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Isolation and characterization of plant growth promoting *Burkholderia* spp

■ M. SIVAJI*, R. VINOTH[1], P. TAMILKUMAR[2], S.SURESH KUMAR, A.CHANDRASEKAR AND M. SYAMALA[3]

Department of Plant Biotechnology, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA
[1]Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA
[2]Department of Seed Science and Technology, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA
[3]Department of Plant Pathology, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA

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*Corresponding author:
Email: sivajibt@gmail.com

ABSTRACT

In this study, a total of eight *Burkholderia* isolates obtained from rhizosphere soil of maize, cotton, rice, sugarcane, groundnut and soybean and tested for the N fixation, phosphate solubilisation and antagonistic activity against *Rhizoctonia solani*. All the eight isolates produced the band for the *nifH* gene in the polymerase chain reaction confirmed the presence of *nifH* gene. Among the 8 isolates, RMS1 produced clear halo zone around 7 mm on surface of HAP medium. CMS1, RMS1 and SMS1 are inhibited the growth of *Rhizoctonia solani* for 43, 40 and 35 per cent, respectively. An isolate RMS1 possessed all the three plant growth promoting traits *viz.*, higher phosphate solubilizing ability, antagonistic activity and presence of *nifH* gene selected for molecular characterization with amplifying and sequencing of 1.46 kb 16S rRNA gene. Based on the BLASTn homology it was found that 16S rRNA sequence of isolate RMS1 having 93 per cent identity with showed 97 per cent homology with *Burkholderia thailandensis.*

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INTRODUCTION

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. As plant roots grow through soil they release water soluble compounds such as amino acids, sugars and organic acids that supply food for the microorganisms. The food supply influence microbiological activity in the rhizosphere which is greater than in soil away from plant roots. In return, the microorganisms provide nutrients for the plants. This activity makes the rhizosphere the most dynamic environment in the soil. (Antoun and Kloepper, 2001).

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. Mostly they are associated with the plant rhizosphere, so they

are called as rhizobacteria. This group of bacteria has been termed Plant Growth Promoting Rhizobacteria (PGPR) and among them are strains from genera such as *Alcaligenes, Acinetobacter, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Paenibacillus, Pseudomonas, Rhizobium* and *Serratia.* The important traits of PGPRs include fixation of atmospheric nitrogen, solubilization of insoluble inorganic phosphates, production of plant hormones, siderophores, bacteriocins etc. These organisms also provide protection to plants against diseases by suppressing deleterious and pathogenic microorganisms. Bioinoculant preparations containing these organisms are very cost effective, pollution free and a potentially renewable source of plant nutrients, making an ideal partner and an excellent supplement to chemical fertilizers (Estrada-de los Santos *et al*., 2001).

Over the past two decades, research on *Burkholderia* species has been steadily expanding. Members of the genus *Burkholderia* are very abundant, occupying diverse ecological niches, including soil (Janssen, 2006). It can be free-living in the rhizosphere as well as epiphytic and endophytic (Compant *et al*., 2008). *Burkholderia* sp. also having many beneficial plant growth promoting activities like controlling the plant pathogens (biocontrol), production of IAA and siderospores for promoting the crop growth (Pandey *et al.,* 2008). These findings have stimulated a growing interest in using *Burkholderia* sp. isolates in agriculture.

MATERIAL AND METHODS

Isolation and morphological characterization of the bacterial isolates :

Soil samples were collected from the rhizospheres of maize, cotton, rice, sugarcane, groundnut and soybean plants from various field location of Tamil Nadu Agricultural University, Coimbatore. A total of 25 composite soil samples were used to isolate phosphate solubilizing *Burkhoderia* sp. by plating on *Burkhoderia* selective BAZ medium with ammonium sulphate as N source. Spread plate method was followed to isolate *Burkholderia* on BAZ agar medium at pH 7.0. The soil samples were serially diluted up to 10^{-4} dilution and one ml of 10^{-4} dilution was transferred to sterile Petriplates and BAZ agar medium was poured and mixed thoroughly. Colonies were observed on the surface of agar plates after 96 hours of incubation.

