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Exploring Quorum Sensing Loci and Biofilm Formation in Bacillus Isolates from Pigeonpea Rhizosphere

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A B S T R A C T

The present study aimed at characterizing the quorum sensing loci of potential *Bacillus* strain from pigeonpea rhizosphere for combating the root rot and wilt diseases of pigeonpea. Screening of 139 *Bacillus* isolates from pigeonpea rhizosphere under *in vitro* conditions revealed that 18 were potential inhibitors of root rot and wilt pathogens. Formation of biofilm by these isolates was observed in solid and liquid media which was quantified by microtitre plate assay. The presence of quorum sensing genes (*comQ, comX, comP* and *comA*) in *Bacillus* strains were detected by PCR following primer designing using the Primer3 software. The ComQXP quorum sensing loci was amplified at approximately 3 kb using forward primer UniComQ and the reverse primer P1. The protein translates of the comX and comP regions of the sequenced nucleotides revealed identity with comX pheromone precursor and histidine kinase sensor protein respectively. The ComA gene of CcB7 was sequenced and the protein translates of which showed identity to response regulation transcription factor. All the sequences are deposited at the GenBank Database.

Introduction

Bacillus is an omnipresent spore-forming gram positive bacterium with the ability to produce a number of lipopeptide molecules with wide range of antimicrobial properties. *Bacillus* has evolved as a successful biocontrol agent during the recent past owing to its strong antibiotic arsenal coupled with high rhizosphere fitness. The lipopeptides produced by *Bacillus* generally belong to the class of iturins, surfactin and fengycins which are highly surface active molecules (Ongena and Jacques, 2008).Success of a biocontrol agent is primarily dependent on its ability to colonize the plant rhizosphere. The basic requirement for plant root colonization by any bacteria is attachment and aggregation in microcolonies/biofilms (Kearns *et al.,* 2004). Thecyclic lipopeptides of the surfactin family is involved in the formation of biofilm in Bacillus. The amphiphilic nature of surfactins accounts for their excellent surface activities.

The production of lipopeptide antibiotics is dependent upon the density of bacterial cells in a particular environment (Grossman, 1995; Msadek, 1999; Tortosa and Dubnau, 1999). High density cultures of *Bacillus subtilis* accumulates ComX pheromone molecules in the culture supernatant, which upon attaining a critical concentration triggers adaptive

cellular responses like surfactin production and biofilm formation. In *B. subtilis*, production of degradative enzymes and antibiotics is a density dependent phenomena regulated by ComQXPA quorum sensing system (Schneider *et al.,* 2002). The *comQXPA* operon controls multiple genes that govern important attributes expressed in the stationary growth phase (Grossman, 1995). Hence in the present study, the bacterial isolate *B. subtilis* CcB7 which showed potential antifungal activity was tested for its capacity to form biofilm in solid and liquid media and an attempt was made to detect the presence of quorum sensing genes and loci in the isolate.

Materials and Methods

Bacillus cultures

The cultures used in this study were isolated from pigeonpea rhizosphere soil by standard serial dilution technique and maintained on nutrient agar slants at 4°C. A total of 18 Bacillus strains which showed potential *in vitro* inhibition of soil-borne fungal pathogens and growth promotion activities were screened for antibiotic production.

DNA isolation and PCR amplification of lipopeptide antibiotic genes

The genomic DNA was isolated from selected strains for antibiotic gene detection through PCR. Genomic DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method described by Knapp and Chandlee (1996), with slight modifications (Melody, 1997).

The antibiotic genes surfactin, iturinA, iturin D, fengycin and bacillomycin D were amplified using gene specific primers as detailed in Table 1.

