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PLANT PATHOLOGY

Isolation and characterization of nitrogen fixing *Burkholderia* Sp.

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Abstract

The bacterium *Burkholderia* has been isolated from the rhizosphere soils of Mimosa, Lemon, Maize, Sugarcane, Sunflower, Rice, Bhendhi, Sunhemp, Cot on and Chilly from the different farms of Tamil Nadu Agricultural University, Coimbatore. Biochemical and physiological characterization was done for the obtained isolates to screen for further studies by using starch hydrolysis, lipid hydrolysis, casein hydrolysis and catalase test, *etc.* The isolates were checked for their ability to fix nitrogen by using different medium like BMGM and N free BMGM medium. The isolates were also checked for nitrogenase activity by using gas chromatography. B1 and R1 isolates showed high nitrogenase activity (7.99 and 7.15 n moles of ethylene /hr./mg of cell protein respectively). Total genomic DNA was isolated and PCR with 16S rRNA gene specific primers carried out to yields amplicons of 1300 bp size in *Burkholderia*. The PCR was carried out with specific primers of *nifH* gene primer for selected isolates. It showed that selected four isolates were found to have *nifH* gene with 400 bp. The present study revealed that some of the *Burkholderia* sp. helps in plant growth promoting activities by fixing nitrogen to enhance the yield of crop plants that can be exploited as bioinoculant in agriculture.

Highlights

- Rhizosphere samples were collected from Mimosa, Lemon, Maize, Sugarcane, Sunflower, Rice, Bhendhi, Sunhemp, Cot on, Chilly from the farms of TNAU, Coimbatore.
- The isolates were checked for their ability to fix nitrogen *i.e.* pH test in BMGM broth. BH1 and RI1 isolates showed remarkable pH increase in medium from 5.7 to 9.01 and 9.05 respectively.
- B1 and R1 isolates showed high nitrogenase activity (7.99 and 7.15 n moles of ethylene / hr. / mg of cell protein respectively).
- Total genomic DNA was isolated and PCR with 16S rRNA gene specific primers carried out to yields amplicons of 1300 bp size in *Burkholderia*.
- PCR with specific primers of *nifH* showed to have *nifH* gene with 400 bp.
- However efficiency of the *Burkholderia* sp. needs to be checked on field level to exploit as bioinoculant for agriculture.

Keywords: *Burkholderia* sp., rhizosphere, nitrogenase activity, bioinoculant

The genus *Burkholderia* comprises 19 species out of which B. *vietnamiensis* is the only N_2-fixing species validly described. For a long time, N_2-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *Burkholderia vietnamiensis* (Gillis *et al.* 1995). The analysis of N_2-fixing bacteria associated with maize and coffee plants grown under field conditions revealed the presence of B. *vietnamiensis,* as well as the richness of novel diazotrophic bacterial species belonging to the genus *Burkholderia* (Estrada-de los Santos *et al.* 2001). Recently, *Burkholderia caballeronis* sp. is also nitrogen fixing species isolated from tomato. (Martinez-Aguilar *et al.* 2013). *Burkholderia* phymatum is highly effective nitrogen-fixing symbiont of *Mimosa* spp. (Elliot *et al.* 2007). Other species of environmental origin were then added to this genus, including Burkholderiagraminis (Viallard *et al.* 1998), B. *caribensis* (Achouak *et al.* 1999), B. *kururiensis* (Zhang *et al.* 2000), B.ubonensis (Yabuuchi *et al.* 2000), B. *caledonica* and B. *fungorum* (Coenye *et al.* 2001), and B. sacchari (Bramer *et al.* 2001). Similarly, B. *vietnamiensis* has at racted interest because of its abilities to promote rice plant growth and grain yield. The main objective of the study is to isolate and characterized the novel nitrogen fixing *Burkholderia* species from the rhizosphere soilswith high efficiency to fix nitrogen.

Materials and Methods

Rhizosphere soils of plants like Mimosa, Lemon, Maize Sugarcane, Sunflower, Rice, Sunhemp and Bhendi from TNAU farms were collected and serial dilution was made. N free BMGM medium was used as selective medium for the isolation of *Burkholderia* sp. (Govindarajan *et al.* 2007). The BMGM plates were incubated at 37^{O}C for 2-3 days for assessing the nitrogen fixing ability of the isolates. From a total of 30 isolates, 10 strains which have the ability to capable of fixing nitrogen were selected for further study listed in Table 1. They were further purified and each isolate was characterized by Gram reaction, motility, morphology, temperature, pH test, salt tolerance tests and biochemical tests.

