

# Genetic characterization of *Burkholderia cepacia* strain from Northeast India: A potential bio-control agent

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## ABSTRACT

This paper reports the characterization of naturally occurring rhizobacteria from NE India for the control of wilt and damping-off diseases in French beans. French bean (*Phaseolus vulgaris* L.) is one of the most important food legumes for direct human consumption which was originated from South and Central America. In NE India wilt and damping-off in beans are serious diseases which are mainly caused by *Fusarium spp* and *Rhizoctonia solani*. Out of 625 bacterial isolates obtained from French bean growing fields of Manipur, 15 potential isolates were selected, whose inhibition zone width were more than 15 mm. Strain IBSD-LKBI exhibited strong antagonistic activity and can inhibit both the pathogens. Besides, this strain also produced extracellular hydrolytic enzymes (protease, chitinase,  $\beta$ -1, 3-glucanase), secondary metabolites (Siderophore, HCN). It also produces plant growth promoting hormone (IAA) and can solubilize organic phosphate. *Burkholderia cepacia* IBSD-LKBI exhibited the highest plant growth promotion and BCA. After 60 days of treatment, plant height, root length, fresh weight and % of disease suppression were significantly ( $P < 0.05$ ) higher than in control plants. Productions of secondary metabolites were detected using gene specific primer. This bacterium was phenotypically characterized and sequence data of 16S rDNA revealed the closest identity with *Burkholderia cepacia* (98%). Multiple sequence alignment & phylogram with closely related sequence accessions using MEGA Software revealed this naturally occurring biocontrol bacteria belongs to *Burkholderia cepacia* complex genomovar I based on the Biolog identification system with 16S rDNA gene sequence analysis. This organism is one of the new reports from NE India for the control of wilt and damping-off diseases

in French beans.

**Keywords:** Antagonistic Activity; *Burkholderia cepacia*; *Fusarium oxysporum*; *Rhizoctonia solani*; French Bean

## 1. INTRODUCTION

One of the obstacles in the production of high quality and yield of food crops is the difficulty to control fungal diseases in plant. Every year, plant pathogenic fungi cause damage to crops, worthy of millions of dollars, all over the world despite the extensive use of chemical pesticides. So the demand for alternative biocontrol techniques, without using any synthetic fungicides, is increasing [1]. Also, environmental concerns and development of resistance in target populations have reduced the availability of effective fungicides. Therefore, the interest in so-called microbial biocontrol agents or their product to limit the attack and damage by phytopathogens has been increased and recommended. In North East region of India the yield of French beans are severely affected due to fungal infection by *Fusarium oxysporum* and *R. solani* causing charcoal rot, wilt and damping off diseases. *Fusarium* and *R. solani* are the most common and destructive plant pathogens and a monotypic soil borne species with a wide host range involving more than 200 species in 75 families [2]. These fungi infect plants from seedling stage to maturity. It is also called summer wilt or dry weather wilt because it often occurs during the summer, when plants are under heat and drought stresses. When severe, the disease reduces yield by killing plants at early reproductive stages. Microsclerotia is a compact mass of hardened mycelium stored with reserve food material that becomes detached and remains dormant until a favourable opportunity for growth occurs.

Continued use of chemical pesticides causes several negative and harmful effects on health and environment

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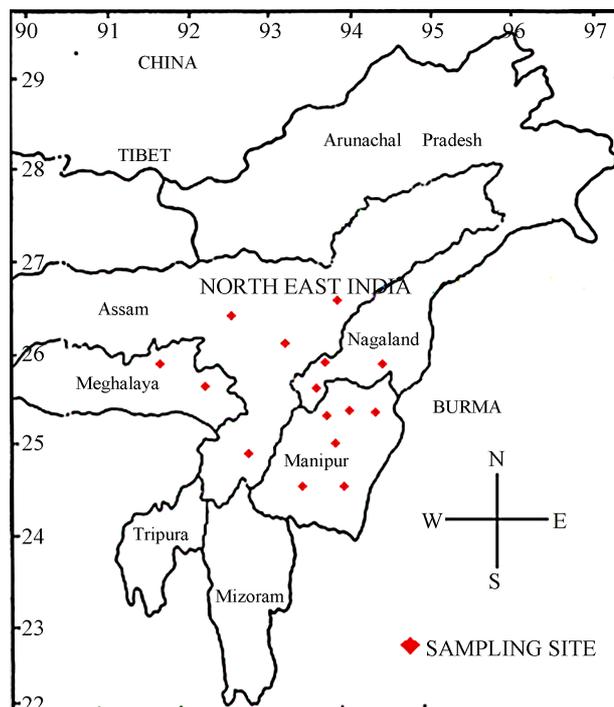
and thereby killing all the beneficial microorganisms from the soil. Due to continuous use of chemical pesticides the pathogens become resistant to a wide spectrum of fungicides applied in the soil. The use of microbial biocontrol agents is an alternative approach for reducing the use of chemicals in agriculture because it is ecofriendly in nature and safe to use in agricultural system to increase crop productivity since the action of such bacteria is highly specific and cost effective. It has been suggested that these bacteria might interact more closely with the host plants and act as effective biocontrol agents in sustainable crop production. Several species of bacteria are known to have beneficial effect on plant growth promotion and disease suppression through production of plant growth promoting regulators and secondary metabolite substances. Organisms such as *Burkholderia cepacia* have shown effective in suppressing many plant diseases and also promote plant growth [3]. Recently, *Burkholderia* species has been classified into 10 genotypically distinct but phenotypically similar species (genomovars) referring to the *Burkholderia cepacia* complex (Bcc) [4].

