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Isolation and identification of potential Plant growth-promoting rhizobacteria strains from groundnut root rhizosphere soil

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Abstract

The Plant growth-promoting rhizobacteria (PGPR) are bacteria, that inoculation into agricultural soil is gaining popularity since this group of bacteria can boost the yield and quality of agriculturally essential crops while also contributing to the stability of agroecosystems. The objectives of our study was to isolate, identify and characterize the PGPR traits (Siderophore production test , IAA test, Ammonia test, Phosphate solubilizing test, HCN production test and Nitrogen fixing test) from rhizosphere of Groundnut (*Arachis hypogaea* L.). The 12 isolates were isolated, out of which four (RB-1, RB-2, RB-3 and RB-5) were selected based on their PGPR traits. Based on the 16S rRNA gene sequencing the identified strains were *Enterobacter* sp. RB1, *Bacillus paramycoides* RB2, *Bacillus cereus* RB4 and *Paenibacillus* sp. RB5. The selected potential strains ability to produce high amount of IAA (19.8 to 39.9 to µg/ml) and all four bacterial strains were positive for HCN, Ammonia test, and siderophore production

Keywords: PGPR; *Enterobacter* sp. RB1; *Bacillus paramycoides* RB2; *Bacillus cereus* RB4 and *Paenibacillus* sp. RB5.

Introduction

Chemical fertilisers are often utilised across the world to provide necessary nutrients to the soil–plant system. Chemical fertiliser pricing, availability, and environmental concerns, particularly with regard to N fertilisers, are, however, serious challenges in today's agriculture. Because of surface runoff and leaching, chemical fertiliser application on sloping terrain with significant annual rainfall, like found in the Hindu Kush Himalayan region, may be ineffective. As a result, substitute actions to promise cheap crop yields, environmental care and security, and long-term ecological stability in the agro-ecosystem urgently needed. In

many regions of the world, the use of PGPR (plant growth promoting rhizobateria) to improve sustainable agricultural production is becoming more common.

PGPR is a natural alternative to artificial fertilisers since it has the potential to solubilize phosphorus, produce plant-growing hormones, and act as a biocontrol agent (**Vessey, 2003; Akhtar et al., 2012**). The rhizosphere is the soil that surrounds the root, and it is a place, where the root and its accompanying microbes have complicated interactions. Rhizobacteria are a type of bacteria that naturally populate the rhizosphere. Beneficial interactions can be maximised if soil microorganisms that boost plant growth after inoculation of seeds or roots, effectively handled. PGPR are helpful bacteria that stimulate plant growth. The PGPR investigated in many crop species (**Burr and Caesar 1983**). PGPR are a functionally varied collection of bacteria with enormous biofertilizer and biopesticide potential. PGPR belonging to *Staphylococcus, Burkholderia,, Pseudomonas, Bacillus,* and *Enterobacter* are the most common-phytate producer (**Hussin et al., 2007**). PGPR are responsible of induced systemic resistance in order to exclude the pathogen in plants (**Bais et al., 2004**). Moreover, in addition to growth promotion, many studies have described the biocontrol potential of PGPR in agriculture

Phosphorus solubilization and uptake by plants, biological nitrogen fixation, siderophores for iron sequestration, synthesis of plant hormones including IAA, gibberellins, and cytokines, and lowering ethylene levels in plants are all direct mechanisms of plant growth via PGPR (**Verma et al., 2010**). PGPR strains like *Azotobacter, Bacillus, Azospirillum,* and *Pseudomonas* have shown to improve plant development, yield, and nutritional content in a variety of crops (**Joseph et al., 2007; Mia et al., 2010**). PGPR bacteria make up about 2–5% of rhizosphere bacteria. Spore-forming bacteria (*Bacilli*) have a benefit over non-spore-forming bacteria like *Pseudomonas.* Because, spores are more robust and resistant to high temperatures and chemical concentrations. Furthermore, biological products based on bacterial spores have a shelf life of 1–3 years. One drawback of employing spores is that it takes time for a vegetative cell to return to its metabolic active state after they applied **(Kumar et al., 2012**). *Bacillus* spp. are considered harmless microbes with extraordinary abilities to synthesise a wide range of useful compounds (**Stein, 2005**). In this study, we isolate and identify the rhizobacteria from rhizome of Groundnut (*Arachis hypogaea* L.)

Materials and Methods

Isolation and Identification of Bacterial Isolates

Groundnut (*Arachis hypogaea* L.) plant samples were collected from groundnut agriculture field, keeranur village, Pudukkottai district, Tamil Nadu, India along with bulk rhizospheric soil samples. This is located between 10.55°N latitude and 78.78°E longitude. The collected soil samples kept in plastic bags, brought to the laboratory and stored in refrigerator for further analysis. By serial dilution plating on Luria–Bertani (LB) agar plates, rhizospheric bacteria were recovered from 1 g soil securely binding to the root (**Somasegaran and Hoben, 1994**). The plates were incubated at 48 hrs at 28±2°C until colonies appeared. Individual colonies were sub cultured and preserved provisionally in 20% glycerol solution at -80°C.

Morphological Characterization

Colony morphology, color, shape and growth pattern observed, after 24 hrs of growth on nutrient agar plates at 28±2°C. The Gram reaction done and as described by **Agbodjato *et al.* (2015).**

Tests for Confirmation of PGPR traits

IAA (Indole acetic acid) Production

Isolated bacterial cultures inoculated in 500-µg tryptophan mL^{-1} enriched nutrient broth and PDB and incubated at 14 days at 27ºC. One ml of culture broth was taken from 20 ml incubating culture broth from every 2 days from the 2^{nd} day to the 10^{th} day and centrifuged at 6000 rpm for 30 min. The concentration of IAA was determined according to **Bric *et al.* (1991)** method. In this experiment, 1ml of the culture supernatant was taken in a test tube and addition with 1 drop of ortho phosphoric acid and 2 ml of Salkowski's reagent. The development of pink colour shown IAA formation, and the amount of IAA was evaluated using a spectrophotometric approach at 530 nm. A pure IAA calibration curve was used as a standard to determine the concentration of IAA in culture.

