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Full Length Research Paper

Isolation, characterization and screening of *Burkholderia caribensis* of rice agro-ecosystems of South Assam, India

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Six isolates of *Burkholderia* were obtained from rhizosphere soils of rice grown in tropical lowlands of South Assam, India. Among the identified *Burkholderia* isolates, SDSA-I10/1 has shown higher nitrogen fixing potential and it was selected for 16S rDNA sequencing. The isolate SDSA-I10/1 showed highest resemblance to *Burkholderia caribensis* MWAP84 (Y17011) and hence, identified as *Burkholderia caribensis* strain SDSA-I10/1 (GU372342). Inoculation of this strain improved the growth and yield parameters of rice significantly over uninoculated control plants, thus it may be used as indigenous microbial inoculant for intensive rice cropping in tropical lowlands.

Key words: *Burkholderia caribensis* strain SDSA-I10/1, 16S rDNA sequence, biofertilizer, Diazotroph, nitrogenase activity, tropical lowland.

INTRODUCTION

The world population is increasing day by day but the expansion of land is limited. Moreover, almost half of the world's population is consuming rice (*Oryza sativa* L.) as the primary food grain, making it the most important food crop currently produced (Cottyn et al., 2001). Hence, to produce higher yields of rice, expensive nitrogenous fertilizers are commonly used. These are used to fulfill the nitrogen demand of rice that can be overcome partially by using biofertilizers when they are scientifically applied. Biofertilizer is important in crop farming systems because it is an inexpensive source of nitrogen for the higher yields of crops. This process diminishes the need for expensive chemical fertilizer. Thus the extensive use of biofertilizers would provide economic benefits to farmers improve the socio-economic condition of people and preserve natural resources.

Diverse diazotrophic and endophytic bacteria have been isolated from rice plants (Engelhard et al., 2000; Gyaneshwar et al., 2001). There is a possibility that all or some of these bacteria could be contributing to the N balance of wetland rice (Malarvizhi and Ladha, 1999; Nieuwenhove et al., 2000). Endophytic bacterial associations with rice are nonspecific and the size of the bacterial population in rice tissues is low (Malarvizhi and Ladha, 1999). The association of rhizospheric *B. vietnamiensis* (Gillis et al., 1995; Tran Van et al., 2000) explains well the biological nitrogen fixation (BNF) observed in certain rice genotypes. In rice, β-proteobacteria of the genus *Burkholderia*, for example, *Burkholderia brasilensis*, *B. vietnamensis* (Gillis et al., 1995) and *Burkholderia* spp. (Muthukumarasamy et al., 2007) have been reported in high numbers. These bacteria colonize the rice plants systemically, although the highest numbers were observed in the roots.

Some of the best studied diazotrophs for nitrogen fixation are *Burkholderia* spp., which fix N_2 when other sources of nitrogen are absent or at low levels (Dobereiner et al., 1993; Kirchhof et al., 1997). In Vietnam, rice grown inoculated with *B. vietnamiensis*

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increased in yield 13 to 22% (Tran Van et al., 2000). An endophytic *Burkholderia* species isolated from rice plants in Brazil fixed 31% of the total nitrogen captured by the plant and resulted in a 69% increase in the rice biomass (Baldani et al., 2000). A yield increase of 54% was observed for the rice inoculated with *B. brasilensis* (Guimaraes et al., 2000). Bacteria belonging to the genus *Burkholderia*, which are very common in soil, water and associated with plants (McArthur et al., 1988), have a wide natural diversity, not only in taxonomy, but also in ecological features. In Brazil, *Burkholderia* species associated with rice plants could fix 31% of the total nitrogen captured by the plant (Baldani et al., 2000) and the inoculation of rice with this endophytic *Burkholderia* species led to a 69% increase in the rice biomass (Baldani et al., 2000). Rice varieties grown in low fertility soil in Vietnam inoculated with the *B. vietnamiensis* TVV75 strain gave yield increases of 13 to 22% (TranVan et al., 2000). Diazotrophs can affect plant growth directly by the synthesis of phytohormones and vitamins, inhibition of ethylene synthesis, improving nutrient uptake, enhancing stress resistance, solubilization of inorganic phosphate and mineralization of organic phosphate (Baldani et al., 2000). Indirectly, diazotrophs are able to decrease or prevent the deleterious effects of pathogenic microorganisms, mostly through the synthesis of antibiotics and/or fungicidal compounds, through competition for nutrients or by the induction of systemic resistance to pathogens (Baldani et al., 2000). In addition, they can affect the plant indirectly by interacting with other beneficial microorganisms.

