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Molecular characterization and *in vitro* evaluation of endophytic bacteria against major pathogens of rice

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Thirty one isolates of endophytic bacteria were collected from different plant sources together with *Bacillus subtilis* var*. amyloliquefaciens* (FZB24) that was obtained from Novozymes South Asia Pvt. Ltd. to screen against major pathogens of rice. All the isolates were characterized on the basis of biochemical and phenotypic analysis. Therefore, following these tests, it can be concluded that 31 isolates exhibited differences and they were subjected to partial 16S-rDNA gene sequencing using polymerase chain reaction for phylogenetic analysis. The molecular characterization through amplification of 16S rDNA fragment to an amplicon size of 546 bp confirmed that the thirty one isolates were *Bacillus*. The PCR analysis showed that all the 31 isolates were found to have the genes for iturin A, 30 isolates for surfactin, 27 isolates for bacillomycin D and one isolate was found to have gene for zwittermicin A. The biosynthetic gene for the production of ACC deaminase was also identified among 11 endophytic *Bacillus* isolates. The random amplified polymorphic DNA (RAPD) analysis showed more similarity among the isolates isolated from the same ecosystem as compared to the isolates collected from different ecosystems. Among the endophytic bacterial isolates tested against the major pathogens of rice *viz*., *Pyricularia grisea, Rhizoctonia solani* and *Xanthomonas oryzae*pv. *oryzae in vitro*, *Bacillus subtilis* var. *amyloliquefaciens* (FZB24) was found to be effective in inhibiting growth of all the three pathogens and it was also found to promote the growth of rice seedling by registering significantly higher vigour index in roll towel method.

Key words: Endophyte, *Bacillus*, antagonistic activity, antibiotic gene, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Plants are constantly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and inside the plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they

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seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). Several bacterial endophytes have been shown to support plant growth and increase nutrient uptake by providing phytohormones (Kang et al., 2007) and biologically fixing nitrogen (Jha and Kumar, 2007). Endophytic bacteria cannot only promote plant growth and act as biocontrol agents, but also produce antibiotics to control plant diseases and reduce disease severity (Senthilkumar et al., 2007). Plants have latent defense mechanism against pathogens, which can be systemically activated upon exposure of plants to stress or infection by pathogens (Baker et al., 1997). This phenomenon is called induced systemic resistance (Tuzun and Kuc, 1991). The classical inducers include pathogens, plant growth promoting rhizobacteria (PGPR), plant growth promoting endophytic bacteria (PGPE), chemicals and plant products. Some endophytes offer increased resistance to pathogens thus making them ideal candidates for biological control (Madhaiyan et al., 2004).

Polymerase chain reaction (PCR) based detection is a favoured approach as it is accurate, rapid and sensitive. The small subunit 16s rDNA sequence has shown to be useful for the detection of bacteria (Stead et al., 1997). Many *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of metabolites with antimicrobial activity is one determinant of their ability to control plant diseases (Silo-suh et al., 1994). Antibiotics from Iturin family, *viz*., fengycin with limited antibacterial activity, show strong antifungal and haemolytic activities which is specific against filamentous fungi (Nishikiori et al., 1986). Surfactin shows antiviral and antimycoplasma activities (Vollenbroich et al., 1997). Zwittermicin A has a broad spectrum activity against certain Gram-negative and eukaryotic microorganisms (Silo-suh et al., 1998). With this background, endophytic bacteria were evaluated *in vitro* against the major rice pathogens viz., *Pyricularia grisea, Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). And also, the antibiotic genes in their genome for the synthesis of antibiotics were detected. Variability among the endonphytic bacteria were studied using RAPD analysis.

MATERIALS AND METHODS

Isolation of endophytes

Source plants from different ecosysytem were manually uprooted and brought to the laboratory. Root, stem and leaf sections (2-3 cm long) were made using a sterile scalpel. The root samples were taken just below the soil line for younger plants and 5-10 cm below the soil line for older plants. Stem samples were first weighed and surface sterilized with hydrogen peroxide (20%) for 10 min. and rinsed four times with 0.02 M potassium phosphate buffer (pH 7.0). Root samples were surface disinfected with sodium hypochlorite (1.05%) and washed in four changes of 0.02 M phosphate buffer solution. Measured quantity of 0.1 ml aliquot from the final buffer wash was removed and transferred into 9.9 ml tryptic soya broth to serve as sterile check. Samples were discarded, if growth was detected in the sterile check within 48 h. Selected samples were triturated in 9.9 ml of buffer in sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution and plated on Tryptic Soya Agar (TSA). Representatives of colony morphology were transferred to fresh TSA plated as pure cultures (McInroy and Kloepper, 1995).

Preparation of bacterial inoculum

Endophytic bacteria were grown on King's B (KB) with constant shaking at 100 g for 48 h at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12,000 g for 15 min and bacterial cells were resuspended in phosphate buffer (PB) (0.01 M, pH 7.0). The concentration was adjusted to approximately 10^8 cfu ml^{-1} (OD595 = 0.3) with a spectrophotometer and used as bacterial inoculum (Thompson, 1996).

Seed bacterization

Rice seeds (cv. ADT 39) were surface-sterilized with 2% sodium hypochlorite for 30 se, rinsed in sterile distilled water and dried overnight under a sterile air stream. Endophytic bacterial strains, inoculated into their respective broths and bacterial suspension was prepared as mentioned above. The required quantity of seeds was soaked in bacterial suspension containing 3×10^8 bacteria ml^{-1} for 2 h and dried under shade.

Plant-growth promotion

The plant-growth promoting activity of the bacterial endophytic strains was assessed on the basis of seedling vigour index as determined by the standard roll towel method (ISTA, 1999). Twenty seeds were kept on presoaked germination paper. The seeds were held in position with another presoaked germination paper strip on top of them and gently pressed. The polythene sheet along with the seeds was then rolled and incubated in a growth chamber for 14 days. Three replications were carried out for each treatment. The root and shoot length of individual seedlings was measured and seed germination percentage calculated. The vigour index was calculated using the formula of Baki and Anderson (1973):

Vigour index = germination (%) × seedling length (shoot length + root length)

Antagonism of endophytic bacterial strains against *P. grisea*

Endophytic bacterial strains were tested for their antagonistic activity against mycelial growth of *P. grisea* and *R. solani* by following the dual culture technique (Dennis and Webster, 1971). Mycelialdisc (8 mm diameter) of seven days old culture of pathogens were placed at one side of the Petri plate containing PDA medium at 10 mm away from the periphery. Bacterial cultures were streaked onto the medium exactly opposite to the mycelial disc 10 mm away from the periphery. The plates were incubated at room temperature (28±2°C) for 10 days. Efficiency of the antagonistic organisms against the sheath blight pathogen was assessed based on the inhibition zone observed.

Antagonism of endophytic bacterial strains against *Xoo*

Cell suspension of *Xoo* was prepared in the sterile distilled water to

Table 1. Sequences of oligonucleotide primers.

Antibiotic gene	Primer	Sequence	Amplicon size (bp)
Iturin A	ITUD1F	5'GATGCGATCTCCTTGGATGT3'	647
	ITUD1R	5'ATCGTCATGTGCTGCTTGAG3'	
Surfactin	SUR3F	5'ACAGTATGGAGGCATGGTC3'	441
	SUR3R	5'TTCCGCCACTTTTTCAGTTT3'	
Zwittermicin A	ZWITF2	5'TTGGGAGAATATACAGCTCT3'	779
	ZWITR1	5'GACCTTTTGAAATGGGCGTA3'	
Bacillomycin D	BACC1F	5'GAAGGACACGGCAGAGAGTC3'	875
	BACC1R	5'CGCTGATGACTGTTCATGCT3'	
ACC deaminase	ACCD F	5'ATGAACCTGCAACGATTC3'	1000
	ACCD R	5'TCAGCCGTCTC GGAAGAT3'	

Table 2. Sequences of RAPD primers used to study the genetic variability among isolates of endophytic *Bacillus*.

S/N	Primer	Sequence
1	OPA 01	5'CAGGCCCTTC3'
2	OPA 08	5'GTGACGTAGG3'
3	OPB 11	5'GTAGACCCGT3'
4	OPB15	5'GGAGGGTGTT3'
5	OPG 5	5'CTGAGACGGA3'
6	OPG 11	5'TGCCCGTCGT3'
7	OPG 16	5'AGCGTCCTCC3'
8	P7	5'GATAGCTCGCTG3'
9	CAG	5'CAGCAGCAGCAGCAG3'
10	GACA	5'GACAGACAGACAGACA3'

a concentration of 10^7 cfu/ml. 1 ml of the bacterial cell suspension (*Xoo*) was mixed with 19 ml of nutrient agar (NA) medium and poured onto the sterile Petri dishes. After solidification, sterile paper discs (6mm diameter) were placed on the surface of the medium at 1 cm away from the side of the Petri dish and 5 µl of the endophytic bacterial culture in NA broth of 4h old was applied to each disc. The plates were incubated at 37±2°C and the inhibition of bacterial growth was measured 48 h after the treatment (Salah et al., 2010).

Statistical analysis

The present experimental data were analyzed using analysis of variance (ANOVA) by Agres Statistical Software Package Version 3.01 (Agres, 1994).