Siderophore Production

The bacterial isolates were cultured in nutritional broth at room temperature for 72 hours on a rotary shaker, then centrifuged at 10000 rpm for 10 minutes and the supernatant was collected. The Arnow test was used to determine the siderophore content. 0.5 ml of the sample, 0.5 ml of 0.5 N HCl (reagent A), 0.5 ml of 10 g sodium nitrite and 10 g sodium molybdate in a final volume of 100 ml (reagent B), and 0.5 ml of 1N NaOH in a final volume of 100 ml (reagent C). A standard curve was prepared using catechol. The assay performed quickly and the sample mixed thoroughly after addition of each reagent. Catechol-containing samples were pink in colour. Absorbance measured at 515 nm **(Arnow, 1937).**

Phosphate Solubilization Activity

The isolated strains plating in the agar comprising precipitated tricalcium phosphate **(Qureshi *et al.*, 2012).** The modified medium of Pikovskaya consist of 10 g glucose, 0.1 g $MgSO_4.7H_2O$, trace amount of

$MnSO_4$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, , 5 g tribasic phosphate and $FeSO_4$, 0.5 g yeast extract, 15 g agar and in 1L distilled water. The cultures streaked on the surface of replicated agar plates. After 96 hrs of incubation, bacterial zone formed around the inoculated area positive test for phosphate solubilization. Based on the diameter of clearance halo zones, solubilization efficiency (SE) and solubilization index (SI) were evaluated using the following formulas

$$\text{Solubilization Efficiency} = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \text{ X } 100$$

$$\text{Solubilization Index} = \frac{\text{Solubilization diameter}}{\text{Colony diameter}}$$

Hydrogen Cyanide Production test

The **Lorck (1948)** method used to screen the hydrogen cyanide generation of isolated bacterial strains. Briefly, bacteria streaked on modified agar plate after nutrient broth was adjusted with 4.4 g glycine/L. In the top of the plate, a Whatman No.1 filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution inserted. Parafilm was used to seal the plates, which were then incubated at 28±2ºC for 96 hrs. HCN production was indicated by the colour changing from orange to red.

Ammonia Production

In peptone water, bacterial isolates were evaluated for the generation of ammonia. In each tube, fresh cultures inoculated in 10 ml peptone water and incubated for 72 hrs at 28±2ºC. Each tube addition with 0.5 mL of Nessler's reagent. Change of brown to yellow color was a positive test for ammonia production **(Cappuccino and Sherman, 1992).**

Nitrogen Fixation Activity

The bacterial strains were subjected to screening of nitrogen fixation activity using glucose nitrogen free minimal medium (G-NFMM), single colony grown on nitrogen free medium was taken and inoculated into G-NFMM containing BTB (Bromothymol blue solution). After one-week incubation, appearance of blue green color change from green color indicated that the isolate had nitrogen-fixing activity **(Lwin *et al*., 2012).**

Biochemical tests

Based on the screening results potential rhizobacteria were selected for a sequence of biochemical tests to distinguish the isolated strains using the standards of **Bergey et al., (1994**).

Molecular identification

Isolation of genomic DNA from selected isolates

In order to extract the DNA from the isolates were inoculated in 5 ml of LB broth, after incubation the well grown culture broth was centrifuged at 13000 rpm for 15 min to pellet down the bacterial cell. Re-suspend the pellet in 500 µl of DNA isolation solution 1 and add 50 µl of lysozyme. Incubate at 37ºC for 15 min. Then add 50 µl of 10% SDS and 5 µl of proteinase K and incubate at 65ºC for 30 min. Add 40 µl of 5M NaCl and 32 µl of CTAB NaCl incubated at 65ºC for 30 min. Then add equal volume of freshly prepared chloroform:

isoamyl alcohol (~677 µl). The isolated DNA was resolved in 1% agarose gel stained with ethidium bromide and their molecular weight was identified using the 100 bp ladder. The electrophoresed gel was visualized under UV- Trans illuminator **(Sambrook et al., 1989).**

16S rRNA Amplification and Phylogenetic Tree Analysis

The potential PGPR was identified by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the specific primers 8F-5'AGAGTTTGATCCTGGCTCAG'3 and 1492R- 5'TACGGCTACCTTGTTACGACTT 3'(**Kumar et al., 2018**). Single reaction of PCR contained 15 µl of master mixer, 1 µl (50 pmol) of each primer, 2 µl (50 ng) of isolated genomic DNA and 15 µl of Milli Q water. The thermal cycler (Applied Biosystems, USA) was used and reactions were carried out under the following conditions. Initial denaturation of 5 min at 94ºC, followed by 32 cycles of denaturation in 1 min at 94ºC, annealing 1 min at 55ºC and 1 min at 72ºC of extension and finally 5 min at 72ºC of final extension for 16S rRNA gene amplification. The resulting PCR product was separated on 1.2% agarose (Himedia, India).As a marker DNA, a typical molecular marker of 1 kb ladder was utilised. Following the completion of the run, the successfully amplified regions purified and sequenced by Genurem Biosciences LLP. A BLAST search was used to find the most closely similar sequence. Finally, the CLUSTALW tool was used to align various sequences. A phylogenetic tree was built using the maximum likelihood method, and tree topologies were assessed using MEGA 6.0 and a bootstrap analysis of 1000 data sets, before being uploaded to the GenBank database.

