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Screening and evaluation of PGPR strains having multiple PGP traits from hilly terrain

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) with multiple beneficial traits serve as potentially, ecofriendly, and cost-effective alternatives to chemical fertilizers and pesticides. They have both direct and indirect affirmative impacts on overall plant growth and health. PGPRs are well known to directly improve the plant growth by phytohormone production and availability of minerals in soil. A total of nine soil samples were taken from near the rhizospheric zone of different crops and 56 rhizobacterial strains were isolated. Only 16 out of 56 rhizobacterial strains were found positive for more than one beneficial trait that included solubilization of phosphate, indole acetic acid (IAA), siderophore, ammonia and H_2S production. Among all PGPR strains, RKM15 was observed having the highest phosphate solubilizing index (3.4), solubilized phosphorus (339 mg L^{-1}) and also siderophore unit (70.54 %). The maximum IAA production was observed by RKM25 strain (35.56 µg ml^{-1}). The most promising RKM15 isolate was identified as *Pantoea dispersa* (MN629239) through 16S rRNA gene sequencing technique. This characterized PGPR strain may be used for the development of biofertilizers to enhance crop productivity and improvement in soil fertility.

1. INTRODUCTION

At present, agriculture practices involve the extensive use of chemical fertilizers that lead to several impacts as groundwater pollution, eutrophication and production of greenhouse gases ultimately leading to environmental pollution and posing several health hazards. Plant growth-promoting rhizobacteria (PGPR) are a diverse collection of rhizobacteria that occur in the rhizospheric zone and interact with the plant's root [1]. PGPRs exhibit different Plant growth promoting (PGP) traits to improve the growth and development of plants. A varied range of rhizobacteria comprising of *Acinetobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Serratia a*nd *Xanthomonas* are known to enhance the growth of plant by various mechanisms such as biological fixation of atmospheric nitrogen, biosynthesis of phytohormones, mineral nutrient solubilization and increasing resistance of the host plant against stress factors [2].

Phosphorus is an important component required for the proper growth of plants because of its involvement in various metabolic processes. A high concentration of phosphate is found naturally in soil due to the application of chemical fertilizers [3], but this phosphate rapidly gets converted into unavailable forms by forming complexes with iron, aluminium, or calcium ions [4]. This unavailable phosphate could be transformed back into available forms by several phosphate solubilizing rhizobacteria.

Indole acetic acid (IAA) is the main phytohormone that is produced by PGPR in addition to host plants. IAA is synthesized throughout the plant body, mainly in meristematic tissues and in buds, including germinating seeds, root and shoot tips. IAA stimulates various important physiological processes such as lateral bud development, differentiation in vascular bundles and tropism responses [5].

Siderophore compounds are chelating agents having a strong affinity for ferric irons [6] and are produced by some PGPRs. This compound chelates to the unavailable form of iron and makes it available to the roots of plant in the adjacent environment [7,8]. Besides, siderophores also control the growth

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of plant pathogens through biological control mechanisms [9]. It has been reported that biocontrol action of siderophores is useful subsequently in many crops [10]. Rhizobacterial strains indirectly support the growth of plant by reducing the growth of phytopathogenic microbes, production of hydrolytic enzymes, hydrogen cyanide (HCN) and hydrogen sulfide (H_2S) in the rhizosphere and minimize the effects of biotic stresses [11].

Potential PGPR isolates can be screened through estimations of phosphate solubilization, production of IAA, siderophore, ammonia and HCN *in vitro* to study their PGP traits [12,13]. PGP traits as phosphate solubilization and ammonia production can directly enhance growth of plants by increasing availability of nutrients while IAA phytohormone increase the root growth [14,15].

In the past few decades, the application of PGPR has increased extensively for sustainable agriculture. More research works show that the application of PGPR inoculants significantly increased growth and yield of crops. The present study was undertaken to screen PGPR strains having various PGP traits and the use of such PGPR strains as bioinoculant.

2. MATERIALS AND METHODS

2.1. Collection of soil sample and isolation of rhizobacteria

Nine soil samples were collected from different sites from the hilly terrain of Harda District in Madhya Pradesh. These soil samples were brought to the laboratory for further study. To isolate the rhizobacteria, 10 g of soil was added to 90 ml of sterile saline distilled water (0.85% NaCl) and placed on an orbital shaker at 120 rpm for 30 minutes. About 100 µl of soil suspension by serial dilution from 10^{-4} to 10^{-6} of each soil sample was spread on nutrient agar medium (NAM) plate and placed in an incubator at 28 ± 2°C for 2 days. Single colonies of rhizobacterial isolates were selected on the basis of faster growth and purified by repeat streaking on the NAM. After purification, these PGPR isolates were screened for PGP traits.