In this study, we have isolated a diazotrophic bacterium, *Burkholderia caribensis* strain SDSA-I10/1from the rhizosphere of cultivated rice varieties grown in rainfed acidic lowlands of South Assam, India. The strain was identified using morphological, physiological, biochemical, and 16S rDNA nucleotide sequence analysis. Finally, the bacterial strain was used in plant inoculation experiment to demonstrate the potential to improve the growth performance and grain yield of rice.

MATERIALS AND METHODS

Assam, the eastern most state of the Indian sub-continent, extends from 22.19$^{/}$ to 28.16$^{/}$ North latitude and 89.42$^{/}$ to 96.30$^{/}$ East longitude. Southern Assam (Barak Valley) comprising three districts, namely Cachar, Karimganj and Hailakandi, is situated between longitude 92.15$^{//}$ and 93.15$^{//}$ East and latitude 24.8$^{//}$ and 25.8$^{//}$ North, covering an area of 6,941.2 sq. km. of land.

The soil samples were collected randomly from 6 locations of South Assam from the rhizosphere of rice growing in acidic lowlands at panicle initiation stage during July, 2009. Three parallel sampling lines were marked out at known distances (depending on the size of the selected rice growing field) from each other in each location. The first line was placed randomly and the others parallel to this line. Each line included four sampling areas (1 m^2) placed at regular distances from each other. Five rectangular soil cores (5 × 3.5 cm^2, 0 to 30 cm deep) were taken from each sampling area involving rhizospheric zone of growing rice crop. The samples taken from the same line were combined and each pooled soil sample, henceforward consisting of 20 cores. The soil samples were placed in sterilized plastic bags, transferred to the laboratory within one day and stored at 4°C prior to isolation. Sterile gloves were used in the soil sampling, working tools were sterilized with ethanol and flamed, and further procedures were performed as aseptically as possible.

Shade-dried, ground and sieved rhizosphere soil samples (1 g) were used for tenfold serial dilution. 10 g soil from each sample was aseptically weighed and transferred to an Erlenmeyer flask with 90 ml sterilized distilled water, and were shaken for 30 min at about 150 rpm. Immediately after shaking, a series of tenfold dilutions of the suspension was made for each sample by pipetting 1 ml aliquot into 9 ml sterilized distilled water. Appropriate serial dilution (10^{-6}) of rhizosphere soil samples was inoculated was inoculated (1 ml) aseptically onto petriplates containing 20 ml melted agar medium at 30°C in triplicate for 5 to 7 days (Burbage and Sasserl, 1982). Quantitative enumeration of the strains from all three pooled soil samples of each location (n=3) was carried out by dilution plate count method in solid N-free modified PCAT medium (specific for isolation of *Burkholderia* sp.) without tryptamine (Estrada-de-los Santos et al., 2001). Colonies growing on the dishes were counted after incubation. Data from each of the three replicates were averaged for a location and expressed as cfu (colony forming units) per g of soil.

The sub-surface pellicles or the turbidity of the entire modified PCAT medium were considered presumptively positive for growth of *Burkholderia* strains. The pellicles/growth was sub-cultured in the same medium for acetylene reduction assay. This assay was carried out by injecting 10% (v/v) acetylene in the head space above the medium and incubated for 1 h (Hardy et al., 1973). Ethylene production was measured using a Systronics Gas Chromatograph with a Poropak Q column and a flame ionization detector connected to a chromatography data computer system. Nitrogenase positive pellicles were sub-cultured in fresh semisolid media and streaked onto solid plates supplemented with yeast extract (100 mg L^{-1}), incubated at 30°C for 5 days.

Colonies were further analysed for taxonomic identity. Identification of *Burkolderia* isolates was done by (a) cultural, (b) morphological, (c) biochemical and physiological study following Bergey's manual of determinative bacteriology (Hensyl, 1994). The isolates were studied following the methods of Cerney (1993) including morphological and physiological features. The physiological and biochemical activities of the isolates were tested through the methods as demonstrated by Collee and Miles (1989).