Characterization of endophytes

Isolation of genomic DNA of endophytes

The genomic DNA from each isolates of endophytes were isolated using the standard protocol of cetyltrimethyl ammonium bromide (CTAB) method proposed by Knapp and Chandlee (1996) with slight modifications (Melody, 1997) from actively grown culture. The genomic DNA was checked by agarose gel electrophoresis and stored at -20°C for further use.

Amplification of 16S rDNA gene was carried out by polymerase chain reaction using an Eppendorf Master cycler, German. Reaction volume of 25 µl, was prepared and mixed in the PCR tubes. Polymerase chain amplification of endophytic bacteria was done by using primers specific BCF 1 (5'CGGGAGGCAGCAGTAGGGAAT3'); and BCR2 (5'CTCCCCAGGCGGAGTGCTTAAT3'). These primers were used to get an amplicon of 546 bp size (Cano et al., 1994). The thermo cycling conditions consisted of a hold of 2 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C and a final extension of 5 min at 72°C. Amplified fragments of DNA were fractionated on a 1% w/v agarose gel during 100 min at constant voltage of 80 V in 0.5×TAE (Tris-Acetat EDTA). A 10-kb reference marker (campany and country) was used to allow standardization. Following staining with ethidium bromide (10 µg ml^{-1}), the gel was visualized using gel doct (company) under UV light to confirm the expected size of the product. Also PCR reactions were carried out using the methodology established by Ramarathnam et al. (2007) with the antibiotic specific primers and ACC deaminase specific primer (Sheehy et al., 1991) (Table 1).

RAPD-PCR analysis

In total, ten primers were used for RAPD analysis (Table 2). All the RAPD primers were purchased from Operon (Operon Biotechnologies, Cologne, Germany) and used as single primers. Amplification was performed in a 20 ml reaction volume consisting of 5 mM each dNTPs, 20 pmol of primer, 0.5 U of Taq DNA polymerase (Bangalore GeneiPvt Ltd, Banglore, India) and 50 ng of template. The PCR was performed, using Eppendorf – Master Cycler ep gradient S (Eppendorf, A G, Hamburg, Germany), with an initial denaturation step for 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C, with a final extension for 10 min at 72°C. Following amplification, 10 ml of each PCR product was separated by electrophoresis in 2% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). A 1Kb ladder was used as a size standard. To visualize DNA, gels were stained with ethidium bromide (0.1 mg/l) and then photographed under transmitted ultraviolet light, using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). All RAPD analyses were repeated at least three times for each primer.

Data analysis

The amplified fragments of each isolate were scored as 1 (present) or 0 (absent). Co-migrating bands were considered homologous characters. Faint bands and bands showing variable levels of intensity were not considered for scoring. A similarity matrix was constructed, using Jaccard's coefficient, and the resulting similarity data were used to construct a dendrogram, using UPGMA and the NTSYS-pc software version 2.02 developed by Rohlf (1990).

Table 3. Biochemical characters of collected endophytic bacterial isolates from different plant sources.

S/N	Isolates	Place	Source	Biochemical tests			Tentatively identified as
				Gram staining	KOH	Growth in 7% NaCl	
1	EPB 1	Coimbatore	Rice leaf	+	-	+	*Bacillus* sp.
2	EPB 2	Coimbatore	Rice leaf	+	-	+	*Bacillus* sp.
3	EPB 3	Coimbatore	*Trianthima* Leaf	+	-	+	*Bacillus* sp.
4	EPB 4	Coimbatore	*Trianthima* Leaf	+	-	+	*Bacillus* sp.
5	EPB 5	Coimbatore	*Trianthima* Leaf	+	-	+	*Bacillus* sp.
6	EPB 6	Bavanisagar	*Acalypha* leaf	+	-	+	*Bacillus* sp.
7	EPB 7	Mettur	Greengram leaf	+	-	+	*Bacillus* sp.
8	EPB 8	Bavanisagar	*Aloe* leaf	+	-	+	*Bacillus* sp.
9	EPB 9	Bavanisagar	Nerinji leaf	+	-	+	*Bacillus* sp.
10	EPB 10	Bavanisagar	Nerinji leaf	+	-	+	*Bacillus* sp.
11	EPB 11	Bavanisagar	*Cactus* leaf	+	-	+	*Bacillus* sp.
12	EPB 12	Coimbatore	Noni leaf	+	-	+	*Bacillus* sp.
13	EPB 13	Bavanisagar	*Opuntia* leaf	+	-	+	*Bacillus* sp.
14	EPB 14	Bavanisagar	*Agave* leaf	+	-	+	*Bacillus* sp.
15	FZB 24	Taegro, Novozymes South Asia Pvt. Ltd.		+	-	+	*Bacillus* sp.
16	EPB 15	Coimbatore	Cotton leaf	+	-	+	*Bacillus* sp.
17	EPB 16	Coimbatore	Cotton leaf	+	-	+	*Bacillus* sp.
18	EPB 17	Coimbatore	Cotton leaf	+	-	+	*Bacillus* sp.
19	EPB 18	Mettur	Redgram leaf	+	-	+	*Bacillus* sp.
20	EPB 19	Mettur	Redgram leaf	+	-	+	*Bacillus* sp.
21	EPC 5	Coimbatore	Cotton root	+	-	+	*Bacillus* sp.
22	EPC 8	Veppankulam	Cotton root	+	-	+	*Bacillus* sp.
23	EPCO 16	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
24	EPCO 26	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
25	EPCO 29	Thangachimadam	Coconut root	+	-	+	*Bacillus* sp.
26	EPCO 30	Thangachimadam	Coconut root	+	-	+	*Bacillus* sp.
27	EPCO 74	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
28	EPCO 78	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
29	EPCO 81	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
30	EPCO 95	Veppankulam	Coconut root	+	-	+	*Bacillus* sp.
31	EPCO 96	Veppankulam	Coconut root	+	-	+	*Bacillus* sp.

RESULTS AND DISCUSSION

In the present study, 31 native endophytic bacteria were isolated from different range of plant sources such as rice, *Trianthema*, *Agave*, *Opuntia*, *Aloe*, greengram, *Tribulus*, cotton, redgram and coconut (Table 3). Also, endophytic bacteria appear to originate from seeds, vegetative planting material, rhizosphere soil and the phylloplane. The source of endophytic bacterial colonization is diverse and bacteria enter seeds and vegetative planting material from the surrounding environment such as rhizosphere and phyllosphere. They are found in numerous plant species with most being members of common soil bacterial genera such as *Pseudomonas, Bacillus*, *Paenibacillus*, *Azospirillum, Gluconacetobacter* and *Herbaspirillum* (Nogueira et al., 2001). Many PGPE strains have been isolated from internal tissues of different crops and tested against the plant diseases by several workers (Reiter et al., 2002; Sabaratnam and Beattie, 2003).

Endophytes *viz.,* EPB 18, EPB 11, EPCO 74, FZB24 and EPB 10 registered vigour index of 3343, 3225, 3127, 3035 and 3023 respectively as compared to untreated control that registered only 1168. This shows that endophytic bacteria were found to increase the vigour index of the rice seedlings *in vitro* as compared to the control (Table 4). The mechanisms by which plant growth is improved may be similar to those exhibited by rhizosphere microorganisms and include the production of phytohormones, promotion through enhanced availability of

Table 4. Growth promoting activity of bacterial endophytes on rice seedlings *in vitro.*

SN	Isolate	Root length (cm)*	Shoot length (cm)*	Germination (%)*	Vigour index
1	EPB 1	20.81	7.95	96	2761
2	EPB 2	19.58	7.93	94	2586
3	EPB 3	20.90	7.20	100	2810
4	EPB 4	20.20	7.70	100	2790
5	EPB 5	21.70	7.50	100	2920
6	EPB 6	21.30	9.33	98	3002
7	EPB 7	21.70	8.00	100	2970
8	EPB 8	18.54	8.21	98	2621
9	EPB 9	19.51	8.52	100	2803
10	EPB 10	22.06	8.17	100	3023
11	EPB 11	23.54	8.71	100	3225
12	EPB 12	19.09	8.49	84	2317
13	EPB 13	20.20	8.39	100	2859
14	EPB 14	19.11	8.12	100	2723
15	FZB 24	21.90	8.45	100	3035
16	EPB 15	20.95	8.06	82	2379
17	EPB 16	15.99	8.31	100	2430
18	EPB 17	20.44	8.00	100	2844
19	EPB 18	23.88	9.55	100	3343
20	EPB 19	19.50	8.60	100	2810
21	EPC 5	19.35	8.66	100	2801
22	EPC 8	19.39	7.90	100	2729
23	EPCO 16	20.15	8.39	100	2854
24	EPCO 26	18.87	8.24	96	2603
25	EPCO 29	15.60	6.41	100	2201
26	EPCO 30	12.91	6.87	86	1701
27	EPCO 74	22.63	8.64	100	3127
28	EPCO 78	16.63	8.64	94	2375
29	EPCO 81	20.99	8.48	100	2947
30	EPCO 95	19.51	9.74	100	2925
31	EPCO 96	12.74	8.13	98	2045
32	Control	8.70	5.90	80	1168
SED		1.60	0.47		112.19
CD (0.05)		3.19	0.94		224.28
CD (0.01)		4.25	1.24		298.18
CV		10.13	7.02		5.04

*Mean of three replications.

nutrients, reduction of ethylene levels, production of antibiotics, induced systemic resistance and out competition of pathogens (Holland, 1997). Several reports have indicated that bacterial endophytes promoted the growth and health of crop plants (Sturz et al., 2000).