Cultural characteristics of the isolates were studied by streaking it on BAZ agar medium in Petriplates. Colony characters like size, margin, elevation and optical characteristics were observed after 48 hours. The morphological characterizations of isolates were made after staining the cultures by gram staining. After staining slides were observed under microscope to visualise the cell morphology (Sivaji *et al*., 2013).

Screening of atmospheric N fixing *Burkhoderia* sp.:

Genomic DNA isolation was carried out from the isolates by following the protocol of Sambrook and Russell (2001). The total genomic DNA isolated from *Burkholderia* isolates was amplified by Polymerase Chain reaction (PCR) with *nifH* gene primers, which was performed using the Eppendorf Master Cycler, Gradient (Eppendorf, Germany) (Suga *et al*., 1995). The details of primers used and temperature profile, time duration of each step for amplification of *nifH* gene are given in Table A and B.

Screening of phosphate solubilising bacteria *Burkhoderia* sp. :

Colonies were taken from the BAZ agar medium and streaked on the Hydroxy apatite (HAP) medium. The pH of the medium was 7.0. The plates were incubated at 30º C and maintained for 3 days in HAP medium to observe the halo zone produced by the isolates in the medium. The isolates with 'P' solubilising ability formed halo zones after 48 to 72 hours incubation. The diameter of the zone of solubilization was measured and expressed in millimetre. The results are expressed as P solubilization (PS) (Nguyen *et al.*, 1992).

PS = Solubilization diameter - Growth diameter

Table A : Details of primers used for amplification of *nifH* gene

Target gene	Primer	Primer sequence (5' – 3')	Reference
nifH	19 Fp	GCIWTYTAYGGIAARGGIGG	Suga *et al*. (1995).
	407 Rp	AAICCRCCRCAIACIACRTC	

Table B : Temperature profile for amplification of *nifH* gene by PCR

Sr. No.	Step	*nifH* gene	
		Temp.	Time
1.	Initial denature	95 °C	5 min
2.	Denature	94 °C	1 min
3.	Annealing	53 °C	40 sec
4.	Extension	72 °C	45 sec
5.	Step 2 to 4	30 cycles	
6.	Final Extension	72 °C	10 min
7.	Hold	4 °C	for ever

Table C : Details of primers used for amplification of 16S rRNA gene

Target gene	Primer	Primer sequence (5' – 3')	Reference
16S rRNA	27f	AGAGTTTGATCCTGGCTCAG	Marchesi *et al*. (1998)
	1492r	ACGGYTACCTTGTTACGACTT	

Table D : Temperature profile for amplification of 16S rRNA gene by PCR

Sr. No.	Step	Temperature	Time
1.	Initial denature	95 °C	5 min
2.	Denature	94 °C	1 min
3.	Annealing	55 °C	45 sec
4.	Extension	72 °C	1 min
5.	Step 2 to 4	30 cycles	
6.	Final extension	72 °C	10 min
7.	Hold	4 °C	For ever

Screening of antagonistic ability of *Burkholderia* isolates :

The antagonistic activity of the isolates was determined by dual culture method, in which two media PDA and BAZ are mixed together to formed dual medium. In this test the *Burkholderia* was streaked on one side and plant pathogenic fungi *Rhizoctonia solani* was kept on another side of plate to check antagonistic activity of *Burkholderia* against the same fungi (Dennis and Webster, 1971). Plates inoculated only with pathogen were maintained as control. The zone of inhibition was recorded as the distance between the fungal pathogen and the area of antagonist growth after 72 h.

$$PI = \frac{C - T}{C} \times 100$$

where,

PI = Per cent inhibition of mycelial growth

C = Radial growth of pathogen in control plates (mm)

T = Radial growth of pathogen in dual culture (mm)

Molecular characterization of the isolate:

PCR amplification was carried out using 16S rRNA universal primer (Marchesi *et al*., 1998). The details of primers used to amplify 16S ribosomal RNA (16S rRNA) gene are given in Table C. Steps followed in PCR, temperature profile and time duration of each step for amplification of 16S rRNA are given in Table D. The amplified 16S rRNA gene PCR product of RMS1 isolate were sent for sequencing to SciGenome Labs Pvt Ltd., Cochin and sequenced through single pass analysis from forward and reverse direction. Sequencing was done by using Automated sequencer.

