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Cloning and Sequencing of Novel Endophytic *Bacillus subtilis* from Coconut for the Management of Basal Stem Rot Disease

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Abstract: In this study, for the management of Basal Stem Rot (BSR) disease sixty endophytic, rhizosphere strains were isolated from coconut, other crops and virgin soils. The strains showed high growth promotion were subjected to *Ganoderma* mycelium inhibition study *in vitro*. The strains EPC5 and EPC8 were showed high growth promotion and strong inhibition to *Ganoderma* pathogen compared to other strains. Both the strains were characterized by biochemical methods and confirmed as *Bacillus*. The *Bacillus* ITS region was amplified by specific primers and EPC5, EPC8 showed amplification of 546 bp products in size. Further, the strains were cloned and sequenced, deposited in NCBI, USA. The sequence showed similarity with *Bacillus subtilis*.

Key words: Endophytes, basal stem rot, *Bacillus subtilis*, cloning and sequencing

INTRODUCTION

Basal Stem Rot (BSR) disease caused by *Ganoderma lucidum* (Leys) Karst. is the most destructive disease and a major limiting factor in coconut production especially in Tamil Nadu, Andhra Pradesh and other coconut growing states of India (Wilson *et al*., 1987; Bhaskaran *et al*., 1989). The disease is also called as Thanjavur wilt, bole rot, *Ganoderma* disease and Anabe roga in different states of India (Vijayan and Natarajan, 1972; Nambiar and Rethinam, 1986; Bhaskaran *et al*., 1996; Srinivasulu *et al*., 2001). Currently, no cost-effective fungicide that gives guaranteed control, although the disease could be delayed by adopting strategic management, which is a labour-intensive procedure. The frequent use of pesticides may lead to the development of tolerance in the target organism. Development of biological control for basal stem rot disease is accepted as a durable and environment friendly alternative for agrochemicals. There is some evidence that endophytes can contribute to the control of plant diseases (Kloepper *et al*., 1992) promote plant growth and yield, suppress pathogens, may help to remove contaminants, solubilize phosphate or contribute assimilable nitrogen to plants (Rosenblueth and Martínez-Romero, 2006). In plant tissues, bacterial endophytes may originate from seeds (Mundt and Hinkle, 1976; McInroy and Kloepper, 1995b), vegetative material (Sturz, 1995), soil (McInroy and Kloepper, 1995a) and the phylloplane (Raaijmakers *et al*., 1995). They are found in numerous plant species (Chanway, 1998) with the most being members of common soil bacterial genera such as *Pseudomonas*, *Bacillus* and *Azospirillum* (Chanway, 1996), many strains can promote plant growth (Chanway, 1998; Hallmann *et al*., 1997). Endophytic bacteria have been shown to control *Fusarium oxysporum* f. sp. *vasinfectum* on cotton (Chen *et al*., 1995), *F. oxysporum* f.sp. *pisi* on pea (Benhamou *et al*., 1996a), *Verticillium alboatrum* and *R. solani* on potato (Pleban *et al*., 1995; Nowak *et al*., 1995) and rice (Krishnamoorthy and Gnanamanickam, 1997), *Sclerotium rolfsii* on bean (Pleban *et al*., 1995) and *Urocystis fagacearum* on oak (Brooks *et al*., 1994).

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Mguni (1996) isolated a number of strains of *Bacillus* sp. with antagonistic potential against black rot of cabbage caused by *Xanthomonas campestris* pv. *campestris*. Wulff (2000) reported that *Bacillus subtilis* strain BB have shown promise as a control agent of black rot under field conditions. *Bacillus subtilis* has great potential uses in agriculture. Its members are able to produce antimicrobial metabolites to control plant pathogens; to fix nitrogen; to form endospores to resist desiccation, heat and UV irradiation and survive in adverse conditions. Endophytic *bacillus* amended with chitin promotes higher growth and suppresses the bacterial blight incidence in cotton under greenhouse conditions (Rajendran *et al*., 2006). With this background, the present study was carried out to isolate, screen and characterize the effective endophytic strains for the management of BSR disease in coconut.