Primer designing and PCR amplification of quorum sensing genes

Primer designing was done using the Primer3 softwareto detect the presence of quorum sensing genes *viz*., *comQ*, *comX*, *comP* and *comA* in the selected isolates. The genome sequence of *B. subtilis* subsp. *subtilis* str. 168 (GenBank accession No. NC000964.3) which showed 99% homology with the promising rhizospheric *Bacillus subtilis* strain CcB7 was used for primer designing. From the whole genome sequence of NC000964.3, the region 3255900 – 3257092 was used for designing primers for ComQ gene, 3256050 - 32555838for ComX, 3255900 – 3253400 for ComP and 3253500 – 3252785 region for ComA gene. The sequences of the primers designed and the expected product size are given in Table 2. The primers were synthesized by Sigma-Aldrich.

The 40 µl PCR reaction mixture contained DNA template 50 ng, 1X Taq buffer, 0.2mM of each of dNTP mixture, 1µM of each primers, 1.5 mM $MgCl_2$ and 2U of *Taq* DNA polymerase (Sigma). PCR amplification was performed in a Mastercycler using the conditions: Initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), annealing temperature (specified in Table 2 for each gene) for 1 min, 72°C for 1 min (primer extension) and final extension 72°C for 10 min.

PCR amplification and sequencing of quorum sensing loci *comQXP*

The entire *comQ* and *comX* genes and the first 1.7 kb of *comP* loci was amplified by PCR using the primer UnicomQ1 (5’ GGGAGGGGGGAAGTCGTTATTG 3’) and P1 (5’ AAGAACCGAATCGTGGAGATCG CG 3’) in a 20 µl reaction mixture. The PCR profile of the *comQXP* locus amplification consisted of 30 cycles of denaturation at 94°C

for 30 s, annealing at 55°C for 45 s, extension at 72°C for 3 min, and final extension at 72°C for 5 min (Tortosa *et al.,* 2001).

The *comQXP* gene was purified from each reaction mixture by agarose (1.2% w/v) gel electrophoresis in TAE 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 QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California) according to the instructions of manufacturer. This purification was performed to remove primer dimers and other residues from the PCR product. The DNA sequencing was performed at Xcelris Genomics Pvt. Ltd. Ahmedabad, India.

Microtitre plate assay of *Bacillus subtilis* biofilm formation

Bacillus subtilis biofilm formation was monitored using a modified version of the microtitre plate assay as described by O'Toole *et al.,* (1999). *Bacillus subtilis* cells were grown in biofilm growth medium (Luria – Bertani (LB) medium plus 0.15 M ammonium sulphate, 100 mM potassium phosphate, pH 7, 34 mM sodium citrate, 1 mM $MgSO_4$ and 0.1% glucose) with shaking to mid-exponential growth and adjusted to an OD600 of 0.01 in fresh biofilm growth medium. Samples of 100 µl of the diluted cells were then pipetted to each well of a 96-well PVC microtitre plate. The microtitre plates were incubated at 37°C under stationary conditions, cultures were mixed by pipetting up and down twice to oxygenate the medium at 12 h after inoculation. The spent growth medium was exchanged for fresh biofilm growth medium 12 h after mixing. This cycle of mixing followed by exchanging the medium every 12 h was repeated for the time course of the experiment. The presence of adhered cells was monitored by staining with crystal violet (CV) at 48 h and 96 h after inoculation. Growth medium and non-adherent cells were removed from the microtitre plate wells followed by rinsing with wash buffer (0.15 M ammonium sulphate, 100 mM potassium phosphate, pH 7, 34 mM sodium citrate, 1 mM $MgSO_4$). Cells adhered to the wells were stained with 1% CV in wash buffer at room temperature for 20 min. Excess CV was then removed, and the wells were rinsed with water. The CV that had stained the cells was then solubilized in 200 ml of 80% ethanol, 20% acetone. Biofilm formation was quantified by measuring the OD570 for each well using a Bio-Rad model 550 plate reader.

Population dynamics of *Bacillus* spp in pigeonpea rhizosphere

A pot experiment was designed to study the population dynamics of *Bacillus* in the pigeonpea rhizosphere. The experiment was conducted in completely randomized block design replicated three times and four pots were maintained for each replication. Surface sterilized seeds of pigeonpea (CoRg7) were sown in pots containing pathogen inoculated potting mixture at the rate of two seeds per pot. Six pots were maintained for each replication. Two *B. subtilis* isolates *viz.,* CaB5 and EPCO16 and a standard chemical Carbendazim were also used as checks.