RESULTS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented under the following heads:

Isolation, cultural and morphological characterization *Burkholderia* sp :

Among the 25 soil samples used to isolate

Table 1 : List of *Burkholderia* isolates obtained in this study

Sr. No.	Crops	Isolate name	Sr. No	Crops	Isolate name
1.	Maize	MMS1	5.	Sugarcane	SMS1
2.	Cotton	CMS1	6.	Sugarcane	SMS2
3.	Rice	RMS1	7.	Groundnut	GMS1
4.	Rice	RMS2	8.	Soybean	SBMS1

Burkholderia, only 8 samples exhibited colonies on BAZ agar medium after 4 days of incubation. Details of the isolates is given in Table 1. Colonies were, Small (1 to 2 mm), circular, pale yellowish brown and translucent at the margins (Fig. 1). All these isolates were found to be rod shaped and Gram negative which showed the possibility of these isolates being *Burkholderia* sp. Similar morphology of *Burkholderia* and Gram reaction were reported by Linu *et al.* (2009).

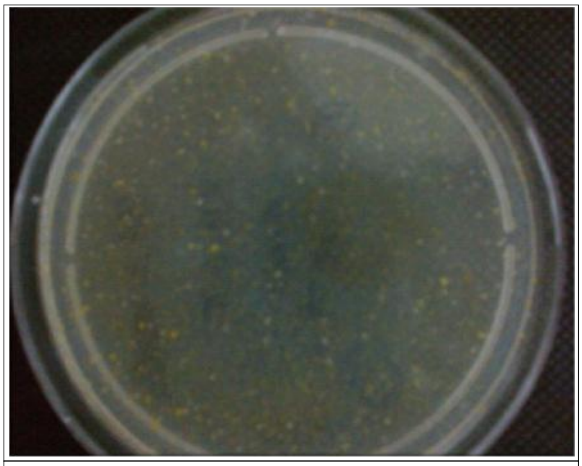
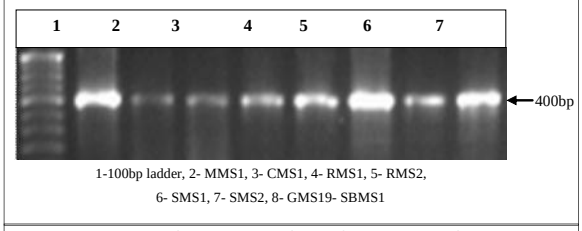


Fig. 1 : Isolation of *Burkholderia* isolates on BAZ agar medium

Amplification of *nifH* gene primers :

All the eight isolates produced the band for the *nif*H gene in the polymerase chain reaction confirmed the presence of *nifH* gene. It was confirmed by run the PCR product on 1 per cent agarose gel (Fig. 2). Estrada-de los Santos *et al*. (2001) reported that genus *Burkholderia* comprises 19 species, including *Burkholderia vietnamiensis*, which is the only known N_2-fixing species of this bacterial genus and in their investigation most of the N_2-fixing isolates were recovered from the environment of field-grown maize and coffee plants. These reports found to be similar with our findings. This report confirms the *nif* gene presence in *Burkholderia* sp. during present investigation.