2.2. Morphological characteristics

After purification of the colony, each isolate was observed for their morphological dissimilar appearances of colonies such as colour, elevation, size, shape and surface, which were recorded on nutrient agar plates to minimize the number of similar isolates. Gram staining of each isolate was initially determined by using crystal violet and safranin staining, according to standard microbiological protocol.

2.3. Determination of PGP traits

2.3.1. Phosphate solubilization

The isolated rhizobacterial strains were checked for their ability to dissolve insoluble tricalcium phosphate as described by Gaur [16]. For this, 5 µl of bacterial suspension (≅3 × 10^8 colony forming unit ml^{-1}) was placed on Pikovskaya's agar medium, incubated at 28 ± 2°C and observed on 7th day after incubation. The solubilization index (SI) was determined by the ratio of total diameter (colony + halo zone) to the diameter of the colony.

Clear halo zone around bacterial colony indicates phosphate solubilization zone. PGPR strains were chosen for further estimation of phosphate solubilization in broth on the basis of SI. The selected PGPR was inoculated in 50 ml of Pikovskaya broth and kept in an orbital shaker at 28 ± 2°C and 120 rpm for 5 days [17]. After the requisite incubation period, rhizobacterial culture was harvested by centrifugation at 10,000 rpm for 15 minutes. The supernatant (1 ml) was mixed with 2.5 ml of Barton's reagent and the volume made up to 50 ml with distilled water and a blank was also prepared. The absorbance of developed yellow colour was measured after 10 minutes at 430 nm in a UV-VIS spectrophotometer (Systronics-2201) and soluble phosphorus present in supernatant was quantified using a standard curve of KH_2PO_4.

2.3.2. Production of IAA

The production of IAA by PGPR isolates was quantified by the method of Bric *et al*. [18]. PGPR isolates were inoculated in 50 ml nutrient broth and incubated at 28 ± 2°C in an orbital shaker at 120 rpm for 48 hours. After the requisite incubation period, rhizobacterial culture was harvested by centrifugation at 10,000 rpm for 10 minutes and supernatant was used for quantification. Salkowski's reagent was mixed in 2:1 proportions with supernatant and placed in the dark for 30 minutes. The developed pink supernatant showed the presence of IAA. The absorbance of this supernatant was measured at 530 nm in UV-VIS spectrophotometer and the amount of IAA was determined using the IAA standard curve.

2.3.3. Siderophore production

PGPR strains were spotted on chrome azurol S (CAS) agar plates and placed in an incubator at 28 ± 2°C for 7 days. The change of colour of medium from blue to yellow-pink around the spot indicated siderophore production.

Further, the quantitative analysis of siderophore was performed using CAS solution. PGPR strain was inoculated in sterile siderophore-inducing medium [20] and incubated on an orbital shaker at 28 ± 2°C at 120 rpm. After 7 days, rhizobacteria were harvested by centrifugation at 10,000 rpm for 5 minutes and 1 ml of supernatant was mixed with 1 ml of CAS solution and 20 µl of shuttle solution (0.2 M 5-sulfosalicyclic acid) was added. After 20 minutes of incubation at room temperature, absorbance was measured at 630 nm in UV-VIS spectrophotometer. Siderophore unit (%) was calculated by the following formula:

$$\text{Siderophore unit (\%)} = \{(Ar - As)/Ar\} \times 100$$

where Ar = reference absorbance at 630 nm
As = sample absorbance at 630 nm

2.3.4. Ammonia production

The purified isolates were grown in 10 ml of peptone broth in test tube and placed at 120 rpm for 48–72 hours at 28 ± 2°C. After incubation, 0.5 ml of Nessler's reagent was added in the culture test tube. The change of colour from yellow to brown indicated a positive test for ammonia production [21].

2.3.5. HCN production

Lorck method [22] was performed to screen the HCN production by PGPR isolates. The modified NAM was prepared by amending it with 4.4 g L^{-1} glycine. PGPR strains were streaked on this medium. Pre-sterilized Whatman filter paper (no. 1) discs impregnated with filter-sterilized solution of picric acid and sodium carbonate and stuck on inside the Petri dish lid. The Petri dish was wrapped with parafilm and placed in an incubator at 28 ± 2°C for incubation of 4 days. The change of colour from yellow to the reddish-brown showed HCN production by PGPR isolates.

