lycopersici* and *R. solani* was carried out by dual plate technique**.** A nine mm mycelial disc of five days old pathogens culture was placed on one side of Petri plate containing PDA medium. The endophytic bacterial isolate was streaked onto the opposite side of the Petri dish. The plates were incubated at room temperature for 96h. The diameter of the mycelial growth in all the treatments was measured and expressed in terms of per cent inhibition over control (Vincent, 1927) which was calculated as I= C-T/C X 100 (I = Per cent inhibition over control, C = Growth in control, T = Growth in treated).

Molecular characterization by 16S rRNA gene sequence

The total genomic DNA from the three promosing isolates was extracted by using the standard cetyl-trimethyl ammonium bromide (CTAB) method given by Melody (1997). DNA was then extracted twice with Phenol-Chloroform, followed by precipitation with 0.6 volume of ice cold isopropanol for 2h at-20^{o}C. DNA was centrifuged at 1200X g for 15 min at 4^{o}C, washed with 70% ethanol and then air dried. Finally, the DNA was resuspended then centrifuged at 1200 X g for 15 min at 4^{o}C. Pellets obtained were dried and resuspended in 50µl of TE buffer. Total The PCR amplification of the target sequence was carried with Primers pF (5'- GGA GAG TTA GAT CTT GGC TCA G- 3') and pR (5' AAG GAG GGG ATC CAG CCG CA-3'), a pair of highly conserved flanking sequences were used to amplify the 16S ribosomal genes.PCR products were visualized on 0.8% agarose gels and final products were viewed and photographed using Alpha imager TM1200 documentation and analysis system.

The PCR product were sent for sequenced at Chromous Biotech Pvt. Ltd., Bangalore and sequenced through single pass analysis from forward and reverse direction. Sequence data was compared with already available sequence data by BLAST analysis in National Center for Biotechnology Information (NCBI) sequence data bank.

Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), using the distance matrix from the alignment. 16S rRNA gene sequence of the following strains was obtained from GenBank. Relevant sequences were collected and data were plotted with PHYLIP software. Selected isolates were identified at genus and species level from the Dendogram drawn.

Results and Discussion

Inhibition of egg hatching

The eight promising endophytic bacterial isolates along with Pf1 were tested for their ovicidal effect against *M. incognita* eggs. Among the isolates EB3, EB16, EB18 and EB19 were found to be the most effective, which caused the highest inhibition of egg hatching (Table 1). In the present study, it is obvious that the inhibition of egg hatching increased with increase in the time of exposure and increase in the concentration of endophtic bacterial isolates. Similar study was conducted by Jonathan and Umamaheswari (2006) where the culture filtrates of *P. fluorescens* showed antagonistic effect on nematode egg hatching. The high degree of ovicidal properties of the endophytic bacterial isolates was attributed due to the presence of the toxin, secondary metabolites and antibiotics and chitin. The breakdown of chitin layer located in egg shell of tylenchoid nematode (Bird and Bird, 1991) by chitinases (produced by PGPE) could cause premature hatch, resulting in fewer viable juveniles (Mercer *et al.,* 1992). The present study has also in line with the above findings.

Juvenile mortality

The results revealed that irrespective of the concentration of culture filtrates, the number of juvenile mortality was increased within the increase in time of exposure. Among the isolates EB3, EB16, EB18 and EB19 were found to be the most effective, which caused the highest nematode mortality (Table 1). In the present study, it is obvious that the juvenile morality increased with increase in the time of exposure and increase in the concentration of endophytic bacteria. Jonathan and and Umamaheswari (2006) found that the culture filtrate of isolates *viz*., Ptbv22, Bbv57 showed significantly higher larvicidal action on *M. incognita* juveniles. The high degree of ovicidal and larvicidal properties of the *P. fluorescens* isolates may be due to the presence of the toxic metabolites and antibiotics *viz*., pyrolnitrin, pyroverdine and 2,4-diacetyl phloroglucinol (Bangara and Thomashow, 1996).

Effect endophytes on fungal pathogens

In the present study, endophytic bacterial isolates *viz*., EB19, EB18, EB16, and EB3 significantly inhibited the growth of *F. oxysporum* f.sp. *lycopersici* and *R. solani* (Table 2). Kye Man Cho *et al*. (2007) reported that the endophytic *Pseudomonas* spp. and *Bacillus* spp. inhibited growth of fungal pathogens *viz*., *R. solani, F. oxysporum* and *Phythium ultimum in vitro*. ACC deaminase producing bacteria showed very strong antagonism against *F. oxysporum* and *R. solani* (Rasche *et al.,* 2006). From the above evidences, it is assumed that production of antibiotics, toxin and secondary metabolites by endophytic bacteria might have inhibited growth of wilt pathogen in the present study also.