Table 1. List of *Burkholderia* isolates obtained from various farms of TNAU

Sr. No.	Crops	Isolate Names
1	Rice	R1
2	Maize	M1, M2
3	Cotton	CO1, CO2
4	Bhendi	B1,B2, B3
5	Sugarcane	S1,S2,S3
6	Lemon	BR1,BR2
7	Mimosa	BM1,BM2
8	Red gram	RG1, RG2
9	Chilli	C1,C2,C3
10	Cow pea	CP1,CP2

Characterization of the obtained isolates

The isolates were purified and checked for Gram reaction and biochemically characterized with the help of tests like Starch hydrolysis, Catalase test, Lipid hydrolysis, Citrate utilization test, Casein hydrolysis, Gelatin liquefaction, Urease activity and Hydrogen sulphide production test were performed for all the isolates and the results were recorded (Pandey *et al.* 2005).

Nitrogen fixing ability

The N_2 fixing ability of the isolates were identified by growing the cultures in a N_2 free BMGM broth (Govindarajan *et al.* 2007) at an initial pH of 5.7 by using malate as a carbon source. The sterilized BMGM broth was inoculated with the test isolates and incubated at 37^{O}C. The alterations in pH were recorded at an interval of every 2 days and results were tabulated. The isolates were further inoculated in nitrogen free BMGM plates and incubated for 3-5 days and colour change of the medium from golden yellow to blue was observed for confirmation of N_2 fixing activity.

Estimation of Nitrogenase activity

The nitrogenase activity was estimated by the acetylene reduction assay based on the reduction of acetylene to ethylene by gas chromatography (Hardy *et al.* 1968). By using sterile syringe, 10% of the air

inside was evacuated and replaced with 6.0 ml of pure acetylene in the glass vials containing 15 ml of *Burkholderia* culture. Glass vials were incubated for 48 hrs. and analyzed for ethylene (C_2H_4). Ethylene was analysed by standard flame ionization detector (FID) gas chromatography standardized with pure ethylene. Peak height of ethylene was measured and recorded. Nitrogenase activity was calculated by using the following formula:

$$\text{Nitrogenase Activity} = \frac{\text{Area count} \times \text{Vol. of flask} \times 0.00446}{\text{Vol. of gas injected} \times \text{hr of incubation} \times \text{mg of cell protein}}$$

Genomic DNA isolation and Molecular characterization of isolates

Genomic DNA isolation was carried out from the isolates by using the phenol/chloroform/ Isoamyl alcohol method (Lazo *et al.* 1987). The total genomic DNA isolated from *Burkholderia* isolates was amplified by PCR, which was performed using the Eppendorf Master Cycler, Gradient (Eppendorf, Germany). PCR amplification was carried out using 16S rRNA universal primer (Marchesi *et al.* 1998). Details of primers used for amplification of 16S rRNA gene given in Table 2.

Table. 2. Details of primers used for amplification of 16S rRNA gene

Target gene	Primer	Primer sequence	Reference
16S rRNA	63f	5'-CAGGCCTAACA CATGCAAGTC-3'	(Marchesi *et al.* 1998)
	1387r	5 ' - G G G C G G W G T GTACAAGGC-3'	

Amplification of nifH gene primer

The total genomic DNA isolated from *Burkholderia* isolates amplified by using *nifH* primer. The details of primer used to amplify *nifH* gene was given in Table 3.