Genomic DNA was isolated by the method of Ausubel et al. (1987), except that the lysate was extracted twice with chloroform to remove residual phenol. The 16S rDNA gene was amplified using degenerative primers according to the direct sequencing method (Hiraishi, 1992). The 16S rDNA sequence of the isolate SDSA-I10/1 as determined in this work was edited manually and aligned with a selection of 16S rDNA sequences from members of the alpha, beta and gamma subclasses of the Proteobacteria. These were retrieved from the sequence collection of the Ribosomal Database Project (Maidak et al., 1996) and from GenBank. Non-resolved positions and gaps were removed prior to the phylogenetic analysis. The sequence was aligned using Clustal V software and homologies of sequences were determined using the basic alignment search BLAST against the NCBI database. Distance matrix was corrected for multiple base changes at single locations by the method of Jukes and Cantor (1969). Phylogenetic tree was constructed from evolutionary distance matrix by the neighbour-joining method of Saitou and Nei (1987) using NEIGHBOR**,** contained in the Phylogenetic Inference Package, PHYLIP 3.51 (Felsenstein, 1978). The phylogenetic tree was constructed using the 10 nearest neighbours and as well as other species available in the database. The analysis of distance matrix, similarity matrix and phylogenetic

tree was done using the neighbor program included in Joe Felsenstein's Phylip 3.51c distribution. Parsimony analysis was performed by using DNAPARS**.** The results obtained by both NEIGHBOR and DNAPARS were subjected to bootstrap analysis by using SEQBOOT in sets of 1000 resamplings. The hierarchy view and the sequence were done using RDP release 10 (ribosomal database release 10 software).

A field experiment was set-up at Kalinjar (24°92′ N, 92°24′ E and 71 m above mean sea level) located 6 km away from Silchar, Assam, India. The field was not cultivated in the last year prior to this experiment. Climate of the region is sub-tropical humid and receives mean annual rainfall 2,431 mm and average rainy days 167 per annum. Total bright sunshine hour (BSSH) is 2,029 h against maximum possible BSSH of 4,242 h per year. Mean relative humidity is 82%. During experimental period in 2009, the mean maximum and minimum temperatures recorded during sali rice (winter, August to November) were 32.6 and 22.3°C, respectively. The length of crop growing period is >200 days in a year in this rice agro-ecological zone of Assam.

The experimental field was divided into two blocks (one for *Burkholderia* treatment and one for control). Within each block, five plots were the replicates, each with an area of 4×5 m^2. Each block was laterally isolated by polythene sheets embedded into the soil to a depth of 30 cm. The experiment was arranged as completely randomized block design.

The initial soil characteristics of the experimental field were determined. The clay loam inceptisol had the following properties: sand 36%, silt 14%, clay 49%, pH (1:2, soil/water) 4.90, total organic C 0.38%, total N 32.5 kg ha^{-1}, total P 27.4 kg ha^{-1}, total K 35.7 kg ha^{-1}, organic matter 0.48%, and electrical conductivity 0.94 dsm^{-1}. The soil of the experimental plot was analyzed as per the standard methods of Jackson (1973).

The plant inoculation experiment was undertaken in sali (winter) cropping season (August to December) and the crop was successfully harvested. Three rice seedlings together (25 days old) were transplanted in the puddled plots at spacing 30×10 cm (between rows × between plants). The two treatments were: *Burkholderia caribensis* or T_1 and control or T_2. The standard inoculum of the strain was prepared by growing the bacteria in 250 ml glucose-peptone broth for 72 h at 28 ± 2°C on a shaker. The cells in active growth stage were harvested by centrifugation at 8000 rpm for 10 min and resuspended the pellet in sterile distilled water to attain a concentration of 10^8 cfu ml^{-1}. Then sterilized charcoal powder was mixed with the aqueous suspension of the diazotroph strain as described by Thakuria et al. (2004) to prepare carrier based inoculant. Plant infection of the strain was done by seedling root dip method. For this purpose, an aqueous slurry of charcoal powder based inoculants of the strain was made in shallow plastic tubs. Twenty five day old seedlings of rice were uprooted carefully and the root portions of the uprooted seedlings were dipped into the aqueous slurry for 12 h to ensure maximum contact of the strain on the root surface. The seedlings treated with aqueous slurry of sterilized charcoal powder but devoid of the *Burkholderia* strain were used as a control. Five replicates were maintained for each treatment. The cfu count of the biofertilizer strain was 10^8 colony forming units (cfu) g^{-1} charcoal. The charcoal based inoculants were applied (at 4 kg ha^{-1}) to rice seedlings by root-dip technique.

At the harvesting stage, the plants were uprooted with intact roots, washed thoroughly to remove the adhered soil and taken for analysis of growth and yield parameters. Plant height (cm $plant^{-1}$), shoot length (cm $plant^{-1}$) and root length (cm $plant^{-1}$) were measured: the average height of five randomly chosen plants from each plot was measured from ground level to the panicle tip. Number of tillers/plant was counted in five randomly chosen plants (average) in each plot. The fresh weight (g $plant^{-1}$) and 100-grain weight (g $plant^{-1}$) were analysed from five randomly taken samples of each plot. Plant dry weight (g $plant^{-1}$) was recorded after drying In an oven for 1 day at 70°C.