2.4. Biochemical characterization of selected PGPR strains

Seven PGPR isolates were selected according to their multiple PGP activity and were further characterized by standard biochemical tests. The selected PGPR strains were biochemically characterized by Gram's staining, oxidase test, catalase test, citrate utilization, amylase assay, urea hydrolysis, methyl red (MR) test, voges proskauer (VP) test, indole test, casein hydrolysis, gelatin hydrolysis [23].

2.5. Molecular characterization of selected isolate

Genomic DNA from selected PGPR strain was extracted as described by Araujo *et al*. [24] with slight modifications and the isolated genomic DNA was used as a template for the amplification of 16S rRNA using primers 27F (5′AGGCCCGGGAAGGCGTATTCAC 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′). PCR was performed in a 50 µl of the final volume of reaction mixture containing 2 µl of DNA template, 4 µl of each forward and reverse primer, 25 µl 2X PCR buffer and 15 µl Milli Q water. PCR was carried out for 30 amplification cycles in a T100™ Thermal Cycler (BioRad, USA). The initial denaturation step was set at 95°C for 3 minutes, followed by 30 amplification cycles at 95°C for 1 minute, annealing temperature at 58°C for 30 seconds and 72°C for 2 minutes and final extension at 72°C for 8 minutes. After detection of PCR product by agarose gel electrophoresis along with 1 kb DNA ladder, the PCR product was further purified for use as a sequencing template and sent for sequencing to Xcelris Labs Limited, Ahmedabad, Gujarat. BLAST program was used to identify nucleotide-related sequence similarities which were obtained from GenBank database and were determined with NCBI databases. A phylogenetic tree was constructed by neighborhood joining bootstrap method among different isolates which includes phylogeny testing using bootstrap analysis with 1,000 replicates using MEGA X Software (Version 10.1.7) [25].

2.6. Statistical analysis

All experiments were performed in triplicate. The obtained data were analyzed by analysis of variance (ANOVA) using SPSS software. The mean values were compared using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05\%$.

3. RESULTS AND DISCUSSION

3.1. *Isolation and primary screening of PGP traits in isolates*

A total of nine rhizospheric representative soil samples were collected and used for the isolation of different PGPR isolates. Out of 56 rhizobacterial isolates, 16 isolates were found positive for different PGP traits like phosphate solubilization, production of siderophore, IAA, ammonia and H2S (Table 1).

3.2. Morphological and biochemical characterization of potential PGPR isolates

A total of seven potential PGPR isolates were selected for their morphological (Table 2) and biochemical characterization (Table 3).

Table 1: Screening of PGPR strains from soil for their multiple PGP traits.

S.No.	PGPR isolates	Cell Shape	Plant growth promoting traits					
			Phosphate solubilization*	IAA**	Siderophore production*	Ammonia production**	H_2S production	HCN production
1	RRM12	Rods	++	–	–	+	+	–
2	RGM14	Cocci	++	–	–	+	+	–
3	RKM12	Rods	+	++	–	–	–	–
4	RKM15	Rods	+++	–	++	+	–	–
5	RSM14	Cocci	+	+	–	–	–	–
6	RSM17	Rods	++	–	–	+	+	–
7	RKM22	Rods	+	–	+	–	–	–
8	RKM25	Rods	++	+++	–	+	+	–
9	RKM32	Rods	+	–	–	+	–	–
10	RKM36	Cocci	+	+	–	–	–	–
11	RKM42	Rods	+	–	+	–	–	–
12	RKM45	Rods	+	+	–	–	–	–
13	RDM13	Cocci	+	–	–	+	+	–
14	RDM16	Rods	++	–	++	+	–	–
15	RRM23	Rods	+	–	–	+	–	–
16	RRM27	Rods	++	–	–	+	+	–

(–) indicates negative result.

*Halo zone >10 mm (+++), 3–10 mm (++), 1–2 mm (+).

