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Lysinibacillus sp. - A Novel Plant Growth Promoting Bacterial Endophyte from Rice

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A B S T R A C T

Rice is a staple food in many countries and faces a variety of production constraints due to pests, diseases, and nutritional deficiencies. Currently, agrochemicals are being used to address these issues in economic perspective. However, their long term uses exhibit serious consequences on health, environment, and sustainability under present food production systems. Considering these facts and to overcome the issues in a much safer way, the use of biological components like endophytic bacteria could be a better choice. The purpose of this research was to extract and characterize endophytic bacteria from rice plants and to bring out their ability to promote plant growth. Endophytic bacterial isolates from different parts of the rice plant were tentatively identified as *Bacillus*, *Klebsiella*, *Pseudomonas*, *Azospirillum*, and *Lysinibacillus* spp. in morphological and biochemical characterization, and *invitro* plant growth promoting activity of different bacterial endophytic isolates from rice were assessed for their efficiency to fix nitrogen and to produce IAA, GA_3, siderophore (iron sequestrating chemical) production, and phosphate solubilization. Among the different isolates compared, the highest IAA production efficiency was recorded with Root Endophytic Bacteria REB 2 (64.19±0.84). The highest quantity of the gibberellic acid (GA_3) production was also recorded with the isolate REB 2 (1.94±0.03) (g/ml). For the ACC deaminase activity, most of the isolates tested positive with the exception of Leaf Endophytic Bacteria LEB 3 and Stem Endophytic Bacteria SEB 2. In case of siderophore synthesis the isolate LEB 3 (16.25±067) ($\mu molmL^{-1}$ CFCF) was at its peak. The ability to solubilize phosphate was investigated, and the isolate REB 2 recorded the maximum solubilization zone of 0.69 mm. After tentative identification of these species by morphological and biochemical methods, the best and most effective isolate identified was REB 2, which performed superior in best production of IAA and GA_3, with maximum phosphate solubilization ability. For siderophore production the best performance was LEB 3 followed by REB 2. Further, characterization at molecular level sequencing found to confirm REB 2 as *Lysinibacillus* sp.

***Key words*:** *Lysinibacillus* sp., Rice, Bacterial endophytes, PGPR activity

Rice (*Oryza sativa*) is the world's most important staple food, feeding more than half of the world's population, and its production must be raised to keep up with rising demand. This must be achieved without the widespread use of synthetic fertilizers and pesticides, which may pollute the environment and harm human health. Exploring alternative options to overcome this problem is essential and the use of endophytic bacteria with favourable properties in rice and other plant growth. Endophytes are wide range of microorganisms that live in host plants without causing diseases [1]. The main goal of endophytic bacterial research is to improve agriculture and agricultural output [2]. Endophytic bacteria, helps the plants directly by plant growth promotion strategies by providing phytohormones to the plant, controlling ethylene levels in the plant by ACC deaminase synthesis [3], and distributing key nutrients like N, P, iron, and other elements from the environment [4]. Endophytic bacteria live in plant tissues without affecting the host or gaining any advantage other than a non-competitive habitat within the host. They have been found in a variety of plant tissues, including stems, roots, flowers, leaves, and seeds, according to several investigations [5]. They are bacteria that can be separated from surface-disinfected tissues or screened from interior plant tissues without harming the plant [6-7]. Endophytic bacteria have been isolated and reported from a wide range of plants, including cotton, wheat, rice, sugarcane, and popular trees, since they are found in almost all plant species [8-12]. They can also promote plant growth and development by activating a variety of novel mechanisms, such as IAA production [13], phosphate solubilizing activity [14], and siderophore activity [15]. Besides enhanced plant

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growth they also protect plant health through different methods. Anticipating these advantages this research was designed and conducted to isolate and characterize endophytic bacteria from rice, that could help in enhancing its productivity and thereby food security. The chosen endophytic bacterial isolates were evaluated for the PGPR activity such as nitrogen fixation, indole acetic acid (IAA) production, GA_3 production, mineral phosphate solubilization, and siderophore (iron sequestering chemicals) production.