The N-content of shoot was estimated by micro-kjeldahl method (Fallik et al., 1988) and the chlorophyll content of leaves was estimated by Arnon method (1949). Protein content of grains was determined by Lowry's et al. (1951) method. Nitrate reductase (NR) activity was assayed by soaking the roots in KNO_3 solution for 48 h. NR was recorded as reduction of KNO_3 to KNO_2 per hour of reaction at 30°C in terms of concentration (μM NO_2 $h^{-1}g^{-1}$ fresh wt.) of the latter (Hewitt and Nicholas, 1964).

Summary statistics were used to obtain the mean, standard error and percent increase over control (Snedecor and Cochran, 1967). The least significant difference (LSD) was calculated following the method of Misra and Misra (1983). The nucleotide sequence of the 16S rDNA gene of the strain SDSA-I10/1 investigated in this experiment was deposited in the NCBI GenBank through the GenBank submission tool BankIt (http://www.ncbi.nlm.nih.gov) under accession number GU372342.

RESULTS

Altogether, six isolates of indigenous *Burkholderia* sp. were obtained from the acidic rice rhizosphere soils of South Assam, India. The data in Table 1 show the cfu count of *Burkholderia* isolates in rice rhizosphere soils at panicle initiation stage in the six locations of South Assam. The isolates of *Burkholderia* showed the highest cfu count in the rhizosphere of acidic submerged rice fields of Cachar district. The isolate SDSA-I10/1 showed highest cell count in the rice fields of Cachar district. The cell count of the isolate SDSA-I10/4 was higher in the lowland rice fields of Karimganj district and that of the isolate SDSA-I10/5 was higher in the lowland rice agro-ecosystems of Hailakandi district. The population of the isolates differed significantly not only between the locations but between the different isolates as well. On an average, SDSA-I10/1 population was highest followed by SDSA-I10/4 and SDSA-I10/5 which are the dominant *Burkholderia* strains in the rice rhizosphere soils of South Assam.

The pure cultures of the isolates of *Burkholderia* were tested for the ability to fix atmospheric N_2 by acetylene reduction technique. The greater the activity of nitrogenase enzyme the greater is the ability of atmospheric N_2-fixation. The maximum nitrogenase activity was observed in SDSA-I10/1 culture (402.3 nM C_2H_4 $hr^{-1}ml^{-1}$ culture). Other isolates also showed good N_2-fixation ability as characterized by ARA activity > 300 nM C_2H_4 $h^{-1}ml^{-1}$ culture (Table 2).

Burkholderia isolates have been identified as per following characteristics: round, entire, convex or raised, smooth, opaque, orange or brown colony, cell rod or oval shaped and occur singly or in pairs, gram negative, cyst and extra-cellular PHB granules present, catalase positive, non sporulating, motile, aerobic or occasional anaerobic, optimum growth temperature 25 to 30°C, pH 3.0 to 5.7, growth at maximum 2.5% NaCl, hydrolyzed urea and nitrate, fermentative, oxidase positive, and acid production from dextrose, galactose, inositol, arabinose, mannitol and sucrose. According to Bargey's Manual of

Table 1. Quantitative enumeration of *Burkholderia* isolates in the rhizosphere of cultivated rice varieties at panicle initiation stage grown under acidic lowland ecosystems.

District	Location	Isolate	Rice variety	*cfu g^{-1} dry soil (× 10^6)
Cachar	Salchapra	SDSA-I10/1	Ranjit	51 ± 4.32
	Narsingpur	SDSA-I10/2	Bahadur	28 ± 3.1
Karimganj	Kaliganj	SDSA-I10/3	Mahsuri	21 ± 1.32
	Patharkandi	SDSA-I10/4	Ranjit	47 ± 2.82
Hailakandi	Lalabazar	SDSA-I10/5	Ranjit	43 ± 1.75
	Panchgram	SDSA-I10/6	Ranjit	17 ± 1.25

± SE, * cfu g^{-1} dry soil is the average of all the locations of the district which was again average of the three pooled soil samples of each location in the district.

Table 2. Estimation of N_2-fixing potential (nitrogenase activity) of *Burkholderia caribensis* isolates by ARA.

Isolate/ Strain	*Nitrogenase activity (nM C_2H_2 h^{-1} ml^{-1} culture)
SDSA-I10/1	402.3
SDSA-I10/2	352.4
SDSA-I10/3	371.7
SDSA-I10/4	312.4
SDSA-I10/5	302.1
SDSA-I10/6	338.5
LSD at 5% significant level	12.25

*Nitrogenase activity is the average of three replicates.