Table 3. Details of primer used for amplification of *nif H* gene

Target gene	Primer	Primer sequence	Reference
*nif*H2	*nifH*-2f	5'-CGCCGGCGCA GTCTTTGCCG-3'	(Frankae *et al.* 1998).
	nifH-2r	5'-CACTCGTTGG AGCTGGTCGG-3'	

Results and Discussion

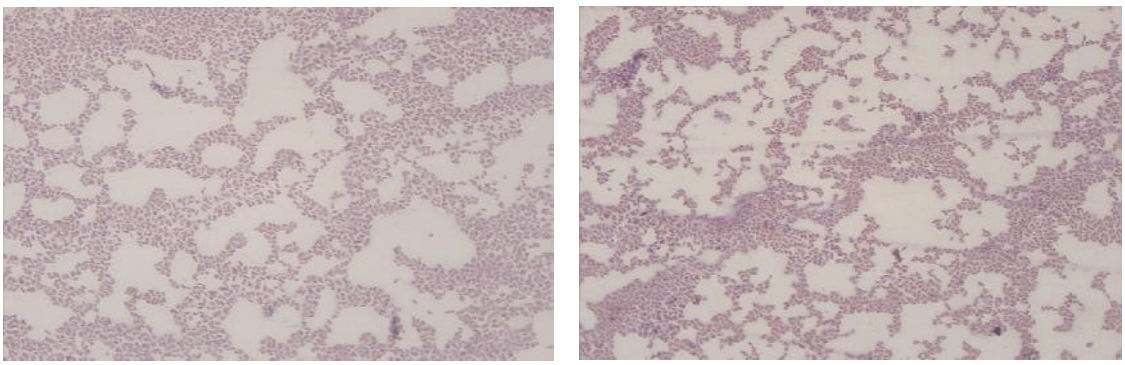
Characterization of the *Burkholderia* isolates

Burkholderia isolates were characterized morphologically and results were given in Table 4.

Table 4. Morphological characteristics of *Burkholderia* isolates on BMGM medium

Character	Result
Colony shape	Circular
Size of colony	2-5 mm
Colour/Pigmentation	White and glistering
Elevation	Convex
Margin	Entire
Colony appearance	Opaque
Motility	Non motile
Bacterium shape	Small Rod shaped
Oxygen demand	Aerobic
Spore formation	Non spore forming
Gram Reaction	Gram negative

Burkholderia isolates were characterized morphologically and results weregiven in Table 4. The cultures were characterized by Gram staining (Figure 1.) salt, pH and temperature tolerance tests and biochemical tests like Starch hydrolysis, Catalase test, Lipid hydrolysis, Citrate utilization Casein hydrolysis, Gelatin liquefaction, Urease test, Hydrogen sulphide production test, etc. were carried out. The obtained results were showed in Table 5. Weber *et al.* (1999) reported similar findings in diazotrophic bacteria from banana and pineapple plants. Cordova-Kreylos *et al.* (2013) also came up with similar characteristics in *Burkholderia rinojensis* sp. isolated from a Japanese soil sample



B1 **R1**

Fig. 1. Microscopic observation (100X) of *Burkholderia* isolates

Table 5. Biochemical characteristics of *Burkholderia* isolates

Sr. No.	Test	Result
1	Starch hydrolysis	Positive
2	Catalase activity	Positive
3	Lipid hydrolysis	Negative
4	Citrate utilization	Positive
5	Casein hydrolysis	Positive
6	Gelatin liquefaction	Positive
7	Urease activity	Negative
8	H2S production	Negative

and demonstrated to have strong insecticidal and miticidal activities in laboratory bioassays. The characters of the isolates were found to be similar as reported in *Burkholderia* from root nodules of *Mimosapudica* by Pandey *et al.* (2005) and in different free-living rhizospheric bacteria by Ahmad *et al.* (2008).

Salt tolerance test

The isolates like M_1, M_2, S_1 and S_2 can tolerate low salt concentration (0.01%-0.05%). All the 10 isolates grow luxuriantly at a salt concentration of 0.2%-0.8% and optimum being 0.3%. The isolates like R_1,B_1, CO_1 and CO_2 can tolerate high salt content viz., 1%, 2% and 3%.

Temperature and pH tolerance test

All the 10 isolates can tolerate a wide range of temperature like 20°C-45°C. However temperature tolerance highly depends on the salt concentration. Also they grow luxuriantly in a wide pH range of 5-9.

