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Research Paper

Multi-phasic Nitrogen Fixing Plant Growth Promoting Rhizobacteria as Biofertilizer for Rice Cultivation

W. R. K. D. W. K. V. Wickramasinghe*[1], D. Girija[2], K. Surendra Gopal[3] and Shilpa Kesevan[4]

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A B S T R A C T

As a suitable alternative to chemical fertilizers, the use of plant growth-promoting rhizobacteria has been increased in recent years due to their potential as biofertilizers. Therefore, this study was aimed to isolation and characterization of plant growth promoting rhizobacteria from rice rhizosphere. In the present work, nitrogen-fixing bacterial strains were isolated from rice rhizosphere and evaluated for their plant growth promoting activities such as production of indole acetic acid (IAA), ammonia, hydrogen cyanide (HCN) and siderophore. These isolates were further screened for their antagonistic ability against two major rice pathogens *Rhizoctonia solani* and *Xanthomonas oryzae*. Eight nitrogen fixers showed positive reaction for three PGP activities such as production of IAA, NH_3 and siderophore. Nitrogen fixing ability of twenty nitrogen fixers were studied and showed highest quantity of nitrogen fixation (9.33 mg of N g^{-1}) by isolate $AkNF_3$ (*Bacillus* sp.) and $PkNF_4$ (*Pseudomonas sp.*). Isolate $KgNF_1$ produced nitrogen fixation amount 8.86 mg of N g^{-1} with highest IAA production and showed antagonism against *X. oryzae* and identified as *Pseudomonas putida*. These findings suggest that some nitrogen fixing strains from rice rhizosphere possess multiple plant growth promoting traits which could be used as PGPR based biofertilizers for rice cultivation.

***Key words*:** Rice cultivation, Nitrogen fixing, Rhizobacteria, Biofertilizer, *Bacillus* sp.

High yielding rice varieties of the "Green Revolution" have provided increased yield, and these yield gains were almost doubled when synthetic fertilizers were used [1]. Nitrogen is one of the primary macronutrients needed for rice growth. Only one third of applied nitrogen fertilizer is used by the rice plants [2]. Nitrogen fertilizers contribute to nitrate contamination of ground water and leads to environmental pollution. One of the alternative technologies for this problem is to use nitrogen fixing bacterial inoculant to increase rice production without adverse effect on environment. Plant growth promoting rhizobacteria (PGPR) are native soil bacteria that colonize in rhizosphere or plant roots resulting in stimulation of plant growth directly or indirectly. The use of PGPR is steadily increasing in agriculture, as it offers an attractive way for reducing the use of chemical fertilizers. Biological nitrogen fixation by bacteria present in the rhizosphere is an important factor contributing to growth of plants [3]. Biological nitrogen fixation has been reported to be exclusively carried out by a few Procaryotes, which could be used as an alternative to chemical fertilizers.

The ability to fix nitrogen is widely distributed among phylogenetically diverse bacteria. Several groups of soil and root- associated nitrogen-fixing microorganisms like Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Achromobacter, Bacillus, Burkholderia, Brevundimonas, Gluconacetobacter and Stenotrophomonas have been found to colonize different crops including rice and stimulate plant growth either directly or indirectly [4]. Biological nitrogen fixation has been estimated to contribute about 30 kg N ha^{-1} per year to rice eco systems [5]. Isolated *Azotobacter* species from different rice fields and reported that *A. vinelandii* fixed higher amount of nitrogen and had multiple plant growth promoting activities viz. production of IAA, siderophore, HCN, ammonia, salicylic acid and phosphate solubilization [6].

Eighty-eight per cent of soils in Kerala are acidic in nature, and acidity impairs the absorption of nutrients and inhibits microbial activity in soil. Currently biofertilizers with multiple plant growth activities are not available for rice crop of Kerala. Wayanad is generally considered as rich in biodiversity because of the unique sub- tropical climatic conditions. Hence there is a need to develop biofertilizers for rice suited to acidic soils of Wayanad district in Kerala. Biological nitrogen fixation of different strains is highly location specific and therefore, resident strains would be better suited [7]. The present study focused on isolation and screening of nitrogen fixing bacteria with multiple PGP traits from rice rhizosphere of Wayanad, which could be developed into biofertilizers.