Systemic Bacteriology, the biochemical and carbohydrate fermentation tests indicated that the characters represented by the isolates were similar to *Burkholderia* spp. (Table 3).

Out of six isolates, the one showing higher N_2-fixation rate (SDSA-I10/1) as envisaged by its higher nitrogenase activity was analyzed for 16S rDNA gene sequence due to feasibility of experimentation. The sequence of the 16S rDNA gene of the isolate investigated in this work has been deposited in GenBank as described in the methods section. The 16S rDNA gene sequence of the isolate SDSA-I10/1 showed close resemblance to *B. caribensis*; MWAP84; Y17011 and *B. caryophylli* (Figure 1). The length of 16S rDNA sequence was approximately 1119bp and showed a high similarity to *B. caribensis* (99.3%) MWAP84 (Y17011). The phylogenetic analysis of the isolate and the type strain of the genus *Burkholderia*, formed one compact cluster with *B. caribensis* str. MWAP84, str. TFD2, *B. caribensis* str. MWAP64, *B. caribensis* str. MWAP71, and *B. caryophylli* str. MCII-8 (Figure 1). 16S rDNA sequence analysis confirmed that this isolate had a 99.3% sequence similarity with *B. caribensis* MWAP84 (Y17011).

The isolate SDSA-I10/1 has showed highest N_2-fixing ability in comparison to other isolates of *Burkholderia* and strain identification of this isolate was carried out on the basis of morphological, physiological, and biochemical study coupled with 16S rDNA gene sequencing. Therefore, this strain was selected for bioinoculation experiment. Since rice is mostly grown in sali (winter) crop during August to December under acidic rainfed lowland ecosystems of South Assam, the bioinoculation effect of this strain in N_2-assimilation of rice was observed on winter cropping season. The popular variety of winter rice in South Assam is *cv*. Ranjit which was selected for bioinoculation experiment.

The data in Table 4 revealed that treatment with the *Burkholderia* strain SDSA-I10/1 significantly increased the growth and yield of rice over untreated control plants in winter season. Almost 13% increase in plant height over uninoculated control was recorded. The shoot length of plants was improved by 6% following inoculation with SDSA-10/1 strain. Root length of rice was improved by 18%. Number of tillers was increased by 29%, fresh and dry biomass by 17% and 34% respectively, and weight of grains by 28% followed by inoculation of winter rice *cv.* Ranjit with *Burkholderia caribensis* strain SDSA-I10/1 in acidic flooded lowlands of South Assam. Inoculation of *B. caribensis* treatment resulted in maximum increase in weight of grains in autumn season among all the treatments.

38% increase in the plant chlorophyll$_a$ content was reported at the harvesting stage of winter rice. The nitrogen content of shoot was improved by 31% following

Table 3. Morphological, physiological and biochemical characteristics of Burkholderia isolates.

Tests	Isolates					
	SDSA-I10/1	**SDSA-I10/2**	**SDSA-I10/3**	**SDSA-I10/4**	**SDSA-I10/5**	**SDSA-I10/6**
Colony morphology	Round, entire, highly raised, opaque, orange colony	Round, entire convex, opaque, colony	Round, entire raised, opaque orange colony	Round, entire highly raised, colourless, colony	Round, entire, convex, colourless colony	Round, irregular Convex, opaque, light brown colony
Cell shape	Oval rod	Oval	Oval rod	Rod	Rod	Spherical
Arrangement	Single	Single	Paired	Single	Single	Single
Gram's reaction	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Spore	Absent	Absent	Absent	Absent	Absent	Absent
PHB granule	Present	Present	Absent	Present	Present	Present
Cyst	Present	Present	Present	Present	Present	Absent
Optimum growth temperature (°C)	25-30°C	25-30°C	25-30°C	25-30°C	25-35°C	25-30°C
Optimum pH	3.0-5.7	3.5-5.7	3.0-6.4	3.5-5.7	4.0-5.7	4.0-5.7
Anaerobic growth	±	±	±	±	±	±
NaCl tolerance (%)	2.5	2.5	2.5	2.5	2.5	2.5
Gas production from glucose	-	-	-	-	+	-
Urea hydrolysis	±	±	±	±	±	±
Starch hydrolysis	-	-	-	-	-	-
Nitrate reduction	±	±	±	±	±	±
Catalase	+	+	+	+	+	+
O/F test	F	F	F	F	F	F
Cytochrome oxidase	±	±	±	±	±	±
H_2S production	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-
Voges Proskauer test	-	-	-	-	-	-
Fructose	-	-	-	-	-	-
Galactose	+	+	+	+	+	+
Inositol	+	+	+	+	+	+
Dextrose	+	-	+	+	+	+
Cellobiose	-	-	-	-	-	-
Arabinose	+	+	+	+	-	+
Mannitol	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-
Sucrose	±	±	±	±	±	-
Xylose	-	-	-	-	-	-