In this study, isolation and characterization of potential indigenous biocontrol bacteria IBSD-LKBI was reported from NE India for the control of two pathogens which caused wilt and damping-off diseases in French bean. The production of different hydrolytic enzymes and production of growth hormone and iron sequestering from soil has been studied. Moreover, this strain played significant functions in promoting growth on beans and this can further lead to the development of a potential bio-inoculants against soil borne fungal pathogens and also to study its ecological role and interactions with plant which can further lead to the development of a potential biocontrol packages/bioinoculants against soil borne fungal pathogens and also to study its ecological role and interaction with plants.

## 2. MATERIAL AND METHODS

### 2.1. Sampling

Twenty five French bean field sites were selected from different locations representing the entire Imphal East and Imphal West valley zone of Manipur, which falls in the Indian region of Indo-Burma diversity hotspot of the world and part of other NE region, for the collection of rhizosphere sample. From each sites, 4 - 5 French bean roots along with the rhizosphere soils from both healthy and infected plants were randomly selected and uprooted during the winter seasons of 2006-2007. The collected samples were carried to the laboratory and stored in the freeze (4°C) prior to isolation of rhizosphere bacteria. The sampling site is given in **Figure 1**.



**Figure 1.** Map of North East India showing sample collection site.

### 2.2. Climatic Condition

Northeast India has humid sub-tropical climate, ranging from hot to humid summers, severe monsoons with an average rainfall recorded around 10,000 mm and above and mild to chilled winter. Therefore, the variation in weather condition adds on to the exploration of indigenous microbes that which could adapt to changes in various environmental conditions and weather being one of the factors for varied applications.

### 2.3. Isolation of Bacteria

Ten grams of the root sample along with the rhizosphere soils were transferred to conical flasks with 90 ml of sterile physiological saline water and homogenized by shaking at 120 rpm for 30 mins. Suspensions from the collected samples were serially diluted up to  $10^{-5}$ . One ml of the homogenates were spread over plates of King's B agar medium, and Luria Bertani agar and incubated at 30°C for 24 - 48 h. Subsequently, the colonies of different colony characteristics and also with microscopically different morphology were selected and purified by dilution streaks. The purified isolates were maintained at -80°C in 30% glycerol stock for further biochemical analysis.

### 2.4. Fungal Strains

The fungal pathogens *Fusarium oxysporum* and *Rhizoc-*

*tonial solani* were isolated from naturally infected French bean and identified by standard protocol based on the morphological and microscopical characteristics using Trinocular fluorescent microscope.

### 3. SCREENING OF ANTAGONISTIC ACTIVITY

The bacterial isolates were tested for their ability to inhibit the growth of *R. solani* and *Fusarium oxysporum* using dual plate assay. Fresh cultures of *Fusarium oxysporum* and *R. solani* were grown in PDA medium (Potato infusion-200; Dextrose-20; agar-18 (g<sup>l</sup><sup>-1</sup>). A plug from the growing edges (5 mm diameter) of the colony was placed at one site of a fresh PDA plate. The bacterial isolates were grown overnight, spotted along the periphery of 90 mm diameter petri disc equidistant from the mycelial plug. These plates (4 replicates for each test organisms) along with uninoculated control were incubated at 30°C for about 72 hrs or until the leading edge of the fungus reached the edge of the plate. Antibiosis was assessed by measuring the diameter of the mycelial growth of *R. solani* and *Fusarium oxysporum*.

### 4. DETERMINATION OF PLANT GROWTH PROMOTION MECHANISMS

Plant growth promotion attributes such as production of Indole acetic acid (IAA) like substances and P-solubilization activity of the fifteen selected isolates were determined in broth culture and agar plate using the modified method given [5]. Bacterial cultures were inoculated in LB broth amended with tryptophan (2 mg/ml) and incubated at 30°C for 2 - 3 days. After centrifugation 2 ml of the supernatant was mixed with 2 drops of orthophosphoric acid along with 4 ml of Solawaski reagents and pink color intensity developed was measured at 530 nm O.D. Level of IAA in culture filtrates was determined from the standard graph obtained from concentration between 0 - 100 ppm IAA (Sigma). The production of P-solubilizing activity was determined in Pikovskaya's agar which contains tri calcium phosphate as insoluble phosphate and cultures plates were grown in shaking conditions at 30°C. Siderophore production was determined by the modified method [6] by plate method using Ternary complex chrome azulol S (CAS), Fe<sup>3+</sup>/Hexadecyl trimethyl ammonium bromide (HDTMA) as an indicator.