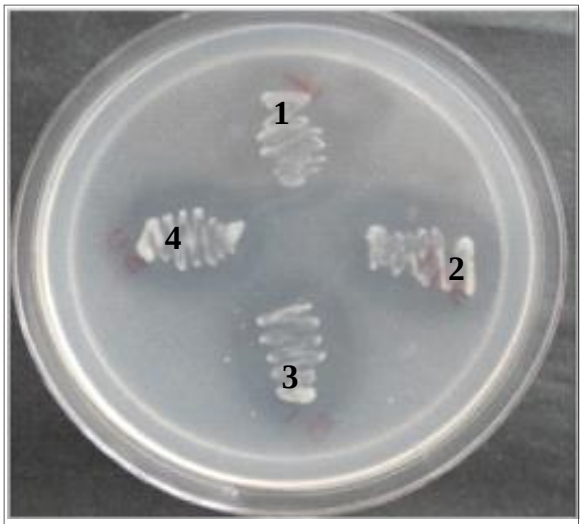


1-100bp ladder, 2- MMS1, 3- CMS1, 4- RMS1, 5- RMS2, 6- SMS1, 7- SMS2, 8- GMS19- SBMS1

Fig. 2 : Amplification of *nifH* gene from all *Burkholderia* isolates

'P' solubilizing *Burkholderia* sp. :

Isolates having phosphate solubilising ability produced halo zone due to solubilisation of insoluble mineral phosphate on HAP after 3 days incubation. Out of 8 *Burkholderia* isolates only three isolates *viz*., RMS1, GMS1 and SMS2 produced clear halo zone (7, 5 and 4 mm, respectively) (Fig. 3) on surface of HAP medium.



1. MMS1, 2. RMS1, 3. GMS1, 4. SMS2

Fig. 3 : Screening of P solubilising *Burkholderia* isolates on HAP medium

It is generally accepted that the mechanism of mineral phosphate solubilisation by PSB strains is associated with the release of low molecular weight

organic acids (Goldstein, 1995 and Halder *et al*., 1990) which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994).

Linu *et al.* (2009) reported high 'P' solubilizing ability in cowpea rhizosphere. This indicates efficacy of 'P' solubilisation by *Burkhloderia* varies from crop to crop. The presence of *Burkholderia* sp. in the rhizosphere of maize (Paulina *et al*., 2002) and sugarcane (Danice *et al*., 2010) was already reported.

Screening of antagonistic ability of *Burkholderia* isolates :

Antagonistic activity by Burkholderia :

Antagonistic activity of *Burkholderia* was tested by dual culture method. Among the 8 isolates CMS1, RMS1 and SMS1 are produced inhibition zone of 43, 40 and 35 per cent, respectively against *Rhizoctonia solani*. No inhibition was found for other isolates (Fig. 4). Sfalanga *et al*. (1999) found new antagonistic *Burkholderia* strain from the rhizosphere of healthy tomato plants that was resistant to several antibiotic substances and suppress the growth of important bacterial and fungal phytopathogens.

Ganyu *et al*. (2009) reported *Burkholderia* bacteria are frequently isolated from the rhizosphere of crops, and are involved in growth promotion of plants and suppression of plant diseases, *Burkholderia contaminans* strain MS14 has a broad range of antifungal activities to plant and human pathogens especially for *Geotrichum candidum*. These reports are in conformation with the findings of present investigation for antagonistic properties of *Burkholderia* against different plant pathogens.