Nitrogen fixation by *Burkholderia*

The N_2 fixing ability of the isolates was identified by growing the cultures in a N_2 free BMGM medium (Figure 2) and BMGM broth at an initial pH of 5.7 by using malate as a carbon source (Figure 3). The sterilized BMGM broth was inoculated with the test isolates and incubated at 37^{O}C. The alterations in pH were recorded at an interval of every 3 days and results were tabulated (Table 6). The isolates were further inoculated in nitrogen free BMGM plates and incubated for 3-5 days and colour change of the medium was observed for confirmation of N_2 fixing activity. In this test four efficient nitrogen fixing Burkholderia B_1, R_1, M_2, and S_2 were able to change pH of BMGM broth from 5.7 to 9.01, 9.05, 8.9 and 9.01 respectively. Nitrogen fixation by *Burkholderia* isolated from root and rhizosphere soil of different plant were earlier in endophytic sugarcane diazotroph by Govindarajan *et al.* (2007) and 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.

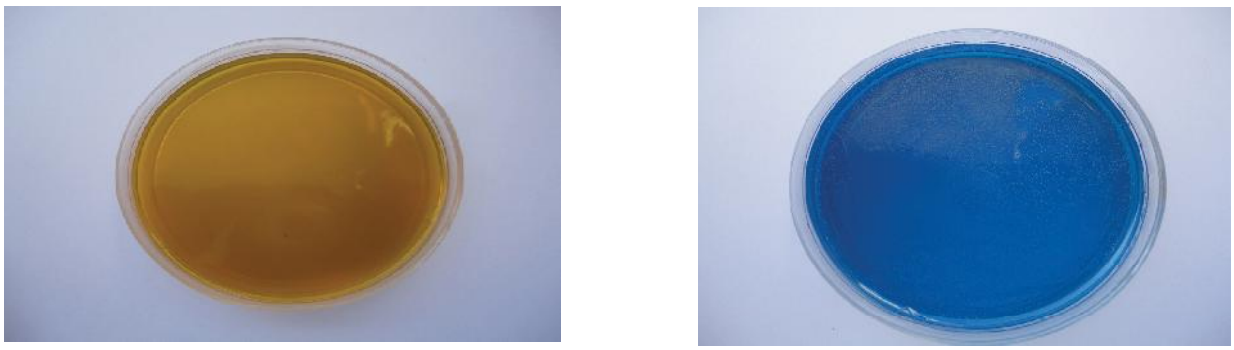


Fig. 2. Change of colour due to pH increase by B1 isolate inoculated in N free BMGM medium

Fig. 3. Nitrogen fixation by *Burkholderia* on N free BMGM broth

Table 6. pH increase in BMGM broth by selected *Burkholderia* isolates.

S. No.	Isolates	2nd day	4th day	7th day	9th day	13th day	17th day	20th day
1.	Control	5.7	5.7	5.7	5.7	5.7	5.7	5.7
2.	B_1	7.8	8.0	8.4	8.5	8.6	8.9	9.01
3.	R_1	7.6	8.0	8.5	8.5	8.6	8.90	9.05
4.	M_1	5.9	6.2	6.5	6.5	6.6	7.0	7.22
5.	M_2	6.9	7.0	7.6	7.8	8.0	8.5	8.90
6.	S_1	7.0	7.3	7.8	8.1	8.3	8.5	8.63
7.	S_2	7.5	8.0	8.3	8.5	8.6	8.7	9.01
8.	CH_2	7.2	7.5	8.0	8.3	8.55	8.6	8.85
9.	CO_1	7.0	7.5	7.9	8.0	8.3	8.5	8.79
10.	CO_2	5.7	6	6.5	6.5	6.6	7.2	7.93

Estimation of nitrogenase activity

Nitrogenase activity of the isolate was studied. The activity varied considerably among the *Burkholderia* isolates (6.00 to 7.99 n moles of ethylene produced mg^{-1}cell protein hr^{-1}). The maximum nitrogenase activity has been observed in B_1 (7.99 n moles), while minimum nitrogenase activity has been recorded in isolate M_2 (6.00 n moles) (Table 7 and Figure 4). Similar finding were reported by many authors in different species of *Burkholderia*; *Burkholderia silvatlantica* sp. (Perin *et al.* 2006), *Burkholderia unamae* sp. (Mellado *et al.* 2004), *Burkholderia tropica* sp. (Reis *et al.* 2004).

Table 7. Nitrogenase activity of *Burkholderia* cultures.