### 5. SCREENING OF DIFFERENT BIOCONTROL MECHANISMS

Different biocontrol mechanisms such as protease, chitinase,  $\beta$  1, 3, glucanase, and hydrogen cyanide production

were assessed.

Protease activity was determined using the method described by [7]. The bacterial cultures were inoculated in Skim Milk Agar plate and incubated at 28°C  $\pm$  2°C for 1 - 2 days and clear zone formation was observed.

Chitinase activity was determined using chitin minimal medium. Cells were streaked on chitin minimal medium supplemented with colloidal chitin (1% w/v) and solidified with 1.5% agar [8].

$\beta$  1, 3, glucanase activity was determined by modified method of Katatny *et al.*, 2001 [9]. For plate screening, carboxy methyl cellulose agar medium amended with laminarin were used. Bacterial cultures were streaked at the centre of the plate. Plates were incubated at 25°C for one day. For gluconolytic activity observations, plates were stained with 0.1% congo red dye for 15 - 20 minutes followed by destaining with 1N NaCl and then with 1N NaOH for 15 minutes for three times.

For quantitative determination of hydrogen cyanide production, bacterial isolates were separately streaked on TSA supplemented with 4.4 g glycine with simultaneous addition of cut filter paper disc soaked with 0.5% picric acid in 1% Na<sub>2</sub>CO<sub>3</sub> and placed in the lid on each petridish by modified method of [10]. In the control tube, no culture was inoculated. These disc were sealed in parafilm and incubated for 4 days at 30°C. The HCN production was determined by colour change observed in yellow colour turning brown to reddish brown. Scoring was done as weak if the colour changes from yellow to light red; moderate-yellow to brown and strong-yellow to reddish brown.

### 6. DETECTION FOR GENES INVOLVED IN ANTIBIOTIC PRODUCTION

PCR based detection of *Burkholderia cepacia* isolates were screened for the presence of biosynthetic loci/gene involved in the production of three known antibiotics with biocontrol activity (2,4, DAPG, pyrrolnitrin, phenazine) using specific PCR amplification from genomic DNA. The production of known antibiotics phenazine, DAPG and pyrrolnitrin (Primer sequences, **Table 1**) were detected using known gene specific primers. The PCR cycling conditions were as per standard protocol [11]. Amplified products were detected at 1.5% gel.

### 7. EVALUATION OF SEED GERMINATION AND DISEASE CONTROL EFFECT

To determine the influence of bacterial strain IBSD-LKBI in French bean growth, a pot experiment was conducted during 2008-2009 for which the O.D of the culture broth was adjusted between 0.3 and 0.5 at 600 nm and mixed with an aqueous solution of 1% carboxy-

**Table 1.** Antibiotic gene specific primer used in this study.

Primer set	Primer name	Primer sequence	Antibiotics related	References
1	PHZ1 PHZ2	GGC GAC ATG GTC AAC GG CGG CTG GCG GCG TAT AT	PCA	Delaney <i>et al.</i> , 2001
2	BPR3 BPR4	GGT GCG ACA TCT TTA ATG GAG TTC CCG CCG GTA TGG AAG ATG AAA AAG TC	2,4-DAPG	McSpadden Gardener <i>et al.</i> , 2001
3	Prn Cf Prn Cr	CCA CAA GCC CGG CCA GGA GC GAG AAG AGC GGG TCG ATG AAG CC	Pyrolnitrin	Mavrodi <i>et al.</i> , 2001

methyl cellulose (CMC) to obtain a cell concentration of  $10^9$  cfu/ml. The standardization broths were used for bacterization of seeds. Fifty sterile French bean (*Phaseolus mungo* L.) seeds of local variety were treated for 30 min with standardized culture broth amended with 1% CMC. Control seeds were treated with mixture of uninoculated LB broth along with 1% CMC. Bacterized seeds were air dried overnight at room temperature. For checking the effectiveness in controlling wilt and damping off diseases in beans inocula of *F. oxysporum* and *R. solani* were prepared by incubating mycelial growth in pre-sterilized oat grain for 7 days at 30°C. Fungal inocula were mixed with soil two days ahead of the seed sowing and the fungal inoculum level was maintained at  $10^5$

cfu/g soil. The pot size was of 10 inches diameter containing 2 kg soil. The experiment was conducted using a randomized plot design with three replicates of each treatment. The treatment combinations of the experiment for evaluation of plant growth and biocontrol effect were 1) Control; 2) IBSD-LKBI and for assessing disease suppression experiment the treatments given were 1) IBSD-LKBI + *Fusarium oxysporum*; 2) *Fusarium oxysporum* infested control plot; 3) IBSD-LKBI + *R. solani*; 4) *R. solani* infested soil. Sterilized seeds were planted at a depth of approximately 1 cm from the soil surface and watered with tap water daily. Percentage of disease incidence (%DI) were calculated at 30<sup>th</sup> day by using the following formula.

$$\%DI = \frac{\text{No. of infected plant in control} - \text{No. of infected plants in treatment}}{\text{No. of infected plants in control}} \times 100$$

The ANOVA approach was used to evaluate the efficiency of biocontrol agent in field. Comparison among treatment and means of appropriate control treatment were made at  $P = 0.05$ . The pool data of the two consecutive seasons were subjected to ANOVA.