In this study, endophytic *Bacillus* isolates *viz.,* FZB24, EPB 13, EPCO 95, EPB 8, EPB 11, EPCO 16, EPCO 26 and EPCO 96 EPCO 78, EPC 5, EPB 7, EPB 9, EPB 10, EPB 4, EPB 3, EPB 17, EPCO 29 and EPCO 81 were found to show more than 35% inhibition over control against rice blast pathogen *Pyricularia grisea in vitro* (Table 5).

The *Bacillus* isolates *viz.,* EPB 13, EPB 18, EPB 14, EPB 8, EPB 17, EPB 3, EPB 19, EPB 9, EPB 16, FZB24, EPB 6 and EPB 4 were found to have an inhibition of more than 35% over control against sheath blight pathogen, *R. solani in vitro*. Similarly, the endophytic isolates of *Bacillus viz*., FZB24, EPB 9, EPB 10, EPCO 29 and EPCO 78 significantly inhibited the growth of *X. oryzae* pv. *oryzaein vitro* by registering a inhibition zone of 20.0 mm diameter (Table 5) over the control. These results are in line with the findings of Bhuvaneswari (2005). She found that endophytic *Bacillus* strains *viz.,*

EPBC 68 and EPBC 73 recorded a inhibition zone of 8.3 and 9.7 mm, respectively, significantly inhibited the growth of *X. axonopodis* pv. *malvacearum* in cotton *in vitro* over the control. Sessitsch et al. (2004) screened 35 endophytic isolates, out of which seven isolates showed antagonistic activity against bacterial pathogens *viz. Streptomyces scabies* (43%) and *Xanthomonas campestris* (29%). Endophytic bacterial strain, EPCO 16 from cotton plants effectively inhibited the mycelial growth of *R. solani in vitro* (Rajendran, 2003).

Ting et al. (2003) identified three endophytic bacterial isolates *viz., P. aeruginosa*, *Serratia marcescens* and *Burkholderia glumae* from wild banana plants showing antagonistic activity against *F. oxysporum.* f.sp*. cubense*. Endophytic bacterial strain EPC 5 showed maximum mycelial inhibition of *Ganoderma lucidum* (Rajendran, 2006). Antagonistic strain of *Pseudomonas putida* (B0) isolated from sub-alpine exhibited antifungal activity against phytopathogenic fungi in Petri dish assays and produced chitinase, β-1,3-glucanase, salicylic acid, siderophore and hydrogen cyanide (Pandey et al., 2006). This inhibition process observed *in vitro* was reported to be the secretion of secondary metabolites and antibacterial agents released (Sessitsch et al., 2004). *Bacillus* species have special characteristics that make them good candidates as biological control agents. *Bacillus amyloliquefaciens* isolates produced surfactin, iturin, bacillomycine and azalomycin F, while *B. subtilis* isolates mostly synthesize surfactin and arthrobactin. Also surfactin, amphomycin, arthrobactin and valinomycin were found in culture extracts of *B. pumilus*isolates. The antagonistic activity found for the metabolites of *Bacillus* spp. associated with the synergistic effect is caused by the combination of antibiotics (Asaka and Shoda, 1996).

Endophytic bacterial strains *viz., Aureobacterium saperdae* and *B. pumilus* showed higher antifungal activity against *Fusarium* wilt in cotton (Chen et al., 1995). Reiter et al. (2002) isolated an endophytic *Clavibacter michiganensis*strain from potato with biocontrol activities against *Erwinia carotovora*. Wulff et al. (2002) reported the antagonistic activity of *B subtilis* and *B. amyloliquefaciens* against black rot of cabbage *in vivo* and the metabolic profiles produced *viz.,* surfactin, iturin, bacillomycin and azalomycin F were responsible for the inhibition of *Xanthomonas campestris* pv. *campestris*. Bhowmik et al. (2002) reported that seed bacterization with endophyte, Endo PR8 was found to be most effective in reducing the cotyledonary infection by *Xam*. With these evidences, it is predicted that production of antibiotics and secondary metabolites by endophytic *Bacillus* isolates might have played a major role in inhibiting the growth of *P. grisea, R. solani*and *X. oryzae*pv*. oryzae invitro*.

PCR based detection of microorganisms is a reliable approach as it is accurate, rapid and sensitive. In the present study, PCR amplification has confirmed that endophytic bacterial strains which were tentatively identified as the *Bacillus* spp. with the phenotypic and biochemical characterization were *Bacillus* spp. (Figure 1), using *Bacillus* genus specific primers which amplified a fragment of approximately 546 bp corresponding to the region of the 16S-23S rRNA intervening sequence for *Bacillus* sp. Similarly, Zinniel et al. (2002) identified six endophytes with the most promising levels of colonization in a range of host plants based on 16S rRNA gene sequence, commercial fatty acid and carbon utilization analyses. Also, Rajendran (2006) have identified that two endophytic isolates EPC5 and EPC 8 were isolated from coconut root to be *Bacillus* sp. with the 16S rDNA gene sequence analysis using gene specific primer.

The primary mechanism of biocontrol agents is the production of antibiotics. *Bacillus* spp. used to produce many antibiotics such as iturin, surfactin, bacillomycin, zwittermicin, fengycin (Athukorala et al., 2009), azalomycin F (Wulff et al., 2002), amphomycin, arthrobactin and valinomycin. In the present study, the presence of antibiotics producing gene is identified by the PCR analysis using gene specific primer. Almost all the isolates are found to contain the iturin and surfactin producing genes (Figure 2a and b). This clearly indicates that these two antibiotics are common to all *Bacillus* spp. This result supports earlier findings in which these two antibiotics were detected from a wide array of *Bacillus* spp. including *B. subtilis*, *B. amyloliquefaciens, B. licheniformis, Bacillus mycoides, B. cereus* and *Bacillus thuringensis* (Athukorala et al., 2009; Thaniyavarn et al. 2003; Ramarathnam, 2007). This implies that surfactin and iturinare among the most common lipopeptide antibiotics produced by *Bacillus* spp. The *Bacillus* strains which had less inhibiting effect *viz*., EPB 15 and EPCO30 were also found to contain the surfactin genes. This confirms the earlier findings of Hofemeister et al. (2004) that the surfactins help bacteria to form biofilms rather than defense functions.

In the present study, one isolate (EPCO 16) has produced zwittermicin (Figure 2c). A very low percentage of bacteria have the ability to produce this antibiotics. Athukorala et al. (2009) reported that among the twenty one isolates, only two isolates were found to produce zwittermicin A. Twenty seven isolates of *Bacillus* sp. produced bacillomycin (Figure 2d). Ramarathnam et al. (2007) reported that bacillomycin D was detected in *B. subtilis* and *B. amyloliquefaciens* respectively. Athukorala et al. (2009) also reported a very low percentage of bacillomycin D, fengycin and zwittermicin A producing bacteria among the different isolates.

There are few points of interest that relate to agricultural uses of PGPE containing 1-aminocyclopropane-1-carboxylate deaminase (ACCD) gene other than biological agent. It has been shown that some PGPE strains are able to counteract flooding problems by reducing the negative effect of irrigation of crops with highly saline water. This is reflected in lowering the plant ethylene levels elevated by salt stress by means of ACCD

Table 5. Effect of bacterial endophytic isolates against major pathogens of rice.

S/N	Isolates	*P. grisea*		*R. solani*		*Xoo*
		Radial growth of (mm)*	% inhibition over control	Radial growth of (mm)*	% inhibition over control*	Inhibition zone (mm)*
1	EPB 1	73.0	18.89	68.0	24.44	15.0
2	EPB 2	69.0	23.33	65.0	27.78	15.0
3	EPB 3	56.0	37.78	55.0	38.89	14.0
4	EPB 4	55.0	38.89	58.0	35.56	12.0
5	EPB 5	73.0	18.89	66.0	26.67	16.2
6	EPB 6	59.0	34.44	58.0	35.56	19.0
7	EPB 7	53.0	41.11	62.0	31.11	19.0
8	EPB 8	47.0	47.78	54.0	40.00	15.6
9	EPB 9	53.0	41.11	57.0	36.67	20.0
10	EPB 10	54.0	40.00	59.0	34.44	20.0
11	EPB 11	49.0	45.56	60.0	33.33	15.0
12	EPB 12	71.0	21.11	81.0	10.00	10.6
13	EPB 13	44.0	51.11	46.0	48.89	17.0
14	EPB 14	53.0	41.11	52.0	42.22	19.0
15	FZB 24	43.0	52.22	58.0	35.56	20.0
16	EPB 15	90.0	0.00	90.0	0.00	0.0
17	EPB 16	62.0	31.11	57.0	36.67	15.0
18	EPB 17	57.0	36.67	54.0	40.00	17.0
19	EPB 18	75.0	16.67	50.0	44.44	14.0
20	EPB 19	70.0	22.22	57.0	36.67	14.2
21	EPC 5	50.0	44.44	72.0	20.00	14.0
22	EPC 8	59.0	34.44	66.0	26.67	17.8
23	EPCO 16	49.0	45.56	65.0	27.78	19.0
24	EPCO 26	49.0	45.56	72.0	20.00	11.0
25	EPCO 29	57.0	36.67	67.0	25.56	20.0
26	EPCO 30	90.0	0.00	90.0	0.00	4.0
27	EPCO 74	68.0	24.44	90.0	0.00	11.0
28	EPCO 78	50.0	44.44	75.0	16.67	20.0
29	EPCO 81	57.0	36.67	74.0	17.78	2.0
30	EPCO 95	46.0	48.89	73.0	18.89	14.6
31	EPCO 96	49.0	45.56	67.0	25.56	7.8
32	Control	90.0	-	90.0	-	0
SED		2.49		2.16		0.38
CD (0.05)		4.97		4.32		0.75
CD (0.01)		6.61		5.74		1.00
CV		5.16		4.06		3.18

*Mean of three replications.

containing PGPE (Mayak et al., 2004a).