MATERIALS AND METHODS

Isolation of endophytic bacteria from rice plants

Leaf, stem and root samples of rice were used for isolating endophytic bacteria. The isolation of endophytic bacteria from rice plants was carried out after surface sterilization with 70% (v/v) ethyl alcohol for 2 minutes, 2 (v/v) NaOCl for 3 minutes, 95% (v/v) ethyl alcohol for 30 seconds and 30% (v/v) H_2O_2 for 1 minutes, respectively, followed by washing four times with sterilized distilled water. Purity check was done by transferring 1 mL of final washing from each set of plant tissue onto LB (Hi Media) plates and incubated at 27±2°C for 24 h and observed for any microbial growth. Randomly selected surface sterilized samples were then transferred to LB for the selective isolation of endophytic bacteria and incubated at 27±2°C for 7 days. After the incubation the isolates were purified and were maintained in LB slants at 4°C.

Designation of different endophytic bacterial isolates

The different endophytic bacteria isolated from different parts of rice plants were designated as Leaf Endophytic Bacteria (LEB 1, LEB 2 and LEB 3), Stem Endophytic Bacteria (SEB 1 and SEB 2) and Root Endophytic bacteria (REB 1 and REB 2).

Morphological characterization of endophytic bacterial isolates

To describe the tentatively identified endophytes, the following morphological tests were performed: cell shape, gram reactions, and motility.

Cell shape

The purified cultures, at log phase were observed microscopically for the cell morphological characteristics.

Gram staining

Gram staining was carried out as per modified Hucker's method. The slides were viewed with the light microscope under oil -immersion. Gram-positive bacteria appear violet and gram -negative bacteria appear pinkish red.

Biochemical characterization of endophytic bacterial isolates

Oxidase test

The endophytic isolates were streaked on Trypticase soy agar medium and incubated at 30°C in an inverted position for 48 h. After the incubation period, 2-3 drops of para-aminodimethyl aniline oxalate solution were added on the streaked area and the plates were observed for the colour change from pink to maroon and finally to purple within 30 seconds indicated a positive reaction.

Nitrate reduction test

The endophytic isolates were inoculated into 10 ml of nitrate broth taken in test tubes and the tubes were inoculated at 30°C. After 14 days, 2 ml of the broth was tested by adding equal amounts of sulfanilic acid and alpha naphthylamine. Development of red color indicated that nitrate had been reduced to nitrite.

Hydrogen sulphide production

Sulfide indole motility (SIM) agar stabs were inoculated with the isolates of endophytic bacteria and incubated at 30°C for 48 hr. Black coloration along the line of stab inoculation indicated H_2S production.

Catalase activity

A loopful of endophytic bacteria maintained at nutrient agar slants for 24 hours was transferred to a glass test tube containing 0.5 ml distilled 0.5 ml three percent H_2O_2 solution and the presence of effervescence was detected.

Indole production

The isolated bacterial endophytes were inoculated into glucose tryptone broth in test tubes and the tubes were incubating at 30°C. After 48 h of incubation, 0.3 ml of Kovacs reagent was added and mixed well. The reddening of the alcohol layer within few minutes indicated indole production.

Methyl red and Voges-Proskauer test

The Methyl red and Voges-Proskauer (MR-VP) broth prepared in two sets were inoculated with the endophytic isolates and incubated for 48 h at 30°C. To the first set of tubes, few drops of an alcoholic solution of methyl red were added. The development of distinct red color was indicative of positive reaction for MR test.

1-naphthol solution (5 per cent solution in 70 per cent ethyl alcohol) was added to the second set of tubes and shaken gently for 15 minutes. The positive reaction of acetyl methyl carbinol production was indicated by development of red color. This indicates positive result for the VP test.

Citrate utilization test

The endophytic isolates were inoculated into Simmons citrate agar medium and incubated for 48 h at 30°C. Simmons citrate agar contained citrate as its only carbon source. The presence of growth and change of color from green to blue due to pH change indicated positive reaction.

Phytohormone production by endophytic bacterial isolates
ACC deaminase activity

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was determined by culturing bacterial isolates on nitrogen-free medium supplemented with 3 mM ACC as a nitrogen source [16]. ACC deaminase activity of bacterial isolates was quantitatively estimated UV spectrophotometrically in terms of α-ketobutyrate production at 540 nm by comparing with the standard curve of α-ketobutyrate.