MATERIALS AND METHODS

Isolation of Bacterial Endophytes

Coconut root samples, cotton and virgin soil were taken and brought to the laboratory. Root sections (2-3 cm long) were made using a sterile scalpel. Root samples were surface sterilized with 1% sodium hypochlorite (NaOCl) in 0.05% triton X-100 for 10 min and rinsed four times in 0.02 M sterile potassium phosphate buffer pH 7.0 (PB). A 0.1 mL aliquot was taken from the final buffer wash and transferred to 9.9 mL Tryptic Soy Broth (TSB) to serve as sterility check. Samples were discarded if growth was detected in the sterility check samples (agitating samples in TSB, Hi Media Code No. M 011, at 28±2°C) within 48 h. Each sample (0.5 g) was triturated with a sterile mortar and pestle in 9.5 mL of the final buffer wash. Serial dilutions up to (10^{10}) of the triturate were made in phosphate buffer. Each dilution of every sample was plated (0.1 mL) on three plates each of three different media; Tryptic Soy Agar (TSA-Hi Media, Code No. M290). Nutrient agar (NA g L^{-1}; peptone 5, beef extract 2 and agar 20, pH 5.0) and King's B Medium (g L^{-1}; proteose peptone 20, K_2HPO_4 1.5, Mg $SO_4.7H_2O$ 1.5, glycerol 20 mL and agar 15, pH 7.2) (King *et al*., 1954). The plates were incubated at 28±2°C for 48-72 h. At each sampling date and for each treatment, one representative of each bacterium, as evident from their colony type and morphology was transferred to fresh King's B medium plates to establish pure cultures.

Preparation of Bacterial Suspension Inoculum

The endophytic bacteria were grown on KB and NA broth with constant shaking at 150 rpm for 48 h at room temperature (28±2°C). The bacterial cells were harvested by centrifugation at 10000 rpm for 15 min and bacterial cells were resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu mL^{-1} (OD_{595} = 0.3) and used as bacterial inoculum (Thompson, 1996).

Seed Bacterization

Coconut being a perennial crop, growth promotion by endophytic and rhizosphere bacteria was tested on rice crop. Rice seeds (cv. ADT 46) were surface sterilized with two per cent sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight under sterile air stream. Endophytic bacterial strains inoculated into respective broth were taken in a conical flask. Required quantity of seeds were soaked in bacterial suspension containing 3×10^8 for 2 h and dried under shade.

Plant Growth-Promotion

Plant growth-promoting activity of bacterial endophytic strains were assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Rice seed bacterization was done as described earlier. Twenty seeds were kept over the presoaked germination paper. The seeds were held in position by placing another presoaked germination paper strip and gently pressed. The

polythene sheet along with seeds were then rolled and incubated in growth chamber for 14 days. Three replications were maintained for each treatment. The root and shoot length of individual seedlings were measured and the germination percentage of seeds was also calculated. Plant growth promotion also tested in pot culture method. Bacterized seeds were sown in pots. Twenty seeds were maintained for each treatment. The root and shoot length of individual seedlings were measured and the germination percentage of seeds was also calculated. The vigour index was calculated by using the formula as described by Baki and Anderson (1973).

$$\text{Vigour index} = \text{Percent Germination} \times \text{seedling length (shoot length + root length)}$$

In vitro* Testing of Endophytic Bacterial Strains on Inhibition of Mycelial Growth of *Ganoderma

Bacterial endophytic strains were tested for their inhibition on mycelial growth of *Ganoderma* by following the dual culture technique (Dennis and Webster, 1971). The bacterial culture was streaked at one side of Petridish (1 cm from the edge of the plate) plated with PDA medium and mycelial disc (8 mm diameter) of seven days old culture of *Ganoderma* was placed on the opposite side in the Petridish perpendicular to the bacterial streak. The plates were incubated at room temperature (28±2°C) for four days and the mycelial inhibition of pathogen was measured in millimeter.

Identification of Endophytic Bacterial Isolates

Various biochemical tests were carried out to identify the isolated endophytic bacterial strains. These include morphological, cultural characteristics on agar plate and biochemical tests. The following are the important bio-chemical tests conducted in the laboratory.

- Simple staining.
- Gram staining.
- Endospore staining.
- KOH test.
- Utilization of citrate.
- Catalase test.
- Starch hydrolysis.
- Gelatin hydrolysis.
- Methyl red test.
- Growth in 7% NaCl (Aneja, 1993; Schaad, 1992).