16S rRNA gene sequencing

The genomic DNA was extracted from three isolates (EB 3, EB 16 and EB 18), documented and presented (Fig. 1). Since the isolates EB 19 has conformed to existing COLR 1 isolate (*Methylobacterium radiotolerans*) of Department of Agricultural Microbiology, TNAU, Coimbatore. The 16S rRNA gene was amplified by using universal eubacterial primers, which could amplify really full length of the 16S rRNA gene about 1500bp (Fig. 2). The gel purified PCR products were sequenced in both directions and the orientation of the sequence was corrected by BioEdit software.

Table.1 Effect of culture filtrate of endophytic bacterial isolates on *M. incognita* juveniles mortality

Isolates	Number of eggs hatched after an exposure in *								Number of juvenile mortality an exposure in #							
	50 %				100%				50 %				100%			
	24h	36h	48h	60h	24h	36h	48h	60h	24h	36h	48h	60h	24h	36h	48h	60h
EB 2	73.33^{f} (43.73)	79.67^{f} (54.65)	83.67^{g} (60.66)	86.33^{f} (67.46)	36.00^{f} (72.38)	38.33^{f} (78.18)	43.67^{g} (79.07)	50.67^{g} (80.90)	29.00 (5.43)^{f}	32.33 (5.73)^{g}	36.00 (6.04)^{f}	40.33 (6.39)^{g}	48.33 (6.99)g	54.67 (7.43)^{g}	58.33 (7.67)^{g}	**59.33 (7.74)^{g}**
EB 3	53.67^{d} (58.82)	58.67^{d} (66.60)	61.67^{d} (71.00)	64.00^{d} (75.88)	20.67^{d} (84.14)	23.67^{d} (86.53)	26.67^{d} (87.22)	30.67^{d} (88.44)	39.33 (6.31)^{d}	45.00 (6.75)^{d}	48.67 (7.01)^{d}	54.67 (7.43)^{d}	63.00 (7.97)^{d}	71.33 (8.48)^{d}	75.67 (8.73)^{d}	**77.33 (8.82)^{d}**
EB 6	89.00^{h} (31.71)	94.33^{g} (46.30)	100.33^{h} (52.82)	103.33^{g} (61.06)	46.67^{g} (64.19)	49.67^{g} (71.73)	54.67^{h} (73.80)	64.67^{i} (75.63)	21.33 (4.67)^{h}	25.67 (5.12)^{i}	28.33 (5.37)^{h}	31.33 (5.64)^{i}	37.33 (6.15)^{i}	43.00 (6.60)^{i}	46.67 (6.87)^{i}	**47.67 (6.94)^{i}**
EB 10	82.67^{g} (36.57)	88.67^{g} (49.53)	94.33^{h} (55.64)	97.33^{g} (63.32)	42.00^{g} (67.77)	45.00^{g} (74.38)	50.67^{h} (75.72)	58.67^{h} (77.89)	24.67 (5.02)^{g}	28.67 (5.40)^{h}	32.00 (5.70)^{g}	36.33 (6.07)^{h}	42.67 (6.57)^{h}	49.33 (7.06)^{h}	52.00 (7.25)^{h}	**53.00 (7.31)^{h}**
EB 11	67.67^{f} (48.08)	73.33^{f} (58.25)	77.00^{f} (63.79)	80.67^{f} (69.60)	31.33^{f} (75.96)	34.33^{f} (80.46)	38.67^{f} (81.47)	44.67^{f} (83.17)	30.33 (5.55)^{f}	36.67 (6.10)^{f}	38.33 (6.23)^{f}	45.00 (6.75)^{f}	53.67 (7.36)^{f}	60.67 (7.82)^{f}	63.33 (7.99)^{f}	**65.67 (8.13)^{f}**
EB 16	32.00^{a} (75.45)	35.33^{a} (79.89)	37.33^{a} (82.45)	38.33^{a} (85.55)	4.33^{a} (96.68)	7.33^{a} (95.83)	8.33^{a} (96.01)	9.67^{a} (96.36)	55.33 (7.47)^{a}	63.00 (7.97)^{a}	67.00 (8.22)^{a}	70.33 (8.42)^{a}	80.33 (8.99)^{a}	88.00 (9.41)^{a}	93.67 (9.70)^{a}	**95.67 (9.81)^{a}**
EB 18	39.67^{b} (69.57)	43.67 (75.14)	45.33^{b} (78.68)	47.33^{b} (82.16)	10.00^{b} (92.33)	13.00^{b} (92.60)	14.67^{b} (92.97)	16.67^{b} (93.72)	50.00 (7.11)^{b}	56.33 (7.54)^{b}	60.67 (7.82)^{b}	65.00 (8.09)^{b}	74.67 (8.67)^{b}	82.67 (9.12)^{b}	86.67 (9.34)^{b}	**89.00 (9.46)^{b}**
EB 19	46.67^{c} (64.19)	51.33^{c} (70.78)	53.33^{c} (74.92)	55.67^{c} (79.02)	15.33^{c} (88.24)	18.33^{c} (89.56)	20.67^{c} (90.10)	23.67^{c} (91.08)	44.67 (6.72)^{c}	52.00 (7.25)^{c}	54.00 (7.38)^{c}	60.33 (7.80)^{c}	68.67 (8.32)^{c}	76.33 (8.77)^{c}	81.00 (9.03)^{c}	**82.67 (9.12)^{c}**
Pf 1	61.00^{e} (53.20)	66.00^{e} (62.43)	69.67^{e} (67.24)	72.33^{e} (72.74)	26.00^{e} (80.05)	29.00^{e} (83.49)	32.67^{e} (84.35)	37.67 ^{e} (85.80)	34.00 (5.87)^{e}	40.67 (6.42)^{e}	42.33 (6.54)^{e}	49.33 (7.06)^{e}	58.33 (7.67)^{e}	66.33 (8.18)^{e}	69.33 (8.36)^{e}	**71.67 (8.50)^{e}**
Broth	112.33^{i} (13.81)	129.67^{h} (26.19)	151.33^{i} (28.84)	191.33^{h} (27.89)	89.67^{h} (31.20)	95.00^{h} (45.92)	106.33^{i} (49.04)	109.00^{j} (58.92)	1.00 (1.22)^{i}	1.67 (1.47)^{j}	1.67 (1.47)^{i}	2.33 (1.68)^{j}	6.67 (2.68)^{j}	7.33 (2.80)^{j}	8.33 (2.97)^{j}	**8.67 (3.03)^{j}**
Control	130.33^{j}	175.67^{i}	212.67^{j}	265.33^{i}	130.33^{i}	175.67^{i}	208.67^{j}	265.33^{k}	0 (0.71)^{j}	0 (0.71)^{k}	0 (0.71)^{i}	0 (0.71)^{k}	0 (0.71)^{k}	0 (0.71)^{k}	0 (0.71)^{k}	**0 (0.71)^{k}**
S Ed	3.11	3.15	3.20	3.18	2.35	2.37	2.12	2.76	0.13	0.11	1.63	0.13	0.16	0.14	0.14	**0.15**
CD (P=0.01)	**8.24**	**8.62**	**8.96**	**8.90**	**6.63**	**6.18**	**6.23**	**8.37**	**0.38**	**0.31**	**4.53**	**0.38**	**0.45**	**0.40**	**0.41**	**0.41**