Cultures	Nitrogenase activity (n moles of ethylene produced / hr./ mg of cell protein)
B1	7.99
R1	7.15
M2	6.00
S2	6.64

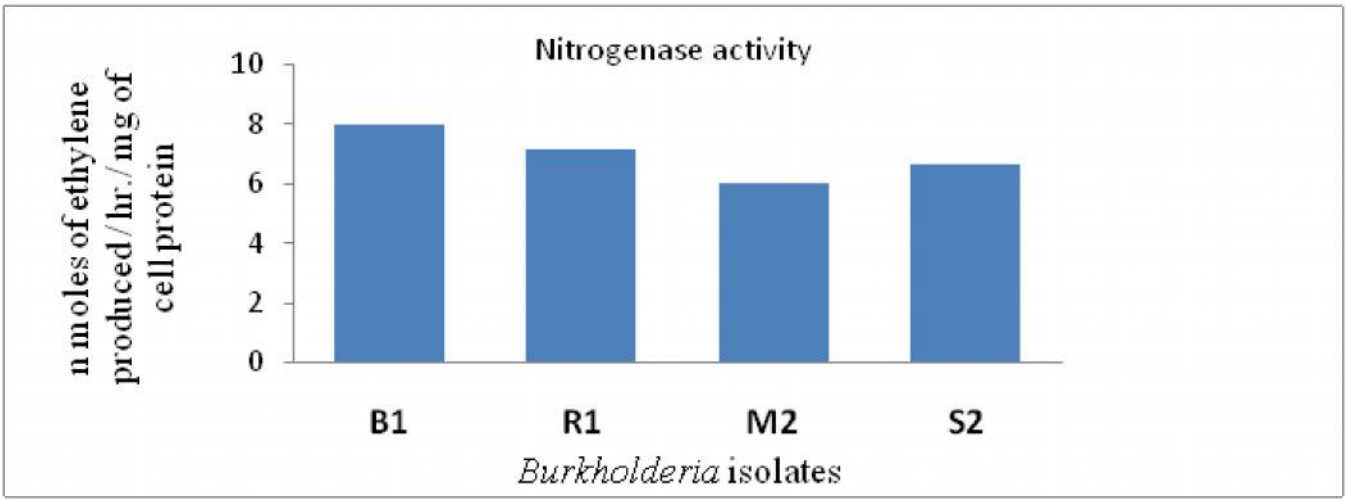


Fig. 4. Nitrogenase activity of *Burkholderia* isolates

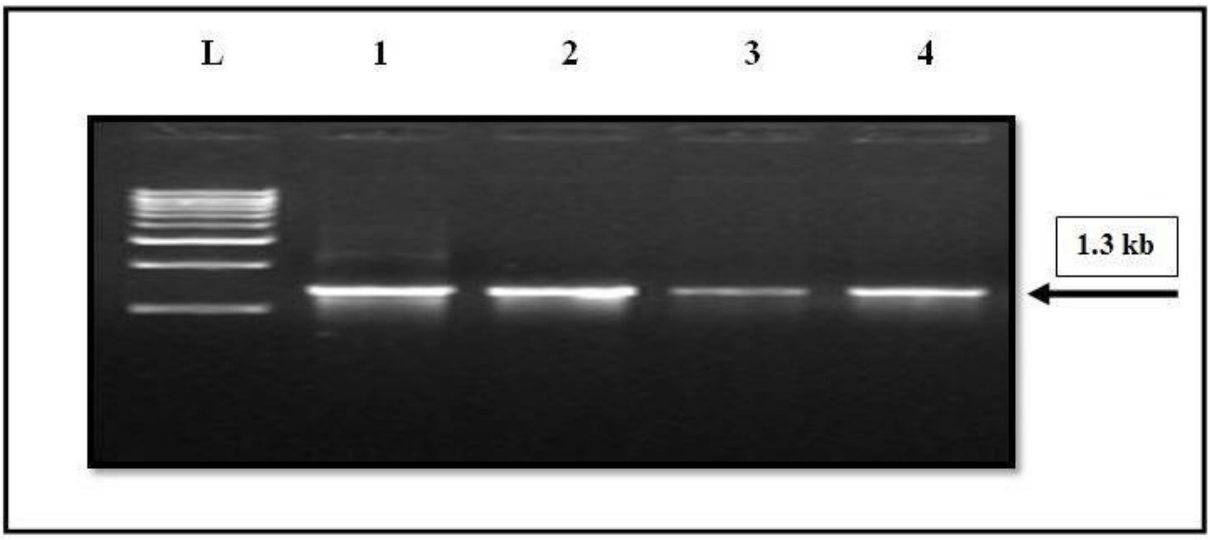


Fig. 5. 16S rRNA gene amplification of *Burkholderi*a isolates

L= 1 kb Ladder, 1=B1, 2=R1, 3=M2, 4=S2.