Isolation of *Bacillus* DNA

Endophytic *Bacillus* sp. was grown in nutrient broth or on nutrient agar plates at 28°C. Total DNA (including chromosomal and plasmid DNA) was extracted as described by Robertson *et al.* (1990) with slight modifications. Cultures grown for 16 h in nutrient broth were centrifuged into a pellet, washed in TE (10 mM Tris pH 7.5/1 mM EDTA pH 8.0) and suspended in 10% sucrose. Cells were incubated at 37°C in lysozyme solution (5 mg mL^{-1} lysozyme, 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0), followed by addition of 20% SDS containing 0.3% beta-mercaptoethanol. DNA was purified by organic extraction and ethanol precipitation. Purified DNA was quantified by UV spectrophotometry.

Detection of *Bacillus* Species Specific Loci in the Endophytic Strains

To confirm strains as *Bacillus* sp., 16S rRNA intervening sequence specific BCF1 (CGGGAGG CAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCTTAAT) primers were used to get an amplicon size of 546 bp (Cano *et al.*, 1994). PCR reactions were carried out in 20 μL reaction mixture containing 10X buffer (with 2.5 mM $MgCl_2$), 2 μL; 2 mM dNTP mixture, 2 μL; 2 M primer,

5 µL; *Taq* DNA polymerase, 3 U; H_2O, 8 µL and 50 ng of template DNA samples were amplified on DNA thermalcycler (Eppendorf Master Cycler Gradient, Westbury, New York) using the PCR conditions 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The total number of cycles was 40 with the final extension time of 10 min. The PCR products were resolved on 2% agarose at 50 V stained with ethidium bromide (0.5 µg mL^{-1}), photographed and analysed using gel documentation system (Alpha Innotech Corporation, USA).

Cloning and Sequencing of 16S rDNA of *Bacillus* sp.

The 16S-23S rDNA of EPC5 and EPC8 were amplified with intervening sequence specific primers, BCF1 (CGGGAGGCAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCT TAAT) primers were used to get an amplicon size of 546 bp (Cano *et al*., 1994). Amplified 16S rDNA was purified from each reaction mixture by agarose (1.2%, w/v) gel electrophoresis in TBE buffer containing 0.5 µg of ethidium bromide per mL. A small agarose slice containing the band of interest (observed under long-wavelength [312 nm] UV light) was excised from the gel and purified by using a QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, California) according to the supplier's instructions. This purification was performed to remove primer dimers and other residues from the PCR amplification. Fragments were cloned into the T/A vector pCR2.1 (Fermentas, St. Leon-Rot, Germany) and transformed into *Escherichia coli* strain DH5α according to the procedure recommended by the manufacturer. Transformants were selected on LB agar amended with ampicillin (75 mg mL^{-1}) and X-Gal (20 mg mL^{-1}). Clones were randomly selected and used as templates in PCR to produce products of required size 546 bp in agarose gel. Clones producing PCR fragments of the appropriate size were then subjected to sequencing. DNA sequencing was performed at Genei Pvt Ltd, Bangalore, India. For sequence determination of the cloned PCR products, a generally applicable sequencing strategy was developed. The sequences for entire cloned PCR products, approximately 546 bp in length were determined by using vector-encoded M13 sequencing primer sites, forward primer (5'-CACGACGTTGTAAAACGAC-3') and reverse primer (5'-GGATAACAATTTCACACAGG-3').

Assignment of Cloned Sequences to Establish Phylogenetic Divisions

Sequence identities of the GenBank database were performed with BLAST analyses (Altschul *et al*., 1990). Average linkage cluster analysis of aligned sequences for construction of phylogenetic trees was performed with Treecon version 1.15. Clustering was determined by UPGMA analysis of pairwise genetic distance values. Amino acid and nucleotide sequences were aligned by using the CLUSTAL X 1.81. Newly obtained sequences were deposited with GenBank database, GenBank, New York, USA.

Statistical Analysis

All the experiments were performed twice with required replicates and arranged in a randomized complete block design for greenhouse experiment. The data were statistically analyzed (Rangasamy, 1995) using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($p<0.05$ and $p<0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Isolation of Endophytic and Rhizosphere Bacteria

Totally sixty isolates of endophytic and rhizosphere bacteria were isolated from healthy coconut roots, cotton stem and root and virgin soils of Periyar reserve and Ooty forest soil (Table 1).