In the present study, ACC deaminase gene was amplified from the 11 isolates in the PCR with the gene specific primers (Figure 2e). Similar results were obtained by Babalola et al. (2003). *P. putida* GR12-12 contains the gene for ACCD, which inhibits ethylene synthesis which is a product of stress. This mechanism is more effective on dicotyledonous plants that are more susceptible to the effects of ethylene especially under stress conditions such as flooding (Grichko and Glick, 2001) drought (Mayak et al., 2004b) and phytopathogens (Wang et al., 2000). Thus, endophytic *Bacillus* strains that possess ACCD activity have the selective advantage over other bacteria during biotic and abiotic stresses.

A total of 31 isolates of endophytic *Bacillus* were tested for their genetic variability by RAPD analysis, using 10

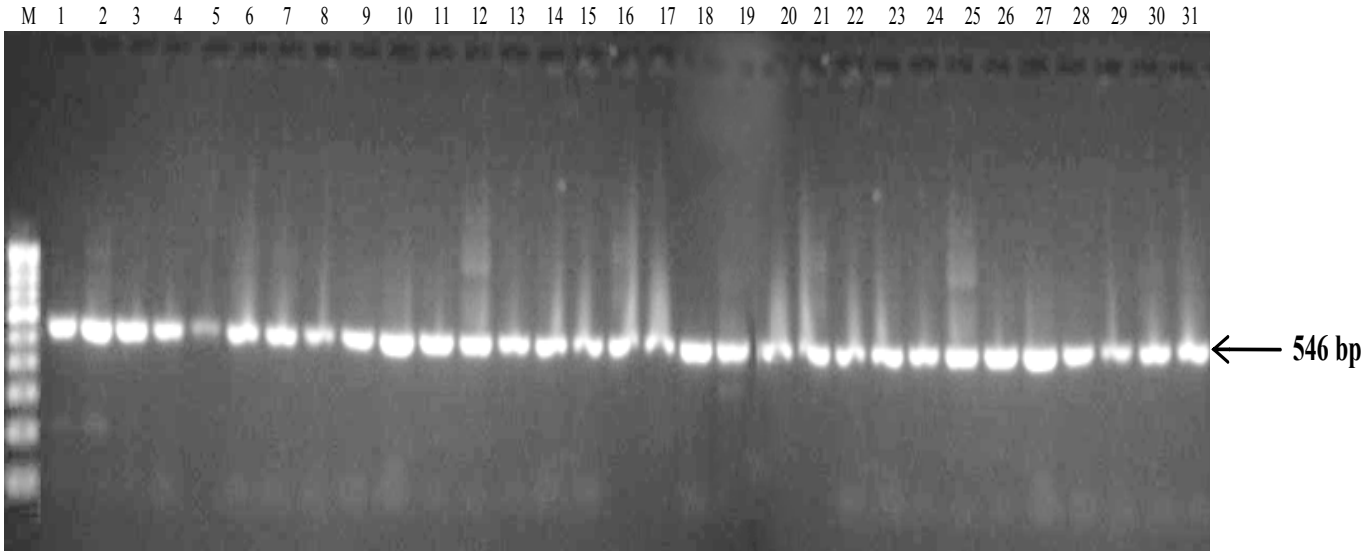

Figure 1. Detection of *Bacillus* species specific loci in the endophytic *Bacillus* strains using specific primers.

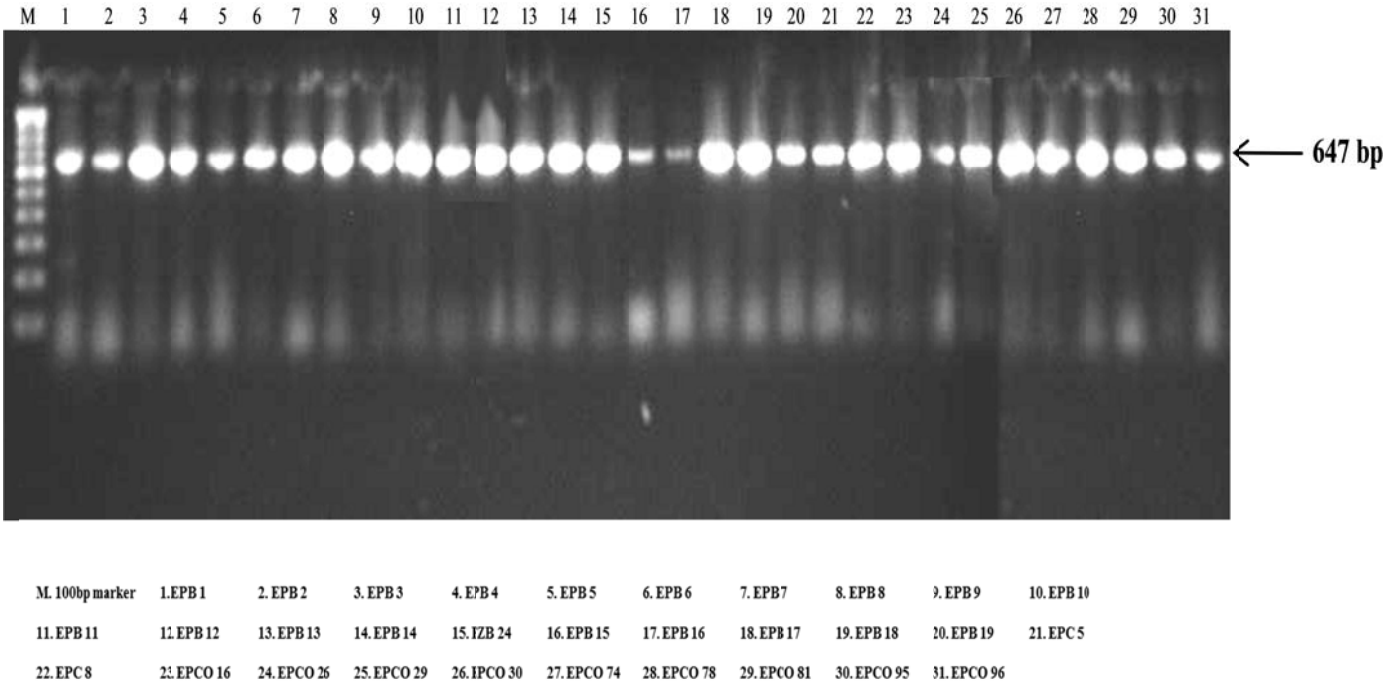

Figure 2a. PCR amplification of antibiotic biosynthetic gene of iturin A from endophytic *Bacillus* isolates.

random primers. Of these, 7 random primers *viz.,* OPA01, OPG 5, OPG 11, OPG 16, CAG and GACA produce easily scorable and consistent banding patterns, which were used for RAPD analysis of thirty one isolates. The number of bands generated by each primer varied from 2 to 6. The amplified products ranged from 150 to 4500 bp, although majority was below 1.5 kb. The RAPD profiles produced with the primers OPA-01, OPG-5, CAG and GACA are shown in Figure 3. Analysis of the genetic coefficient matrix (Table 6), derived from the scores of RAPD profile, showed that minimum and maximum percent similarities among the endophytic *Bacillus*