Production of indole-3-acetic acid (IAA)

The production of indole-3-acetic acid (IAA) was measured in Luria–Bertani (LB) broth supplemented with 5 mM L-tryptophan [17]. Bacterial cultures (10^{-7} CFU ml^{-1})

were inoculated in LB broth and maintained at 30°C in a shaking condition for 36 hours at 120 rpm. The culture was centrifuged at 10,000 rpm for 15 minutes at room temperature after the incubation period. 2 ml Salkowski reagent (2 per cent 0.5 M $FeCl_3$ in 35 percent perchloric acid) was added to one millilitre of supernatant. Two drops of orthophosphoric acid were also added, and the mixture was maintained in the dark for colour development. After 2 hours, the optical density was measured at 530 nm.

Siderophore production

The siderophore production was determined on Blue agar Chrome Azurol S (CAS) medium-containing CAS and hexadecyl trimethyl ammonium Bromide (HDTMA) as indicators by incubating at 28°C for 24 h. Siderophore production (μmolmL^{-1} CFCF) was quantitatively estimated spectrophotometrically employed the procedure [18].

Phosphorus solubilization

The isolates were inoculated on the center of the plate containing Pikovskaya's medium and then it is evaluate the plates after a week of incubation to determine the halo zone around the colony. In Pikovskaya's media, isolates displayed varying phosphate solubilizing capacity. P solubilization by bacterial endophytes has been reported in a number of studies. The findings match those of [19-20], who identified *Lysinibacillus* sp. as a possible P solubilizer.

Molecular identification of endopytic bacterial isolates

A phylogenetic analysis of the 16S rDNA gene was used for identification of the chosen isolate. For PCR amplification, the 16S rDNA universal primers Forward 27F 5′- AGAGTTTGATCMTGGCTCAG - 3′ and Reverse 1492R 5′- GGTTACCTTGTTACGACTT - 3′ were used. The isolate's genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Sequence Scanner Software v1 was used to check the sequence quality (Applied Biosystems). MEGA 7 was used to perform sequence alignment and any necessary modification of the acquired sequences. The amplified sequences were validated to be 16S rRNA by using the NCBI's BLAST program's similarity index. Based on the higher percentage similarity against the reference species, the species utilized in this study was assigned.

RESULTS AND DISCUSSION

Isolated bacterial entophytes were characterized morphologically and biochemically. The results were tabulated in (Table 1) and the morphological characterization with different shapes viz., rod, short rod and spiral shapes of the bacteria were identified. Using the biochemical characterization, the endophytic bacterial isolates from different parts of rice plant were identified tentatively as *Bacillus, Klebsiella, Pseudomonas, Azospirillum* and *Lysinibacillus* sp. and the details are enclosed in (Table 2).

Table 1 Morphological characterization of different rice endophytic bacterial isolates

Location	Isolates	Colony characters	Gram staining	Cell shape	Endospore	Motility
Leaf	LEB 1	White flat wrinkled colonies with irregular margins	+	rods	+	+
	LEB 2	White slimy raised colonies	-	Short rods	-	-
	LEB 3	White flat wrinkled colonies with irregular margins	+	rods	+	+
Stem	SEB 1	Slimy white raised colonies	-	rods	-	+
	SEB 2	Pinkish white raised slimy colonies	-	rods	-	+
Root	REB 1	White flat slimy colonies	-	Spiral	-	+
	REB 2	Circular colonies	+	Rod-shape	-	+

Table 2 Biochemical characterization of different rice endophytic bacterial isolates

Location	Isolates	Oxidase test	Nitrate test	H_2S production	Catalase	Indole production	MR test	VP test	Citrate utilization	Tentative identification
Leaf	LEB 1	+	+	+	+	-	+	-	+	*Bacillus*
	LEB 2	-	+	-	+	-	+	-	+	*Klebsiella*
	LEB 3	+	+	-	+	-	+	-	+	*Bacillus*
Stem	SEB 1	+	+	-	+	-	+	-	+	*Pseudomonas*
	SEB 2	+	+	-	+	-	+	-	+	*Pseudomonas*
Root	REB 1	+	+	-	+	-	+	-	+	*Azospirillum*
	REB 2	+	-		+	-	-	-	+	*Lysinibacillus*

