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Recent Studies on N_2 Fixing *Burkholderia* Isolates as a Biofertilizer for the Sustainable Agriculture

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A B S T R A C T

In this study thirteen isolates of *Burkholderia* sp. were isolated from the root, stem and leaf samples of four different crops *viz.*, rice, maize, sugarcane and blackgram, using N-free *Burkholderia* Azelaic acid (BAz) semi solid medium. Based on growth performance on different media the following isolates *viz.*, RB_1 (Rice *Burkholderia* 1), MB_2 (Maize *Burkholderia* 2), SB_3 (Sugarcane *Burkholderia* 3) and BB_4 (Black gram *Burkholderia* 4) were selected for further studies. Among the four isolates, BB_4 isolate showed the maximum nitrogenase activity (81.74 n moles ethylene produced h^{-1} /mg of cell protein) followed by SB_3, RB_1 and MB_2. Interestingly the cell protein content (1.54 mg g^{-1}), ammonia excretion (10.04 µg ml^{-1}), polysaccharide production (28.82 mg g^{-1}) and hydrogen cyanide production (44.42 µg ml^{-1}) were also observed more in BB_4 than others. Further the Pot culture experiment was carried out to evaluate the effect of best N_2 fixing selected isolate (BB_4) on the growth of maize. The maximum germination percentage (83.3 per cent) was observed in the maize when it was treated with *Burkholderia* + 100% N + P fertilizer. At the same time after 60th day of sowing higher shoot (59.7cm) and root (32 cm) growth of maize was observed when it was treated with *Burkholderia* + 100 % N + P, which was on par with the treatment with *Azospirillum* + Phosphobacteria +100 % N and P. Therefore it is clear from the above observation that *Burkholderia sp* (BB_4) may be useful as a cost effective bio fertilizer to promote sustainable agriculture.

Introduction

By the year 2050, world population is expected to double from its current level of more than 5 billion. It is reasonable that the demand of fixed nitrogen for crop production is also expected to double. If this is supplied by industrial sources, synthetic nitrogenous fertilizer use will shoot to about 160 million tonnes of nitrogen per year, which is about equal to that produced by the biological process. This amount of nitrogenous fertilizer will require burning of around 270 million tonnes of coal or its equivalent. However, expanded exploitation of Biological Nitrogen Fixation (BNF) could reduce and in the longer term substantially, replace the need for industrially produced fertilizer nitrogen. Many N_2 fixing microorganisms like *Rhizobium, Azospirillum, Azotobacter* etc.,

have been well studied and well described in the past. The latest addition to this N_2 fixing group of microorganism is *Burkholderia* sp. This genus is very attractive because of its wide spread geographical distribution and physiological characteristics. The presence of this organism in environments such as water, soil and plant rhizosphere (Parke and Gurian-Sherman, 2001) has been reported. Several N_2 fixing *Burkholderia* species are known to be associated with roots, stems and leaves of many plant species. The ability to fix atmospheric nitrogen has been established in several *Burkholderia* sp. including *B. vietnamiensis, B. kururiensis, B. tropicalis, B. sacchari* and *B. brasilensis* in association with various parts of banana, pineapple, maize, sorghum, cotton, rice, coffee and sugarcane. *B. cepacia* is recognized for its abilities to promote maize growth, to enhance crop yields and to suppress many soil borne pathogens

Nitrogenase activity associated with the roots in kallar grass first reported by Malik *et al.,* (1980; 1982). Silva *et al.,* (1981) confirmed the nitrogen fixation with in the roots by using tritiated Acetylene Reduction Technique and Electron Micro Auto Radiography. Growth and acetylene reduction to ethylene is observed with different carbon sources in N-free semi solid media (Caballero-Mellado *et al.,* 1995). Rhizosphere is the region of active nutrient excretions and successful ecological niche for the omnipotent bacteria like *Pseudomonas,* (Karthikeyan and Kulakow, 2003). So there could be more chances for getting some relative genera of *Pseudomonas* like *Burkholderia*. This leads the scientists to thoroughly screen among the rhizosphere bacteria for the presence of this genus. Phylogenetically, the latter species belongs to the *B. cepacia* complex. The widespread soil bacteria, *Burkholderia* and fluorescent *Pseudomonas* are very efficient root colonizers even in the presence of indigenous microorganisms (Schroth and Hancock, 1982). Hebbar *et al.,* (1994) and Carole *et al.,* (2013) reported that *B. cepacia* was closely associated with *Zea mays* roots, representing over 4 per cent of the total culturable rhizobacteria in maize rhizosphere.

Estrada-De Los Santos *et al.,* (2001) tentatively named species like *B. tropicalis* and *B. brasilensis* which have not been formally described but strains of these putative novel species have been recovered from various parts of banana and pineapple plants. Recently a few strains of *Burkholderia* have been isolated from the rhizosphere region of maize they were named as new species namely *B. unamae* (Caballero-Mellado *et al.,* 2004). The ability of each *Burkholderia* strain to fix nitrogen was estimated by using the acetylene reduction assay and *B. vietnamiensis* strains isolated from the rhizosphere of rice plants were positive for nitrogenase activity. Using the N^{15} isotope dilution technique, it was estimated that kallar grass might fix up to 26 per cent of its N content (Geoffrey *et al.,* 2006; Malik *et al.,* 1997).