MATERIALS AND METHODS

Ten rice growing tracts of Wayanad District, Kerala, India were selected for soil sample collection. These locations

*W. R. K. D. W. K. V. Wickramasinghe
wickramasinghekalani@yahoo.com

[1-4]Department of Microbiology, College of Agriculture, Kerala Agricultural University, Thrissur - 680 656, Kerala

included Thanivayal, Edakkal, Kolagappara, Nellara, Kuppamudi, Ambalavayal, Marathat, Ambukuthi, Malavayal and Pottankoli. Rhizosphere soil samples were collected with intact root systems from two-months-old rice plants and brought to the laboratory in polythene bags. Isolation of rhizosphere bacteria were carried out by using serial dilution followed by spread plating. Jensen's agar and Ashby's agar medium were used for enumerating nitrogen fixing bacteria. Isolates which were obtained from nitrogen free media were further screened for plant growth promoting (PGP) activities including production of IAA, NH_3, HCN and siderophore by using standard procedures.

IAA production of the isolates was detected by the method of Salkowski [8]. The selected isolates were inoculated in sterile Luria-Bertani supplemented with tryptophan at the rate of 1 mg ml^{-1} and incubated in the dark for seven days. After incubation, supernatant was mixed with 4 ml of Salkowski reagent. Development of pink colour in the solution indicated IAA production qualitatively. The quantity of IAA produced was determined by spectrophotometry and expressed in µg ml^{-1} by referring to a standard graph of IAA, prepared from a series of IAA solutions of known concentrations.

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in to sterile 4% peptone broth and after 48 hours, 0.5 ml of Nessler reagent was added to each tube. The development of yellowish-brown colour was recorded as positive for ammonia production.

Production of HCN was determined using standard protocol described by [9]. Bacterial isolates were streaked on nutrient agar medium amended with glycine (4.4 g l^{-1}) and Whatman. No. 1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plates and sealed with parafilm and incubated for 48 h. A change in colour of filter paper from yellow to reddish-brown was considered as indication of HCN production.

Production of siderophore was tested by Chrome Azurol Sulfonate (CAS) assay [10]. Freshly grown cultures were spot-inoculated on CAS blue agar plates and incubated at room temperature for three to four days. Formation of yellow-orange halo around the colony indicated production of siderophore.

Selected nitrogen fixing isolates were also screened for antagonistic activity against two rice pathogens *Rhizoctonia solani* and *Xanthomonas oryza*e following dual culture method [11] by growing them on potato dextrose agar (PDA) and nutrient agar plates respectively.

Isolates which showed multi-phasic PGP activities were further subjected to quantification of nitrogen fixation by micro-Kjeldahl method of [12] and [13]. Sterilized Jensen broth was inoculated with selected nitrogen fixing isolates and incubated for fourteen days at room temperature. After incubation, 10 ml of Jensen broth of respective inoculated organisms were withdrawn and digested with 10 ml concentrated sulfuric acid. In distillation, 40% NaOH and 4% Boric acid with mixed indicator was used for trapped NH_3. After distillation it was titrated with 0.01N HCl. Total nitrogen was calculated using following formula and expressed as mg N fixed per gram of sucrose utilized. Data were analysed by using WASP 2.0 software.

$$\text{mg of N fixed g}^{-1}\text{ of C Source} = \frac{TV - BV \times N \times 0.014}{Y} \times 1000$$

Where, TV = Titre value
BV = Blank value
N = Normality of HCL
Y = Weight of carbon source

Selected nitrogen fixers were grown on nutrient agar medium and characterized their colony morphology such as size, shape, colour and elevation. Further these isolates were subjected to the Gram staining and observed their Gram reaction and cell morphology.

Most efficient nitrogen fixing isolates which had multiphasic PGP activities were further identified by 16S rDNA gene sequencing. Amplification of 16S rRNA gene was done by using colony PCR [14] by using universal primers 8F and 1522R and Mastermix (Takara, Japan) in Eppendorf Mastercycler. The PCR product were purified and sequenced at Agri Genome labs Pvt. Ltd, Kochi with primer 8F and 1522R, using Sanger's method. The BLAST (Basic Local Alignment Search Tool) programme of NCBI (National Centre for Biotechnology Information) was used for sequence analysis and nucleotide homology alignment of each isolates. The sequences of all the isolates were deposited in the GenBank of the NCBI and accession numbers were obtained. Phylogenetic analysis was carried out for studying the relationship of the isolates collected during the present study with already existing accessions of relevant species. The 16S rDNA nucleotide sequence of six isolates were aligned with the best ten closely related sequences of 16S rDNA gene retrieved from NCBI database. The sequences were aligned using MAFFT software and a phylogenetic tree was constructed employing the MEGA 7 software.