## 8. MOLECULAR IDENTIFICATION

### 8.1. Preparation of Genomic DNA

Genomic DNA was extracted using the modified method [12]. A single colony was inoculated to 5 ml of LB broth (Hi-media, M1245) at 30°C, overnight. Culture broth of 50D at  $A_{600}$  nm was centrifuged at  $10,000 \times g$  for 5 min (Allegra 22R, Beckman-Coulter, USA). The cell pellet was washed twice with sterile distilled water and after removal of the supernatant, the pellet was suspended with 200  $\mu$ l sterile deionised water (TKA water purification system, Germany) with 10  $\mu$ l of lysozyme (1 mg·ml<sup>-1</sup>) GeNei, B3, (Bangalore, India) by vortexing and incubated at 37°C for 30 min followed by boiling for 5 min and centrifuged at  $10,000 \times g$  for 5 min. The supernatant was carefully pipetted out and used for PCR analysis either immediately or stored at -20°C until required. Purity of the DNA was measured using Nanodrop spectrophotometer by reading the value between 260/280 nm.

### 8.2. 16S-23S rDNA Gene Sequencing

Genomic DNA of IBSD-LKBI was amplified using the primers: 16SF-R2 (5'-CGCGGGATCCTTGTACA-CACCGCCCGTC-3') and 23SR-R10 (5'-GGCCGTCGACCCTTTCCCTCACGGTACTG-3') 0.5  $\mu$ l (2U) of Taq DNA polymerase (Promega, M1901) and 19.0  $\mu$ l of autoclaved deionised water (Millipore water purification system). The amplification was performed with a total of 36 PCR cycles in a thermal cycler Biorad, USA) at 94°C for 10 min. These were then subjected to 36 cycles consisting of 94°C for 30 sec, annealing at 55°C for 1 min and elongation at 72°C for 2 min and amplification product cooled at 4°C. PCR products were purified and sequenced at Institute of Microbial Technology (IMTECH).

### 8.3. Nucleotide Sequence Accession Number

The nucleotide sequence of the 16S rDNA gene of IBSD-LKBI strain is shown to have 98% similarity with *Burkholderia cepacia* and it has been deposited in GenBank under accession number GQ927326.

### 8.4. Genomovar Identification

The sequences of the genomovar were compiled from

NCBI nucleotide database. DNA sequence homology search were performed using the online BLAST search engine with megablast option in GenBank (available at <http://www.ncbi.nlm.nih.gov>) [13]. Comparative consistency analysis performed using TCOFFEE advance search engine [14]. Phylogenetic molecular evolutionary analysis was conducted using MEGA Version 5.05 [15].

## 9. RESULTS

### 9.1. Isolation & Screening of Antagonistic Activity

Out of the total 625 bacterial isolates collected from French bean rhizosphere soil of Imphal East and West different districts of Manipur using King's B and LB agar, 15 isolates were selected based on their significant reduction in the fungal growth against *F. oxysporum* and *R. solani* where their zone width is more than 15 mm diameter after 4 days of incubation on dual plate assay and most potent bacteria were IBSD-LKBI, IBSD-56 and IBSD-38 (**Table 2**). Among the three bacterial strains IBSD-LKBI exhibited the highest inhibition zone diameter with significant fungal growth inhibition to control the two test fungal pathogens. Therefore, strain IBSD-LKBI was selected for further studies.

### 9.2. Determination of Plant Growth Promotion Assay

The production of plant growth promotion hormone such as Indole acetic acid (IAA) like substances were assessed in culture filtrate supplemented with tryptophan. Level of IAA like substances ranges from 22.1 - 65.2 µg/ml cul-

ture filtrate (**Table 1**) and IBSD-LKBI produced significant level of IAA at the rate of 58.4 µg/ml culture filtrate. Similarly, in addition to this P-solubilization and siderophore production was determined after 24 - 48 hr of incubation of the culture. Out of the 15 selected potential isolates 67% showed P-solubilization activity. In case of siderophore activity formation of bright zone with yellowish fluorescens in dark blue medium were observed. Out of the total 15 isolates only 6 isolates showed siderophore production with highest zone produced by isolate IBSD-P-50 with a zone diameter of 15 mm (**Table 2**).