In field trials *Burkholderia* sp. was capable of reducing fertilizer-N input by 25-30 kg N per ha. In pot culture experiment, *B. vietnamiensis* inoculation with rice resulted in a final 20 per cent yield increase through positive effects of most yield components (Lammel *et al.,* 2013; Tran Van *et al.,* 1994). It significantly increased several yield components, resulting in a final 13 – 22 per cent increase in grain yield. Garcia de Salomone *et al.,* (1996) demonstrated that the amount of N derived from BNF by maize plants inoculated with different *Azospirillum* sp. was related with the varieties of maize used. In particular, *B. cepacia* seemed to be strictly associated with maize roots, probably being are of the most representative bacterial species in the maize rhizosphere. Furthermore, colonization of the maize

rhizosphere by *B. cepacia* was not significantly affected by plant development (Di Cello *et al.,* 1997). Barraquio *et al.,* (1997) also suggested that rice stem are more suitable niches for N fixing endophytes than roots because more photosynthates are available to the bacteria.

In the current study isolation, screening and physiological studies of *Burkholderia* sp were carried out and the application of this bacterium as a biofertilizer onto the maize crop was used to compete with the commercially exploited biofertilizer for the sustainable agriculture.

Materials and Methods

Soil and seed for pot culture

The pot culture experiments were conducted using the soil collected from garden land of Tamil Nadu Agricultural University and maize seeds for pot culture study were obtained from the Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore.

Reference strains

The reference strains of *Burkholderia* sp. *viz.*, *Burkholderia vietnamiensis* and *Burkholderia tropicalis* were obtained from Prof. P. Vandamme, Gent University, Belgium and Dr. J. Balandreau, Institute of Research for Development, South Africa respectively.

Isolation and screening of *Burkholderia* sp. from crop plants

Collection of plant samples

The plant samples of rice, maize, sugarcane and blackgram were collected from the Tamil Nadu Agricultural University farm, Coimbatore for the isolation of *Burkholderia* isolates. The plants were carefully uprooted without damaging the root system and collected in separate polybags and brought to the laboratory for immediate use.

Isolation of *Burkholderia* sp.

The method of isolation of endophytes described by Zinniel *et al.,* (2002) and Hallmann *et al.,* (1997) were adopted for the isolation of *Burkholderia* from the root, stem and leaves of plant samples collected. The fresh plant samples were carefully separated into stems, roots and leaves. Each part was cut in to sections of 2-3 cm lengths and dried on absorbent towels. One gram of each part was taken and was surface sterilized for l min with 70 per cent ethyl alcohol and 1 per cent chloramines-T. The plant parts were washed thoroughly with sterile distilled water thrice to remove the traces of chloramines-T and homogenized in a sterile pestle and mortar in laminar chamber. The homogenate was transferred into 10 ml sterile distilled water and serially diluted upto 10^{-3}. One ml of homogenate was transferred into tubes containing sterile N-free BAz semisolid (*Burkholderia* Azelaic acid), BAc (*Burkholderia* Azelaic acid citrullin) and PCAT (*Pseudomonas cepacia* Azelaic acid and Tryptamine) medium separately. The inoculated tubes were incubated at 28±2°C for three days. The number of positive tubes in each medium was observed.

Screening of *Burkholderia* isolates using tryptose soya agar and growth of the isolates in different selective medium

To differentiate *Burkholderia* sp. from *Pseudomonas* sp. tryptose soya agar medium was used. Exactly 50 ml of tryptose soya agar was prepared in 100 ml conical flask sterilized and poured in the sterile petriplates. The isolated culture strains were streaked on the solidified media and the plates were

incubated at room temperature. After 24 hrs, the growth of the cultures was observed. The isolates which have not produced the fluorescent pigmentation were selected as *Burkholderia*. The selected colonies of isolates were grown in different selective media *viz.,* BCSA (*Burkholderia cepacia* selective agar), BA_Z (*Burkholderia* Azelaic acid), BA_C (*Burkholderia* Azelaic acid citrullin), BMGM (Modified BAz) and PCAT (*Pseudomonas cepacia* Azelaic acid and Tryptamine) and the growth was observed.

Physiological characteristics of *Burkholderia* isolates

Nitrogenase activity

In vitro nitrogenase activity of the isolates was assayed as per the method of Hardy *et al.*, (1968).The BAz medium was prepared and dispensed in 40 ml quantities in 130 ml glass bottles and sterilized. The medium in the bottles were inoculated with 1ml of log phase cells of isolates and incubated at 22°C under static conditions. After 7 days of growth, the cotton plugs in the vials were replaced with subaseal rubber stopper. The vials were flushed with nitrogen gas to exhaust oxygen in the gas phase. Using an air tight syringe, 3 ml of pure acetylene gas was injected after withdrawing same volume of air from the bottle and incubated for 24 hrs at room temperature. Control was also maintained without inoculation of cultures.

After the incubation period, 0.5 ml of the gas sample was withdrawn from the bottle and injected into gas chromatograph (Varian CP 3800) with FID detector system having 80-100 mesh porapak N-column. The column temperature was maintained at 85°C, oven temperature at 70°C and ionization temperature at 100°C. Nitrogen was used as a carrier gas with a flow rate of 30 ml/sec and for flame ionization hydrogen and zero air at the rate of 30 ml sec^{-1} respectively. The area of ethylene peak was recorded for each culture.