Values are mean of three replications, *****Figures in parentheses are per cent decreased over control and # √n+0.5 transformed value
In column means followed by a different letters are significantly different from each other at 1 per cent level by DMRT

Table.2 *In vitro* inhibition of growth of fungal pathogens by endophytic bacterial isolates (Dual plate technique)

S. No.	Isolates	Inhibition of *F. oxysporum* f.sp. *lycopersici*		Inhibition *R. solani*	
		Mycelial growth (cm)	Growth inhibition %	Mycelial growth (cm)	Growth inhibition %
1	EB 2	5.33^{c}	40.74^{de}	6.13^{de}	**31.85^{e}**
2	EB 3	4.23^{b}	52.96^{b}	4.57^{bc}	**49.26^{c}**
3	EB 6	6.40^{d}	28.89^{f}	6.63^{de}	**26.30^{e}**
4	EB 10	6.20^{d}	31.11^{ef}	6.30^{de}	**30.00^{e}**
5	EB 11	5.27^{c}	41.48^{cd}	5.93^{d}	**34.07^{de}**
6	EB 16	2.90^{a}	67.78^{a}	3.27^{a}	**63.70^{a}**
7	EB 18	3.17^{a}	64.81^{a}	3.50^{a}	**61.11^{ab}**
8	EB 19	4.13^{b}	54.07^{b}	4.23^{b}	**52.96^{bc}**
9	Pf 1	4.40^{b}	51.11^{bc}	5.07^{c}	**43.70^{cd}**
10	Control	9.00^{e}	-	9.00^{f}	**-**
	S Ed	0.39	4.62	0.33	**3.89**
	CD (P=0.01)	**1.12**	**12.31**	**0.92**	**11.07**