Table 1: List of endophytic bacterial strains and source

Isolates	Place	Source
EPC 1	Kallikadu	Coconut root
EPC 2	Kallikadu	Coconut root
EPC 3	Kallikadu	Coconut root
EPC 4	Coimbatore	Coconut root
EPC 5	Coimbatore	Coconut root
EPC 6	Coimbatore	Coconut root
EPC 7	Coimbatore	Coconut root
EPC 8	Veppankulam	Coconut root
EPC 9	Coimbatore	Coconut root
EPC 10	Kallikadu	Coconut root
EPC 11	Aliyar	Coconut root
EPC 12	Aliyar	Coconut root
EPC 13	Aliyar	Coconut root
EPC 14	Aliyar	Coconut root
EPC 15	Aliyar	Coconut root
EPC 16	Aliyar	Coconut root
EPC 17	Aliyar	Coconut root
EPC 18	Aliyar	Coconut root
EPC 19	Kallikadu	Coconut root
EPC 20	Veppankulam	Coconut root
EPC 21	Aliyar	Coconut root
EPC 22	Aliyar	Coconut root
EPC 23	Aliyar	Coconut root
EPC 24	Aliyar	Coconut root
EPC 25	Aliyar	Coconut root
EPC 26	Aliyar	Coconut root
EPC 27	Veppankulam	Coconut root
EPC 28	Veppankulam	Coconut root
EPC 29	Thangachimadam	Coconut root
EPC 30	Thangachimadam	Coconut root
EPC 31	Rameshwaram	Cotton root
EPC 32	Thangachimadam	Coconut root
EPC 33	Rameshwaram	Cotton stem
EPC 34	Coimbatore	Coconut root
EPC 35	Coimbatore	Coconut root
EPC 36	Coimbatore	Coconut root
EPC 37	Coimbatore	Coconut root
EPC 38	Coimbatore	Coconut root
EPC 39	Coimbatore	Coconut root
EPC 40	Coimbatore	Coconut root
EPC 41	Muthankurichi	Coconut root
EPC 42	Muthankurichi	Coconut root
EPC 43	Coimbatore	Coconut root
EPC 44	Muthankurichi	Coconut root
EPC 45	Muthankurichi	Coconut root
EPC 46	Muthankurichi	Coconut root
EPC 47	Kanniankadu	Coconut root
EPC 48	Kanniankadu	Coconut root
EPC 49	Aliyar	Coconut root
EPC 50	Aliyar	Coconut root
EPC 51	Aliyar	Coconut root
EPC 52	Aliyar	Coconut root
EPC 53	Aliyar	Coconut root
EPC 54	Aliyar	Coconut root
EPC 55	Aliyar	Coconut root
EPC 56	Kambam	Coconut root
EPC 57	Kambam	Coconut root
Isolate 58	Periyar reserve	Virgin soil
Isolate 59	Periyar reserve	Virgin soil
Isolate 60	Ooty forest soil	Virgin soil