The acetylene reduction activity was determined, by the following formula.

ARA = Peak height (mm) X Attenuation range X Constant X Volume of acetylene gas injected in to the vial / Hours of incubation X Volume of gas injected into gas chromatograph X Milligram of cell protein

The factor was arrived by the use of standard ethylene. The ARA values are expressed in terms of nmoles of ethylene produced h^{-1} mg^{-1} of cell protein.

Estimation of ammonia excretion

Estimation of ammonia excretion was estimated by the method of Solarzano, 1969. The isolated endophytic cultures were grown in BAz medium. Five ml of culture filtrate was taken and to that 0.2 ml of phenol reagent was added and mixed. Then 0.5 ml of oxidizing reagent was added and mixed thoroughly. It was incubated for one hour in dark with intermittent shaking. Blue colour was developed and the intensity of colour was measured in Spectrophotometer at 640 nm. The standard graph was drawn using ammonium chloride (1-20 µg ml^{-1}). The results were expressed in µg ml^{-1}.

Estimation of cell protein

The protein content of all the isolates was determined following the method of Lowry *et al.,* (1951). The BSA was used as a standard.

Estimation of polysaccharide production

The polysaccharide production in the cells was determined using the methods of Dubois *et al.,* (1951). Glucose was used as a standard.

Estimation of HCN production

HCN production by the isolates was detected by the method of Bakker and Schipper (1987). BAz medium amended with 4.4 g^{-1} l^{-1} glycine was prepared, sterilized and plated. After solidification, the isolates were streaked on the respective plate and pre-soaked Whatman no.1 filter paper disc (9 cm in diameter) in 0.5 per cent picric acid and 2 per cent Na_2CO_3 was placed in the lid of each plate.

The plates were sealed with parafilm and incubated at 28±2°C for 4-7 days. Uninoculated plates served as control for comparison of results. The colour was eluted by placing the paper in a clean petriplates containing 10 ml of distilled water and read in Spectrophotometer at 625 nm. Standard curve was prepared with KCN and hydrogen cyanide content of the sample was expressed as µg ml^{-1} of culture.

Evaluating the performance of *Burkholderia* isolates under pot culture condition

A pot culture experiment was conducted to know the effect of individual inoculation of *Burkholderia* isolate (BB_4) in comparison with *Azospirillum* and phosphobacteria on maize.

Experiment details

Crop: Maize

Pot size: 30 cm X 30 cm

Design : CRD (Completely Randomized Design)

Number of treatments: 11

Number of replications: 3

The pots were filled with 15 kg of the garden soil collected from Eastern block, Tamil Nadu Agricultural University, Coimbatore. Before filling, the soil was completely sterilized at 20 pounds (square inch)$^{-1}$ for 2 h.

Treatment details for maize

T_1 - Uninoculated control with 100 % N + 100 % P

T_2 - *Burkholderia* alone

T_3 - *Azospirillum* + phosphobacteria

T_4 - *Burkholderia* + 100 % N + 100 % P

T_5 - *Azospirillum* + phosphobacteria + 100 % N + 100 % P

T_6 - Uninoculated control with 75 % N + 75 % P

T_7 - *Burkholderia* + 75 % N + 75 % P

T_8 - *Azospirillum* + phosphobacteria + 75 % N + 75 % P

T_9 - Uninoculated control with 50 % N + 50 % P

T_{10} - *Burkholderia* + 50 % N + 50 % P

T_{11} - *Azospirillum* + phosphobacteria + 50 % N + 50 % P

Preparation of inoculants

The *Burkholderia* isolates (BB_4) were multiplied in BAz broth, upto log phase with a cell load of 10^8 ml^{-1} and used for inoculant preparation. Lignite was used as a carrier material. The lignite was powdered and the pH was brought to pH 6.0 by adding appropriate amount of $CaCO_3$ and sterilized at 15 lbs pressure for 15 min. The log phase

culture (10^8 cells / ml) of the *Burkholderia* isolate (BB_4) was mixed with sterile carrier, adjusting to 40 per cent water holding capacity. The inoculant was kept for curing in shallow trays for 24 hrs in aseptic room and packed in high-density opaque polythene bags (300 gauge) at the rate of 200 g bag^{-1} and sealed. *Azospirillum* and phosphobacteria inoculant packets were obtained from the biofertilizer production laboratory of the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

Biofertilizer inoculation

The maize seeds were treated with lignite based culture of *Burkholderia*, *Azospirillum* and phosphobacteria as per treatment at 5 g 100 g^{-1} of seeds using rice gruel as a sticking agent. The seeds were mixed with the inoculum 30 min prior to sowing and dried under shade.

Calculations of germination per cent, shoot and root length

Total number of germinated shoots was recorded on 15^{th} day after planting and expressed in per cent.

The shoot length of the plant was measured from the ground level to the tip of the plant and expressed in cm. The root length was measured from the ground level to tip of the root and expressed in cm.

Estimation of available soil nitrogen and Phosphorus

Available soil nitrogen was estimated by Alkali Permanganate Method (Subbiah and Asija, 1956) and expressed as Kg ha^{-1}. Available soil phosphorus was estimated by Calorimetric method (Olsen *et al.,* 1954) and expressed as Kg ha^{-1}.