The treatments used are furnished below. One gram of rhizosphere soil was collected from treated plants at monthly interval till four months after sowing. The population dynamics of *Bacillus* was assessed through serial dilution technique at 10^{-6} dilution in Nutrient agar medium.

T1 - Seed treatment @ 10 g kg-1 + basal application of *B. subtilis* CcB7 @ 2.5 kg ha-1
T2 - Seed treatment @ 10 g kg-1 + basal

application of *B. subtilis* CaB5 @ 2.5 kg ha^{-1}

T3 - Seed treatment @ 10 g kg^{-1} + basal application of *B. subtilis* EPCO16 @ 2.5 kg ha^{-1}

T4 - T1 + soil application of *B. subtilis* CcB7 @ 2.5 kg ha^{-1} at 45 DAS

T5 - T2 + soil application of *B. subtilis* CaB5 @ 2.5 kg ha^{-1} at 45 DAS

T6 - T3 + soil application of *B. subtilis* EPCO16 @ 2.5 kg ha^{-1} at 45 DAS

T7 - Seed treatment + soil drenching with carbendazim 0.1% at 45 DAS

T8 - T1 + carbendazim 0.1% soil drenching at 45 DAS

T9 - T2 + carbendazim 0.1% soil drenching at 45 DAS

T10 - T3 + carbendazim 0.1% soil drenching at 45 DAS

T11 - Control

The population of *Bacillus* spp in all the treatments were enumerated by serial dilution technique at monthly interval up to 120 days.

Statistical analysis

All the experiments were analysed independently.

The treatment means were compared by Duncan's Multiple Range Test (DMRT).

The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics unit, The Philippines.

Results and Discussion

Screening of 18 promising isolates for the presence of lipopeptide antibiotic genes was done by PCR. The culture CcB7 tested positive for the presence of all the tested five antibiotic genes tested *viz.*, surfactin, iturin A, iturin D, fengycin D, and bacillomycin D (Table 3). The bacterial isolate CcB7 was confirmed as *Bacillus* at the genus level based on the PCR amplification of the 16s rRNA using ITS primers BCF1 and BCR2 which yielded an amplicon size of approximately 546 bp. The partial sequence of 16s rRNA gene showed 99% similarity to *B. subtilis* strains in the NCBI database and therefore designated as *B. subtilis* strain CcB7. The sequence was submitted to GenBank with Accession No. KR265028.1.

Amplification of quorum sensing genes

PCR amplification using the primers designed for quorum sensing genes produced amplicons of size 544 bp for ComQ, 175 bp for ComX, 996 bp for c*omP* gene and 464 bp for *comA* gene (Plate 1). The quorum sensing loci *comQXP* was amplified in *Bacillus subtilis* strain CcB7 by PCR using the forward primer UniComQ and the reverse primer P1. The amplicon size obtained was approximately 3 kb (Plate 2). The PCR product was excised from gel and purified using quick gel extraction kit and sequenced by Primer Walk at Xcelris Genomics Pvt. Ltd., Ahmedabad, India. The consensus sequence of 2775 bp was generated from the sequence data obtained. Sequence homology searches performed using the BLAST program of NCBI revealed 94% similarity with the quorum sensing locus of *B. subtilis* isolate RO-F-3 (AF456137). The *comX* gene was detected with a homology of 99% (*e* value=0.00) similarity to *B. subtilis* strain RO-F-3 quorum sensing gene locus and the protein translate revealed identity with ComX

pheromone precursor. The *comP* gene sequence showed 95% similarity to *B. subtilis* strain RO-F-3 quorum sensing gene locus and the protein translate revealed identity with histidine kinase sensor protein. The *comA* gene sequence showed 98% similarity to comA gene of *B. subtilis* and the protein translate revealed identity with response regulation transcription factor. The nucleotide sequences of *comX*, *comP* and *comA* have been deposited at the GenBank with accession numbers KT335526, KT335525 and KT335524 respectively.