Table 2: Effect of bacterial endophytes on Rice seedling growth

	Vigour index	
Isolates	Pot culture study	Roll towel method
1	1483.49^{y}	1221.08^{V}
2	1097.47^{M}	979.98
3	864.48^{X}	1016.48^{Y}
4	1043.49^{Q}	1451.48^{P}
5	1943.37^{d}	2053.08^{e}
6	1615.35^{o}	2021.78^{w}
7	1056.48^{P}	1146.08^{X}
8	1934.39^{e}	2182.88^{m}
9	1139.43^{K}	1416.98^{R}
10	1671.48^{m}	1431.68^{Q}
11	1487.38^{x}	1625.08^{L}
12	1862.48^{f}	3524.48^{b}
13	1826.33^{g}	3549.48^{a}
14	1455.30^{r}	2007.48^{x}
15	1711.47^{j}	2035.78^{uv}
16	1294.47^{F}	1602.48^{M}
17	1575.46^{q}	2093.78^{p}
18	1431.45^{B}	1803.48^{F}
19	1663.48^{n}	2056.88^{r}
20	1518.35^{v}	2035.08^{v}
21	1807.47^{h}	3028.88^{d}
22	1532.46^{t}	2004.98^{y}
23	1592.28^{p}	1924.28^{z}
24	1448.48^{A}	2006.38^{xy}
25	1061.48^{O}	1711.68^{l}
26	904.50^{y}	1002.68^{Z}
27	1224.34^{H}	1629.08^{K}
28	1336.45^{D}	2121.88^{n}
29	2006.28^{b}	3379.48^{c}
30	1322.45^{E}	1851.68^{D}
31	1357.48^{C}	1673.88^{J}
32	1943.43^{d}	2258.48^{i}
33	1695.46^{i}	2825.48^{g}
34	1490.48^{w}	2097.88^{o}
35	1103.46^{L}	1854.48^{C}
36	1574.46^{q}	2049.08^{t}
37	1175.40^{J}	2454.48^{g}
38	1013.47^{R}	1232.98^{U}
39	1061.32^{O}	1518.08^{N}
40	1217.48^{I}	1875.48^{A}
41	1280.40^{G}	1831.48^{E}
42	764.44^{Y}	979.48
43	1615.42^{o}	2252.18^{j}
44	1224.37^{H}	1756.48^{H}
45	989.46^{S}	1859.38^{B}
46	1140.41^{K}	1452.08^{P}
47	983.48^{T}	1797.08^{G}
48	1084.50^{N}	1473.48^{O}
49	889.50^{W}	1157.48^{W}
50	1525.39^{u}	2200.98^{l}
51	1703.34^{k}	2037.18^{u}
52	1979.48^{c}	2653.58^{f}
53	1539.42^{s}	2216.28^{k}
54	1759.50^{i}	2201.08^{l}
55	1560.49^{r}	2085.48^{q}
Pf1	2024.43^{a}	2368.28^{h}
Control 1	939.46^{U}	1403.48^{S}
Control 2	1013.37^{R}	1390.88^{T}

Values are mean of two replications; Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at p = 0.05

Table 3: *In vitro* antagonistic activity of bacterial endophytic isolates of Coconut against *Ganoderma*

Isolates	Percent inhibition over control
Pseudomonas fluorescens (Pf-1)	40.67 (39.62)[f]
Bacillus (EPC5)	33.80 (35.54)[e]
Bacillus (EPC8)	29.16 (32.68)[d]
Pseudomonas (EPC15)	27.78 (31.80)[d]
Pseudomonas (EPC32)	4.75 (12.58)[c]
Pseudomonas (EPC52)	2.65 (9.37)[b]
Control	0.00 (0.0)[a]

Values are means of three replications; Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at p = 0.05; Values in parentheses are arcsine transformed

Table 4: Identification and characterization of bacterial endophytic strains by biochemical characteristics

	Isolate No.		
	EPC5	EPC8	Pf1
Source	Coconut root	Coconut root	Black gram rhizosphere
Simple staining	Short rod	Rod	Rod
Gram's staining	+ ve	+ ve	- ve
KOH test	- ve	- ve	+ ve
Catalase test	+ ve	+ ve	+ ve
Starch hydrolysis	+ ve	+ ve	+ ve
Gelatin hydrolysis	+ ve	+ ve	+ ve
Growth in 7% NaCl	+ ve	+ ve	- ve
Citrate utilization test	+ ve	+ ve	- ve
Methyl red test	- ve	- ve	+ ve
Tentatively identified as	*Bacillus* sp.	*Bacillus* sp.	*P. fluorescens*

Efficacy of Bacterial Endophytic Strains on Plant Growth Promotion

Coconut being a perennial crop, growth promotion by endophytic and rhizosphere bacteria was tested on rice crop. The growth promotion by bacterial endophytes and rhizosphere isolates was assessed by treating the rice seeds with all the 60 bacterial isolates separately by their suspension. Endophytic bacterial isolates EPC5, EPC8, EPC12, EPC13, EPC15, EPC21, EPC29, EPC32, EPC52 and *Pseudomonas fluorescens* strain Pf1 were found to increase the vigour index of rice seedlings significantly when compared to untreated control (Table 2).

***In vitro* Screening of the Endophytic Bacterial Strains Against the Pathogen**

Out of 60 endophytic and rhizosphere bacterial strains, ten isolates were selected in preliminary screening for growth promotion. These isolates were tested for their efficacy by dual plate technique against *G. lucidum* along with Pf1. Among the ten isolates, five strains were found to inhibit the growth of *G. lucidum in vitro*. The strain, Pf1 showed high inhibition to *G. lucidum* followed by EPC5 (coconut root isolate) and EPC8 (coconut root isolate). The per cent inhibition was significantly higher in plates streaked with Pf1 (40.67 %), EPC5 (33.80 %) and EPC8 (29.16 %) against control plates (Table 3).