**Colour intensity high (+++), medium (++), low (+).

3.3. Secondary screening of PGP traits in isolates

3.3.1. Phosphate solubilization

Phosphorus, an essential macronutrient, plays a key role in plant growth and development. PGPR isolates have the capability to solubilize insoluble phosphate and make it accessible for the plants [26,27]. In this study, 16 of 56 isolates were able to solubilize phosphate by making more than 3 mm clear halo zone around the colonies. On the basis of solubilizing zone, seven PGPR strains were preferred for the quantitative determination of phosphate solubilization. Maximum SI and solubilized phosphorus were shown by isolates such as RKM15 (3.4 and 339 mg L^{-1}) and RGM14 (2.43 and 321.66 mg L^{-1}) (Figs. 1 and 3). Minimum SI and solubilized phosphorus were observed in the case of RSM17 (1.35 and 186.67 mg L^{-1}) (Fig. 3). Similarly, Tomer *et al*. [28] isolated a total of 133 isolates from soil and one isolate showed the highest solubilization efficiency of 713.11 mg L^{-1} which resembles with its largest SI as shown on Pikovskaya agar plate. Baliah *et al*. [29] observed that among 10 strains, *Pseudomonas fluorescens* (CTP2) released a maximum of 46.0 mg L^{-1} phosphorus followed by TP1 (40.6 mg L^{-1}) in medium containing tricalcium phosphate. Liu *et al*. [30] reported phosphate solubilization efficiency in the range from 213.19 mg L^{-1} to 315.72 mg L^{-1}. Karpagam *et al*. [31] reported that of 37 bacterial isolates, only six isolates showed higher SI (1.13–2.23). Phosphate-solubilizing rhizobacteria produce various types of organic acids, namely, butyric acid, fumaric acid, adipic acid, acetic acid, glyconic acid, oxalic acid, malonic acid, lactic acid, succinic acid and malic acid [32,33]. Even though organic acids may disturb the rhizosphere pH, these acids increase accessible phosphorus content in soil [34].

3.3.2. IAA production

IAA is the most common phytohormone that is synthesized by bacteria. Out of 56 isolates, only five PGPR isolates were found positive for the production of IAA without the addition of L- tryptophan in nutrient broth and were selected for further quantitative analysis. The data in Figure 4 shows maximum IAA production in RKM25 (35.56 µg ml^{-1}), whereas minimum IAA production was observed in RKM36 (7.01 µg ml^{-1}). Comparable results of IAA by PGPR isolates were reported by other researchers as well [35,36]. Pandey and Gupta [37] observed IAA production in a range from 10.96 to 37.78 µg ml^{-1} by PGPR isolates in the presence of L-tryptophan. Several studies showed that some rhizobacteria are able to synthesize IAA without L-tryptophan precursor. IAA is mainly synthesized by tryptophan (Trp) precursor by two pathways (dependent and independent) in both plants and bacteria. Bacteria have been found to be able to produce IAA through more than two pathways [38,39].

3.3.3. Siderophore production

PGPR isolates are also known to release iron-chelating compounds that increase the availability of iron to plants in iron-limiting soils [40]. Four isolates were found positive, that produced siderophore. Among them, RKM15 showed a large yellow halo zone on CAS agar plate (Fig. 2).

CAS agar assay confirmed the production of siderophore. The siderophore chelated to the iron of medium resulted in change of colour from blue to yellow-pink [41]. Four selected PGPR isolates

Table 2: Morphological features of selected PGPR strains.

Morphology of colony	PGPR Isolates						
	RRM12	RGM14	RKM15	RSM17	RKM25	RDM16	RRM27
Colour	Brownish	Yellowish	White	White	White	White	White
Shape	Circular	Irregular	Circular	Circular	Circular	Circular	Circular
Surface	Wavy	Wavy	Shining	Smooth	Smooth	Smooth	Smooth
Margins	Entire	Undulate	Entire	Entire	Entire	Entire	Entire
Structure	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Degree of growth	Profuse	Profuse	Profuse	Profuse	Profuse	Profuse	Profuse

Table 3: Biochemical characterization of selected PGPR strains.

Biochemical test	PGPR isolates						
	RRM12	RGM14	RKM15	RSM17	RKM25	RDM16	RRM27
Gram stain	–	+	–	–	–	–	–
V.P. test	+	–	+	+	+	+	+
M.R. test	–	+	–	–	–	–	–
Catalase test	+	+	+	+	+	+	+
Casein hydrolysis	+	+	–	+	–	–	+
Amylase assay	–	–	–	–	–	–	–
Citrate utilization	+	+	+	+	+	+	+
Urea hydrolysis	–	–	–	–	+	–	+
Oxidase test	–	+	–	–	+	–	–
Indole test	+	+	–	–	+	–	+
Gelatin hydrolysis	–	+	–	+	–	–	+

were evaluated quantitatively of which, RKM15 (70.54%) and RDM16 (64.25%) showed the highest production of siderophore (Fig. 5). Similarly, Gupta and Gopal [42] reported siderophore production in the range from 21% to 70% by six isolates, from which *P. fluorescens* showed maximum siderophore production. Pahari and Mishra [43] screened 31 isolates, of which only two BGBA-1 and BRBA-1 produced maximum siderophore units. Ghavami *et al*. [44] reported that of 50 isolates, 10 bacterial isolates were able to produce siderophore.