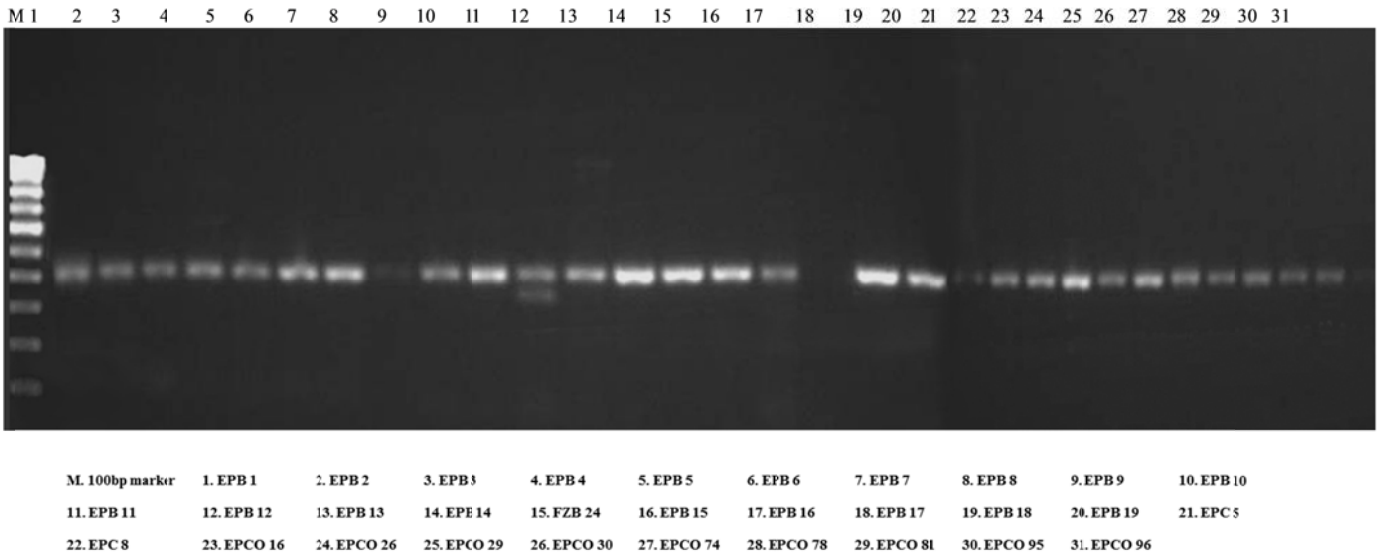

Figure 2b. PCR amplification of antibiotic biosynthetic gene of surfactin from endophytic *Bacillus* isolates.

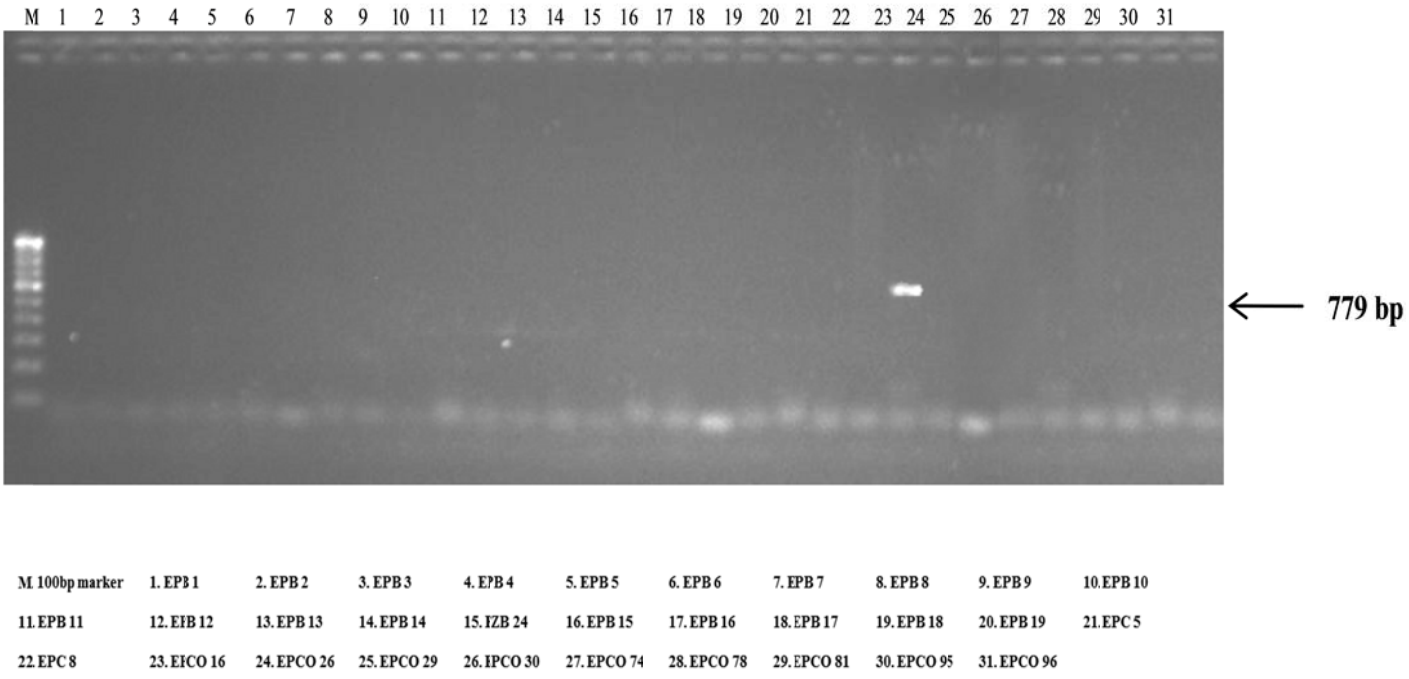

Figure 2c. PCR amplification of antibiotic biosynthetic gene of zwitermicin A from endophytic *Bacillus* isolates.

isolates were in the range of 7 to 73%, respectively (Table 4). Cluster analysis, using UPGMA, clearly separated the isolates into 2 clusters (I and II) confirming some level of genetic diversity among the isolates of endophytic *Bacillus* (Figure 4). Cluster I consisted of 12 isolates and cluster II consisted of 19 isolates. Interestingly, most of the coconut isolates which were isolated from the roots were clustered under the group 1. All the remaining isolates were clustered under group 2. The isolates which were isolated from the same plants

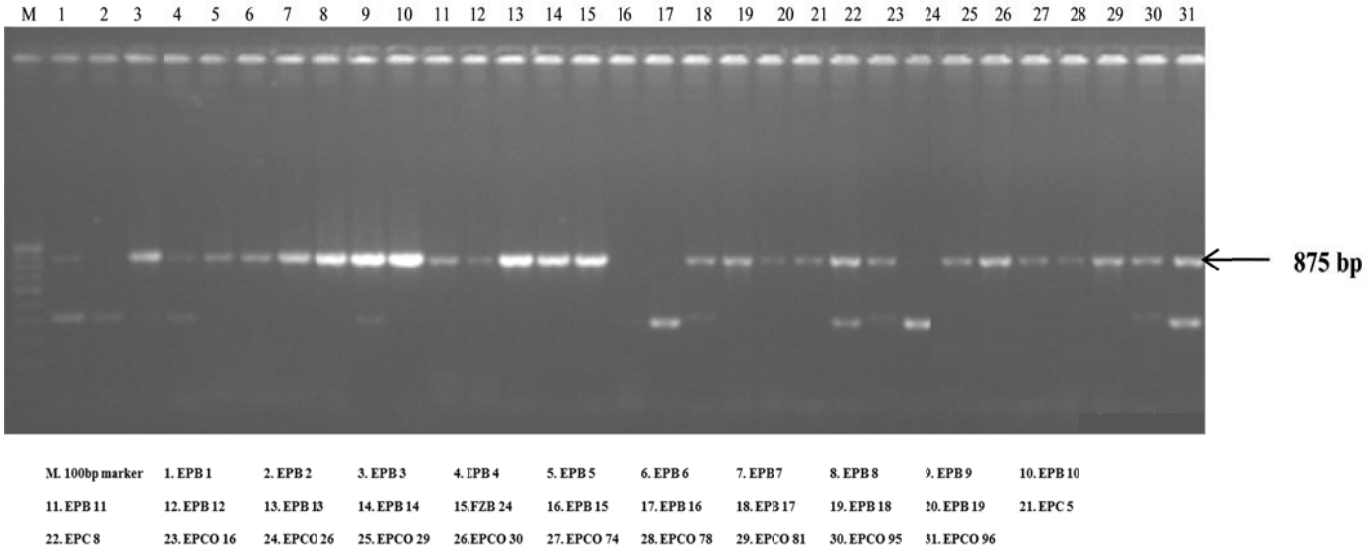

Figure 2d. PCR amplification of antibiotic biosynthetic gene of bacillomycin D from endophytic *Bacillus* isolates.