+, Tests positive; ±, tests variable; -, tests negative.

inoculation with *B. carinbensis* strain SDSA-I10/1. The protein content of grains was also increased over the uninoculated plants. 25% increase in protein content of matured grains was observed in the plant inoculation test. The NR activity of rice roots was improved by 16% over uninoculated control in winter crop (Table 5).

DISCUSSION

Six isolates of N_2-fixing heterotrophic *Burkholderia* sp. were observed in the rhizosphere region of cultivated rice varieties grown in the tropical rainfed lowlands of South Assam, India. The occurrence of N_2-fixing *B. caribensis* strain in the root region of rice grown in acidic rainfed submerged lowlands of South Assam, India is reported for the first time in this present investigation. Enumeration, isolation and identification of diazotrophs from rhizosphere soil of Korean rice varieties revealed three groups of N_2-fixing bacteria belonging to the genera *Azospirillum*, *Burkholderia* and *Gluconacetobacter* (Kang, 2006). Agronomically important paddy rice varieties in

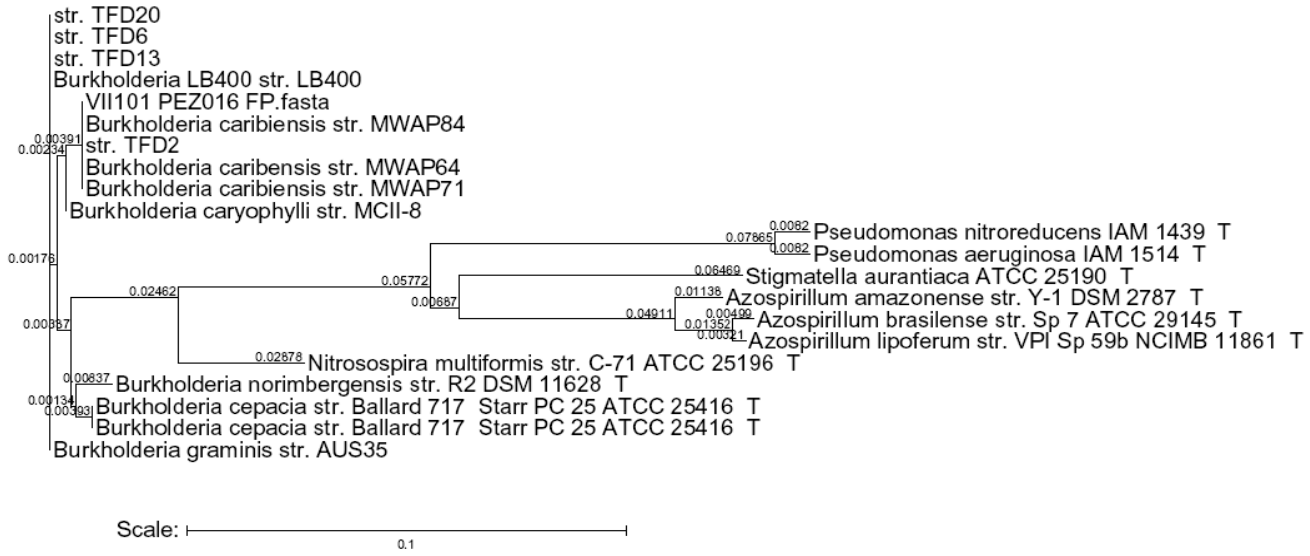

Figure 1. Phylogenetic tree based on 16S rDNA gene sequence comparison showing the position of *B. caribensis* strain SDSA-I10/1 (VII101_PEZ016_FP.fasta) and other related strains of the family Burkholderiaceae.

Table 4. Effect of *B. caribensis* strain SDSA-I10/1 on the growth and yield of winter (sali) rice cv. Ranjit in the absence chemical fertilizers.