Results and Discussion

Isolation of soil microbes

Based on the morphology of the microorganisms, grown in Nutrient agar, Casein starch agar and Potato dextrose agar (**Figure 1**). Totally 12 different microorganisms were isolated from plates, in that five bacterial strains, six actinomycetes and one fungal strain (**Figure 2**). All the microorganisms examine to evaluate their PGPR characteristics.

Screening of potential PGPR strains

The Plant Growth Promoting Traits was confirmed following assays: Siderophore production test, IAA test, Ammonia test, Phosphate solubilizing test, HCN production test and Nitrogen fixing test.

Siderophore production test

Iron is required for the growth of all existing cells. The bioavailability of iron on plant root surfaces or in the soil encourages soil microbes to compete (Persmark et al. 1990). The role of bacterial siderophores was encouraging plant growth by increasing iron availability in the rhizosphere was described by researchers (**Kloepper et al. 1989**). The isolated strains were evaluated for the production siderophores was quantitatively by Arnow assay. In our study, this assay results revealed that the bacterial strains such as RB1, RB2, RB4 and RB5 had ability to produce the siderophores. The highest amount of siderophores were produced by RB2 and RB5 (**Figure 3**).

Indole acetic acid test

The IAA thought to be the most significant auxin that regulates plant growth and development. Tryptophan, an amino acid, played a key part in rhizobacteria's synthesis of IAA (**De La Torre-Ruiz et al. 2016**). RB1, RB2, RB4 and RB5 isolates were showed the positive IAA production property and great variation was observed (Table 1). All the isolates utilized tryptophan as a precursor for their growth and produced IAA. The maximum of RB5 isolate showed 39.9 µg/ml of the IAA in 10^{th} day and followed by RB4 showed 24.9 µg/ml, RB1 showed 19.8 µg/ml and RB2 by 16.06 µg/ml of IAA. Other isolates showed no IAA production (**Figure 4**). According to **Barazani and Friedmann** (1999) value of IAA exceeded above 13.5 $\mu g.ml^{-1}$, which confirms that it is deliberated PGPR. So, in our study results confirms that all the four stains are PGPR, because these strains ability to produce more than 13.5 µg/ml Crop development and yield are boosted by strains that produce a lot of IAA and acetamide indole in the soil. Plants are believed to be primarily reliant on exogenous sources of phytohormones, particularly those generated by bacteria, when faced with specific restrictions. Root exudates are a natural source of L-tryptophan for rhizosphere bacteria. Production is influenced by species, culture conditions, growth phase, and substrate availability, in addition to the tryptophan dose (**Silini - Cherif et al., 2016**).

HCN production test

HCN production was thought to serve a role in plant growth promotion by inhibiting plant diseases at first (**Voisard et al., 1989**). This notion, however, has recently evolved. HCN synthesis is thought to boost phosphorus availability indirectly by chelating and sequestering metals, as well as indirectly increasing nutrient availability to rhizobacteria and host plants (**Rijavec & Lapanje, 2016**). Because PGPR produce HCN regardless of genus, they can be oemployed as biofertilizers or biocontrols to boost crop production. In our study, Out of 12 isolates, the RB1, RB2, RB4 and RB5 isolates were found positive for HCN production (**Figure 5)**. Among the isolates, RB5 was potent isolate for the production of HCN **(Table 1).**

Ammonia test

The formation of ammonia (NH_3) by PGPR is a key trait that indirectly boosts plant growth. It helps plants grow by accumulating and supplying nitrogen to their hosts (**Kumar et al., 2016**). Various investigations have shown that rhizobacteria produce ammonia (**Hyder et al., 2020**). In this study, all bacterial isolated strains were tested for the production of ammonia in peptone water. In that, all the bacterial strains (RB1, RB2, RB3, RB4 and RB5) were showed positive for ammonia production, remaining isolates shown negative results (**Table 1**).

Biochemical tests for selected PGPR

Based on the selection of plant growth promoting (PGP) traits, rhizobacterial strains RB1, RB2, RB4 and RB5 are as having the potential PGP activity. These four potential rhizobacterial strains were taken further for their morphological identification. PGPR mainly belong to the genera *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Clostridium* and *Serratias* (**Gupta et al., 2015**). In our study, based on the morphological results have revealed that the selected PGPR strains were belonging to the

genus *Enterobacter* sp., *Bacillus* sp., *Bacillus cereus* and *Paenibacillus* sp.. According to **Garbeva et al. (2003)**, the bulk of soil gram-positive bacteria (morethan 90%) are members of the *Bacillus* genus resembling to *Paenibacillus*.. *Bacillus* sp. is the common genus in the rhizosphere, and *Bacillus* sp. are well-adapted to a variety of environmental circumstances thanks to their genetic and metabolic diversity (**Saxena et al., 2020**). In this study results confirmed that, all isolates are motile, rod shaped and reacted gram positive and negative respectively for gram staining. RB1 and RB4 isolates reacted positive for VP test, Citrate test and followed by RB2 showed positive for Methyl red test and VP test and finally the RB5 showed positive for Methyl red test, VP test and Oxidase test. Other biochemical tests were reacted negatively for all the isolates (**Table 2**).