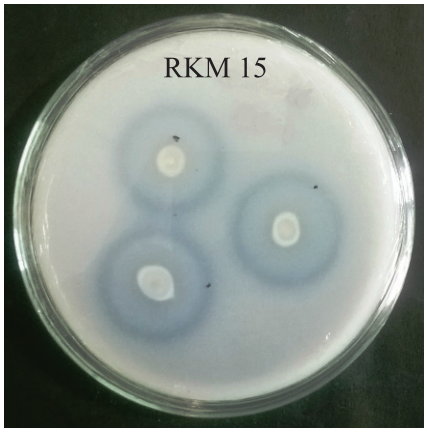

Figure 1: Solubilization of tricalcium phosphate by rhizobacterial isolate RKM15 on Pikovskaya agar medium.

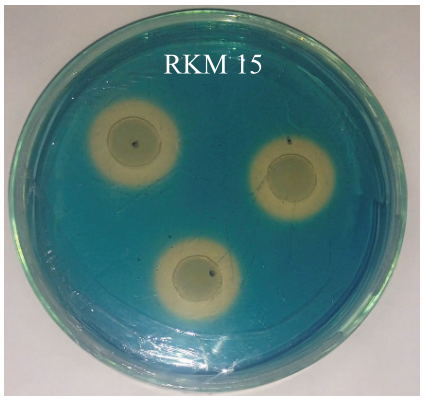

Figure 2: Production of siderophore by rhizobacterial isolate RKM15 on CAS agar medium.

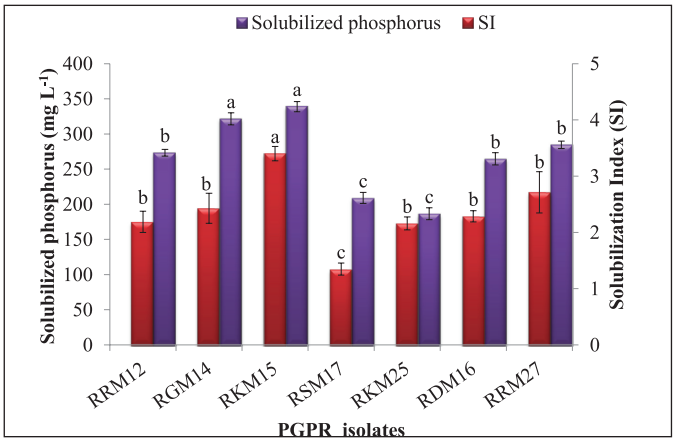

Figure 3: Phosphate solubilization by selected rhizobacterial isolates. Bars sharing the same letter do not differ significantly ($p \leq 0.05$) and error bars (┬) show SE.

3.4. Molecular characterization of potential PGPR isolate

RKM15 isolate was found to be the most prominent among all PGPRs. Hence, this isolate was selected for further characterization through 16S rRNA gene sequencing. According to the sequencing result, RKM15 demonstrated sequence homology with *Pantoea dispersa,* as presented in phylogenetic tree (Fig. 6). This isolate showed maximum similarity (96.25%) with *P. dispersa.* Accession number MN629239 of RKM15 isolates was obtained by submitting the sequences to GenBank database of NCBI.