Assay on biofilm formation by *Bacillus* strains

Bacillus subtilis strain CcB7 was found to form robust pellicles or floating biofilms and solid surface associated biofilms in standing culture (Plate 3). They formed highly structured colonies on agar medium that were strikingly mucoid in the centre (Plate 4). The formation of biofilm was also quantified by microtitre plate assay (Plate5). At 48 and 96 h after incubation, the OD values for the *Bacillus subtilis* strain CcB7 was 3.35 and 3.75 respectively which were significantly higher compared to other strains tested (Table 4).

Population dynamics of CcB7 in rhizosphere soil

Population dynamics study revealed that there was a gradual increase in the *Bacillus* spp population in all the treatments wherein *Bacillus* was amended (*i.e*. except carbendazim and untreated check). At 120 days after treatment, the population of *Bacillus* spp. was $78x10^8$cfu/g of rhizosphere soil treated with seed treatment @ 10 g kg^{-1} seed along with basal application of *B. subtilis* strainCcB7 @ 2.5 kg ha^{-1} followed by application of CcB7 at 45 DAS (T_4). This was significantly superior to the treatments which included single basal application of *Bacillus* spp. (Fig. 1). The results clearly indicate that there is enhanced *Bacillus* population in all treatments except standard chemical check and untreated control. It also indicates that the pigeonpea rhizosphere favoured CcB7 strains which were initially collected from the respective rhizosphere soil.

Table.1 Details of the primers used for amplification of antibiotic genes

Antibiotic	Primer name	Primer sequence (5'-3')	Annealing temp.	Amplicon size (bp)	Reference
Surfactin	SUR3F SUR3R	ACAGTATGGAGGCATGGTC TTCCGCCACTTTTTCAGTTT	57ºC	441	Ramarathnam *et al.*, 2007
Iturin A	ITUD1F ITUD1R	GATGCGATCTCCTTGGATGT ATCGTCATGTGCTGCTTGAG	60ºC	647	Ramarathnam *et al.*, 2007
Iturin D	ITUD-F1 ITUD-R1	TTGAAYGTCAGYGCSCCTTT TGCGMAAATGGSGTCGT	57ºC	482	Chung *et al.*, 2008
Fengycin D	FEND 1F FEND 1R	TTTGGCAGCAGGAGAAGTTT GCTGTCCGTTCTGCTTTTTC	60ºC	964	Athukorala *et al.*, 2009
Bacillo-mycin D	BACC1F BACC1R	GAAGGACACGGCAGAGAGTC CGCTGATGACTGTTCATGCT	62ºC	875	Ramarathnam *et al.*, 2007

Table.2 Details of the primers designed for amplifying quorum sensing genes

Gene	Primer	Description	Annealing Temp.(°C)	Amplicon size
comQ	Forward	GTC TTG CAT CTT GTA TCCC T	63	544 bp
	Reverse	ACG TAA ACG AGG CCA TGCAA		
comX	Forward	TCA CCC CAT TGA CGG GTT T	62	175 bp
	Reverse	GGG GAT ACA AGA TGC AAG CC		
comP	Forward	TCG CAA TCA AAA CCG CTT C	62	996 bp
	Reverse	GGA TCT GAA TCT AGG CGG CG		
comA	Forward	CGC CTC GTT CAC CAA CTT TC	61	464 bp
	Reverse	TAA GTG CAG GCG GAC CAT TT		

Table.3 PCR-based detection of antibiotics producing genes in the *Bacillus* strains