Biochemical Tests for Endophytic Bacteria

The isolates which promoted plant growth and inhibitory to *G. lucidum* were characterized by biochemical methods for identification. The methods viz., Gram's staining, Endospore staining, Catalase test, Starch hydrolysis, Gelatin liquefaction, Growth at 7% NaCl and HCN test were carried out (Table 4).

PCR Amplification of Endophytic *Bacillus* Genus Specific Loci

For the further confirmation of *Bacillus* strains, polymerase chain reaction was performed using gene specific primers. The ITS primers amplified a fragment size of 546 bp corresponding to the region of the 16s-23s rRNA intervening sequence for *Bacillus* sp. Best isolates from different ecosystems

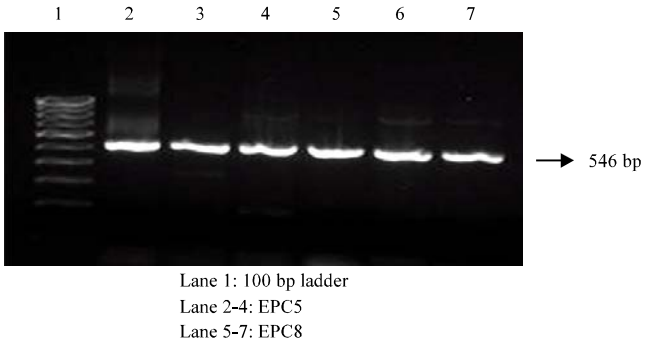

Fig. 1: Detection of endophytic *Bacillus* by gene specific primer

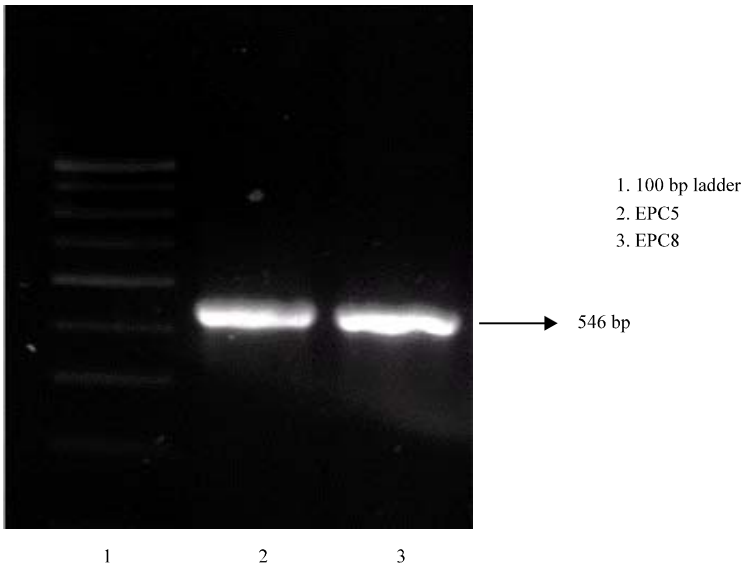

Fig. 2: Colony PCR for *Bacillus*

of Tamil Nadu were examined for the amplification of the 16s-23s rRNA region and EPC5 and EPC8 isolates of endophytic bacterial collection showed amplified product with the size of 546 bp. The results of PCR amplification has further confirmed that these isolates were pertaining to the group of *Bacillus* (Fig. 1).

Cloning and Sequencing of 16s rDNA of *Bacillus* sp.

The 16s-23s rDNA fragments of EPC5, EPC8 were cloned into the T/A vector pTZ57R/T and transformed into *Escherichia coli* strain DH5α. Transformants on LB agar amended with ampicillin were randomly selected and used as templates in PCR to produce the products of required size of 546 bp in agarose gel (Fig. 2). Complete sequence of *Bacillus* ITS region was obtained and submitted in the NCBI, GenBank, New York, USA. They were compared with nucleotide and aminoacid sequences from the GenBank database of different *Bacillus* isolates from various countries (Table 5).