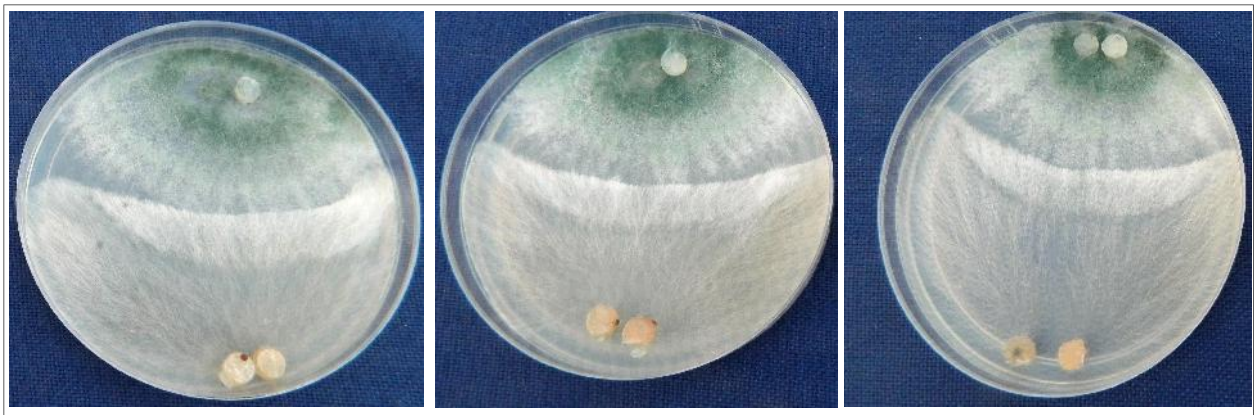
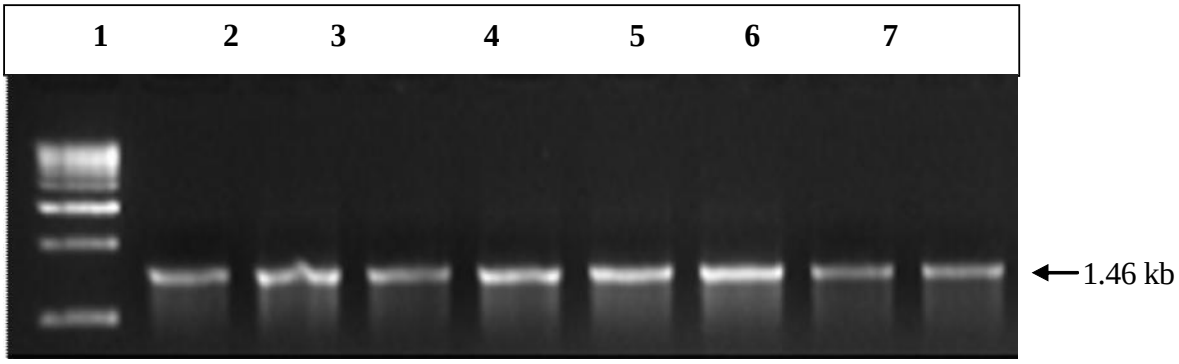


Fig. 4 : Inhibition zone of *Burkholderia* isolates against *R. solani*



1-1kb ladder, **2-** MMS1**, 3-** CMS1**, 4-** RMS1**, 5-** RMS**2, 6-** SMS1**, 7-** SMS2**, 8-** GMS1
9- SBMS1

Fig. 5 : Amplification of 16S rRNA from all *Trichoderma viridi (fungi)*

Molecular characterisation of isolates:

The genomic DNA of *Burkholderia* isolates were amplified by PCR using 16S rRNA primers, they yielded 1.46 kb band which was notified by running the PCR product on 1.2 per cent agarase gel (Fig. 5). Nucleotide sequence of the 16S rRNA region of the genome of the isolate RMS1 was sequenced by using automated sequencer. The sequences were blasted against NCBI database. Based on the BLASTn homology it was found that isolate RMS1 having 93 per cent identity with showed 97 per cent homology with *Burkholderia thailandensis* (Govindarajan *et al*., 2007 and Salles *et al*., 2002)

Conclusion :

Identification a new effective plant growth-promoting bacteria and characterization of its PGPR traits is an important process to develop the new multifunctional biofertilizer. This will lead the higher crop productivity and also help to reduce the usage of chemical fertilizers, pesticides and environment polluting substances. In this study, a total of 8 *Burkholderia* isolates obtained from rhizosphere soil of maize, cotton, rice, sugarcane, groundnut and soybean plants. Isolate RMS1 has multiple PGP traits like N fixation, phosphate solubilisation and biocontrol activity against plant pathogens. This can be exploited as a new bioinoculant that have multiple benefits for the agricultural crops.

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