S.No	*Bacillus* strain	Surfactin	Iturin A	Iturin D	Fengycin A	Bacillomycin D
1	CcB4	-	+	+	-	-
2	CcB7	+	+	+	+	+
3	CcB13	-	+	+	-	-
4	CcB14	-	+	+	-	-
5	CcB15	+	+	+	+	-
6	CcB17	+	-	+	-	-
7	CcB20	+	+	-	+	-
8	CcB27	+	-	+	+	-
9	CcB30	-	+	-	-	-
10	CcB33	+	+	-	-	-
11	CcB34	+	-	-	-	-
12	CcB37	+	-	+	-	-
13	CcB55	+	+	+	-	-
14	CcB58	+	-	+	-	-
15	CcB64	-	-	+	-	-
16	CcB68	-	+	+	-	-
17	CcB69	-	-	+	-	-
18	CcB96	-	-	+	+	-

(+) Present (-) Absent

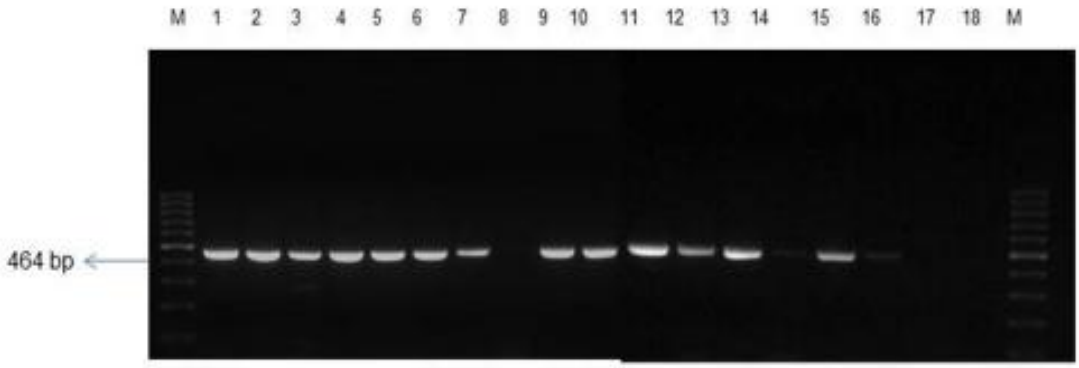
Table.4 Quantification of biofilm formation by *Bacillus* using Microtitre plate assay

***Bacillus* strain**	**OD Value at 600 nm * (48 h after incubation)**	**OD Value at 600 nm* (96 h after incubation)**
CcB4	2.20^{f}	3.73^{ab}
CcB7	3.35^{a}	3.75^{ab}
CcB13	1.81^{h}	2.65^{hi}
CcB14	2.16^{fg}	2.34^{j}
CcB15	2.33^{ef}	3.15^{ef}
CcB17	2.50^{cde}	3.08^{efg}
CcB20	0.98^{n}	2.31^{j}
CcB27	1.23^{lm}	3.23^{de}
CcB30	3.11^{b}	3.46^{cd}
CcB33	2.65^{c}	3.77^{ab}
CcB34	2.43^{de}	2.89^{fgh}
CcB37	1.10^{mn}	2.42^{ij}
CcB55	1.99^{gh}	2.68^{hi}
CcB58	2.20^{f}	3.65^{bc}
CcB64	1.55^{ij}	2.83^{gh}
CcB68	1.63^{ij}	1.76^{k}
CcB69	2.50^{cde}	2.96^{efg}
CcB96	1.00^{n}	2.64^{hi}

*Values are mean of three replications

Means in a column followed by same superscript letters are not significantly different according to DMRT.

