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Isolation, Characterization and Evaluation of PGPRs for their Potential to Improve Growth Parameters in *Vigna radiata*

UPASANA SAHU, RICHA VAISHNAV, RENU[1], S. R. MOHANTY[2], SWATI MOHAPATRA, AJIT VARMA AND SWATI TRIPATHI*

Amity Institute of Microbial Technology, Amity University, Noida-201 313 (Uttar Pradesh), India
**(e-mail: swatitri@gmail.com; stripathi2@amity.edu; Mobile: 77479 58222)*

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ABSTRACT

Sustainable organic farming to fulfil the requirements of food for the growing population across the globe as well as the growing concerns of impact of chemical fertilizers and climate change need to be addressed using Plant Growth Promoting Rhizobacteria (PGPR) to maintain soil health and enhance crop productivity. Rhizospheric soil was collected from the upland rice field of Raigarh village in Pusaur dist. Raipur, Chhattisgarh. A total of 146 isolates obtained were screened qualitatively and quantitatively for plant growth promoting (PGP) activities like phosphate, potassium and zinc solubilization, IAA production, siderophore production, ammonia production and nitrate reduction. The isolates were further subjected to biochemical and molecular characterization for the identification of potential PGPRs. Ninty three out of 146 isolates, were found positive in both qualitative and quantitative PGP characteristics. The range of insoluble phosphate solubilization varied from 19 to 60 µg/ml. Siderophore production of bacterial isolates ranged from 12 to 31% and the IAA ranged from 10 to 54 µg/ml. The potential isolates RIGA-65, RIGB-93, RIGB-130 and RIGB-146 were proved to be efficient in seed germination and pot experiments. Among four isolates, *Bacillus subtilis* strain (RIGA-65) was found to be the most potent PGPR with maximum insoluble phosphate solubilization (60 µg/ml), siderophore production (31%) and IAA production (54 µg/ml). The highest germination was also recorded in *Bacillus subtilis* strain RIGA-65 (97%) followed by RIGB-93 (83%), RIGB-130 (81%) and RIGB-146 (22%) as compared to the untreated control (22%).

Key words: Rhizosphere, PGPR, phosphate solubilization, potassium solubilization, IAA production, siderophore production

INTRODUCTION

The microbe-plant interaction in the root rhizosphere niches can be beneficial, neutral, or harmful to plant growth (Yuan *et al.*, 2018). PGPRs can affect plant growth either directly or indirectly (Chrouqi *et al.,* 2017; Prasad *et al.*, 2019). The direct promotion of plant growth by PGPR is because of the production of plant growth regulators (Cassán *et al.*, 2014), and enhanced availability of nutrients to the host plant by the production of siderophores, solubilizing phosphate, and atmospheric nitrogen fixation (Goswami *et al.*, 2016; Kalayu, 2019). Several studies have demonstrated the potential of rhizobacteria to synthesize auxins *in vitro* (Jha and Saraf, 2015; Lebrazi *et al.*, 2020). PGPR's increases available phosphorus for the plant through the production of organic acids (Israr *et al.*, 2016; Bechtaoui *et al.,* 2020). They also play a significant role in producing siderophores, which enhance the availability of Fe to higher plants (Saha *et al.,* 2016; Riaz *et al.*, 2021). The indirect promotion of plant growth occurs when PGPR reduces or prevents the deleterious effects of one or more phytopathogenic organisms (Kumar *et al.*, 2015; Das *et al.*, 2023).

It is an established fact that rhizobia fix nitrogen and play a crucial role in improving plant growth (Mabrouk *et al.*, 2018) and yield of legume crops (Stambulska *et al.*, 2018). Due to a variety of growth-enhancing mechanisms like additional infection sites by PGPRs that is later occupied by rhizobium, the production of antibiosis in the rhizosphere and production

[1]ICAR-National Bureau of Agriculturally Important Microorganism, Kushmaur, Mau Nath Bhanjan-275 103 (Uttar Pradesh), India.
[2]ICAR-Indian Institute of Soil Science, Nabibagh, Berasia Road, Bhopal-462 038 (Madhya Pradesh), India.