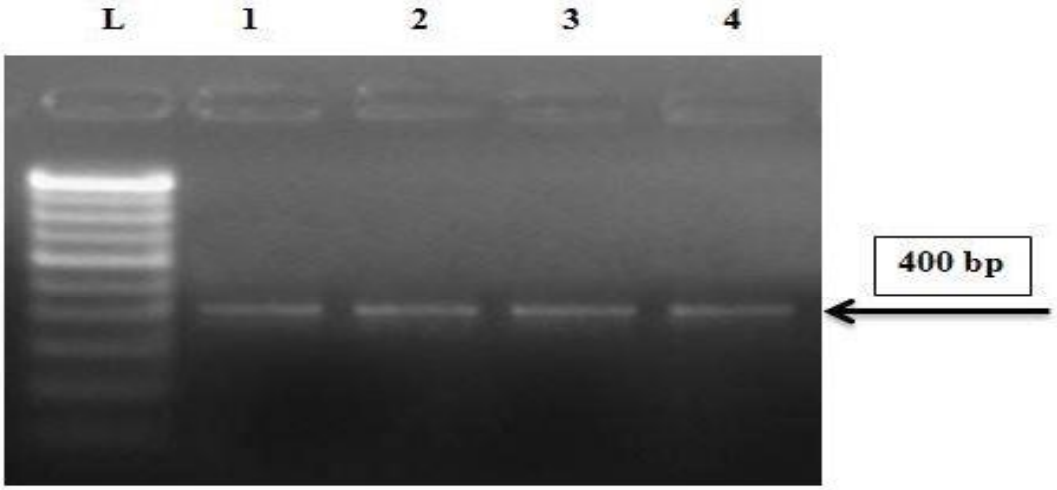


Fig. 6. *NifH* gene amplification in *Burkholderia* isolates
L=100bp ladder, 1=B1, 2=R1, 3=M2, 4=S2

16S rRNA gene amplification of *Burkholderia* isolates

Amplification of the 16S rRNA gene of the four isolates was done by using primers mentioned earlier. When the 16S rRNA region of the each of the five isolates was amplified by PCR, a major amplification band of 1.3 kb was observed and it was notified by PCR product on 0.8% agarose gel (Figure 5). Similar findings were reported by Gee *et al.* (2003) in *B. pseudomallei* and *B. mallei.* Taghavi *et al.* (1996) obtained similar result in *Burkholderia solanacearum* and *Pseudomonas syzygii*.

NifH gene amplification of *Burkholderia* isolates

Amplification of the *nifH* gene of the 4 isolates was done by using the primers as mentioned earlier. When the *nifH* region of each of the five isolates was amplified by PCR, a major amplification band of 400 bp was observed, and it was notified by run the PCR product on 1 per cent agarose gel (Figure 6). The nifH DNA region of *Burkholderia species* located, sequenced and identified the regulatory DNA elements involved in *nifH* expression by many authors. *NifD* and *NifK* genes *Burkholderia* endosymbiont of the arbuscular mycorrhizal fungus *Gigaspora margarita* reported by Minerdi *et al.* (2001). Twenty *Mimosa* nodulating bacterial strains isolated from Brazil and Venezuela reported to have *nodA* and *nifH* genes by Chen *et al.* (2005).

Conclusion

The present study was a preliminary at empt to identify and characterize *Burkholderia* associated with the rhizosphere of different crops. The isolates were checked for their ability to fix the atmospheric nitrogen. Some isolates like B_1, R_1, M_2, S_2 found to have great potential, further field testing is required to promote these isolates as bioinoculant for nitrogen source.

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