Plate.1 PCR amplification of ComA gene of 18 isolates of *Bacillus*



M- 100 bp marker
1 - CcB4
2 - CcB7
3 - CcB13
4 - CcB14
5 - CcB15
6 - CcB17
7 - CcB20
8 - CcB27
9 - CcB30
10- CcB33
11- CcB34
12- CcB37
13- CcB55
14 - CcB58
15 - CcB64
16 - CcB68
17 - CcB69
18- CCb96

Plate.2 PCR amplification of ComQXP loci of *B. subtilis* strain CcB7

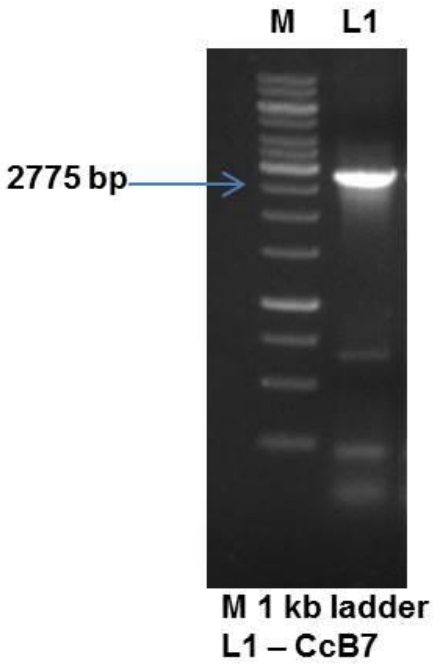


Plate.3 Pellicle formation by *B. subtilis* CcB7 in liquid media

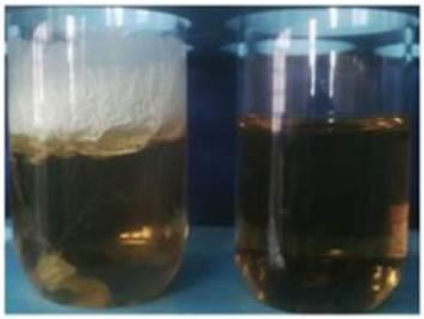


Plate.4 Formation of robust biofilm on solid media

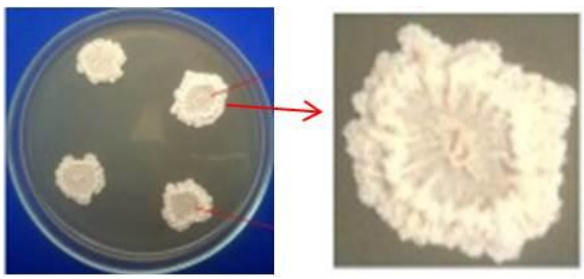
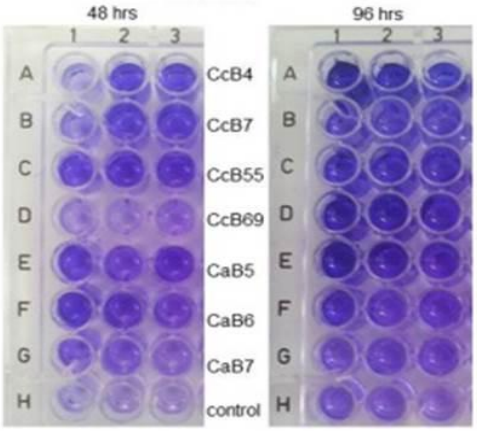
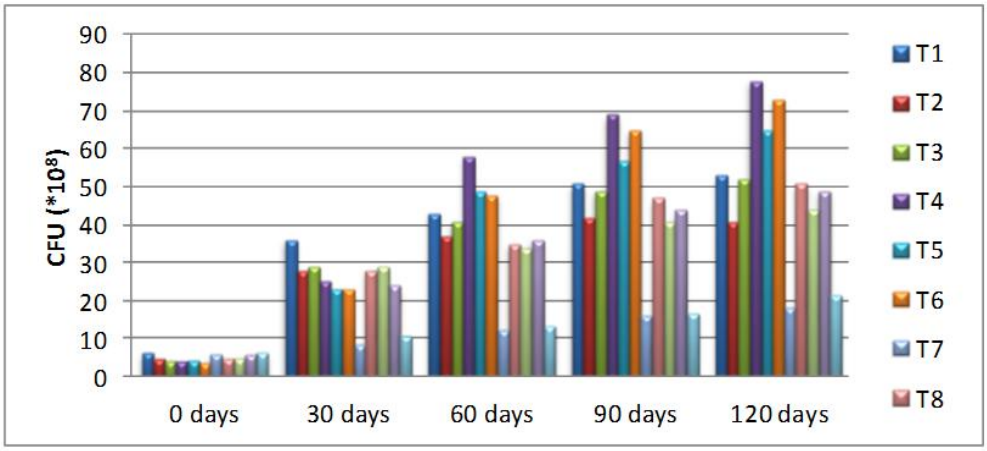