Statistical analysis

The data generated from the experiment were statistically analysed as per the procedure suggested by Panse and Sukhatme (1976). Critical differences were worked out at 5% probability level and presented.

Results and Discussion

Isolation of *Burkholderia* sp. from different crop plants

Sixteen isolates were obtained from the root, stem and leaves of rice, maize, sugarcane and black gram using N-free BAz semi solid medium, BAc and PCAT media with an isolation frequency of 50 per cent. The results are presented in Table 1.

These isolates produced pale yellow surface pellicles on N-free BAz semi solid medium. The isolated cultures, when grown on N-free BAz agar medium turned it from green to blue colour. While grown on BAz broth, they turned it to the yellow colour (Fig.1).

Screening of *Burkholderia* isolates using tryptose soya agar and its growth in different media

All the 16 isolates were grown on tryptose soya agar medium to differentiate *Burkholderia* sp. from *Pseudomonas* sp. Out of that, 3 isolates were found to produce fluorescent pigment and they considered belonging to *Pseudomonas* sp. (Fig.2).

Remaining 13 isolates were grown on different media *viz.,* BCSA (*Burkholderia cepacia* selective agar) (Fig.3), BAz (*Burkholderia* Azelaic acid), BAc (*Burkholderia* Azelaic acid Citrullin), BMGM (Modified BAz medium) and PCAT (*Pseudomonas cepacia* Azelaic acid and Tryptamine). Among the isolates, RB_1, MB_2,

SB_3 and BB_4 were found most effective as they grew well in N_2 fixing *Burkholderia* medium (BAz, BAc and BMGM) as shown in Table 2.

Physiological characteristics of *Burkholderia* isolates

Nitrogenase assay

All the *Burkholderia* isolates and reference strains showed the nitrogenase activity when grown in N-free BAz medium. Among the isolates, BB_4 recorded higher nitrogenase activity of 81.74 nmoles of ethylene produced mg^{-1} protein h^{-1} followed by SB_3, which recorded 69.59 nmoles. The reference strain *B. vietnamiensis* recorded the maximum nitrogenase activity of 85.15 nmoles (Table 3).

Ammonia excretion

All the *Burkholderia* isolates and reference strains showed the ammonia excretion. Among the isolates, BB_4 recorded higher ammonia excretion of 10.04 µg ml^{-1} followed by reference strain *B. vietnamiensis*, which recorded 8.65 µg ml^{-1}. The results are shown in Table 3.

Cell protein

The cell protein content of the *Burkholderia* isolates and reference strains are presented in Table 3. Among the isolates, BB_4 recorded higher cell protein of 1.54 mg g^{-1} followed by reference strain *B.vietnamiensis* (1.25 mg g^{-1}) and RB_1 (1.24 mg g^{-1}).

Polysaccharide production

All the *Burkholderia* isolates and reference strains showed the polysaccharide production. Among the isolates, BB_4 recorded higher polysaccharide production (28.82 mg g^{-1}) followed by reference strain *B.vietnamiensis* (28.20 mg g^{-1}). The results are presented in Table 3.

HCN production

The results are presented in Table 3. All the *Burkholderia* isolates and reference strains showed the HCN production. The isolate BB_4 recorded maximum HCN production (44.42 µg ml^{-1}) followed by RB_1 (36.4 µg ml^{-1}). The reference strain *B.vietnamiensis* recorded 35.15 µg ml^{-1} of HCN production.

Effect of *Burkholderia* inoculation on growth of maize under pot culture condition

Germination percentage of maize

The bioinoculants and fertilizer application does not significantly influence the germination percentage of maize seeds. Maximum germination percentage (83.3 per cent) was recorded in the maize treated with *Burkholderia* with 100 per cent N and P fertilizer application. The results are presented in Table 4.

Available nitrogen

Biofertilizers and fertilizer N applied treatments had significantly influenced the available soil N when compared to control. However, inoculation of biofertilizers alone recorded lower values than in combination with fertilizer N. Among the biofertilizer treatments, *Burkholderia* (BB_4) inoculation recorded maximum available N of than *Azospirillum* + Phosphobacteria inoculated treatments. Application of 100 per cent N with *Burkholderia* registered the maximum soil nitrogen (206 kg ha^{-1}) and it was on par with 100 per cent N + *Azospirillum* + Phosphobacteria and 75 per cent N + *Burkholderia* (Table 5)

Table.1 Isolation of *Burkholderia* sp. from different parts of crop plants

S.No.	Location	Crop	Sample	Medium used			*Burkholderia* isolate number
				BAz***	BAc***	PCAT**	
1.	Paddy Breeding Station*	Rice	Root	++	++	+	RB_1, RB_2
			Stem	++	++	+	RB_3
			Leaf	++	++	+	RB_4
2.	Western block*	Maize	Root	++	++	+	MB_2, MB_4
			Stem	++	++	+	MB_3
			Leaf	++	++	+	MB_1
3.	Eastern block*	Sugarcane	Root	++	++	+	SB_3, SB_2
			Stem	++	++	+	SB_1,
			Leaf	++	++	+	SB_4
4.	Millet Breeding station*	Black gram	Root	++	++	+	BB_4, BB_1
			Stem	++	++	+	BB_2, BB_3
			Leaf	-	-	-	-