RESULTS AND DISCUSSION

A total of 60 nitrogen fixing bacteria were isolated from the rice field soils from ten different locations of Wayanad district of Kerala, India. Thirty-two nitrogen fixers were selected from preliminary screening based on the growth of nitrogen free medium and they were screened for plant growth promoting (PGP) and antagonistic activities against rice pathogens *R. solani* and *X. oryzae*. Based on the PGP and antagonistic activities, twenty isolates were selected that had multiple PGP characters for quantification of nitrogen fixation. Plant growth promoting and antagonistic activities of selected isolates are depicted in (Table 1).

Fourteen isolates out of twenty were positive for production of IAA. Indole acetic acid is the phytohormone known to enhance growth in terms of root and stem length of the plant. These fourteen isolates were further subjected to the quantification of IAA production. Estimation of IAA production ranged from 2.50 to 34.83 µg ml^{-1} and were significantly different at $P \leq 0.01$. Highest value was recorded by $KgNF_1$ (34.83 µg ml^{-1}) followed by $AvNF_4$ (25.00 µg ml^{-1}) and $AkNF_3$ (23.16 µg ml^{-1}). Our results are in agreement with previous studies by [15], ten isolates tested for IAA production and all the isolates produced significant amount of IAA which ranged from 12.68 to 46.88 µg ml^{-1}. Nitrogen fixing PGPR from rice rhizosphere and results revealed that, out of 166 isolates 115 isolates were positive for IAA production and it ranged between 0.04 to 231 µg ml^{-1} [16]. IAA production by PGPR can vary among different species and isolates, and it is also influenced by culture condition, growth stage and substrate availability.

All selected isolates were positive for production of ammonia. Ammonia production and nitrogen fixation of rhizosphere bacteria from maize rhizosphere and reported that

all nitrogen fixing isolates had ability to produce ammonia [17]. Thirteen isolates out of twenty were positive for production of siderophore. Five isolates were medium siderophore producer. Siderophore production of PGPR helps in plant growth promotion by two mechanisms. One method is direct supply of iron to plants [18] and other is indirectly depriving iron for plant pathogens [19]. All the isolates were negative for production of HCN.

Out of four PGP activities screened IAA, NH_3, HCN and siderophore production, eight isolates ($KgNF_1$, $AvNF_2$, $AvNF_3$, $AvNF_4$, $AkNF_3$, $PkNF_2$, $PkNF_3$ and $PkNF_4$) recorded positive reaction for three PGP activities (production of IAA, NH3 and siderophore) except for production of HCN. Isolated PGPR from wheat rhizosphere and evaluated their PGP activities *in-vitro* [15]. They reported some PGPR as multi-trait PGPR those are having more than one trait of plant growth promotion activities. In this study, multi-trait N fixers were found which could be used as bioinoculants.

Two isolates, namely $KpNF_5$ and $MtNF_4$ exhibited antagonistic activities against *Rhizoctonia solani* and isolate $KgNF_1$ and $KpNF_5$ showed antagonistic activities against *Xanthomonas oryzae*. Isolate $KpNF_5$ showed antagonistic against both the tested pathogens. HCN and siderophore acted as a biocontrol agent and protected plant from biotic stresses. This in turn can indirectly enhance the plant growth by keeping plant healthy and disease free.

Amount of N fixation by selected isolates varied from 1.86 to 9.33 µg ml^{-1} and showed significant difference ($P \leq 0.01$) among the selected strains (Table 1). Highest N fixed was 9.33 mg N fixed g^{-1} of sucrose in the case of $AkNF_3$ and $PkNF_4$ followed by $KgNF_1$ (8.86 mg N fixed g^{-1} of sucrose). Nitrogen fixing ability of best five PGPR isolates from maize rhizosphere by Micro-Kjeldahl method and amount of nitrogen fixation varied from 6.3 mg N fixed g^{-1} of sucrose to 11.4 mg N fixed g^{-1} of sucrose [20]. Further reported that *Pseudomonas syringae pv. syringae* strain HS191, *Bacillus cereus* strain 20UPMNR, and *Pseudomonas aeruginosa* strain ZSL-2 fixed 6.3, 8.2, and 9.4 mg N fixed g^{-1} of sucrose, respectively and the present results are also in agreement with these results.