Plate.5 Microtitre plate assay for quantifying biofilm formation



Intensity of violet colour is proportional to biofilm forming capability

Fig.1 Effect of formulation on the population dynamics of *Bacillus* spp. in pigeonpea rhizosphere



The use of *Bacillus* in the biological control of plant diseases is well documented (Asaka and Shoda, 1996; Narasimhan and Shivakumar, 2015). The colonization of plant organs that are infection sites for the pathogen is a key feature for biocontrol success (Mikiciński *et al.*, 2016). Results of the present study showed that native *Bacillus* strain CcB7 was more efficient in colonizing the pigeonpea rhizosphere compared to strains isolated from other rhizospheres. An important trait of plant rhizobacteria is their ability to effectively colonize the rhizosphere and maintain a stable relationship with the surface of plant roots which again depends on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions. The microorganisms isolated from the rhizosphere of a specific crop may show better adaptation to that crop and may provide better control of diseases than organisms isolated from other plant species. Such plant associated microorganisms make better biocontrol agents because they are already closely associated with and adapted to the plant and the particular environmental conditions in which they must function. The selection of bacterial strain having the potential to synthesis surfactin will facilitate in biofilm formation and better rhizosphere colonization thereby excluding the pathogens. Further

production of antibiotics like iturin and fengycin will also inhibit the growth of pathogenic organisms.

In the present investigation, the *Bacillus* strain CcB7 formed highly structured colonies which indicated biofilm formation. Microtitre plate assay showed the adherence of bacterial cells to the plates indicating formation of biofilm by all *Bacillus* isolates. Quorum-sensing regulated biofilm formation allows the organism to create a niche, into which, it then secretes secondary metabolites protecting the rhizosphere. It is possible that surfactin and biofilm formation may allow *B. subtilis* to efficiently colonize plant roots and also provide protection to their host.

Previous studies have reported that by modifying cell surface properties, surfactin and iturin positively influence cell spreading, swarming and biofilm formation (Bais *et al.*, 2004; Leclere *et al.*, 2006) and thus may globally favour plant root colonisation. Bais *et al.*, (2004) observed that lipopeptide production and biocontrol activity are directly related to the ability of *B. subtilis* to form stable biofilms on plant roots. Surfactin produced early in the growth cycle of the bacterium rapidly increase the surface motility and accelerates the development of multicellular communities called as biofilms (Rudrappa *et al.*, 2008). The involvement of cyclic lipopeptides in biofilm formation by *B. subtilis* has been reported previously (Branda *et al.*, 2001; Hofemeister *et al.*, 2004). Nihorimbere *et al.*, (2011) showed that surfactin synthesis by S499 cells forming biofilm is very effective compared to iturins and fengycins as observed or the cLP pattern secreted in planta. The development of *Bacillus* cells as root-adhering microcolonies may thus be a crucial factor to explain the differential production of the three cLP families. Saha *et al.*, (2012) studied the biofilm formation by *B. subtilis* isolates AI01 and AI03 and found that the isolate AI01 was a more efficient producer of biofilm. They also noted that the level of adherence to polystyrene plates increased with increase in incubation period.

To conclude, the *Bacilllus subtilis* strain CcB7 isolated from the rhizosphere of pigeonpea was found to have three major antibiotic genes, viz., surfactin, iturin and fengycin and very good biofilm forming ability. It also survived well in the soil during the entire crop period and will definitely be an excellent biocontrol agent for reducing the incidence of root rot and wilt disease.

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