(BAz – *Burkholderia* Azelaic acid medium), (BAc – *Burkholderia* Azelaic acid Citrullin medium), (PCAT – *Pseudomonas cepacia* Azelaic acid Tryptamine medium), (++ - Formation of yellowish surface pellicle), (+ - Formation of whitish surface pellicle), (* - Tamil Nadu Agricultural University Campus), (** - All *Burkholderia* sp. grown), (*** - Nitrogen fixing *Burkholderia* sp. grown)

Table.2 Growth of *Burkholderia* isolates in different selective media and comparison with *Pseudomonas* sp. using tryptose soya agar medium

Burkholderia isolates	BCSA	BAz	BAc	BMGM	PCAT	Tryptose soya agar	
						Burkholderia sp.	*Pseudomonas* sp.
Rice						.	
RB_1	+	++	++	++	+	+ve	-ve
RB_2	+	+	+	+	+	+ve	-ve
RB_3	+	+	+	+	+	+ve	-ve
RB_4	+	+	+	+	+	+ve	-ve
Maize							
MB_1	+	+	+	+	+	+ve	-ve
MB_2	+	++	++	++	+	+ve	-ve
MB_3	+	+	+	+	+	-ve	+ve
MB_4	+	+	+	+	+	+ve	-ve
Sugarcane							
SB_1	+	+	+	+	+	+ve	-ve
SB_2	+	+	+	+	+	+ve	-ve
SB_3	+	++	++	++	+	+ve	-ve
SB_4	+	+	+	+	+	+ve	-ve
Blackgram							
BB_1	+	+	+	+	+	+ve	-ve
BB_2	+	+	+	+	+	-ve	+ve
BB_3	+	+	+	+	+	-ve	+ve
BB_4	+	++	++	++	+	+ve	-ve

(+ - Moderate growth; ++ - Good growth), (BCSA – *Burkholderia cepacia* Selective Agar), (BAz – *Burkholderia* Azelaic acid medium), (BAc – *Burkholderia* Azelaic acid Citrullin medium), (BMGM – Modified medium of BAz), (PCAT – *Pseudomonas cepacia* Azelaic acid Tryptamine medium)

Table.3 Physiological characteristics of *Burkholderia* isolates

Burkholderia isolates	Nitrogenase activity (n moles ethylene produced h^{-1} mg cell protein) $^{-1}$	Ammonia excretion (µg ml^{-1})	Cell protein (mg g^{-1})	Polysaccharide production (mg g^{-1})	HCN production (µg ml^{-1})
RB_1	52.12	7.45	1.24	27.55	36.4
MB_2	40.97	7.72	1.17	27.28	24.75
SB_3	69.54	8.31	0.94	27.43	26.65
BB_4	81.74	10.04	1.54	28.82	44.42
*B. vietnamiensis**	85.15	8.65	1.25	28.20	35.15
B. tropicalis *	80.95	8.10	1.17	27.52	25.64
SEd	2.87	0.15	0.09	0.07	0.23
CD (5%)	6.25	0.34	0.20	0.16	0.51

(* - *Burkholderia vietnamiensis* and *Burkholderia tropicalis* - Reference strains)

Table.4 Effect of *Burkholderia* inoculation in comparison with *Azospirillum* and Phosphobacteria on the germination of maize

Treatments	Germination of maize seeds (%)
T_1 100 % N + P	66.6
T_2 *Burkholderia* alone	50.0
T_3 *Azospirillum* + phosphobacteria	58.3
T_4 *Burkholderia* + 100% N + P	83.3
T_5 A*zospirillum* + phosphobacteria + 100 % N + P	66.6
T_6 75 % N + P	58.3
T_7 *Burkholderia* + 75 % N+ P	66.6
T_8 *Azospirillum* + phosphobacteria + 75 % N + P	66.6
T_9 50 % N + P	50.0
T_{10} *Burkholderia* + 50 % N+ P	58.3
T_{11} *Azospirillum* + phosphobacteria + 50 % N + P	58.3
SEd	2.5
CD (5%)	5.3

Table.5 Effect of *Burkholderia* inoculation in comparison with *Azospirillum* + Phosphobacteria on the available soil nitrogen and phosphorus to maize under pot culture condition

Treatments	Available soil nitrogen (Kg ha^{-1})	Available soil phosphorus (Kg ha^{-1})
T_1 100 % N + P	171	36.3
T_2 *Burkholderia* alone	193	36.9
T_3 *Azospirillum* + phosphobacteria	190	36.9
T_4 *Burkholderia* + 100% N + P	206	37.0
T_5 A*zospirillum* + phosphobacteria + 100 % N + P	205	36.9
T_6 75 % N + P	175	36.5
T_7 *Burkholderia* + 75 % N+ P	204	36.9
T_8 *Azospirillum* + phosphobacteria + 75 % N + P	203	36.8
T_9 50 % N + P	176	36.7
T_{10} *Burkholderia* + 50 % N+ P	181	36.8
T_{11} *Azospirillum* + phosphobacteria + 50 % N + P	183	36.8
SEd	1.5	0.3
CD (5%)	3.1	NS

Table.6 Effect of Biofertilizer inoculation on the growth of maize under pot culture condition