### 9.3. Extra Cellular Cell Wall Degrading Enzyme Assay

The potential for the production of different fungal cell wall degrading enzymes such as chitinase, protease, β-1, 3-glucanase activity were assessed for the selected potential strains and evaluated based on different enzymatic activities. All the isolates were inoculated in colloidal chitin supplemented medium for chitinase activity and skim milk agar plates for protease activity and later the plates were incubated at 30°C for 2 - 3 days and observed for the clear zone formation around the colonies. A total of 10 isolates showed chitin activity ranging from 7 mm to 14.1 mm zone diameter whereas for protease activity the zone diameter ranges from 7.5 - 15.5 mm of which IBSD-LKBI exhibits highest activity of both chitinase and protease activity. In addition to this 9 isolates showed β-1, 3-glucanase activity. HCN production was observed only in three isolates (**Table 2**).

**Table 2.** Evaluation of different antagonistic and biocontrol potential of the bacterial isolates.

Treatment	Antifungal activity (diameter in mm)		Cell wall degrading enzyme & secondary metabolite production assay				Plant growth proportion attributes		
	<i>Fusarium oxysporum</i>	<i>R. solani</i>	Chitinase	Protease	Glucanase	HCN	IAA like substances (µg/ml)	Siderophore	P-solubilization assay
IBSD-324	15.5	17.52	10.56 ± 1.1	12.0 ± 1.6	+	+	+	12 ± 1.2	+
IBSD-59	17.8	18.4	11.16 ± 1.5	14.2 ± 0.76	-	-	+	-	+
IBSD-18	15.5	16.8	10.3 ± 0.4	11.1 ± 0.02	-	-	+	13 ± 0.58	+
IBSD-LKBI	19.5	20.4	14.1 ± 0.64	15.5 ± 0.57	+	+	+	14 ± 0.08	+
IBSD-59	16.2	18.8	12.9 ± 0.86	10.2 ± 0.58	+	-	+	11 ± 0.03	+
IBSD-56	18.6	19.0	-	9.56 ± 0.03	+	-	+	-	+
IBSD-11	16.4	15.4	9.56 ± 1.5	8.0 ± 0.28	-	-	-	-	+
IBSD-23	17.9	19.0	7.6 ± 0.04	10.0 ± 1.33	+	-	+	-	+
IBSD50	15.0	17.9	-	11.5 ± 0.43	-	-	+	15 ± 0.86	-
IBSD-38	18.5	18.2	-	10.0 ± 1.15	+	-	+	-	+
IBSD-338	17.5	17.8	7.0 ± 0.57	12.0 ± 0.86	+	-	+	14 ± 0.09	-
IBSD-62	16.2	15.6	10.5 ± 1.15	9.5 ± 0.57	+	-	+	-	+
IBSD-161	17.3	17.4	-	8.5 ± 1.5	-	-	-	-	-
IBSD-TOR	15.5	18.4	-	7.56 ± 1.2	+	+	-	-	-
IBSD-111	17.8	18.2	12.0 ± 1.73	11.56 ± 1.15	-	-	-	-	-
LSD (P = 0.05)	1.284	0.389	1.38	2.88			-	4.82	

\*Values are the means of four replicates.

#### 9.4. Biochemical Characteristics of IBSD-LKBI

This bacterial isolates were gram negative, motile, rod shaped bacteria and exhibited positive catalase, oxidase and arginine hydrolysis. It can utilize dextrose, galactose and manose. Based on the above characteristics the isolates could be identified as genus *Burkholderia* sps.

#### 9.5. Detection of Biosynthetic Genes for Antibiotic Production

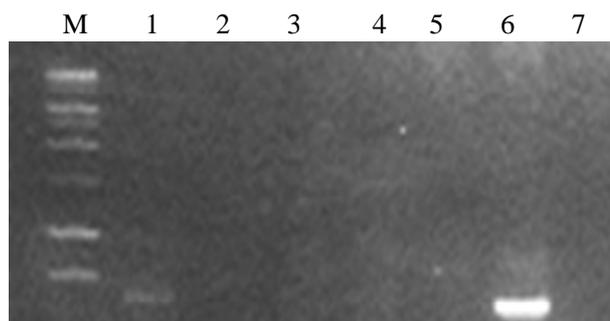
During the study on production of genes involved in antibiotic productions such as 2,4 DAPG, pyrrolnitrin and phenazine based on gene specific primers, PCR based detection confirms that *B. cepacea* IBSD-LKBI produces genes responsible for phenazine production (Figure 2).

#### 9.6. Impact Assessment of IBSD-LKBI on Plant Growth Promotion

The effect of plant growth promotion using IBSD-LKBI was assessed in French beans healthy soil during 2008 & 2009. The rate of seed germination percentage was found to significantly increase (88%) as compared to the control plant (68%). After two months of planting the plant height and root length treated with IBSD-LKBI were found to remarkably increase at  $40.0 \pm 3.0$  &  $21.0 \pm 1.0$  respectively and  $34.0 \pm 2.0$  and  $14.0 \pm 1.5$  in case of untreated plants. Whereas the seedling biomass was found to be  $27.86 \pm 1.2$  in case of treated plant against the control untreated plant ( $16.62 \pm 2.0$ ) which is good sign to be used as a strong plant growth promoter in French bean (Table 3).