Molecular characterization of Rhizobacterial strains

The taxonomy of the rhizobacterial strains was determined using molecular and phylogenetic analysis. 16S rRNA gene sequencing was used to identify bacteria from soil rhizosphere fractions. This technique offers a culture-independent method for tracking dominant bacterial populations in soil **(Sultana et al., 2020).** Genomic DNA was isolated from the bacterial isolates and validated using a 1% agarose gel (Figure 6). Using 8F and 1492R primers, the 16s rRNA gene of the bacterial isolate was amplified, and the amplified product was observed at 1500 bp in a 1.2 % agarose gel using genomic DNA as a template. The 16S rRNA gene amplified product acquired from Rhizobacterial strains were shown in **Figure 7**. The similarity searching of sequences in nBLAST results shown that, four Rhizobacterial strains belonging to the 3 different genera: *Enterobacter*, *Bacillus* and *Paenibacillus*. Similarly to our study, *Bacillus* and *Enterobacter* strains were isolated from Rice (**Sherpa et al., 2021**). Phylogenetic trees built from 16S rRNA sequences, which exhibited that the selected isolates are members of genus *Bacillus*, *Enterobacter*, and *Paenibacillus*. The amplified 16s gene sequence were, after sequencing to deposited in GenBank on following accession numbers OM362912, OM392020, OM439625 and OM442896 (**Table 3**).

Phylogenetic analysis for Rhizobacterial strains

The rhizobacterial sequences *Enterobacter* sp. strain RB1, *Bacillus paramycoides* strain RB2, *Bacillus cereus* strain RB4 and *Paenibacillus* sp. strain RB5 were aligned with closely related sequences from NCBI, which shows above 98 % similarity with other retrieved sequences. The maximum likelihood technique was used to infer the evolutionary relationship among the organisms. The ideal tree is displayed, with the total of branch lengths equal to 0.05. The branch lengths are in the same units as the evolutionary distances used to estimate the phylogenetic tree, and the tree is rendered to scale. The analysis involved 23 nucleotide sequences. Gaps and missing data were removed from all positions. The phylogenetic tree results showed having one major and two minor phylogenetic clades. The first clade consist of clusters of *Bacillus* genus with different species and matching percentages. In first clade contains the *Bacillus paramycoides* strain RB2 (OM392020) closely related with the strain *B.tropicus* (OM281423) and ***Bacillus cereus* strain RB4** (OM439625) closely related with the strain *B.cereus* (KF624695). The second clade consist of clusters of ***Paenibacillus*** genus with different species and matching percentages. In second clade contains the *Paenibacillus sp.* strain RB5 (OM442896) closely related with the strain *Paenibacillus* sp. (MF403053).

Finally, the third clade consist of clusters of *Enterobacter* genus and mixed kind of species. In third clade contains the *Enterobacter* sp. strain RB1 (OM362912) is closely related with the strain *E. cloacae* (KF228926) (**Table 3**). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (**Figure 8**).

Conclusions

In the current study, isolation, identification and characterization of PGPR from groundnut plant rhizosphere studied. According to the findings of this study, out of twelve, four bacterial strains found positive for HCN, Indole-3-acetic acid (IAA), Ammonia test, and siderophores production. The identified rhizobacterial strains revealed 98 to 100% identity with close similarity and belonging to *Bacillus*, *Enterobacter* and *Paenibacillus* genera, according to 16S rRNA sequence analysis.

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Figure legends

Figure 1: Isolated strains from groundnut rhizosphere soil

Figure 2: Purified microbial colony strains isolated from groundnut rhizosphere soil

Figure 3: Siderophore production of RB1, RB2, RB4 and RB5

Figure 4: Quantification of IAA production of RB1, RB2, RB4 and RB5

Figure 5: HCN production of RB1, RB2, RB4 and RB5

Figure 6: Isolation of genomic DNA from selected rhizobacterial strains

Figure 7: PCR 16 s rRNA amplified products of rhizobacterial strains

Figure 8: Phylogenetic analysis of 16s rRNA gene for RB1, RB2, RB4 and RB5 strains