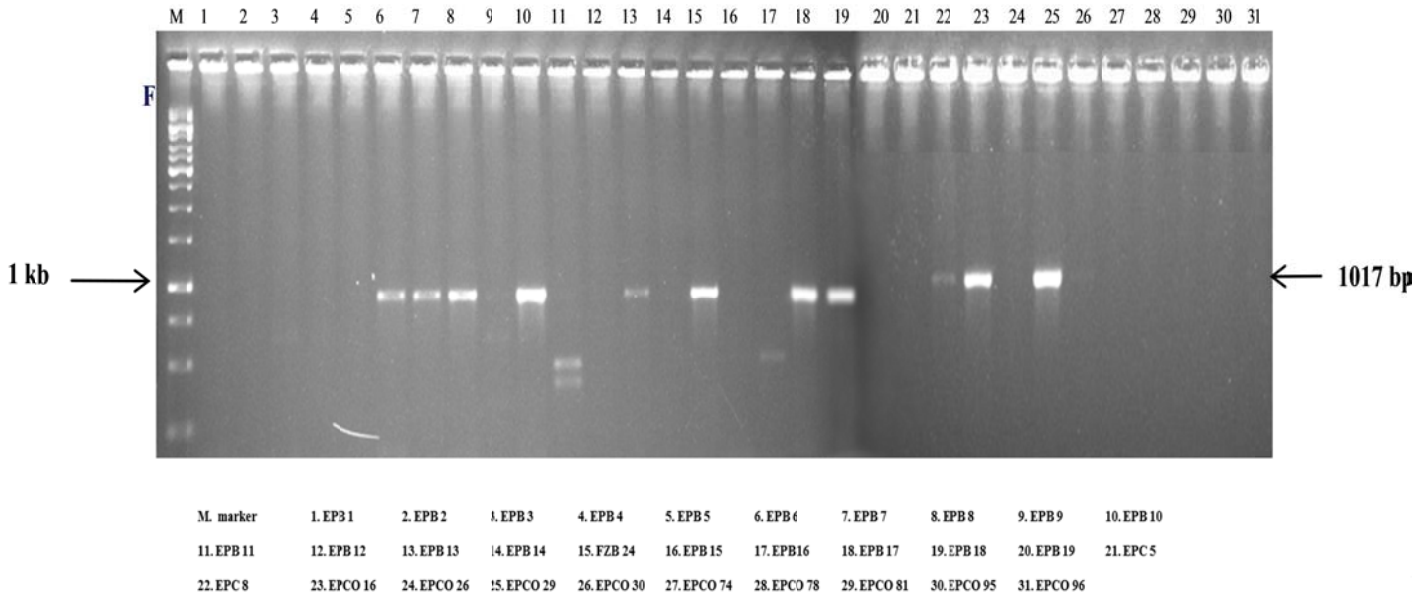

Figure 2e. Detection ACC deaminase biosynthetic gene in the endophytic *Bacillus* isolates using gene specific primer.

were also closer and they formed single clusters. Saveetha (2009) reported that typing of fluorescent pseudomonads using RAPD-PCR indicated more similarity between the DAPG producing strains than the non-producers. Also, Radjacommare (2004) had characterized several *Pseudomonas fluorescens* isolates from rice and vanilla ecosystems based on carbon source utilization, protein profiling, RAPD and ARDRA with Alu I and Hae III analysis.

Conclusion

Among all endophytic bacterial isolates tested against the major pathogens of rice *viz., P. grisea, R. solani*and *X. oryzae*pv. *oryzae in vitro*, endophytic *Bacillus* strains viz., FZB 24, EPB 13, EPB 8, EPB 9, EPB 7, EPB 10 and EPB 17 were found to be effective in inhibiting growth of all the three pathogens and it was also found to promote the growth of rice seedling *in vitro*. Also, it was found to

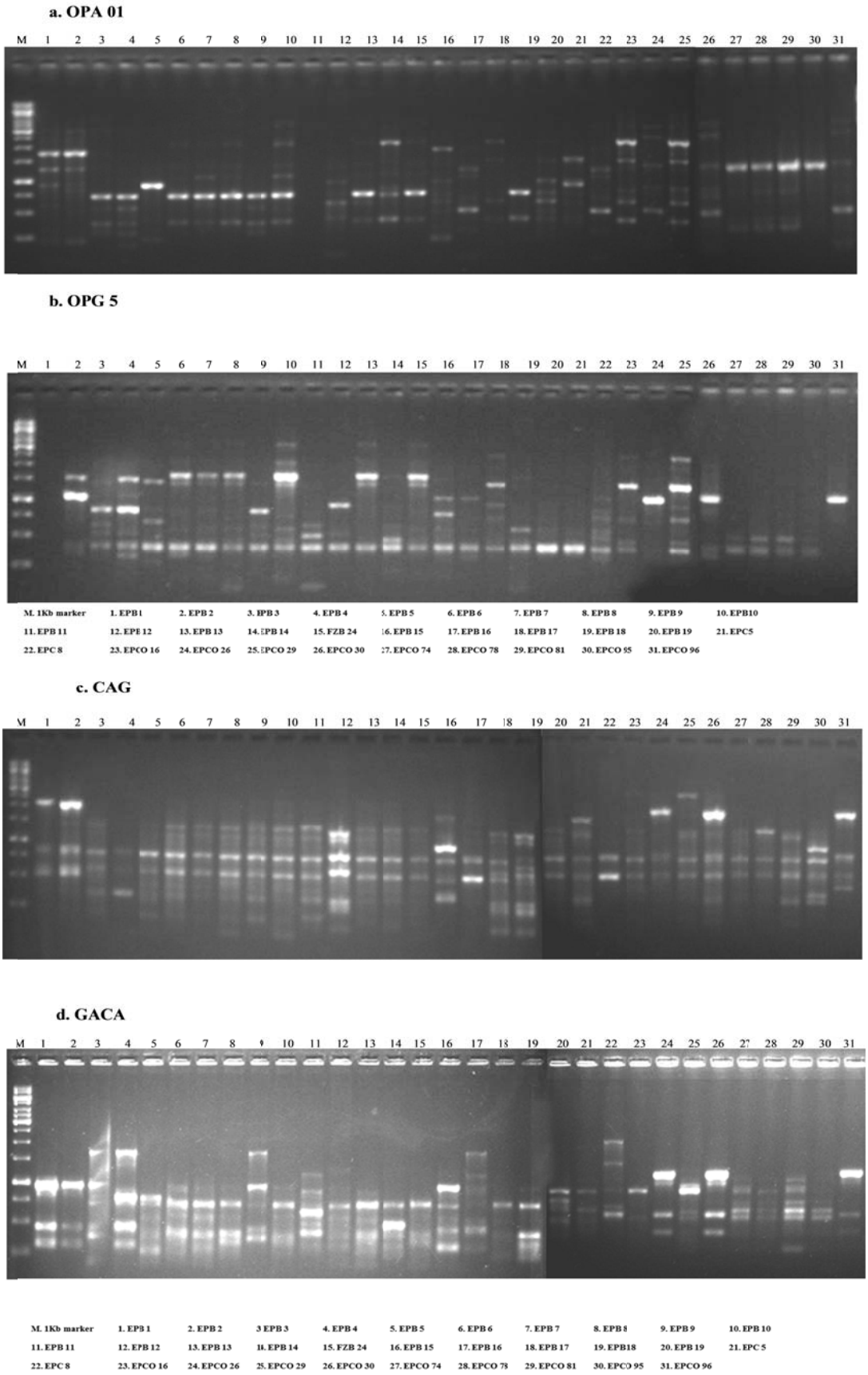

Figure 3. Random amplified polymorphic DNA analysis of endophytic *Bacillus* isolates with random primers.