Treatments	30th DAS		45th DAS		60th DAS	
	Root Length (cm)	Shoot Length (cm)	Root Length (cm)	Shoot Length (cm)	Root Length (cm)	Shoot Length (cm)
T_1 100 % N + P	9.8	18.6	14.5	26.4	21.6	32.6
T_2 *Burkholderia* alone	8.7	20.6	13.7	26.4	22.9	33.9
T_3 *Azospirillum* + phosphobacteria	9.4	21.4	12.9	27.6	22.0	33.7
T_4 *Burkholderia* + 100 % N + P	18.9	30.4	26.8	42.6	32.4	59.7
T_5 A*zospirillum* + phosphobacteria + 100 % N + P	14.6	28.6	22.3	40.8	31.6	58.4
T_6 75 % N + P	11.3	19.7	17.5	24.8	22.4	32.5
T_7 *Burkholderia* + 75 % N+ P	13.8	26.4	19.7	33.7	28.7	42.3
T_8 *Azospirillum* + phosphobacteria + 75 % N + P	15.3	25.9	18.9	31.5	26.9	40.9
T_9 50 % N + P	8.5	16.4	13.6	23.9	19.6	28.3
T_{10} *Burkholderia* + 50 % N+ P	10.4	17.8	15.9	24.6	21.7	29.8
T_{11} *Azospirillum* + phosphobacteria + 50 % N + P	10.2	16.4	16.3	23.9	22.1	30.1
SEd	0.50	0.92	0.73	1.28	1.02	1.62
CD (5%)	1.04	1.91	1.51	2.64	2.12	3.37

Fig.1 Growth of *Burkholderia* sp. on BAz broth

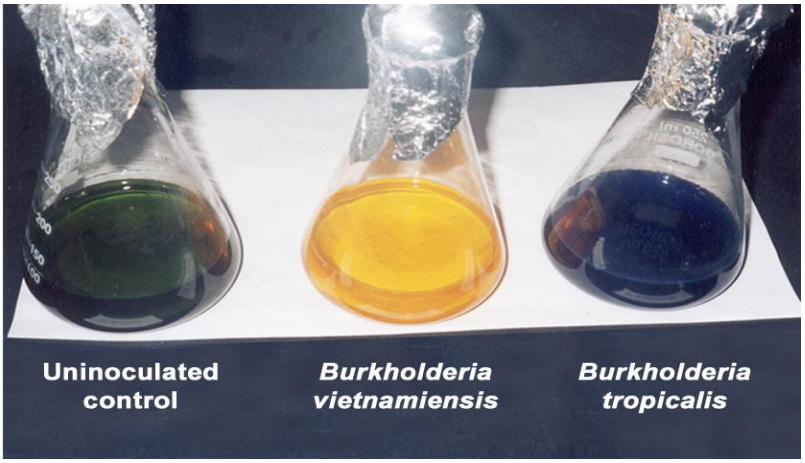

Fig.2 Growth of *Burkholderia* sp. isolates on tryptose soya agar

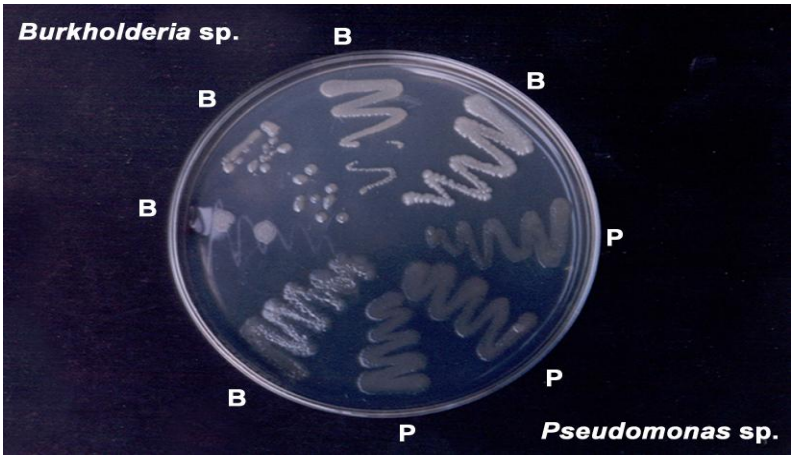

Fig.3 Growth of *Burkholderia* sp. on BCSA agar

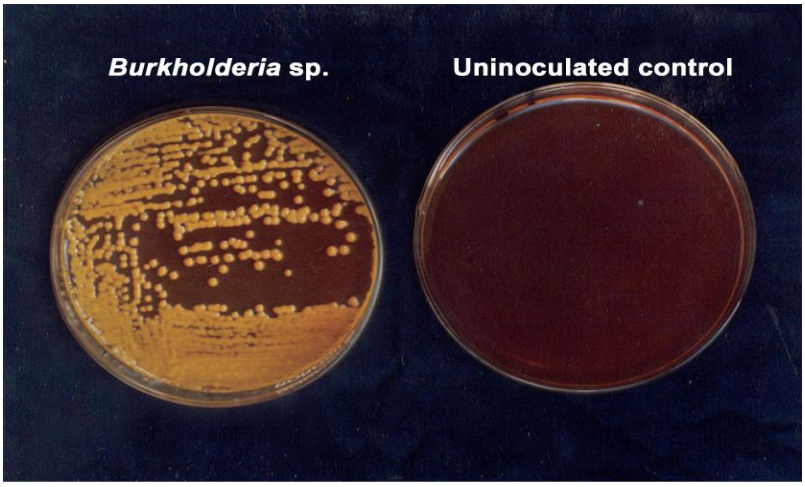

Fig.4a Pot culture view of two month old crop

Fig.4b Experimental view of *Burkholderia* inoculated maize under pot culture condition

Available phosphorus

In general, the inoculants or fertilizer application did not significantly influenced the soil phosphorus when compare to control. The treatments 100 per cent N + *Burkholderia* and *Azospirillum* + phosphobacteria recorded the highest soil phosphorus content (37.0 and 36.9 Kg ha^{-1} respectively). The results are presented in Table 5.