Figure 1

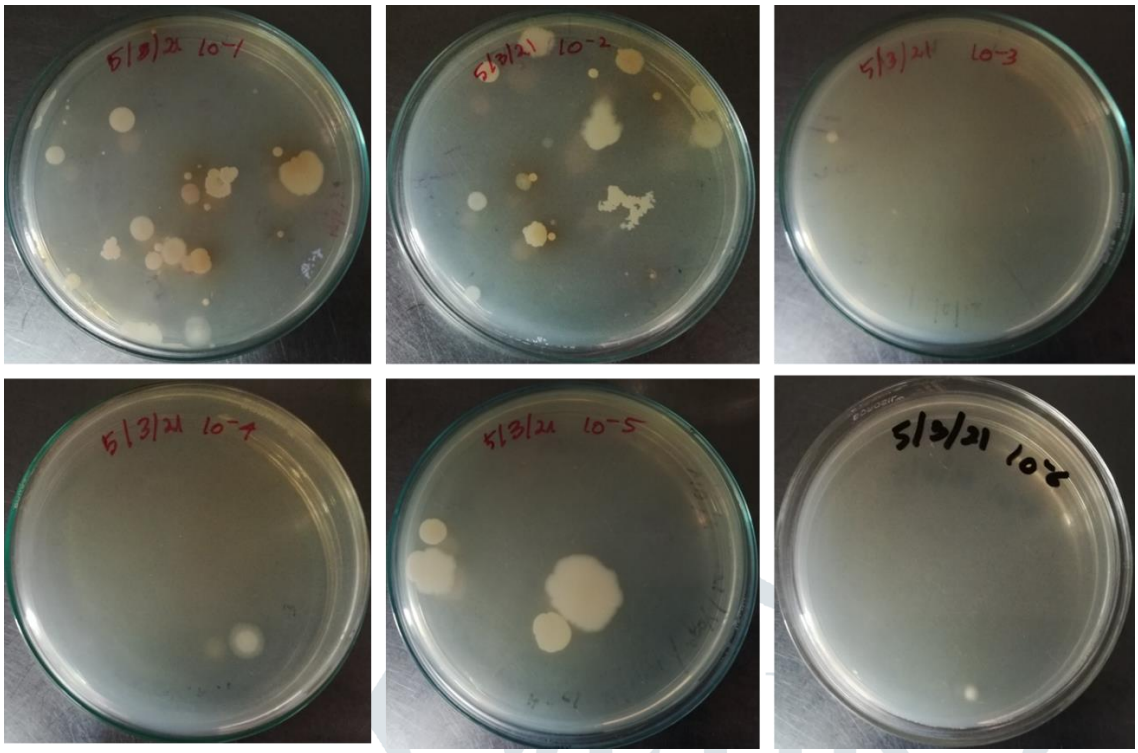


Figure 2

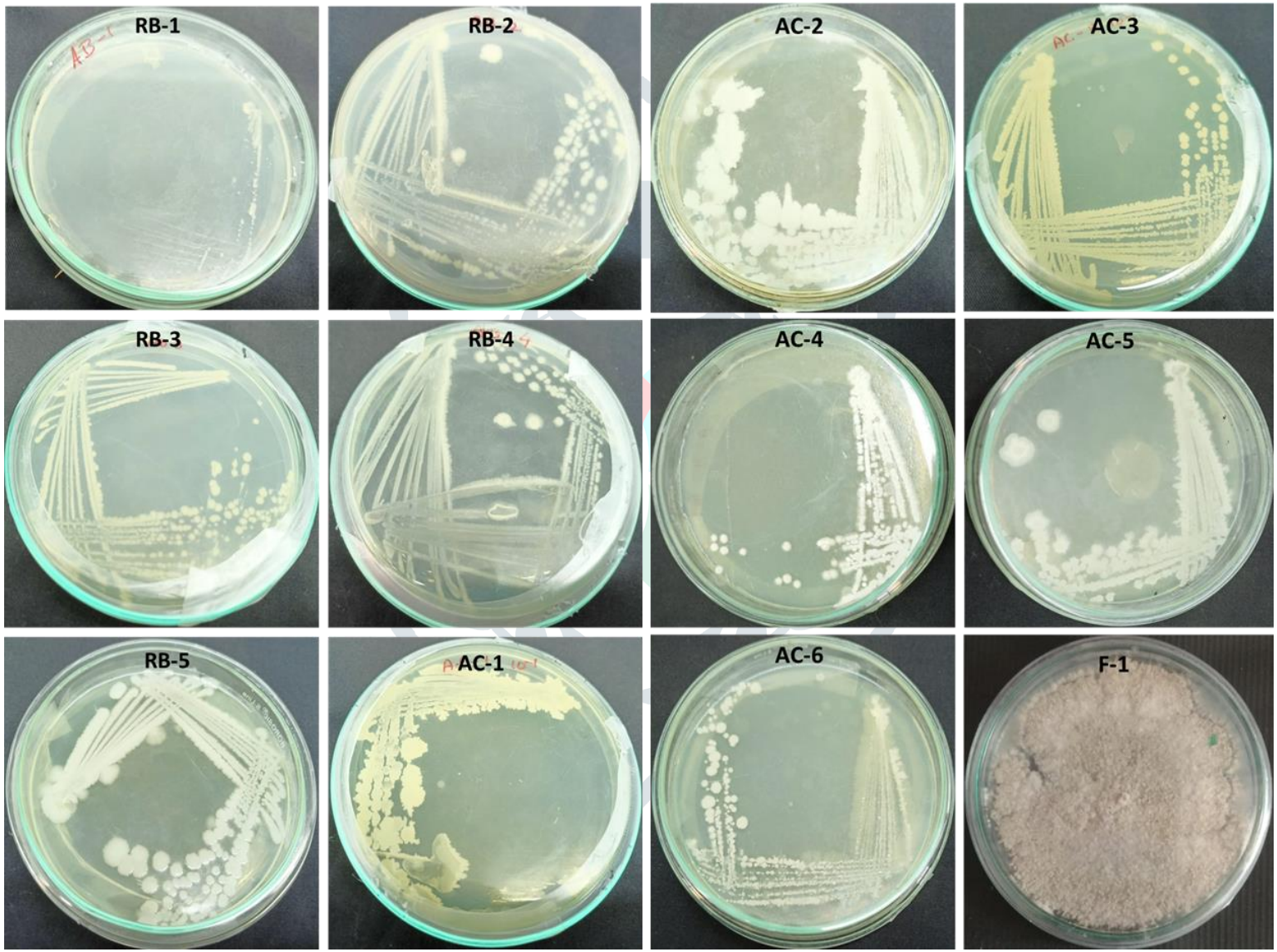


Figure 3

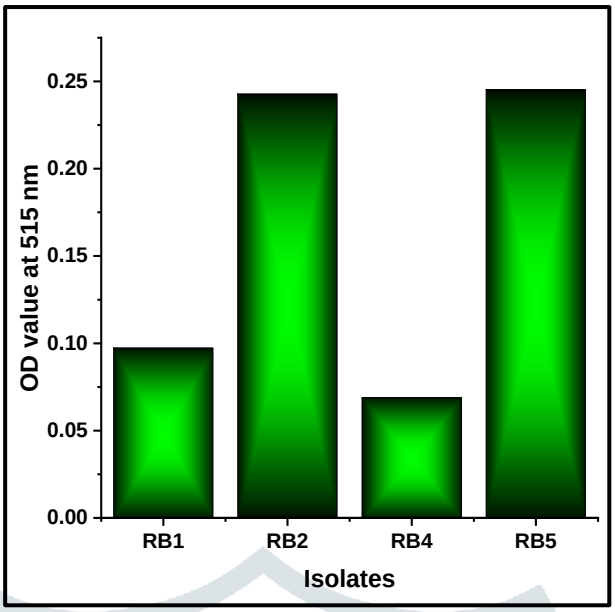