of siderophores, which chelate insoluble cations or colonize root surfaces, it is highly likely that the interactive effect of rhizobia and free-living soil bacteria could be more beneficial (Burghardt, 2020; Djebaili *et al.*, 2020). Few studies have indicated that inoculating seed with mixed cultures has a significant positive effect on plant growth as compared to inoculating single strains (Akinrinlola *et al.*, 2018; Djebaili *et al.*, 2020). Unfortunately, rhizobacteria that stimulate plant growth directly have received lesser attention than biocontrol rhizobacteria. Therefore, this study aimed at evaluating the capability of the isolated bacteria for plant growth promoting traits, the biochemical and molecular characterization and their evaluation for enhancing growth parameters and yield productivity in mungbean crop.

MATERIALS AND METHODS

Rhizospheric soil samples were collected in triplicates from five sites of the upland rice field of Raigarh village Pusaur dist. Raipur, Chhattisgarh, India [21°48'14.01" N; 83°22'14.46" E] and kept in a polythene bag at 4°C till further processing.

The rhizobacterial strains were isolated on different media like Yeast Mannitol Agar (YEMA), Pseudomonas Isolation Agar (PIA), Actinomycetes Isolation Agar (AIA), Jenson's media (JM), Pikovskaya's Agar (PKV) and Nutrient Agar (NA) (HiMedia Ltd., India). Plates were incubated at 37±1 °C for 24-48 h, colonies were isolated, purified by repeated streaking, coded, and examined to study the colony morphology and Gram's character. Colony Forming Units (CFU) were calculated per gram of dry soil. The isolates were maintained on NA slants and preserved at 4°C.

The isolates were assessed for IMViC tests, urease, amylase, protease and starch hydrolysis activities by the standard procedures. The genomic DNA of rhizobacterial strains was extracted from 24 h old pure culture using a DNA extraction kit (Wizard®, Promega, USA) and kept at -20°C for sequencing. Molecular identification of the bacterial isolates was done by amplifying 16S rRNA using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACG GCTACCTTGTTACGA-3'). Multiple Sequence Alignment was performed with the ClustalX (1.83). Thompson e, and Mega 5 software was used to construct a phylogenetic tree using the neighbour-joining (NJ) DNA distance algorithm according to the standard method. Bootstrap analysis of NJ data sets was used to evaluate the resulting tree topologies. The sequence data of all the isolates were analyzed and submitted to the NCBI GenBank sequence database.

The rhizobacterial isolates were screened for qualitative and quantitative phosphate solubilization on PKV agar and National Botanical Research Institute's Phosphate (NBRIP) broth containing 0.5% tricalcium phosphate (TCP) with slight modifications. Cultures were inoculated and incubated at 35±2 °C for 3 to 7 days, to find a distinct clear zone around the colony. The Phosphate Solubilization Index (PSI) was measured using the formula:

$$PSI = (\text{Diameter of the clear zone}) / (\text{Diameter of the colony})$$

For quantitative estimation, flasks containing 50 ml NBRIP media were inoculated in triplicates with 500 µl bacterial culture and incubated in an incubator shaker at 30±2°C at 180 rpm for 7 to 10 days along with uninoculated control. The cultures were harvested by centrifugation at 10,000 rpm for 10 min. Phosphorus concentration in the supernatant was determined using the vanadate-molybdate method at 430 nm after 10 min. The total soluble phosphorus was calculated from the regression equation of the standard curve and expressed as µg/ml over the control.

IAA production was detected in Luria Broth (LB) supplemented with and without filter sterilized solution of 100 mg/ml L-tryptophan at 35±2°C for 48 h according to the standard protocol. The medium was inoculated with 0.1 ml bacterial cultures adjusted to an optical density (OD) of 0.5 measured at 660 nm. The fully grown cultures were centrifuged at 8000 rpm for 10 min. The supernatant and Salkowski reagent were mixed in a 1:2 ratio and two drops of orthophosphoric acid. The pink colour indicated a positive result for IAA production and OD was measured at 530 nm. Concentrations of IAA production by the rhizobacterial cultures were estimated using IAA standard in mg/ml.

Freshly grown cultures were inoculated in 10 ml peptone water and incubated for 48-72 h at

37±2°C. Nessler's reagent (0.5 ml) was added to each tube. The development of a brown-to-yellow colour indicated a positive test for ammonia production.