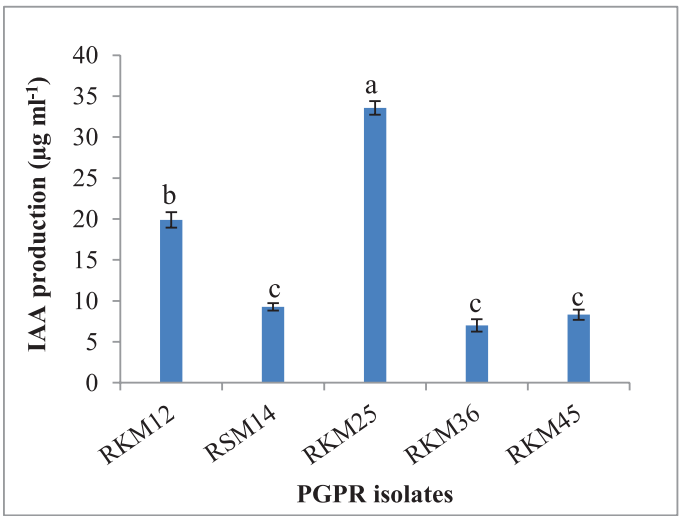

Figure 4: IAA production by selected rhizobacterial isolates. Bars sharing the same letter do not differ significantly ($p \leq 0.05$) and error bars (┬) show SE.

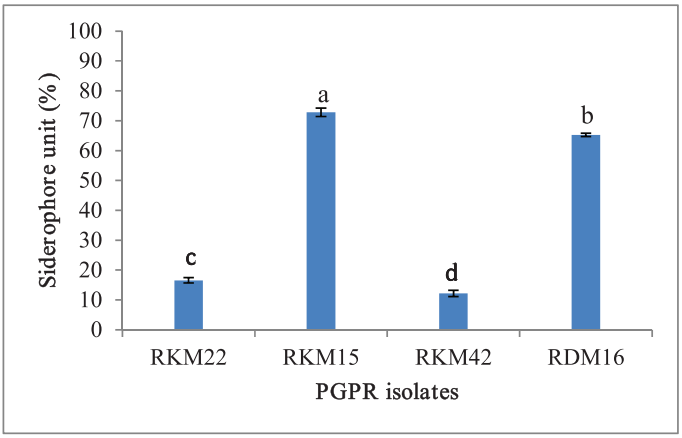

Figure 5: Siderophore production by selected rhizobacterial isolates. Bars sharing the same letter do not differ significantly (p≤0.05) and error bars (┬) show SE.

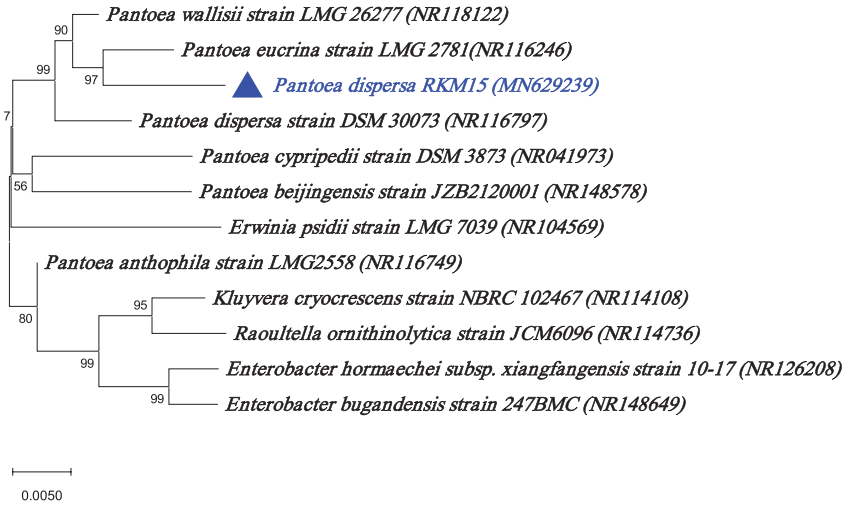

Figure 6: Phylogenetic analysis of the RKM15 isolates using MEGA X version 10.0.5 by neighbor-joining method at bootstrap replicate value of $n = 1000$. Phylogenetic tree was constructed with branch lengths in the same units that inferred evolutionary distances. The PGPR isolates identified in the present study have been highlighted, and 16S rRNA gene accession numbers are given within brackets.

4. CONCLUSION

Finally, the present study emphasized on the capacity of the rhizobacteria isolated from soils of hilly regions of Harda District in Madhya Pradesh, having PGP traits such as phosphate solubilization, production of siderophores and ammonia. The RKM15 strain may be concluded as a very efficient phosphate solubilizer along with other PGP traits such as siderophore and ammonia production and showed maximum similarity with *P. dispersa*. RKM15 strain may be used as a bioinoculant to enhance plant growth by increasing nutrient availability through diverse mechanisms in soil.

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CONFLICTS OF INTEREST

Authors declare that they do not have any conflicts of interest.

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None.

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