Figure 4

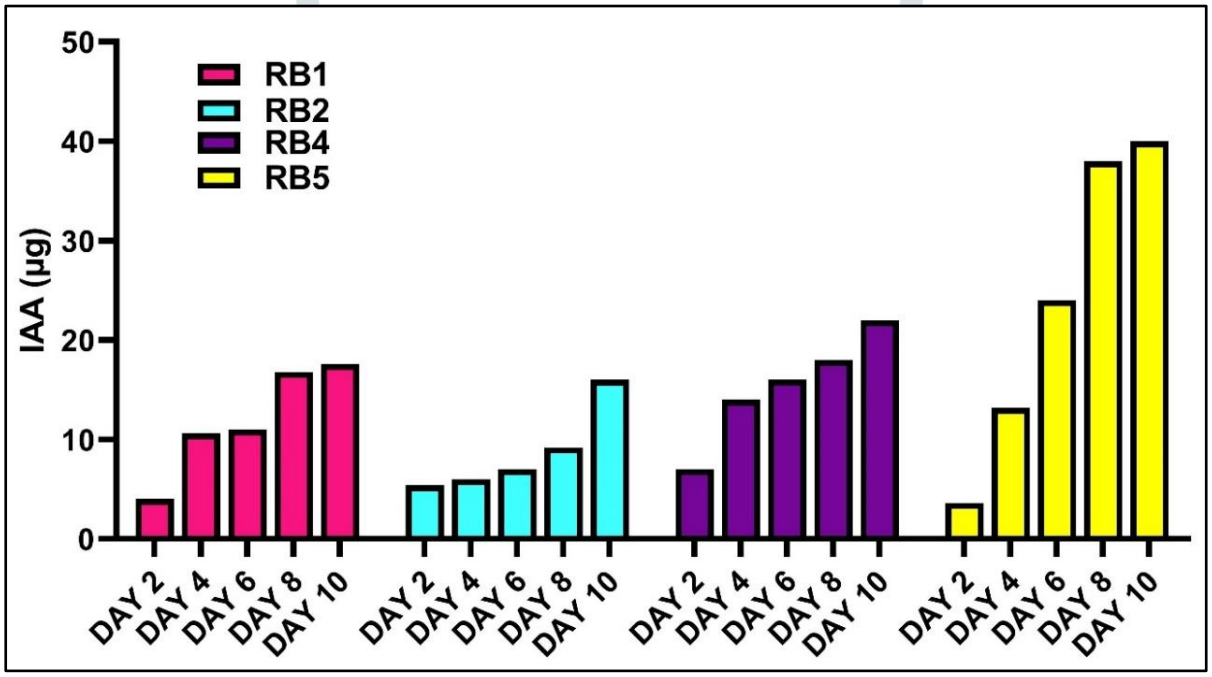


Figure 5

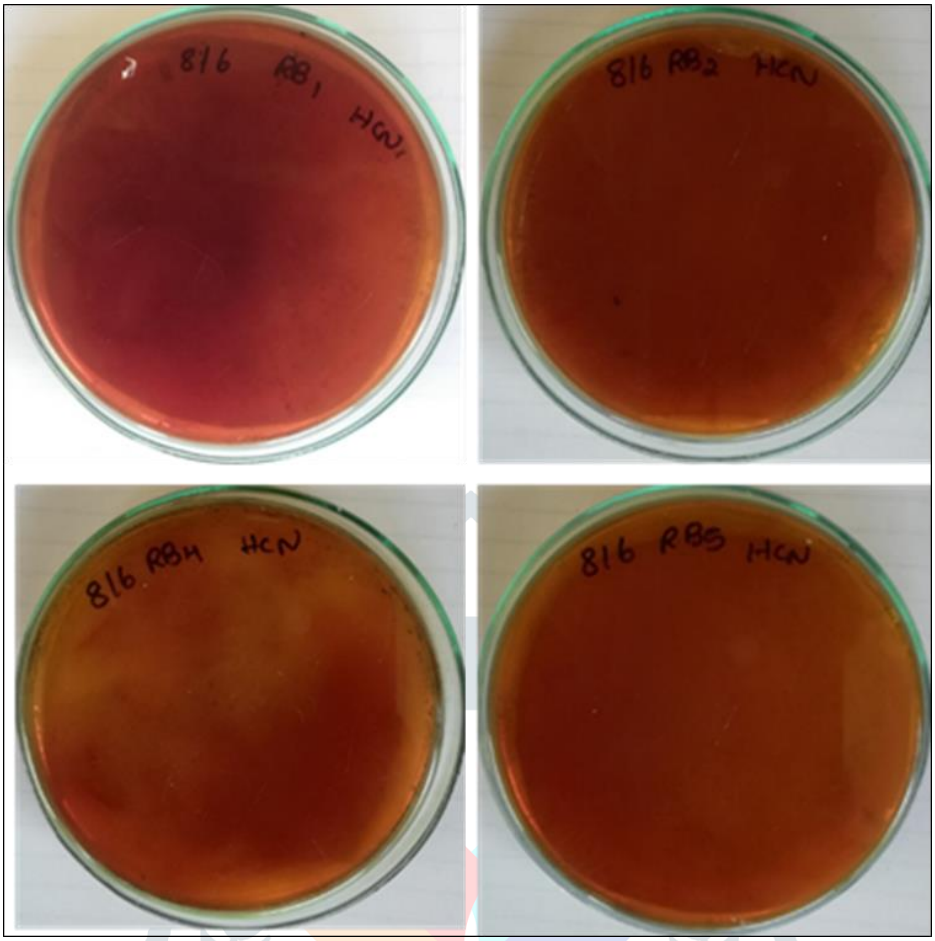


Figure 6