Shoot length

The results are presented in Table 6. Individual inoculation of biofertilizers *viz.*, *Burkholderia* and *Azospirillum* + Phosphobacteria had no significant influence on the shoot length of maize over the uninoculated control during the growth periods. However, in combination with 100 per cent N and P, the cultures recorded significantly higher shoot length at all the three stages of growth (30^{th}, 45^{th} and 60^{th} DAS). Among the biofertilizers, the inoculation of *Burkholderia* with 100 per cent N and P recorded higher shoot growth, however the treatment is on par with *Azospirillum* + phosphobacteria + 100 per cent N and P treatment (Fig.4a & Fig.4b).

Root length

All the treatments except individual inoculation of *Burkholderia* and *Azospirillum* + phosphobacteria registered significant influence over the root growth when compared to uninoculated control. Inoculation of *Burkholderia* with 100 per cent N and P recorded the maximum root length and it was on par with 75 per cent N + P *Burkholderia*. This was followed by 75 per cent N + P + *Azospirillum* and phosphobacteria treatment (Table 6).

Group of beneficial rhizobacteria especially *Rhizobium*, *Azospirillum*, *Azotobacter* etc. have been used extensively worldwide as bacterial inoculant for higher crop productivity (Sindhu *et al.*, 1997). In recent years there are encouraging reports about the use of dinitrogen fixing bacteria in combination with phosphate solubilizing and plant growth promoting rhizobacteria for meeting the nutritional requirements of crops and supplementing the expensive renewable inorganic fertilizers (Natarajan and Subramanian, 1995). Members of the genus *Burkholderia* are versatile organisms that occupy a surprisingly wide range of ecological niches. These bacteria are exploited for biocontrol, bioremediation and plant growth promotion purposes (Coenye and Vandamme, 2003). For a long time, N_2 fixing ability of the genus *Burkholderia* was recognized only in the species *B. vietnamiensis* (Gillis *et al.*, 1995). Recently, *B. kururiensis*, which has endophytic association with maize, sorghum and coffee plants also showed the nitrogen fixing ability (Estrada-De los Santos *et al.*, 2001).

In the present study, *Burkholderia* sp. was isolated from the different parts of crop plants using different media *viz.*, PCAT, BAc and BAz semi solid. Bacterial growth on N-free semi solid BAz media showed the formation of surface pellicles with different characteristics. The pellicles were yellowish and dense and diffuse. In general, the pH changes were not observed in N-free BAz media, but the growth of some isolates result in a slightly raised pH as indicated by change of colour of the medium from green to blue.Earlier N-fixing isolates were detected when tested in the N-free semi solid BMGM (modified BAz) medium and many N-fixing isolates formed white colonies, while other formed in whitish or yellowish colonies on BAc agar (Estrada-De Los Santos *et al.*, 2001). However, all the colonies were round and smooth with entire margins varying in diameter from 1 to 2 mm. White colonies

were flat or slightly convex and these isolates turned the medium from green to deep blue. While, isolates whitish or yellowish colonies were convex and turned the medium a light blue colour as observed in the present investigation.

In the present investigation sixteen isolates were selected from the four crop plants and screened for *Pseudomonas* by growing on tryptose soya agar. Tryptose soya agar medium was used for differentiating *Burkholderia* from *Pseudomonas* (Vermis *et al.,* 2003). Out of the 16 isolates, three isolates (MB_3, BB_2 and BB_3) produced fluorescent pigment and considered as *Pseudomonas* sp. The remaining 13 isolates were considered as *Burkholderia* sp. and subjected to grow on different media *viz.,* BCSA, BAz, BAc, BMGM and PCAT.

The presence of nif H gene in *B. vietnamiensis* revealed the potentiality of its nitrogen fixing ability (Gillis *et al.,* 1995 and Minerdi *et al.,* 2001). *B. tuberum* and *B. phymatum* are novel species (Vandamme *et al.,* 2002a) reported to be capable of nitrogen fixation and nodulation in tropical legumes plant (Moulin *et al.,* 2001). Van Borm *et al.,* (2002) recently showed that the micro flora inhabiting a pouch shaped organ at the junction of the midgut and the intestine of Tetraponera ants partially consists of *Burkholderia* species, which are most likely involved in the oxidative recycling of nitrogen rich waste. Growth and acetylene reduction was observed in *B. unamae* with different carbon sources in N-free semi solid BAz (Caballero-Mellado *et al.,* 2004). In the present experiment, acetylene reduction assay in the isolates revealed that all the isolates showed nitrogenase activity and nitrogen fixation. The reference strains and the isolate BB_4 recorded comparatively more ARA (Acetylene Reductase Activity) indicating their potentiality in nitrogen fixation. *B. vietnamiensis* was studied for better ability to fix atmospheric nitrogen (Gillis *et al.,* 1995).Geoffrey *et al.,* (2006) has also reported that *Burkholderia phymatum* produce ARA in *Mimosa affinis* legume crop.