Rhizobacterial cultures were screened for siderophore production by using spectrophotometry according to the standard protocol which was further confirmed by the CAS agar method and Universal Chemical Assay (CAS). Siderophore production was indicated by orange halos around the colonies after the incubation at 28°C. The quantitative estimation was done by CAS shuttle assay. The rhizobacterial strains were grown on a Succinate medium and incubated for 24-30 h at 37°C at 120 rpm. After incubation, the cultures were centrifuged at 10,000 rpm at 4°C for 10 min and 0.5 ml CAS solution was added to the cell-free supernatant. The blue colour observed was measured at 630 nm with a reference containing 0.5 ml uninoculated succinate medium and 0.5 ml CAS solution. The proportion of CAS colour shifted as using the percentage of siderophore was estimated by the formula:

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

Where, Ar was the absorbance of reference and As was the absorbance of the sample.

Rhizobacterial isolates were inoculated onto the modified PKV medium with pH, 7.0±0.2 containing 1% ZnO. The plates were incubated for 48 h at 32±2°C. The clear zone around the colony was measured and considered positive for zinc solubilization.

Rhizobacterial isolates were spot inoculated onto a modified Aleksandrow medium. Plates were incubated for three days at 32±2°C. A clear zone around the colony was considered positive for potassium solubilization.

Nitrate broth was prepared and inoculated with the rhizobacterial cultures with uninoculated broth as a negative control. These were incubated at 37 °C for 48 h. To each test, one drop of sulfanilic acid and α-naphthylamine was added. Colour change to red indicated a positive result.

The compatibility among the selected isolates was done by cross streak method. Development of formulation using all four isolates RIGA-65, RIGB-93, RIGB-130 and RIGB-46 was done using talc as carrier. Seed coating of mungbean seed var. Samrat was done with this bioformulation. Four bacterial isolates giving the best PGP activity were further taken up for seed germination assay by modified plate method. Twelve bioformulation coated seeds were placed on germination paper with blotting paper in the Petri dish in triplicates and incubated at 37°C. Finally, germination of seed, germination percentage, root length, shoot length, and fresh and dry weight were recorded.

The percentage germination was calculated using the formula:

$$\text{Per cent germination} = 100 \text{ X } [(\text{Number of germinating seeds})/(\text{Total number of seeds})]$$

The vigour index was calculated by using the standard formula:

$$\text{Vigour Index I} = (\text{Mean root length} + \text{Mean shoot length}) \times \text{Germination\%}$$

$$\text{Vigour Index II} = (\text{Mean root dry wt.} + \text{Mean shoot dry weight}) \times \text{Germination\%}$$

All the experiments were conducted in triplicates and the results obtained were expressed in terms of the mean of three biological replicates with standard deviation. The means were compared with the Least Significant Difference (LSD) and the levels of significance were represented with the *p*-value.

RESULTS AND DISCUSSION

In total, 146 rhizobacterial isolates were obtained from the field of Raigarh village, Pusaur dist. Raipur, Chhattisgarh, India on different media and studied for their colony characteristics and morphology. The colonies were round, pointed, convex, flat, creamy, smooth, rough, shiny, transparent, off-white, white and yellow. The colony size ranged from small (0.1-1 mm) to large (2.5-3 mm). The isolates were positive for different biochemical tests such as urease production (46%), indole test (52%), MR and VP test (50%), citrate utilization (38%), starch hydrolysis (27%), amylase production (37%) and protease activity (83%). The 146 isolates were further evaluated for PGP activities. Based on their qualitative and quantitative estimation, 87 (59%) were found positive for IAA production, 20 (13.69%) for potassium solubilization, 64 (43.83%) for