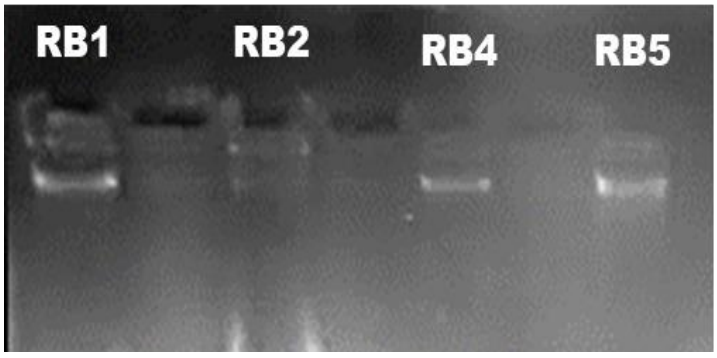


Figure 7

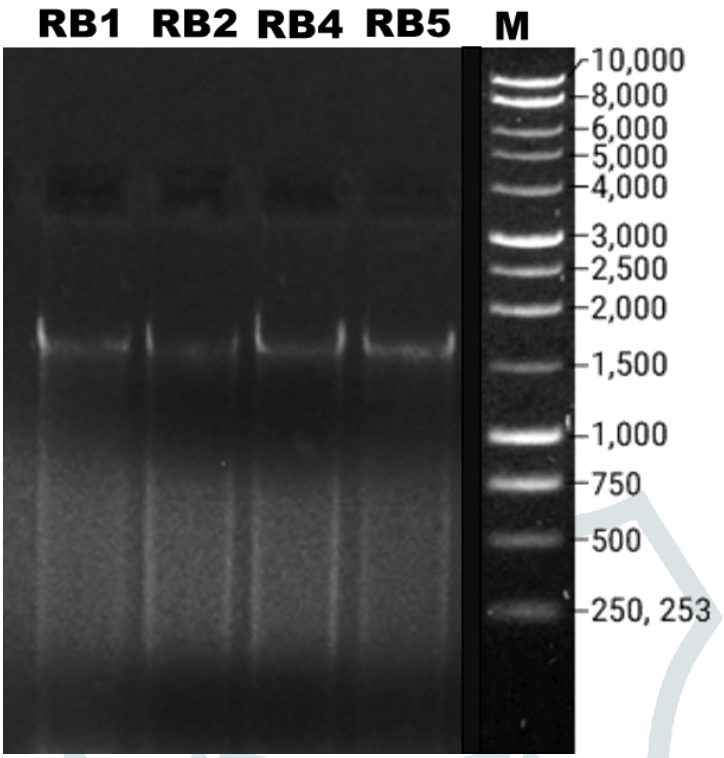
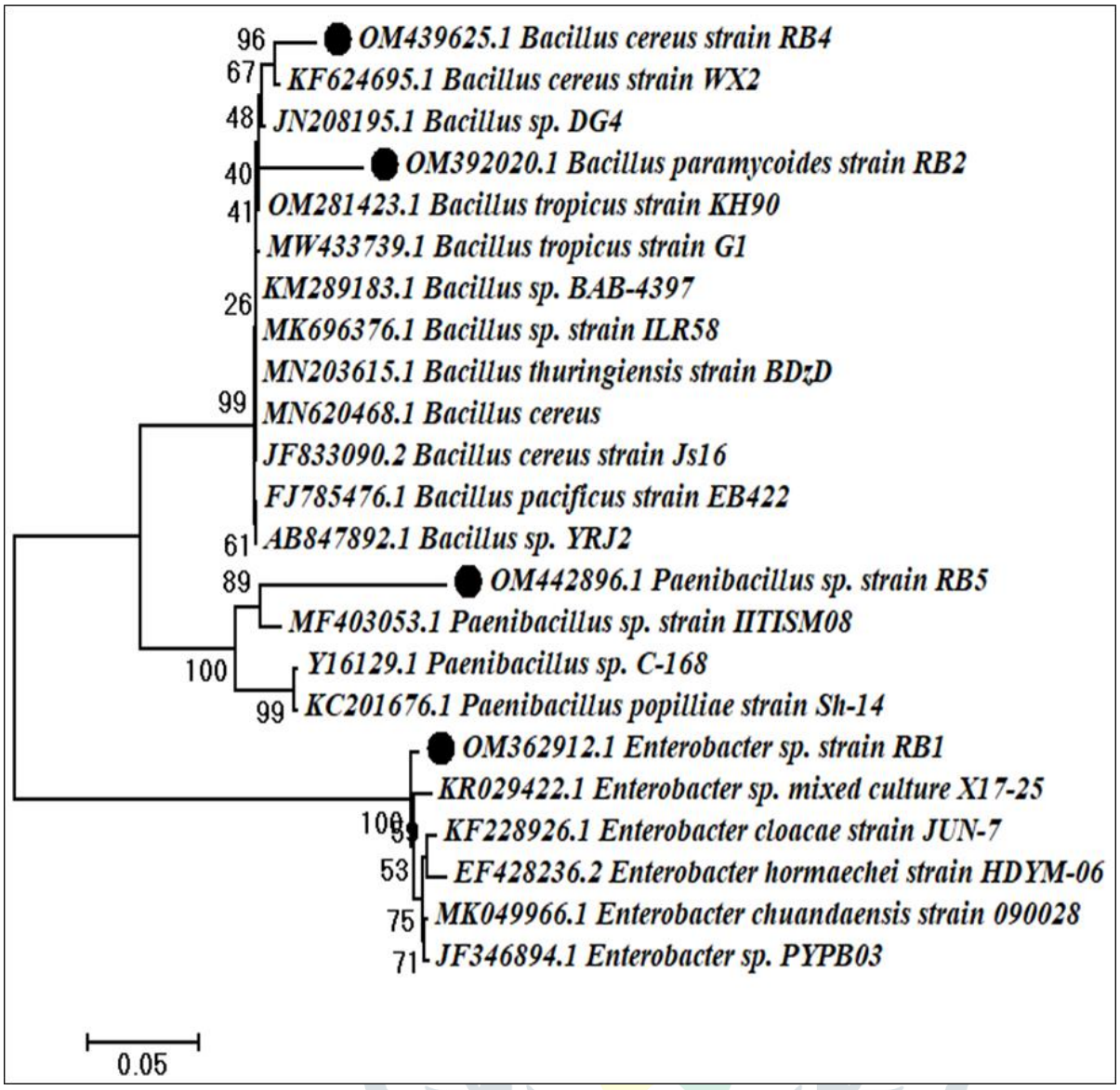