In vitro plant growth promoting activity of different bacterial rice endophytic isolates were analysed for its efficiency to produce IAA, GA_3, siderophore production and phosphate solubilization ability. Based on the efficiency of the endophytic bacterial isolates to produce IAA production the isolate REB 2 (64.19 ± 0.84) (μg/ml) recorded the highest IAA production, and this was followed by (28.87 ± 0.34) on LEB 1 and the least was recorded by LEB 3 (11.19 ± 0.84). Highest amount of GA_3 was produced by the isolate REB 2 (1.94 ± 0.03) (μg/ml) and the least GA_3 was produced by SEB 1 (0.96 ± 0.03). ACC deaminase activity was positive for most of the isolates except LEB 3 and SEB 2. The siderophore production was maximum on LEB 3 (16.25 ± 067) (μmolmL^{-1} CFCF) followed by the isolate REB (216.07 ± 0.05) and the least siderophore production was recorded by the isolate SEB 2 (09.85 ± 0.04). Phosphate solubilization ability was analyzed and the maximum solubilization of 0.69 mm was noticed in the isolate REB 2, which was followed by 0.32 mm of zone by LEB 3 and 0.31 mm of zone by SEB 2 isolates. Further the results for *invitro* plant growth promoting activity of different bacterial rice endophytic isolates were tabulated in Table 3. The best and effective strain REB 2 which performed better in maximum IAA, GA_3, siderophore production and phosphate solubilization after tentative identification of the species by morphological and

biochemical was characterized at molecular level sequencing to confirm as *Lysinibacillus* sp. The sequenced gene was submitted to NCBI Gene Bank and accession number was also obtained (OK036788) and the detail such as FASTA Sequence of the organism and Phylogenetic tree is enclosed below.

Table 3 *In vitro* plant growth promoting activity of different rice endophytic bacterial isolates

Isolates	IAA (µg/ml)*	GA_3 (µg/ml)*	ACC deaminase activity	Siderophore production (µmolmL^{-1} CFCF)*	Phosphate solubilization efficacy (mm)
LEB 1	28.87±0.34	1.32±0.03	Positive	13.85±0.04	0.23
LEB 2	21.42±0.51	1.41±0.07	Positive	11.07*0.05	0.00
LEB 3	11.19±0.84	1.36±0.05	Negative	16.25±067	0.32
SEB 1	18.87±0.34	0.96±0.03	Positive	13.25±067	0.12
SEB 2	12.42±0.51	1.21±0.07	Negative	09.85±0.04	0.31
REB 1	21.19±0.84	1.05±0.05	Positive	10.07±0.05	0.00
REB 2	64.19±0.84	1.94±0.03	Positive	16.07±0.05	0.69