Although the ability to fix nitrogen is reported as a common feature among the known species of the genus *Burkholderia*, it was noticed this bacteria grown rich in diazotrophic condition, as was demonstrated with ARA assay and confirmed with the presence of nifHDK genes (Estrada De los Santos *et al.,* 2001). The present experiment also confirmed that diazotrophism is a common property among this bacteria. Ammonia excretion was also reported in all the isolates of *Burkholderia* studied. Similar study has been reported recently by Arthee and Marimurthu, 2017 from *Burkholderia* sp for the excretion of ammonia.

Effect of inoculation of *Burkholderia* isolates on the growth of maize under pot culture condition. Being a potential nitrogen fixer and endophytic colonizer, many species of *Burkholderia* were used as plant growth promoting rhizobacteria in different crops (Di Cellio *et al.,* 1997 and Tran Van *et al.,* 2000). The population of such organism was about 10^6 cfu g^{-1} of rhizosphere soil and 10^4 cfu g^{-1} fresh tissue of roots contribute significantly higher quantity of nitrogen to the crop plant (Vandamme *et al.,* 1997). The selected isolate of *Burkholderia* was inoculated individually and in combination with 100, 75 and 50 per cent fertilizer nitrogen and phosphorus and their effect on the growth of maize was studied under pot culture condition. Much variation was noticed in the germination per cent between the treatments, showing that the treatment *Burkholderia* + 100 per cent N particularly influenced the germination per cent of maize. Biofertilizer application did not have any influence on the available soil phosphorus. However, they had a significant

influence over the available soil nitrogen. This is in agreement with the earlier report of Bershova (1954). Inoculation of *Burkholderia* with 100 per cent fertilizer nitrogen recorded maximum available soil nitrogen, indicating its supremacy over the other biofertilizers tested. *B. ambifaria* promoted plant growth significantly when inoculated with seeds (Cicillio, 2002). Many *Burkholderia* sps performed better when compared with non-inoculated control treatment fertilized with N and the overall N_2 fixing performance was far better compared with the performance reported by Lammel *et al.,* 2013. The growth parameters like root and shoot length were influenced significantly by the application of inorganic nitrogen or biofertilizers indicating the response of maize to nitrogen. In a greenhouse experiment performed with the maize rhizosphere isolate *B.cepacia*, increased maize growth, excerting positive effect on both the shoots and roots of young plants was observed (Silmar *et al.,* 2016; Bevivino *et al.,* 2000). The progressive increase in the *B.cepacia* number with respect to the number of total culturable micro flora during maize growth confirmed that the association established by *B.cepacia* with maize roots appear to be closer as the plant developed (Di Cellio *et al.,* 1997).

In maize, the new species *B.tropicalis* is thought to be an ancient symbiotic partner, lost during exportation of maize to Europe. But still *Burkholderia* sp. able to bring out significant improvement in plant productivity when inoculated into seeds. In addition, the ability of introduced *B.cepacia* to actively colonize the maize root system in the presence of large number of other indigenous rhizosphere microorganisms, causing only short-term perturbations in the microbial community of maize rhizosphere, as reported by Nacamulli *et al.,* 1997), is further evidence that this bacterial species is rhizosphere-competent on maize. *B. vietnamiensis* inoculation (Tran Van *et al.,* 2000) has already shown the potential of to use as plant growth promoting rhizobacteria.

The present pot culture experiment revealed that *Burkholderia* inoculation along with fertilizer nitrogen significantly increased the plant growth, available soil nutrients and rhizosphere colonization in maize. The experiment also revealed that the *Burkholderia* inoculation is superier than the *Azospirillum* and phosphobacterial dual inoculation. The *Burkholderia* inoculation could have potentially colonized inside the maize plant and effectively transferred the nitrogen as ammonia to maize plant, in addition to its plant growth promoting activities, resulting in the enhanced plant growth.

Sixteen isolates of *Burkholderia* sp. were obtained from root, stem and leaf samples of crop plants *viz.,* rice, maize, sugarcane and black gram using N-free BAz semi solid medium, BAc and PCAT media. Isolated cultures were compared with *Pseudomonas* sp. using tryptose soya agar medium and three isolates showed positive result for *Pseudomonas* were screened out. The remaining isolates were grown in different media *viz.,* BAz, BAc, BCSA, PCAT and BMGM. The isolates RB_1, MB_2, SB_3 and BB_4 recorded maximum growth and were selected for further characterization. The isolate BB_4 recorded higher nitrogenase activity among the four isolates and was statistically on par with reference strains which showed maximum. But interestingly the cell protein content, ammonia excretion, polysaccharide production and HCN production were observed more in BB_4 than reference strains. Maximum germination percentage (83.3 per cent) was recorded in the maize treated with *Burkholderia* with 100 per cent N and P fertilizer application. Available soil nitrogen was influenced by the treatments with

maximum recorded in 100 per cent N and P with *Burkholderia* combination. Results of the pot culture trial showed that the response of maize was better for *Burkholderia* than *Azospirillum* + phosphobacteria as the former increased the root and shoot growth than the other two biofertilizers.

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