Treatment	Plant height (cm)	Shoot length (cm)	Root length (cm)	No. of tillers/ plant	Plant fresh weight (g)	Weight of 100 grains/ plant (g)	Dry/ biomass plant (g)
B. caribensis, SDSA-I10/1 (T1)	125.3 (113)	100.3 (106)	25.0 (118)	17.3 (129)	137.3 (117)	2.66 (128)	44.05 (134)
Control (T2)	110.4 (100)	94.2 (100)	16.2 (100)	13.4 (100)	117.1 (100)	2.08 (100)	32.82 (100)
LSD (P=0.05)	9.45	7.13	4.47	2.99	7.22	0.43	3.77

*Each variable is the average of five replicates; figures in bracket indicate percent increase over control; LSD (P<0.05) = Least significant difference at 5% level of significance.

Table 5. Effect of *B. caribensis* strain SDSA-I10/1 on the biochemical parameters of winter (sali) rice cv. Ranjit in absence of chemical fertilizers.

Treatment	Chlorophylla content of leaves (mg g^{-1} fr wt.)	Nitrogen content of shoot (mg g^{-1} dry wt.)	Protein content of root grains (%)	Nitrate reductase activity (µM NO_2 $h^{-1}g^{-1}$ fr wt.)
B. caribensis, SDSA-I10/1(T1)	1.08 (138)	0.59 (131)	9.73(125)	9.78(116)
Control (T2)	0.78 (100)	0.45 (100)	7.80 (100)	8.42 (100)
LSD (P=0.05)	0.06	0.05	0.28	1.08

*Each variable is the average of five replicates; figures in bracket indicate percent increase over control; LSD (P<0.05) =Least significant difference at 5% level of significance.

Korea harbour different diazotrophic isolates such as *Azospirillum* spp., *Herbaspirillum* spp., *Burkholderia* spp. and *Gluconacetobacter* spp. (Kang, 2006). The identification experiments revealed the association of N_2 fixing *Herbaspirillum* spp., *Gluconacetobacter diazotrophicus* and *Burkholderia* spp. in Korean rice varieties for the first time apart from the association of *Azospirillum* spp. and *Pseudomomnas* spp.

This present work revealed that the number of cultivable N_2-fixing *B. caribensis* in the root region of rice at panicle initiation stage in most of the sampling sites of South Assam was over 10^6 cfu g^{-1}dry soil confirming the number found by Watanabe et al. (1978) in paddy soil at the International Rice Research Institute. This may be due to the fact that the number of N_2 fixers is strongly governed by soil organic matter content (Xie et al., 2003)

and rice agro-ecosystem soils of South Assam are rich in organic matter. The population count of the isolate SDSA-I10/1 was highest followed by SDSA-I10/4 in the acidic rice field soils of South Assam. Watanabe and Barraquio (1979) revealed that nitrogen-fixing bacteria are present in greater numbers in the roots of wetland rice. All the isolates are more or less acid tolerant and therefore they are prevalent in the acidic rice agro-ecosystems of South Assam. All the six isolates formed the typical orange yellow sub surface pellicles on semisolid selective modified PCAT medium which showed the micro aerobic nature of the organism (Cavalcante and Dobereiner, 1988). Rennic and Vose (1983) used single nitrogen free medium for isolating nitrogen-fixing bacteria and showed that at the higher dilutions 75% of the isolates exhibited acetylene reduction. The results confirmed that the isolates were of *Burkholderia* spp. The studied isolates used galactose, inocitol, dextrose, arabinose, mannitol, and sucrose like that of recently reported *Burkholderia phytofirmans* (Sessitsch et al., 2005).

Recently studies have been carried out in different parts of the world to investigate the role of diazotrophic inoculation in crop growth and yield to develop alternative sources of chemical fertilizers. Nitrogenase activity of maize rhizospheric bacteria was detected in 19 isolates ranging from 21.8 to 3624 nM C_2H_2 h^{-1} ml^{-1} culture (Naureen et al., 2005). In this present study, the nitrogenase activity range (302.1 to 402.3 nM C_2H_4 h^{-1} ml^{-1} culture) of the isolates fell within that of rhizospheric bacteria as detected by Naureen et al. (2005). Reis et al. (2004) reported variation in nitrogen fixing efficiency in different strains under different conditions. In this present study, six isolates of *Burkholderia* sp*.* from rhizosphere of rice were designated as SDSA-I10/1, SDSA -I10/2, SDSA-I10/3, SDSA-I10/4, SDSA-I10/5, and SDSA-I10/6 respectively. The isolates showed appreciable amount of nitrogenase activity and the strain SDSA-I10/1 showed highest ARA and hence the strain was selected for 16S rDNA sequencing and plant inoculation test. Bacteria were enumerated by cfu count and the isolates were identified based on morphological, physiological, and biochemical characteristics. This work has led to the identification of a novel strain of culturable N_2-fixing heterotrophic *B. caribensis* SDSA-I10/1 of the class β-proteobacteria. The isolate SDSA-I10/1 has the highest 16S rDNA similarity to the members of the family Burkholderiaceae and are in the same clade of *B. caribensis* (99.3%). This present investigation has led to the molecular characterization of one indigenous diazotroph strain, *B. caribensis,* GU372342 from acidic lowland rice agro-ecosystem of South Assam, India which has shown higher N_2-fixing capacity and can be used as microbial fertilizer for rice.