RB1
RB2
RB4
RB5
M
10,000
8,000
6,000
5,000
4,000
3,000
2,500
2,000
1,500
1,000
750
500
250, 253

Figure 8


96
67
48
40
41
26
99
61
89
100
99
100
53
75
71
OM439625.1 Bacillus cereus strain RB4
KF624695.1 Bacillus cereus strain WX2
JN208195.1 Bacillus sp. DG4
OM392020.1 Bacillus paramycoides strain RB2
OM281423.1 Bacillus tropicus strain KH90
MW433739.1 Bacillus tropicus strain G1
KM289183.1 Bacillus sp. BAB-4397
MK696376.1 Bacillus sp. strain ILR58
MN203615.1 Bacillus thuringiensis strain BDzD
MN620468.1 Bacillus cereus
JF833090.2 Bacillus cereus strain Js16
FJ785476.1 Bacillus pacificus strain EB422
AB847892.1 Bacillus sp. YRJ2
OM442896.1 Paenibacillus sp. strain RB5
MF403053.1 Paenibacillus sp. strain IITISM08
Y16129.1 Paenibacillus sp. C-168
KC201676.1 Paenibacillus popilliae strain Sh-14
OM362912.1 Enterobacter sp. strain RB1
KR029422.1 Enterobacter sp. mixed culture X17-25
KF228926.1 Enterobacter cloacae strain JUN-7
EF428236.2 Enterobacter hormaechei strain HDYM-06
MK049966.1 Enterobacter chuandaensis strain 090028
JF346894.1 Enterobacter sp. PYPB03
0.05

Tables

Table 1: Selected plant growth promoting characteristics of isolates

Strain name	Siderophore production test	IAA test	Ammonia test	Phosphate solubilizing test	HCN production test	Nitrogen fixing test
RB1	+	++	+	-	+	-
RB2	+++	+	+	-	+	-
RB3	-	-	+	-	-	-
RB4	+	++	+	-	+	-
RB5	+++	+++	+	-	+	-
AC1	-	-	-	-	-	-
AC2	-	-	-	-	-	-
AC3	-	-	-	-	-	-
AC4	-	-	-	-	-	-
AC5	-	-	-	-	-	-
AC6	-	-	-	-	-	-
F1	-	-	-	-	-	-

+ indicates the presence of trait; ++ indicates the presence of medium level; +++ indicates the presence of high level; - indicates absence of traits

Table 2: Gram staining, growth characteristics and biochemical characteristics of Rhizosphere isolates

S.No	Parameters	RB1	RB2	RB4	RB5
1	**Colour**	White	Off-white	Off-white	Milky white
2	**Configuration**	Circular	Circular	Circular	Circular
3	**Pigmentation**	-	-	-	-
4	**Growth**	Good	Good	Good	Good
5	**Simple staining**	Rod	Rod	Rod	Rod
6	**Gram straining**	Negative	Positive	Positive	Positive
7	**Endospore staining**	Negative	Positive	Positive	Positive
8	**Indole test**	Negative	Negative	Negative	Negative
9	**Methyl red test**	Negative	Positive	Negative	Positive
10	**VP test**	Positive	Positive	Positive	Positive
11	**Citrate test**	Positive	Negative	Positive	Negative
12	**Oxidase test**	Negative	Negative	Negative	Positive

Table 3: Molecular identification of rhizobacteria strains isolated from groundnut root soil

S.No	Rhizobacterial names	Isolates	Accession No	Closest sp. (% homology)	Accession number (closely related organism)
1	*Enterobacter* sp.	RB1	OM362912	98.47%	KF228926 (*E. cloacae*)
2	*Bacillus paramycoides*	RB2	OM392020	95.76%	OM281423 (*B.tropicus*)
3	*Bacillus cereus*	RB4	OM439625	97.52%	KF624695 (*B.cereus*)
4	*Paenibacillus* sp.	RB5	OM442896	89.23%	MF403053 (*Paenibacillus* sp.)