#### 9.7. Assessment of Biological Control Efficacy

The strain IBSD-LKBI had good control effects on French bean under potconditions in disease infested soils (Table 4). In the *Fusarium* and *R. solani* treated plots, % of disease incidence were found to be 65.5% and 68% respectively. As per disease control effect exhibited by IBSD-LKBI treated plant with a control value of 86.9% and 67.4% against the wilt and damping-off diseases in French bean caused by *Fusarium oxysporum* and *R. solani* respectively. Evaluation of IBSD-LKBI treated plant showed that its control effects were equivalent to that of inhibition of mycelial growth of the pathogens *in vitro* conditions. Therefore, these pooled data clearly indicates that indigenous strains IBSD-LKBI could significantly reduce the severity and development of wilting and damping-off disease in French beans and could be used as a potential bio-control agent.



**Figure 2.** Detection of band for antibiotic using PCR gene specific primer for Phenazine-1-carboxylic acid. Lane M: 100 bp DNA marker, 1: IBSD-38, 2: IBSD-56 3: IBSD-11, 4: IBSD-50, 5: IBSD-TOR, 6: IBSD-LKBI, 7: RFP-18.

**Table 3.** Effect of seed bacterization with IBSD-LKBI strain on French bean seedling and plant growth promotion.

Treatment	Seed germination (%)	Shoot length (cm)	Root length (cm)	Seedling biomass (g/plant)
		60 days		
IBSD-LKBI	88	$40.0 \pm 3.0a$	$21.0 \pm 1.0a$	$27.86 \pm 1.2a$
Control	68	$34.0 \pm 2.0b$	$14.0 \pm 1.5b$	$16.62 \pm 2.0b$

\*Variances between pot experimental trials were homogenous and thus data were pooled and statistically analyzed. Values followed by same letter in each vertical column are significantly different ( $P > 0.05$ ) according to analysis of variance ANOVA using SPSS statistical packages.

**Table 4.** Effect of seed bacterization with IBSD-LKBI strain on percentage of disease incidence of French bean seedling in *F. oxysporum* and *R. solani* infested soil.

Sl.No.	Treatment	% Disease incidence	Control value (%)
1	IBSD_LKBI + <i>Fusarium oxysporum</i>	$17.0 \pm 1.5$	86.9
2	<i>Fusarium oxysporum</i> infested soil (Control)	$65.25 \pm 0.25$	-
3	IBSD_LKBI + <i>R. solani</i>	$22.17 \pm 0.35$	67.4
4	<i>R. solani</i> infested soil (Control)	$68.0 \pm 0.15$	-

\*Values in the parenthesis are of six replicates in two separate sets of experiments.

### 9.8. Comparison of Sequence Identity among the *Burkholderia* Species Belonging to Different Genomovars

Until now no clear distinction has been established between the ten genomovars of Bcc, but rDNA and *recA* gene derived primer pairs are available for the identification of *B. cepacia* genomovar (I), *B. multivorans* (II), *B. cenocepacia* (III), *B. stabilis* (IV), *B. vietnamiensis* (V), *B. dolosa* (VI), *B. ambifaria* (VII), *B. anthina* (VIII), *B. pyrrocinia* (IX), *B. ubonensis* (X), [16-18]. In present study Sixteen (16) sequences of 16S rDNA of Bcc and one (1) *recA* gene sequence of *Burkholderia dolosa* (AF323971) were studied for molecular and evolutionary analysis [19-21].

The isolated sequence was compared for consistency analysis [14] of 16S rDNA sequences of isolates from India as well as all representative isolates from other part of the world belonging to various *Burkholderia* genomovars [6] (Table 5). The Phylogenetic analysis based on 16S rDNA sequence with neighbor joining method at bootstrap values based on 1000 replications establish a clear cut genomovar clusters of Bcc [15] Figure 3. Notably these clusters were further strengthens the consistency analysis for Bcc.