Table 6. Similarity matrix for the endophytic *Bacillus* isolates generated through the RAPD primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	1.00																														
2	0.63	1.00																													
3	0.37	0.30	1.00																												
4	0.27	0.27	0.53	1.00																											
5	0.42	0.38	0.30	0.27	1.00																										
6	0.27	0.24	0.38	0.33	0.38	1.00																									
7	0.24	0.21	0.39	0.28	0.31	0.69	1.00																								
8	0.24	0.21	0.38	0.28	0.34	0.72	0.81	1.00																							
9	0.25	0.22	0.48	0.32	0.32	0.50	0.62	0.66	1.00																						
10	0.22	0.19	0.33	0.25	0.22	0.51	0.49	0.51	0.43	1.00																					
11	0.29	0.26	0.33	0.27	0.32	0.41	0.46	0.49	0.43	0.30	1.00																				
12	0.21	0.21	0.33	0.27	0.24	0.34	0.38	0.38	0.36	0.33	0.30	1.00																			
13	0.21	0.21	0.33	0.30	0.27	0.62	0.59	0.57	0.43	0.52	0.39	0.43	1.00																		
14	0.18	0.21	0.30	0.30	0.24	0.62	0.59	0.62	0.43	0.45	0.36	0.54	0.67	1.00																	
15	0.18	0.18	0.33	0.38	0.20	0.51	0.53	0.56	0.42	0.48	0.35	0.36	0.56	0.56	1.00																
16	0.30	0.27	0.27	0.35	0.30	0.41	0.38	0.38	0.36	0.31	0.43	0.30	0.40	0.43	0.42	1.00															
17	0.21	0.19	0.28	0.31	0.19	0.29	0.29	0.29	0.33	0.26	0.37	0.28	0.31	0.34	0.33	0.40	1.00														
18	0.18	0.18	0.21	0.22	0.21	0.35	0.36	0.39	0.30	0.34	0.33	0.31	0.38	0.45	0.40	0.34	0.35	1.00													
19	0.25	0.19	0.29	0.29	0.32	0.36	0.41	0.39	0.38	0.27	0.34	0.41	0.41	0.41	0.34	0.41	0.30	0.29	1.00												
20	0.22	0.22	0.26	0.30	0.33	0.34	0.39	0.34	0.36	0.30	0.36	0.33	0.33	0.33	0.29	0.37	0.34	0.38	0.52	1.00											
21	0.21	0.21	0.22	0.28	0.28	0.36	0.33	0.32	0.31	0.23	0.38	0.26	0.32	0.32	0.31	0.38	0.39	0.36	0.37	0.53	1.00										
22	0.21	0.21	0.19	0.20	0.18	0.20	0.23	0.23	0.24	0.18	0.24	0.17	0.20	0.20	0.28	0.26	0.39	0.26	0.27	0.35	0.37	1.00									
23	0.25	0.25	0.26	0.23	0.22	0.33	0.31	0.33	0.31	0.30	0.25	0.29	0.36	0.39	0.38	0.23	0.24	0.41	0.24	0.25	0.31	0.24	1.00								
24	0.30	0.30	0.24	0.22	0.17	0.16	0.19	0.19	0.23	0.15	0.26	0.22	0.19	0.19	0.21	0.22	0.31	0.24	0.17	0.26	0.22	0.32	0.29	1.00							
25	0.21	0.24	0.17	0.20	0.18	0.20	0.17	0.20	0.20	0.19	0.16	0.18	0.22	0.22	0.27	0.15	0.16	0.31	0.18	0.21	0.23	0.20	0.56	0.19	1.00						
26	0.32	0.32	0.18	0.15	0.21	0.16	0.16	0.16	0.17	0.15	0.21	0.17	0.20	0.14	0.14	0.14	0.20	0.19	0.21	0.21	0.19	0.26	0.24	0.61	0.20	1.00					
27	0.20	0.20	0.18	0.17	0.24	0.18	0.15	0.15	0.16	0.17	0.17	0.16	0.13	0.13	0.13	0.13	0.13	0.18	0.14	0.24	0.23	0.23	0.19	0.21	0.19	0.32	1.00				
28	0.22	0.22	0.16	0.18	0.26	0.20	0.21	0.20	0.21	0.21	0.22	0.21	0.18	0.18	0.20	0.24	0.18	0.24	0.22	0.34	0.28	0.28	0.18	0.19	0.21	0.29	0.63	1.00			
29	0.16	0.16	0.18	0.22	0.23	0.18	0.18	0.18	0.22	0.20	0.20	0.19	0.16	0.16	0.18	0.21	0.16	0.21	0.20	0.26	0.22	0.22	0.19	0.18	0.21	0.25	0.52	0.73	1.00		
30	0.18	0.18	0.23	0.21	0.18	0.24	0.28	0.27	0.25	0.21	0.19	0.21	0.21	0.21	0.26	0.24	0.18	0.24	0.15	0.22	0.21	0.21	0.25	0.23	0.21	0.29	0.48	0.62	0.65	1.00	
31	0.18	0.18	0.16	0.08	0.08	0.08	0.11	0.11	0.11	0.09	0.16	0.12	0.07	0.09	0.09	0.15	0.12	0.11	0.13	0.11	0.14	0.14	0.14	0.19	0.15	0.20	0.15	0.17	0.19	0.17	1.00

1, EPB 1; 2, EPB 2; 3. EPB 3; 4, EPB 4; 5, EPB 5; 6, EPB 6; 7, EPB 7; 8, EPB 8; 9, EPB 9; 10, EPB 10; 11, EPB 11; 12, EPB 12; 13, EPB 13; 14, EPB 14; 15, FZB 24; 16. EPB 15 17; EPB 16; 18, EPB 17; 19, EPB 18; 20, EPB 19; 21, EPC 5; 22, EPC 8; 23, EPCO 16; 24, EPCO 26; 25, EPCO 29; 26, EPCO 30; 27, EPCO 74; 28, EPCO 78; 29, EPCO 81; 30, EPCO 95; 31. EPCO 96.

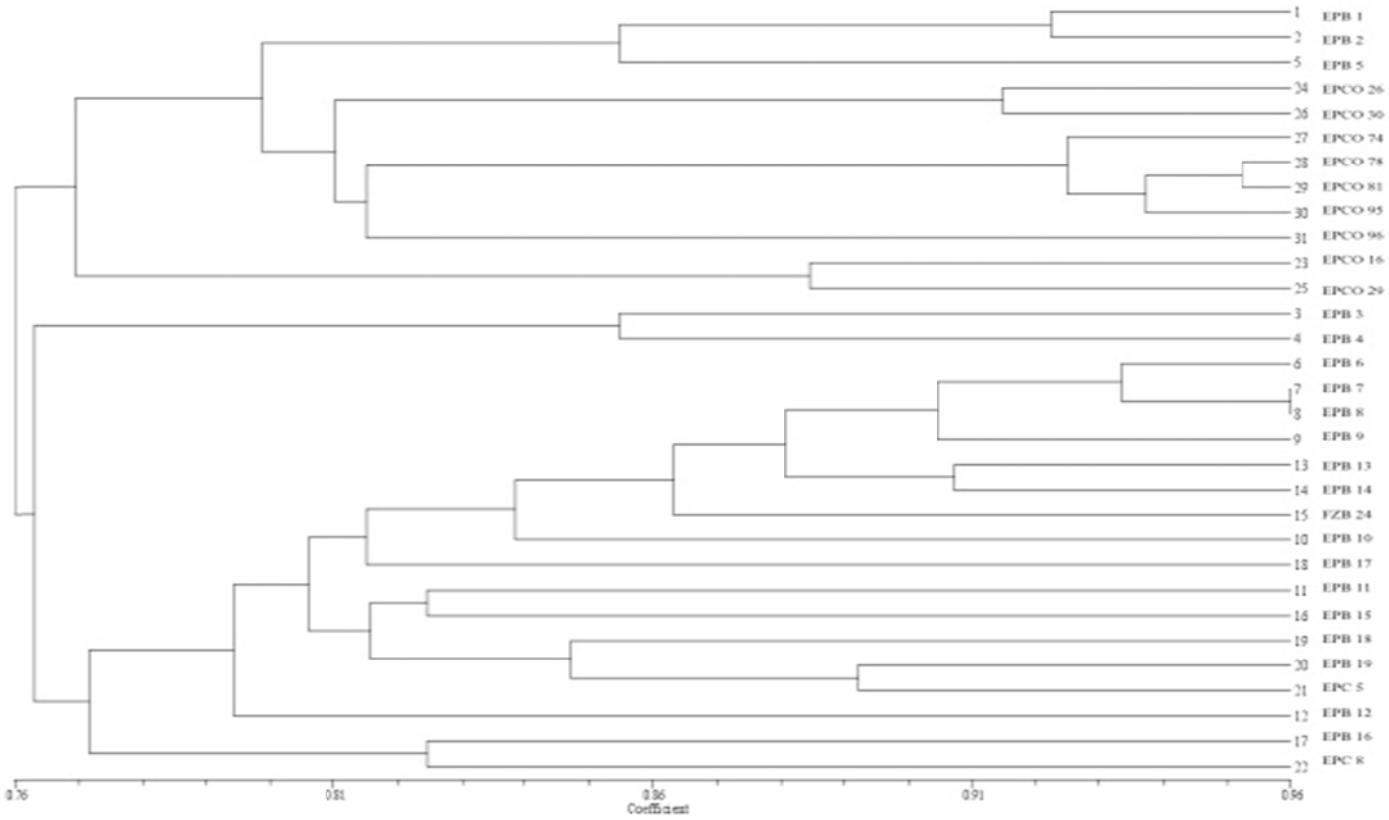

Figure 4. Dendrogram showing the molecular variability of the endophytic *Bacillus* isolates.

harbor antibiotics biosynthetic gene such as iturin, surfactin, bacillomycin and ACCD. So these strains of *Bacillus* sp. can be exploited under glasshouse and field conditions for the management of major rice diseases.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Asaka O, Shoda M (1996). Biocontrol of Rhizoctonia solani damping-off of tomato with *Bacillus subtilis* RB 14. Appl. Environ. Microbiol. 62:4081-4085.

Athukorala SNP, Fernando WGD, Rashid KY (2009). Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Can. J. Microbiol. 55: 1021-1032.

Babalola OO, Osir EO, Sanni AI, Odhiambo GD, Bulimo WD (2003). Amplification of 1-amino-cyclopropane-1-carboxylic (ACC) deaminase from plant growth promoting rhizobacteria in *Striga*-infested soil. Afr. J. Biotechnol. 2 (6): 157-160.

Baker B, Zambryski P, Staskawicz B, Dinesh S (1997). Signalling in plant-microbe interactions and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. 4: 343-350.

Baki AAA, Anderson JD (1973).Vigour determination in soybean seed by multiple criteria. Crop Sci. 13:630-633.

Bhowmik B, Sing RP, Jayaram J, Verma JP (2002). Population dynamics of cotton endophytic *Pseudomonas*, their antagonism and protective action against the major pathogens of cotton. Phytopathology 55(2):124-132.

Bhuvaneswari R (2005). Endophytic *Bacillus* mediated induced systemic resistance against bacterial blight (*Xanthomonas axonopodis* pv.*malvacearum*) and bollworm (*Helicoverpa armigera*) in cotton. M.Sc.(Ag.,) Thesis, Tamil Nadu Agricultural University, Coimbatore, India. pp.124.

Cano RJ, Borucki MK, Higby-Schweitzer M, Poinar HN, Poinar GO, Pollard KJ (1994). *Bacillus* DNA in fossil bees: an ancient symbiosis?. Appl. Environ. Microbiol. 60(6): 2164-2167.