Table 1 Plant growth promoting and antagonistic activities, quantification of IAA production and nitrogen fixation of selected nitrogen fixers under *in vitro* conditions

Isolates	PGP activities				Antagonistic activity		IAA Production (µg ml^{-1})	N fixed (mg of N g^{-1} of sucrose utilized)
	IAA	NH3	HCN	Siderophore	*R. solani*	*X. oryzae*		
$KgNF_1$	+++	++	-	+	-	+	34.83 ^{a}	8.86 ^{ab}
$KgNF_9$	+	+++	-	-	-	-	3.50 ^{g}	6.06 ^{defg}
$KpNF_2$	++	+	-	-	-	-	14.83 ^{cd}	7.93 ^{abcd}
$KpNF_4$	+	+	-	-	-	-	5.00 ^{fg}	4.66 ^{fgh}
$KpNF_5$	-	++	-	++	+	+	-	8.40 ^{ab}
$KpNF_6$	+++	+	-	-	-	-	19.33 ^{bc}	4.26 ^{gh}
$KpNF_7$	++	+	-	-	-	-	15.83 ^{cd}	3.73 ^{hij}
$AvNF_1$	-	+	-	++	-	-	-	6.53 ^{cdef}
$AvNF_2$	+++	+	-	++	-	-	18.83 ^{bc}	2.33 ^{ij}
$AvNF_3$	+	+	-	+	-	-	6.16 ^{efg}	5.60 ^{efgh}
$AvNF_4$	+++	+	-	+	-	-	25.00 ^{b}	1.86 ^{j}
$MtNF_3$	+	++	-	-	-	-	2.50 ^{g}	4.20 ^{ghi}
$MtNF_4$	-	++	-	-	+	-	-	7.00 ^{bcde}
$AkNF_2$	-	++	-	++	-	-	-	7.93 ^{abcd}
$AkNF_3$	+++	++	-	++	-	-	23.16 ^{b}	9.33 ^{a}
$AkNF_5$	-	++	-	+	-	-	-	4.16 ^{ghi}
$PkNF_1$	-	+	-	+	-	-	-	6.06 ^{defg}
$PkNF_2$	++	++	-	+	-	-	11.16 ^{def}	4.66 ^{ghi}
$PkNF_3$	++	+	-	+	-	-	12.33 ^{de}	6.06 ^{defg}
$PkNF_4$	+++	++	-	+	-	-	20.66 ^{bc}	9.33 ^{a}

Positive reactions (High +++, Medium ++, Low +), Negative reactions (-), NF: Nitrogen fixers, Th: Thanivayal, Ed:Edakkal, Kg:Kolagappara, Nr: Nellara, Kp: Kuppamudi, Av: Ambalavayal, Mt: Marathat, Ak: Ambukuthi, Mv: Malavayal, Pk: Pottankoli

The N_2-fixing ability of bacteria is very important for plant as a potential alternative to reduce the application of chemical fertilizer. Seven isolates with multiple abilities were selected based on the quantity of fixed nitrogen for characterization. Cultural and morphological characters of selected nitrogen fixers are presented in (Table 2). One isolate ($AkNF_3$) produced large colony size and others are small or medium. All cells were circular in shape with cream or creamy white colour. Two isolates were produced flat colonies and others raised colonies. Isolate $AkNF_3$ was Gram-positive, rod and all others were Gram negative, short rods.

Seven selected N fixers were subjected to the molecular characterization based on 16S rRNA gene sequencing. However, PCR amplified sequences were obtained only for six isolates and they were analysed using BLASTn, to find out the similarity with sequences available in NCBI databank. The accession sharing maximum homology with the query sequences was considered for identification of isolate. 16S rRNA sequences of six nitrogen fixers were submitted to the GeneBank of NCBI and obtained accession numbers (Table 3). Phylogenetic trees of six nitrogen fixers are shown in (Plate 1).