## 10. DISCUSSION

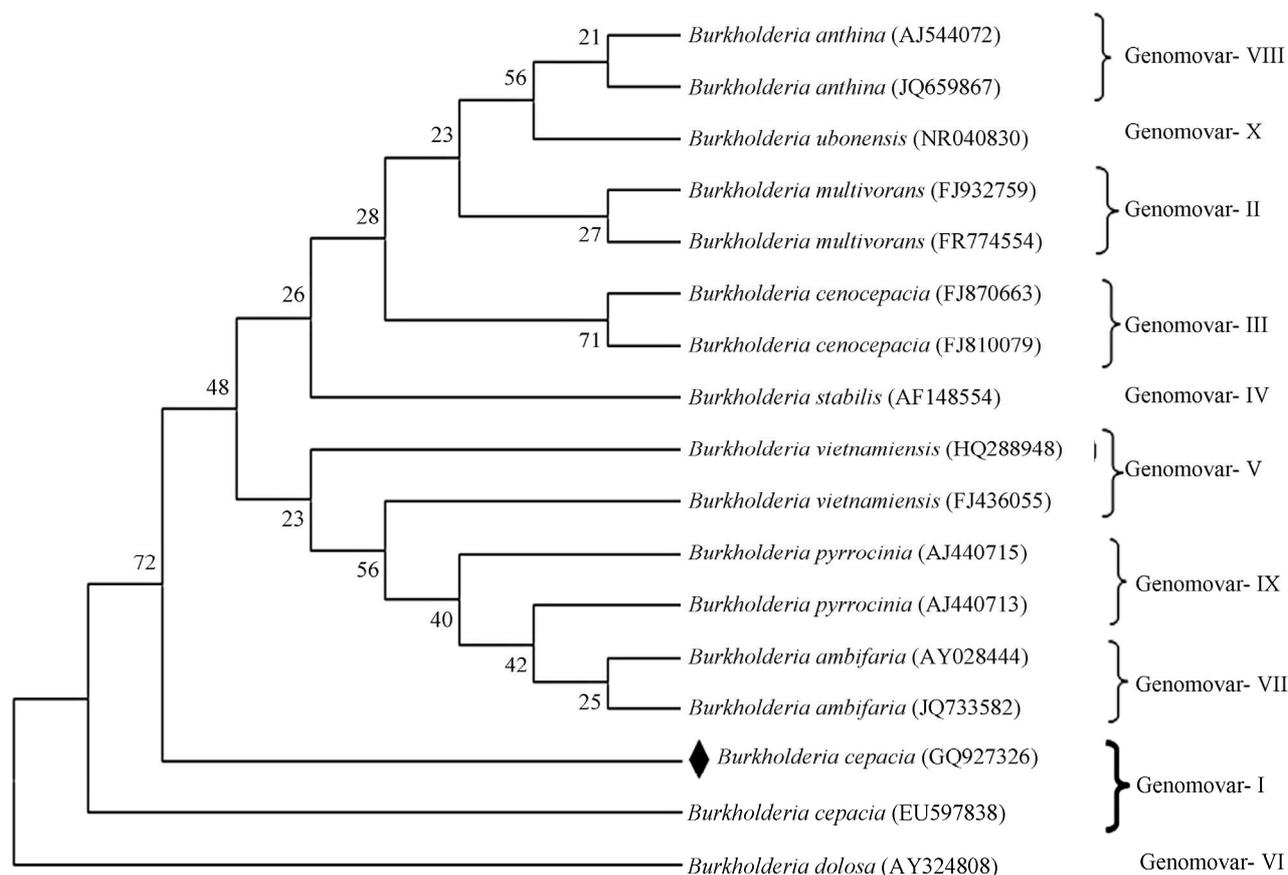
Antagonistic plant-associated bacteria are an important functional group of beneficial bacteria responsible for the

control of soil-borne pathogens (Weller, 1988). *B. cepacia* was first described by Burkholderia in 1950 isolated from putrefactive onions [22]. Several *B. cepacia* species are considered to be beneficial in nature and are widespread in nature particularly in plant environment. *B. cepacia* is a bacterial complex with 10 genomic species which have similar phenotypical traits and different genetic ones and are called genomovars [23,24]. The *Burkholderia cepacia* complex (Bcc) consists of several species of closely related and extremely versatile gram-negative bacteria found naturally in soil, water, and the rhizosphere of plants. Strains of Bcc have been used in biological control of plant diseases and bioremediation in many cases, while some strains are plant pathogens or opportunistic pathogens of humans with cystic fibrosis. Members of genomovars I, III, VII and IX had been confirmed as having good biocontrol ability, especially effective against *Pythium* and *Rhizoctonia* [25,26].

Sergio de los reported *Burkholderia cepacia* XXVI as a promising biological control agent against the causal agent of anthracnose, *C. gloeosporioides*, through the production of hydroxamate siderophore [27]. Zaiton established *Burkholderia cepacia* strain UPM B3, isolated from symptomless palms and its potential as a biocontrol agent against *Ganoderma* [28]. The ecological versatility of these bacteria is likely due to their unusually large genomes, which are often comprised of several (typically two or three) large replicons, as well as their ability to use a large array of compounds as sole carbon

**Table 5.** The LKB1 isolate from India (GQ927326) is compared at the nucleotide level using TCOFFEE advanced search engine with representative isolates of *Burkholderia* from India and other part of the world using consistency score.