One of the probable causes of poor rice production may be due to low (<50%) agronomic use efficiency of nitrogenous fertilizers in acidic rice field soils under high rainfall (>2000 mm) conditions (Ladha et al., 1998).

Therefore, most of the rice field soils of South Assam are deficient in N and the farmers are using excessive amount of chemical N fertilizers. Since the long term use of chemical nitrogenous fertilizers depletes the soil organic matter and poses a threat to the survival of indigenous soil micro-flora, biological nitrogen fixation (BNF) technology can play an important role in substituting the use of chemical N fertilizers in rice cultivation.

In this present study, gnotobiotic assays were conducted to test the inoculation effect of *B. caribensis* (GU372342) on biological and biochemical parameters of rice. Seedlings treated with the strain showed a considerable increase in plant height, shoot length, root length, number of tillers, fresh weight, grain weight, dry biomass, chlorophyll$_a$ content, N and protein content, and NR activity compared with the control. In a similar study conducted under gnotobiotic conditions, Baldani et al. (2000) reported that the inoculation of rice with *Herbaspirillum seropedicae* enhanced plant dry weight by 71.5%. These results could be explained by the fact that inoculated plants absorbed more nutrients which reflect on growth activity, nitrogenous compound assimilation, forming more growth substances (IAA), more cell division and enlargement, more forming of tissues and organs. However, any practical application of these results should be preceded by further evaluation under field conditions. Besides exploring the potential for BNF and other promising PGP functions carried out by free-living diazotroph strains, it is also important to ensure that the bacteria are well adapted to environmental conditions before they are utilized as inoculant strains. The isolated free-living and/or associative *B. caribensis* strain could be very useful in the formulation of new microbial inoculants and could be applied most profitably to enhance the growth and yield of rice in tropical rainfed acidic lowlands.

Bacteria such as *Pseudomonas* spp., *Burkholderia caryophylli*, *Achromobacter piechaudii* were shown to lower the endogenous ethylene level in planta by producing a degradative enzyme 1-aminocyclopropane- 1-carboxylic acid (ACC)-deaminase (Shaharoona et al., 2007). The effects of ACC-deaminase producing rhizobacteria on plants included increased root growth, and improved tolerance of salt and water stress (Shaharoona et al., 2007).

Elcoka et al. (2008) hypothesized that microbial inoculants will replace mineral fertilizer; rather many studies, for example Adesemoye et al. (2009), have shown that microbial inoculants are good and reliable supplements to fertilizer. This present experiment revealed that inoculation of winter rice with acid tolerant *B. caribensis* strain improved plant height, shoot length, root length, number of tillers, fresh weight, weight of grains, dry biomass, chlorophyll content, N-content, protein content and NR activity by almost 20 to 30% over control and this is in conformity with the findings of Deb Roy et al. (2009) who studied the inoculation effect of three native diazotroph strains of South Assam namely,

Azotobacter chroococcum, *Azospirillum amazonense* and *Beijerinckia indica* on the growth and yield of summer (ahu) rice *cv.* IR-36 grown in acidic flooded lowland ecosystem. There were repeated beneficial effects on rice plants inoculated with a *Burkholderia vietnamiensis* strain on early and late components in low fertility sulfate acid soil of Vietnam (Tran Van et al., 2000). The nitrogen fixation by the inoculated strain per se seems not to be the sole cause of increased plant growth as indicated from the present data on percent N content of inoculated samples. It is presumed that the bacterium used for inoculation in this present study appears to act as that of plant growth promoting rhizobacteria (PGPR). It would have effected plant growth by the synthesis of phytohormones and vitamins, inhibition of plant ethylene synthesis and improved nutrient uptake as reported recently (Dobbelaere et al., 2003).

Conclusion

The isolates of *Burkholderia* spp. are indigenous and best suited to the ecophysiological condition of lowland rice agro-ecosystems of South Assam which have showed higher N_2 fixation rate and one of the isolates SDSA-I10/1 (GU372342) might be used as efficient microbial fertilizer strain for growing rice, the major staple food of the people in tropical rainfed acidic lowlands.

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