Chen C, Bauske EM, Musson G, Rodrfguez-Kabana R, Kloepper JW (1995). Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. Biol. Control 5:83 -91.

Dennis C, Webster J (1971). Antagonistic properties of species groups of *Trichoderma*: Production of non-volatile antibiotics. Trans. Br. Mycol. Soc. 57: 25-39.

Grichko VP, Glick BR (2001). Amelioration of flooding stress by ACC deaminase-containing plant growth promoting bacteria. Plant Physiol. Biochem. 39:11-17.

Hofemeister J, Conrad B, Adler B, Hofemeister B, Feesche J, Kucheryyava N (2004). Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide- like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. *Mol.* Genet. Genomics

272(4): 363-378.
Holland MA (1997). Occam's razor applied to hormonology: Are cytokinnins produced by plants? Plant Physiol. 115: 865-868.
ISTA (1999). International Rules for Seed Testing. Seed Science and Technology, 27:31.
Jha PN, Kumar A (2007). Endophytic colonization of *Typhaaustralis*by a plant growth promoting bacterium *Klebsiellaoxytoca* GR3. J. Appl. Microbiol. 103: 1311-1320.
Kang SH, Cho HS, Cheong H, Ryu CM, Kim JF, Park SH (2007). Two bacterial endophytes eliciting boot plant growth promotion and plant defense on pepper (*Capsicum annuum* L.). J. Microbiol. Biotechnol. 17: 96-103.
Knapp J, Chandlee JM (1996). Rapid, small-scale dual isolation of RNA and DNA from a single sample of orchid tissue. Biotechniques 21:54-55.
Kobayashi DY, Palumbo JD (2000). Bacterial endophytes and their effects on plants and uses in agriculture, *In* Microbial endophytes. Bacon, C.W. and White, J.F. (Ed.), Marcel Dekker, Inc., New York, USA. pp.199-233.
Madhaiyan M, Poonguzhali S, Senthilkumar M (2004). Growth promotion and induction of systemic resistance in rice cultivar Co-47 (*Oryzasativa* L.) by *Methylobacterium* sp. Bot. Bull. Acad. Sin. 45: 315-324.
Mayak S, Tirosh T, Glick BR (2004a). Plant growth promoting bacteria that confer resistance in tomato and pepper to salt stress. Plant Physiol. Biochem. 167:650-656.
Mayak S, Tirosh T, Glick BR (2004b). Plant growth promoting bacteria that confer resistance to water stress in tomato and pepper. Plant Sci. 166: 525-530.
McInroy JA, Kloepper JW (1995). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton.Can. J. Microbiol. 41: 895-901.
Melody SC (1997). *Plant Molecular Biology - A laboratory manual*. Springer-Verlag, New York. pp.121-154.
Nishikiori T, Naganwa H, Muraoka Y, Aoyagi T, Umezawa H (1986). Plipastatins: new inhibitors of phospholipase A2, produced by *Bacilluscereus* BMG302-fF67. III. Structural elucidation of plipasatins. J. Antibiot. (Tokyo) 39(6): 755-761.
Nogueira EM, Vinagre F, Masuda HP, Vargas C, de Padua VLM, da Silva FR, dos Santos RV, Baldani JI, Ferreira PCG, Hemerly AS (2001). Expression of sugarcane genes induced by inoculation with *Gluconacetobacterdiazotrophicus*and *Herbaspirillumrubrisubalbicans*. Genet. Mol. Biol. 24(1-4): 199-206.
Pandey A, Trivedi P, Kumar B, Palani LS (2006). Characterization of a Phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Curr. Sci. 87: 687-692.
Radjacommare R (2004). Molecular and biochemical markers aided selection of effective Bio-control microbial strains for the eco-friendly management of major diseases in Rice and Vanilla. Ph.D., (Ag) Thesis, Tamil Nadu Agricultural University, Coimbatore, India, 226 p.
Rajendran L (2003). Bacterial endophytes mediated induced systemic resistance against major pests and diseases in cotton. M.Sc. (Ag.,) Thesis, Tamil Nadu Agricultural University, Coimbatore, India. 95p.
Rajendran L (2006). Biotechnological tools and methods for early detection and sustainable management of basal stem rot disease in coconut plantation using microbial consortia.Ph.D Thesis, Tamil Nadu Agricultural University, Coimbatore, India. 200 p.
Ramarathnam R (2007). Phyllosphere bacterial biological contol of *Leptosphaeria maculans*, the blackleg pathogen of canola (*Brassica napus* L.): screening for potential antibiotic producers, investigation of the mechanism of control, biochemical detection of the antifungal compounds and establishment of the role of antibiosis. Ph.D. Thesis, University of Manitoba, Winnipeg, Manitoba.
Ramarathnam R, Bo S, Chen Y, Fernando WGD, Xuenwen G, de Kievit T (2007). Molecular and biochemical detection of fengycin and bacillomycin D producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Can. J. Microbiol. 53(7): 901-911.
Reiter B, Pfeifer U, Schwab H, Sessitsch A (2002). Response of endophytic bacterial communities in potato plants to infection with *Erwiniacarotovora* subsp. *atroseptica.* Appl. Environ. Microbiol. 68(5): 2261-2268.
Rohlf FJ (1990). NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System, Version 2.02. New York, Exeter soft- ware.
Sabaratnam S, Beattie GA (2003). Differences between *Pseudomonas syringae* B728a and *Pantoeaagglomerans* BRT98 in epiphytic and endophytic colonization of leaves. Appl. Environ. Microbiol. 69(2):1220-1228.
Salah EK, Valluvaparidasan V, Ladhalakshmi D, Velazhahan R (2010). Management of bacterial blight of cotton using a mixture of *Pseudomonasfluorescens* and *Bacillus subtilis*. Plant Prot. Sci. 46(2): 41-50.
Saveetha K (2009). Interactive genomics and proteomics of plant growth promoting rhizobacteria (PGPR) for the management of major pests and diseases in rice.Ph.D Thesis, Tamil Nadu Agricultural University, Coimbatore, India. pp. 253.
Senthilkumar M, Govindasamy V, Annapurna K (2007). Role of antibiosis in suppression of charcoal rot disease by soybean endophyte *Paenibacillus* sp. HKA15. Curr. Microbiol. 55:25-29.
Sessitsch A, Reiter B, Berg B (2004). Endophytic bacterial communities of field grown potato plants and their plant growth promoting and antagonistic abilities. Can. J. Microbiol. 50:239-349.
Sheehy RE, Honma M, Yamada M, Sasaki T, Martineau B, Hiatt WR (1991). Isolation, sequence and expression in *Escherichia coli* of the *Pseudomonas* sp. strain ACP gene encoding1-amino cyclopropane-1-carboxylate deaminase. J. Bacteriol., 173:5260-5265.
Silo-Suh LA, Letherbridge BJ, Raffel SJ, He Clardy J, Handelsman J (1994). Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environ. Microbiol. 60(6):2023-2030.
Silo-Suh LA, Stabb EV, Raffel SJ, Handelsman J (1998).Target range of zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. Curr. Microbiol. 37(1):6-11.
Stead DE, Hennessy J, Wilson J (1997). Modern methods for identifying bacteria. In: Pathogen and microbial management in micropropagation. (Ed. Cassells, A.C.), Kluwer, Dordrecht. pp. 61-74.
Sturz AV, Christie BR, Nowak J (2000). Bacterial endophytes: Potential role in developing sustainable systems of crop production. Crit. Rev. Plant Sci. 19: 1-30.
Thaniyavarn J, Roongaswang N, Kameyama T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2003). Production and characterization of biosurfactants from *Bacilluslicheniformis* F2.2. Biosci. Biotechnol. Biochem. 67(6): 1239-1244.
Thompson DC (1996). Evaluation of bacterial antagonist for reduction of summer patch symptoms in Kentucky blue grass. Plant Dis. 80: 856-862.
Ting ASY, Meon S, Kadir J, Son R, Gurmit S (2003). Potential role of endophytes in the biocontrol of *Fusarium* wilt. SecondInternational symposium on *Fusarium* wilt on banana held at Salvador de Bahia, Brazil. p. 32.
Tuzun S, Kuc J (1991) Plant immunization: An alternative to pesticides for control of plant diseases in greenhouse and field, *In* : The Biological Control of Plant Diseases - Proceedings of the International Seminar "Biological Control of Plant Diseases and Virus Vectors". (Ed, J. Bay-Patersen.), Food and Fertilizer Technology Center, Taipei, Republic of China. pp. 30-40.
Vollenbroich D, Pauli G, Ozel, Vater M (1997) Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillussubtilis*. Appl. Environ. Microbiol. 63(1): 44-49.
Wang C, Knill E, Glick BR, Defago G (2000). Effect of transferring 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHAO and its gacA derivative CHA96 on their growth-promoting and disease suppressive capabilities. Can. J. Microbiol. 46(10):898-907.
Wulff EG, Mguni CM, Mansfeld-Giese K, Fels J, Lubeck M, Hockenhull J (2002) Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, B. *subtilis* and B. *pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris*. Plant Pathol. 51:574-584.
Zinniel DK, Lambrecht P, Beth Harris N, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbiol. 68(5): 2198-2208.