Table 2 Colony morphology and staining reaction of selected nitrogen fixers

Characters		Selected nitrogen fixers						
		$KgNF_1$	$AkNF_3$	$PkNF_4$	$KpNF_2$	$KpNF_5$	$AkNF_2$	$MtNF_4$
Colony morphology	Size	Small	Large	Small	Medium	small	Small	Medium
	Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
	Color	Cream	Creamy white	Cream	Cream	Cream	Cream	Cream
	Elevation	Raised	Flat	Raised	Flat	Raised	Raised	Raised
Staining	Gram reaction	-	+	-	-	-	-	-
	Cell morphology	Short rods	Rods	Short rods	Short rods	Short rods	Short rods	Short rods

Small: colony diameter < 0.2mm; Medium: colony diameter 0.2-0.5 mm; Large: colony diameter 0.5-0.7mm
Positive reactions (+), Negative reactions (-)

Table 3 Details of identity and accessions numbers of selected nitrogen fixers in GenBank of NCBI database

Isolate	Accession number in GenBank	Identity based on 16S rRNA gene sequence
$AkNF_3$	MW288141	*Bacillus* sp. strain AkNF3
$PkNF_4$	MW269608	*Pseudomonas* sp. strain PkNF4
$KgNF_1$	MW288152	*Pseudomonas putida* strain KgNF1
$KpNF_5$	MW307349	*Alcaligenes faecalis* strain KpNF5
$MtNF_4$	MW307253	*Burkholderia* sp. strain MtNF4
$AkNF_2$	MW307254	*Brevundimonas naejangsanensis* strain AkNF2

Phylogenetic analysis of *Bacillus* sp. strain AkNF3 revealed that this isolates closely related to the *Bacillus* sp. (Fig a). This strain showed high amount of nitrogen fixation with higher level of IAA production (23.16 µg ml^{-1}). Further this strain was positive for production of ammonia and siderophore. Tested 19 different *Bacillus* strains and reported that 16 isolates had *nifH* gene [21]. Highest nitrogenase activity of *B. cereus* among 42 different strains of *Bacillus* sp. isolated from sunflower rhizosphere [22]. Diversity of nitrogen fixing bacteria associated with sugarcane and revealed that the occurrence of various strains of *Bacillus* genus as plant-growth promoting and they were screened for nitrogen fixation and reported CY5 (*Bacillus megaterium*) and CA1 (*Bacillus mycoides*) were the most prominent [4].