***** Values are mean of three replications
In column means followed by a common letter are not significant at 1 per cent level by DMRT

Table.3 Species identification of endophytic bacteria by 16S rRNA gene sequence homology

Endophytic bacterial isolates	Source	16S rRNA gene sequence homology			
		Species identified[a]	NCBI Accession no.	No. of bases sequenced	Per cent homology[b]
EB 3	Banana	*Brevundimonas diminuta*	EU593764	1-1338	**96**
EB 16	Chilli	*Bacillus cereus*	DQ289077	1-1451	**98**
EB 18	Papaya	*Bacillus pumilus*	EU982474	1- 659	**95**
EB 19	**Paddy**	***Methylobacterium radiotolerans***	**FJ624148**	**1-1448**	**99**

[a] Species identified based on 16S rRNA gene similarity of endophytic bacteria
[b] Per cent similarity of the sequence in BLAST result

Fig.1 Genomic DNA of promising endophytic bacteria

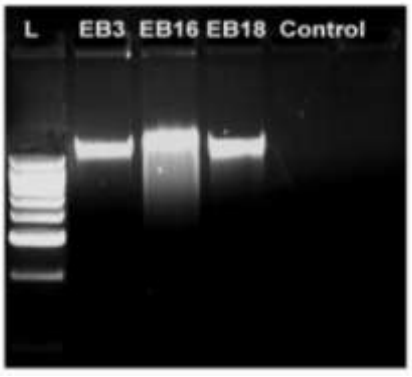


Fig.2 PCR amplification of 16S r RNA of promising endophytic bacteria

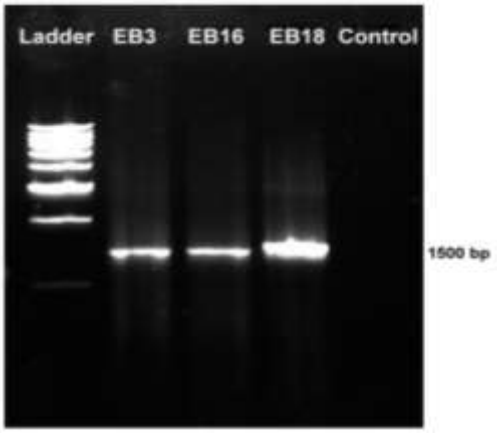
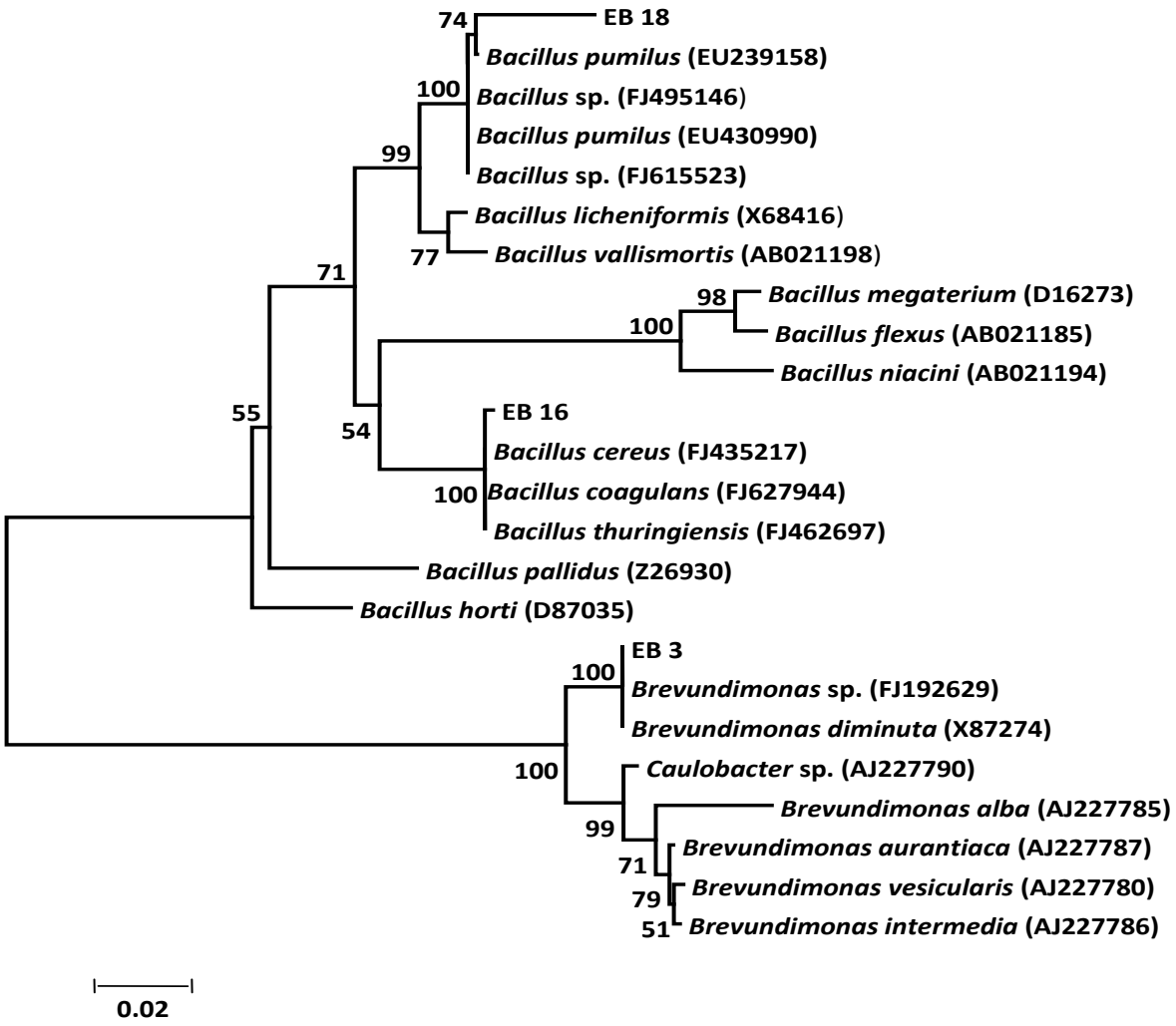