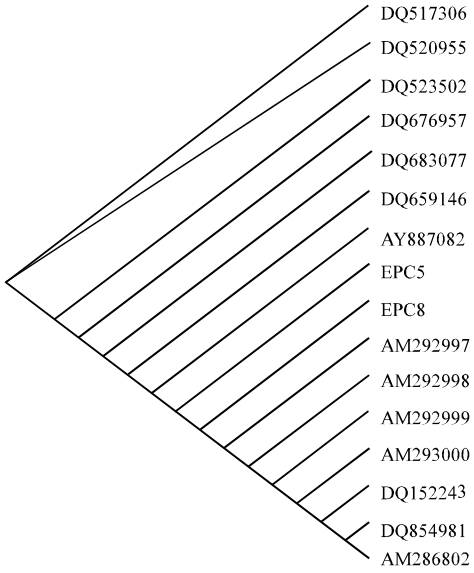

Fig. 3: UPGMA tree of 16s rDNA of *Bacillus* with other nucleotide sequences form GenBank

Table 5: Internal Transcribed Spacer (ITS) sequences of *Bacillus* isolates used in this study

Isolates	Accession No.
Bacillus subtilis	AY887082
Bacillus subtilis strain GB03	DQ683077
Bacillus subtilis strain Jinran 14	DQ676957
Bacillus subtilis strain GR011	DQ659146
Bacillus subtilis strain B432	DQ523502
Bacillus subtilis strain B-FS01	DQ520955
Bacillus subtilis strain B237	DQ517306
Bacillus sp. GPTSA100-6	DQ854981
Bacillus cereus strain JUN 7	DQ152243
Bacillus sp. JG-B41	AM293000
Bacillus sp. JG-B37	AM292999
Bacillus sp. JG-B35	AM292998
Bacillus sp. JG-B12	AM292997
Bacillus sp. A8S3	AM286802

The unweighted pair group method with arithmetic means (UPGMA) tree resulting from the analysis of nucleotide and aminoacid sequences of DNA gene using the sequences from the GenBank were compared shown in Fig. 3. Genetic distance among the sequences from the GenBank and from the cloned sequences of DNA based on UPGMA was shown in Table 6.

DISCUSSION

Coconut palms were affected by many pests and diseases. Among them, BSR disease caused by *Ganoderma lucidum* is the most destructive one which is widespread in nature. Basal stem rot in coconut is a serious disease in India and in severely infected areas, incidence as high as 80% was recorded (Ramadoss, 1991). Basal stem rot can be contained effectively by management practices in the early stages of disease development. The use of cultural practices and toxic chemicals has both advantages and disadvantages. Managing this disease by biological methods has become increasingly important. Among biological control methods, endophytic bacteria are an alternative to chemical pesticides that can be more reliable and ecologically as well as economically sustainable. The efficacies

Table 6: Sequence identity similarity matrix for *Bacillus* EPC5 and EPC8 isolates based on 16S rDNA ITS sequence data with GenBank database reference isolates

Isolates	EPC8	AY887	DQ683	DQ676	DQ659	DQ523	DQ520	EPC5	DQ517	DQ854	DQ152	AM293	AM292	AM292	AM292	AM286
EPC8	1.000	0.891	0.536	0.904	0.904	0.904	0.904	0.897	0.904	0.848	0.851	0.848	0.848	0.848	0.848	0.517
AY887		1.000	0.565	0.949	0.949	0.949	0.949	0.926	0.949	0.886	0.888	0.886	0.886	0.886	0.886	0.524
DQ683			1.000	0.515	0.515	0.515	0.515	0.512	0.515	0.489	0.489	0.489	0.489	0.489	0.489	0.925
DQ676				1.000	1.000	1.000	1.000	0.928	1.000	0.925	0.926	0.925	0.925	0.925	0.925	0.482
DQ659					1.000	1.000	1.000	0.928	1.000	0.925	0.926	0.925	0.925	0.925	0.925	0.482
DQ523						1.000	1.000	0.928	1.000	0.925	0.926	0.925	0.925	0.925	0.925	0.482
DQ520							1.000	0.928	1.000	0.925	0.926	0.925	0.925	0.925	0.925	0.482
EPC5								1.000	0.928	0.877	0.881	0.877	0.877	0.877	0.877	0.499
DQ517									1.000	0.925	0.926	0.925	0.925	0.925	0.925	0.482
DQ854										1.000	0.990	1.000	1.000	1.000	1.000	0.509
DQ152											1.000	0.990	0.990	0.990	0.990	0.517
AM293												1.000	1.000	1.000	1.000	0.509
AM292													1.000	1.000	1.000	0.509
AM292														1.000	1.000	0.509
AM292															1.000	0.509
AM286																1.000