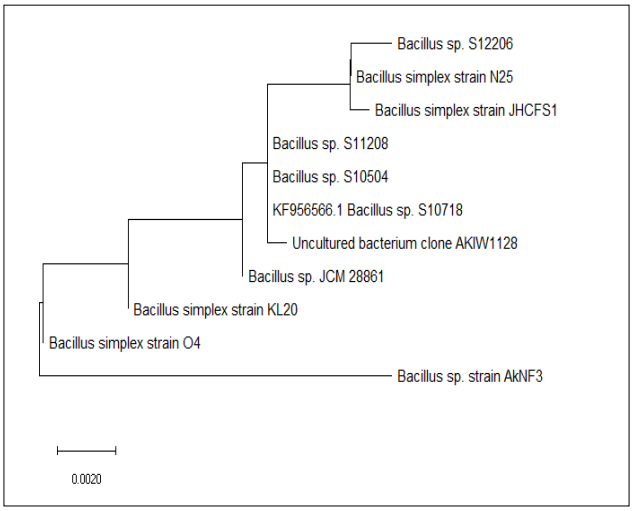

Fig a. *Bacillus* sp. AkNF3

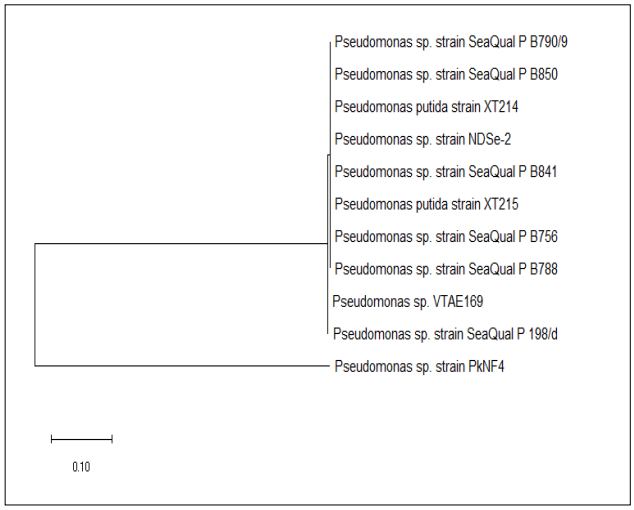

Fig b. *Pseudomonas* sp. PkNF4

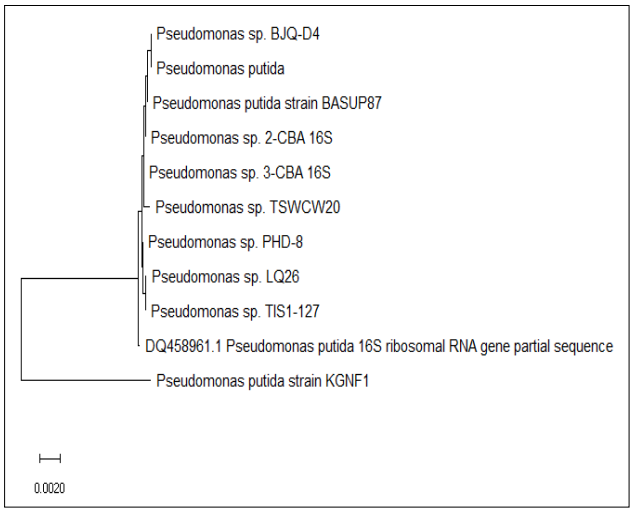

Fig c. *Pseudomonas putida* KgNF1

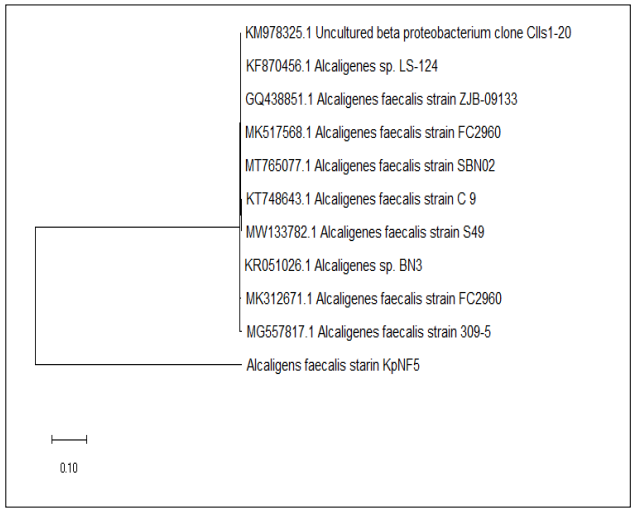

Fig d. *Alcaligenes faecalis* KpNF5

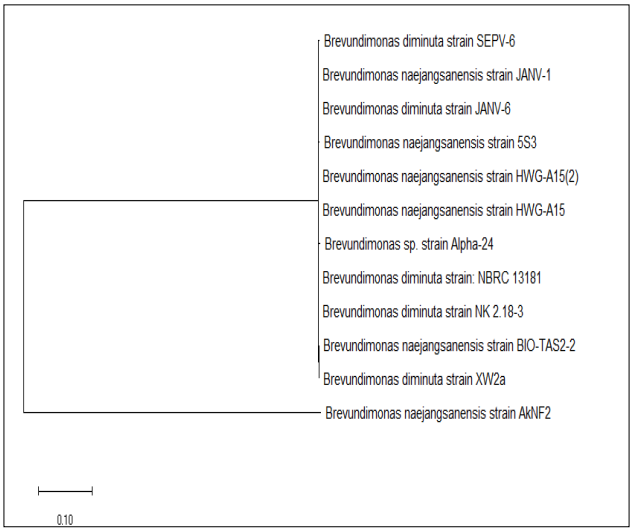

Fig e. *Brevundimonas naejangsanensis* AkNF2

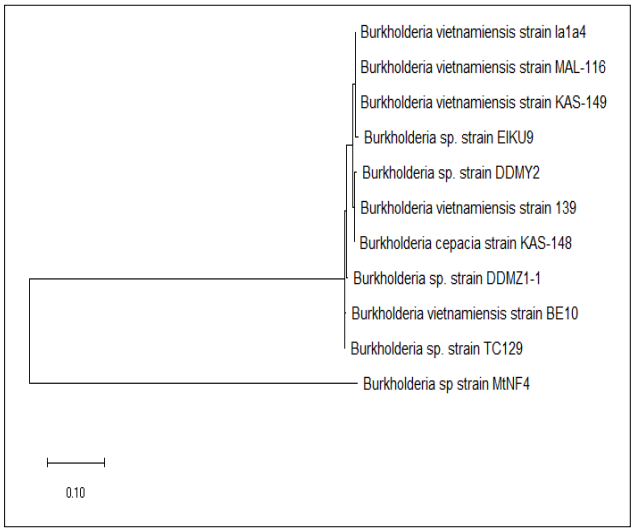

Fig f. *Burkhoderia* sp. MtNF4

Plate 1 Maximum likelihood phylogenetic tree produced using multiple alignment of 16S rRNA gene sequence of selected nitrogen fixers with those of other bacterial strains found in GeneBank database