Accession no.	Species	Strain	Location	Genomovar	Score
AF148554	<i>Burkholderia stabilis</i>	LMG 14294	Belgium	IV	100
AY028444	<i>Burkholderia ambifaria</i>	MVPC1/4	Italy	VII	100
AJ440713	<i>Burkholderia pyrrocinia</i>	R11805	Belgium	IX	99
AJ440715	<i>Burkholderia pyrrocinia</i>	R1904	Belgium	IX	99
JQ659867	<i>Burkholderia anthina</i>	R7-112	Singapore	VIII	99
NR_040830	<i>Burkholderia ubonensis</i>	GTC-P3-415	USA	X	100
AJ544072	<i>Burkholderia anthina</i>	BHS1	Germany	VIII	99
FJ810079	<i>Burkholderia cenocepacia</i>	11806	China	III	100
EU597838	<i>Burkholderia cepacia</i>	G63	China	I	99
GQ927326	<i>Burkholderia cepacia</i>	LKB1	India	I	98
AY324808	<i>Burkholderia dolosa</i>	LMG 21443	Belgium	VI	96
FJ436055	<i>Burkholderia vietnamiensis</i>	SYe-6586	Mexico	V	99
HQ288948	<i>Burkholderia vietnamiensis</i>	G8	Netherlands	V	99
FJ932759	<i>Burkholderia multivorans</i>	103104	China	II	98
FJ870663	<i>Burkholderia cenocepacia</i>	PP9Q	Malaysia	III	99
FR774554	<i>Burkholderia multivorans</i>	L-bf-R2A-15	Finland	II	97
JQ733582	<i>Burkholderia ambifaria</i>	YCJ01	China	VII	99



**Figure 3.** Phylogenetic tree based on 16S rDNA (except AY324808 genomic DNA) sequences, representing the relatedness among the *Burkholderia* genomovars. Distance and clustering was performed by using the software package MEGA 5.05. Boot strap values based on 1000 replication are listed at the branching point. The sequence of present study dotted in black.

sources. *B. cepacia* has been reported to promote growth of maize, enhance crop yield [29], and degrade diverse pesticides [30].

In the present study, several indigenous bacterial strains were isolated from Imphal East and West districts of French bean growing area and characterized by phenotypic and genotypic approach and investigated for their ability to inhibit the growth of *Fusarium oxysporum* and *Rhizoctonia solani*. To the best of our knowledge, indigenous native isolates performs best in terms of its specificity in a particular environment where it survive [31]. The use of indigenous bacterial isolates such as *Pseudomonas* and *Burkholderia* species has not been reported so far from NE India for the control of soil borne diseases in french beans.

To find new biocontrol agents antagonistic to the soil-borne fungus *Fusarium oxysporum* and *R. solani*, 625 rhizobacterial isolates were evaluated using a combination of different screening steps. As a result of the first screening, isolates were found to produce detectable inhibition zones against the two test fungus on agar plate assay. The production of clear zones in dual plate-culture screens is due to the production of cell wall degrading

enzymes and toxic metabolites.

Because of the discrepancies, *in vitro* and *in vivo* studies, the assay should be combined with other investigations as was done here. In the present study, we found examples for a good correlation between *in vitro* and *in vivo* results e.g. IBSD-LKBI. The identification of an antagonistic bacterium is another important step in the assessment of the BCA.

Production of cell wall degrading enzymes such as glucanase, chitinase, protease, and production of secondary metabolites (Siderophore and HCN) are common mechanisms that bacteria use to inhibit fungal pathogens [5,32,33]. In this study, IBSD-LKBI found to produce different cell wall degrading extracellular enzymes such as protease, glucanase, chitinase and stipulated its role in biological control. The result also supported with the earlier studies [34,35]. This biocontrol strain also produces phenazine when assessed with gene specific primers (Figure 2).

IBSD-LKBI isolates also shows a strong plant growth promotion activity as supported by the production of plant growth hormone such as IAA. Several soil microorganisms have the ability to solubilise insoluble phos-

phate and maintain soil health and quality. IBSD-LKBI showed the capacity to solubilize inorganic phosphate. In addition to this it can produce Fe<sup>+</sup> chelating siderophore which are a good sign of PGP activity.

To select bacterial isolates with highest antagonistic properties for approaches in biocontrol application, a multistep screening for biocontrol activity against *Fusarium oxysporum* and *R. solani* and PGR combined with its genotypic and phenotypic characterization was performed. All of these data were integrated into an assessment scheme to identify the best antagonist. Based on this assessment scheme, IBSD-LKBI strain could be considered good biocontrol candidates which can control wilting and damping-off diseases in French bean pathosystems. *B. cepacia* (*Pseudomonas cepacia* formerly) has been reported to prevent *Pythium* diseases of cucumber and peas and *R. solani* stem rot of poinsettia [26].

Rhizosphere plant associated bacteria are able to interact more closely with the host plant and fungal pathogens living in soil and plant tissue [36]. Our findings confirm that they could be efficient BCAs. More efficient methods on characterization and identification of BCC are now available for precise evolutionary relationship. Notably, these methods enable the study of fastidious or pathogenic species without employing tricky or expensive microbiological methods. Consistency and distance values prove *B. cepacia* (IBSD-LKBI) relates to the genomovar I phylogenetic tree of BCC genomovar relatedness based on 16S r DNA are shown in **Figure 3**. The rhizosphere associated *B. cepacia* IBSD-LKBI is an interesting candidate for biological control. In further experiments, these selected BCAs will be evaluated in field trial to select the best candidate for commercialization. *B. cepacia* strains reported in this study as a strong BCA is required to study their ecological role in soil and may be used as a biocontrol agent in sustainable agricultural practices.

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