of the biocontrol agents Pf1 and *T. viride* has been studied individually for the management of *Ganoderma* (Karunanithi *et al*., 2004). Sixty endophytes were isolated from coconut root, cotton stem and roots. Colonization of plant roots with certain beneficial microbes causes the induction of a unique physiological and biochemical state in plants called priming. Primed plants display either faster, stronger or both activation of the various cellular defense responses that are induced following attack by either pathogens or insects or in response to abiotic stress (Conrath *et al*., 2006). Similar reports obtained by Cho *et al*. (2003) stated that endophytic colonization of balloon flower by *Bacillus* sp. CY22 without any harm to the root. The endophytes could actively dissolve the cell wall components to gain entry (Zinniel *et al*., 2002). In the present study, endophytic bacteria from the roots of coconut palms EPC5, EPC8, EPC15, EPC29 and EPC52 were found to increase the vigour index of rice seedlings significantly when compared to untreated control. Hallmann *et al*. (1997) reported that most of the endophytic bacterial strains are capable of promoting plant growth. Endophytic bacteria colonize a broad spectrum of plant species and plant parts (Sturz *et al*., 1997). *Bacillus* species are among the most common bacteria found to colonize plants endophytically (Lilley *et al*., 1996; Mahafee and Kloepper, 1997) and it is likely that their endophytic ability could play an important role in the biocontrol of vascular plant pathogens. In our study, most of the isolates were characterized as *Bacillus* sp. whereas some *Pseudomonas* sp. has also been identified. Several reports indicate the role of bacterial endophytes in the management of plant pathogens. Barka *et al*. (2002) reported that *in vitro* bacterization of grapevine with plant growth promoting rhizobacterium, *Pseudomonas* strain PSJN reduced the incidence of grey mold caused by *Botrytis cinerea* when compared to non bacterized controls. In the present study, Pf1 and endophytic bacterial strain EPC5 showed maximum mycelial inhibition of *G. lucidum*. Antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine exhibited antifungal activity against phytopathogenic fungi in Petri dish assays and produced chitinase, β-l,3-glucanase, salicylic acid, siderophore and hydrogen cyanide (Pandey *et al*., 2006). The inhibitory action may be due to the production of antifungal or antibacterial agents (Maurhofer *et al*., 1998). Out of 905 bacterial isolates from rhizosphere of healthy avocado trees, eight strains produced antibiotics viz., phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN) inhibitory to *Dematophora necatria* white root rot (Cazorla *et al*., 2006). EPC5 and EPC8 isolates were Gram-positive, spore forming bacteria, able to grow at 45°C and in the presence of 7% NaCl. They utilize citrate as sole carbon source. Both strains were catalase positive, efficiently hydrolyzed starch and gelatin. The result obtained by analyzing primary character and carbon source utilization of different endophytic bacteria revealed that they belong to *Bacillus* spp. Rapid, sensitive and selective methods are necessary for identification and characterization of bacteria at the species and strain level. In order to overcome the problem involved in phenotypic characterization, some of the molecular methods are utilized nowadays. The use of FAME and UP-PCR fingerprinting profiles were generally helpful in the identification of *Bacillus* spp., making these features useful for the classification of genus at species level. Tilak and Reddy (2006) identified isolates from maize as *Bacillus cereus* and *B. circulans* by biochemical characteristics and profile of fatty acids. Wulff *et al*. (2002) characterized fifty one *Bacillus* isolates by universal primer polymerase chain reaction fingerprinting and clustered into three different groups viz., *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus*. EPC5 produced endospore in stress condition which supports the work of Ongena *et al*. (2005) where *B. subtilis* M4 produced endospore which are tolerant to extreme pH values, more resistant to drying process for powder formulation and provided control against *Colletotrichum lagenarium* in cucumber. Our study confirms the strains as *Bacillus subtilis* by cloning and sequencing. Hill *et al*. (2004) characterized more than 300 *Bacillus* isolates by fluorescent Amplified Fragment Length Polymorphism (AFLP) which revealed extensive diversity within *B. thuringiensis* and *B. cereus* compared to *B. anthracis*. Thus molecular tools such as PCR and sequencing of 16SrDNA genes proved to be powerful tools for a rapid characterization of microbial communities for the management of destructive diseases.

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