*Values of mean of three replications ± SD

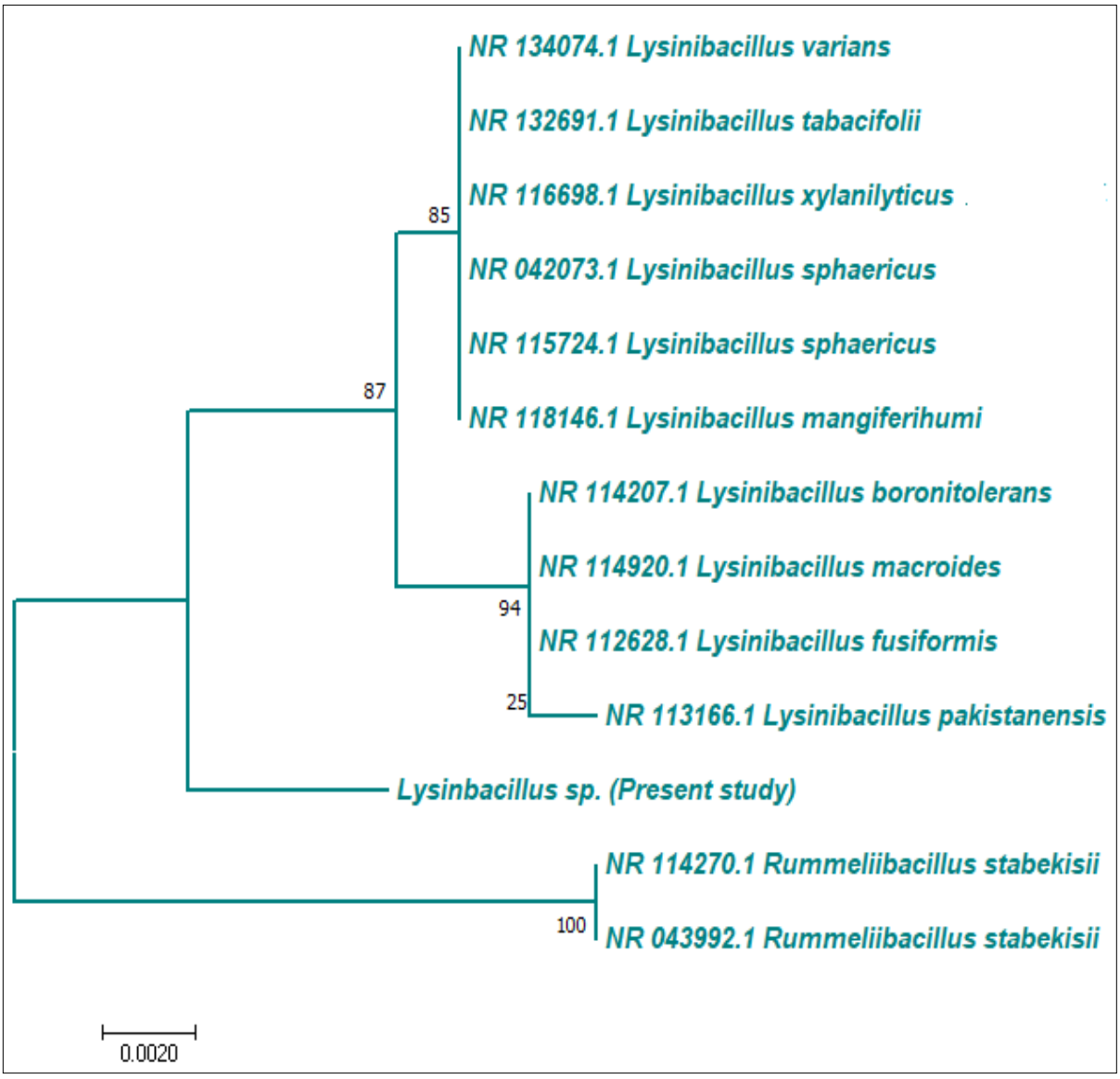

Fig 1 Phylogenetic tree indicating the relatedness of *Lysinibacillus* sp. with other close relatives

>*Lysinibacillus* sp.

TAGAGATAGGGTTTCCCCTTCGGGGACAACGGTGACAGGTGGTGCATGGTTTTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGACGATACAAAGGGTTGCCAACTCGCGAGAGGGAGCTAATCTCATAAAGTCGTTCTCAG
TTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG

Naureen *et al.* [21] had studied *Lysinibacillus sphaericus* ZA9 in greater detail and shown its potential for plant growth promotion and biotic stress management. They indicated production of higher quantity of IAA (697µg/ml), siderophore, hydrolytic enzymes and HCN. This bacterium had shown potential solubilization of phosphorus, potassium and silicon. They had shown enhanced shoot growth in tomato and cucumber due to application of this bacterium. It is also reported to produce 2-pentyl-4-quinolinecarboxylic acid and 1- methyl cyclohexene which are strong antagonist to most of the fungi.

There are several such reports indicating potential of *Lysinibacillus* sp. for plant growth promotion like phosphate solubilization and phytohormone production [22].

CONCLUSION

The isolates of endophytic bacteria viz., *Bacillus*, *Klebsiella*, *Pseudomonas*, *Azospirillum*, and *Lysinibacillus* sp. from different parts of rice plant parts were subjected to morphological and biochemical characterization and *in vitro* plant growth promoting activity were assessed for its efficiency to produce IAA, GA_3, siderophore production, and phosphate solubilization. From the studies, it could be concluded that among the different bacterial endophytes REB 2 (*Lysinibacillus* sp.) isolated from rice root was found to perform best in producing IAA, GA_3, and phosphate solubilization with better siderophore production, and hence this could be recommended as an effective plant growth promoting bioinoculant for crop plants.

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