Fig.3 Phylogenetic relationship of endophytic bacteria based on 16S rRNA gene sequences



The sequence analysis supports this phylogenetic position of the endophytic bacteria by boot strap method. In the sequence analysis, the endophytic bacterial isolate EB16 revealed 98 per cent sequence similarity with *Bacillus cereus*, isolate EB3 showed 96 per cent similarity with *Brevundimonas diminuta* and EB18 showed similarity 95 per cent with *Bacillus pumilus* (Table 3). They form very close clustering in phylogenetic tree of 16S rRNA gene by neighbor- joining method.

Phylogenetic relationship

In the study from phylogenetic tree inferred from 16S rRNA gene sequence showed that the isolate *viz*., EB16, EB18 and EB3 very close to *B. cereus, B. pumilus* and *B. diminuta* and respectively (Fig. 3). They form very close clustering in phylogenetic tree of 16S rRNA gene by neighbor -joining method. The first isolated EB 19 identified as *M. radiotolerans* produced indole acetic acid able to utilize ACC deaminase as sole carbon source, which regulates ethylene production by metabolizing ACC into ά ketobutyrate and ammonia (Glick *et al.,* 1998) and this ammonia is toxic to nematodes.

The second isolate EB16 showed the close similarity to *Bacillus cereus* and it was isolated as endophyte from chilli roots. *B. cereus* plays an important role in plant growth promoting bacterium by ACC deaminase which could suppress disease development by production of two chitinases which inhibit activity against fungal pathogens (Huang *et al.,* 2005), antagonistic to phytonematodes. It produced bacteriocins or bacteriocin like substances and antibiotics *viz*., oligomycin A, kanosamine, zwittermicin A, and xanthobaccin (Milner *et al.,* 1996). The third isolate EB3 was close related to *Bacillus pumilus* and it was isolated as endophyte from papaya roots. *B. pumilus* plays an important role in plant growth promotion by gibberellins (Probanza *et al.,* 2002) and has EglA gene which encodes a β-1,4-endoglucanase capable of hydrolyzing cellulose (Lima *et al.,* 2005) and antimicrobial activity.

The fourth isolate EB3, was close by related to *Brevundimonas diminuta*, is the new nomenclature for former *Pseudomonas diminuta* based on a new genus name due to short wavelength polar flagella, restricted biochemical activity, different polyamine and ubiquinone patterns as well as different fatty acid composition (Segers *et al.,* 1994). This group of bacterium is also able to degrade aerobically isoquinoline, a toxic compound used in pesticides, antioxidants and reproducible control of *M. incognita* by *B. vesicularis* (Hallmann *et al.,* 1997) *B. diminuta* produces extracellular metallo and serine proteases (Chaia *et al.,* 2000). The results indicated that the four endophytic bacterial isolate studied have better plant growth promotion activity and serves as a potential biocontrol agent against root knot nematode. There is a vast scope for development of suitable cost effective and efficient bioformulations based on these isolates.

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