Phylogenetic analysis revealed that isolate $PkNF_4$ was closely related to the *Pseudomonas* sp. (Fig b). This strain fixed 9.33 mg N g^{-1} of sucrose utilized and was positive for the production of IAA (20.66 µg ml^{-1}), ammonia and siderophore. Nitrogen fixing phytohormone-producing bacterial isolate from Kallar grass (strain A) and identified as *Pseudomonas sp.* by 16s ribosomal RNA gene sequencing analysis [23].

Phylogenetic tree of isolate $KgNF_1$ proved that this strain closely related to the *Pseudomonas putida* (Fig C). This strain showed antagonistic activity against rice pathogen *X. oryzae* and further showed ability to produce IAA (34.83 µg ml^{-1}), ammonia and siderophore. Nitrogen fixing ability of *Pseudomonas putida* as reported by [24]. All diazotrophs isolated from paddy soils proved nitrogen fixing potential and among them seven isolates were better nitrogen fixers and *Pseudomonas putida* showed ARA activity 280 ethylene/h/mg protein [24]. *Pseudomonas putida* showed high level of IAA (110 µg ml^{-1}) production and high levels of IAA excreted by *P. putida* was most consistent in enhancing plant growth [25].

Isolate $KpNF_5$ showed antagonistic activity against both rice pathogens *R. solani* and *X. oryzae*. Biocontrol activities of five rhizobacteria strains and showed that *Alcaligenes faecalis* strains (P1 and BK1) were able to unanimously suppress the mycelial growth of *Rhizoctonia solani* and *Magnoportha oryzae* [26]. Phylogenetic tree of this strain also suggested this strain belonged to the *Alcaligenes faecalis* (Fig d).

The isolate $AkNF_2$ had nitrogen fixation ability (7.93 mg N fixed $g^{-1}$of sucrose) with multiple PGP characters. Our results also agreed with [3] also reported that bacterial strain isolated from the sugarcane rhizosphere was a multiple PGP activity which exhibited nitrogen-fixing ability and production of indole-3-acetic acid as well as ammonia and identified as *Brevundimonas* sp. on the basis of phenotypic, biochemical, phylogenetic and 16S rDNA gene sequencing data. Phylogenetic tree construction of isolate $AkNF_2$ with available sequences of NCBI revealed this strain closely related to the *Bruvendimonas naejangsanensis* (Fig e).

Phylogenetic tree of isolate $MtNF_4$ proved that isolate closely related to the *Burkholderia* sp. (Fig f). This strain produced fixed nitrogen 7.00 mg g^{-1} of sucrose and had ability to produce ammonia. Further this isolate showed antagonistic effect against rice pathogen *R. solani*. Diazotrophic bacteria on growth and yield of traditional rice varieties and revealed that diazotrophic *B. vietmaniensis* strain AR1122 was a good biofertilizer candidate for inoculation of traditional rice varieties [2]. Members of the genus *Burkholderia* are very abundant, occupying diverse ecological niches including soil and having many beneficial plant growth activities like controlling the plant pathogens (biocontrol), production of IAA and siderophores for promoting the crop growth [27].

CONCLUSIONS

Most of the nitrogen fixing isolates had at least two or more PGP traits apart from nitrogen fixation. Besides nitrogen fixation, the presence of one or more growth-promoting trait enhances crop growth. The present study revealed that nitrogen fixing strains of *Bacillus* sp. AkNF3, *Pseudomonas* sp. PkNF4, *Pseudomonas putida* KgNF1, *Alcaligenes faecalis* KpNF5 *Brevundimonas naejangsanensis* AkNF2 and *Burkholderia sp*. MtNF4 from rice rhizosphere possessed multiple plant growth promoting traits which could be used as PGPR inoculant for rice cultivation. However further studies are required to evaluate their performance on crop growth and yield of rice crop.

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