phosphate solubilization, 33 (22.60%) for Zn solubilization, 41 (28.08%) for siderophore production, 68 (46.57%) for nitrate reduction and 69 (47.26%) for ammonia production (Table 1). The sequences were compared to the nucleotide database NCBI for molecular characterization using BLAST (Fig. 1). The *Bacillus subtilis* strain (RIGA-65) showed 100% similarity and the 16S rRNA genome sequence was submitted to GenBank with the accession number KX758996.1. Most of the isolates showed PGPR traits, mainly *Bacillus subtilis* strain RIGA-65, RIGB-93, RIGB-130 and RIGB-146. The highest phosphate solubilization was found in RIGA-65 (60 µg/ml) followed by RIGB-146 (54 µg/ml), RIGB-93 (21 µg/ml) and RIGB-130 (19 µg/ml). The highest siderophore production was recorded in RIGA-65 (31.17%) followed by RIGB-130 (23%), RIGB-93 (13%) and RIGB-146 (12.47%). Similarly, the maximum IAA production was found in RIGA-65 (54 µg/ml) followed by RIGB-146 (39 µg/ml), RIGB-93 (12 µg/ml) and RIGB-130 (10 µg/ml) (Table 2). The highest germination was recorded in mungbean seeds treated with RIGA-65 (97%) compared with control (53%). Similarly, the highest shoot and root length was observed in RIGA-65 (11.2 and 5.5 cm) as compared with control (9.1 and 3.4 cm). The maximum fresh and dry weight was recorded in RIGA-65 (5.7 and 0.9 g) compared with control (3.1 and 0.4 g). The highest vigour index I of 1619.9 was recorded for RIGA-65 as compared with control (662.5) and RIGA-65 (87.8) recorded the highest vigour index-II as compared to the control (19.6). A recent study has also reported biopriming with *B. subtilis* improving seed vigour (Ibanhes *et al.* 2021) (Table 3).

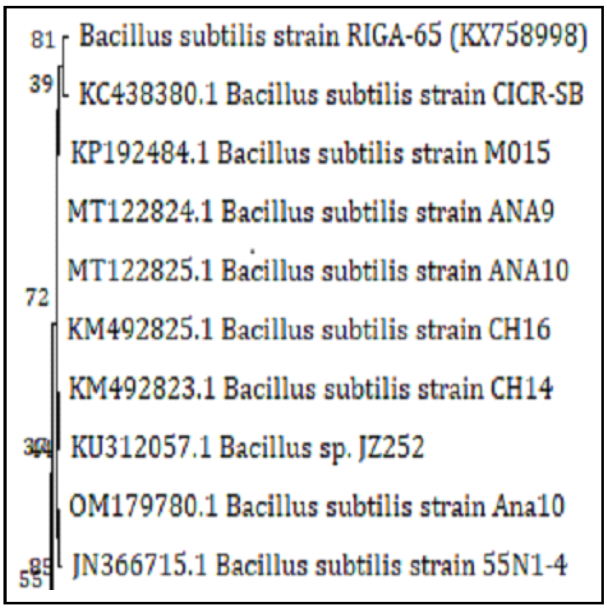

Fig. 1. Phylogenetic tree of the rhizobacterial strain *Bacillus subtilis* RIGA-65 and known plant growth promoting rhizobacterial species.

The present research work was undertaken to evaluate and screen PGPRs for their ability to maximize the rate of germination and thereby crop yield. The outcomes of this study

Table 1. Percentage of rhizobacterial isolates positive for different biochemical and PGPR traits

S. No.	Percentage of positive isolates (%)			
	Biochemical traits		PGPR traits	
1.	Urease	46	IAA production	59
2.	Methyl-red	50	Potassium solubilization	13.69
3.	Voges-Proskauer's	50	Phosphate solubilization	43.83
4.	Indole	52	Zinc solubilisation	22.6
5.	Citrate	38	Siderophore production	28.08
6.	Starch hydrolysis	27	Nitrate reduction	46.57
7.	Amylase	37	Ammonia production	47.26
8.	Protease	83		

Table 2. Qualitative and quantitative estimation of PGPR traits shown by the rhizobacterial isolates

S. No.	Isolates	Qualitative tests						Quantitative tests		
		Nitrate reduction	Ammonia production	Siderophore production	Phosphate solubilization	Potassium solubilization	Zinc solubilization	Phosphate solubilization concentration of P µg/ml)	IAA production concentration of IAA (µg/ml)	Siderophore production (µg/ml)
1.	RIG-65	++	++	+++++	++	+	++	60	54	31
2.	RIG-93	+++	+++	++++	++	+++	+++	21	12	13
3.	RIG-130	+++	+++	++	++	++	++	19	10	23
4.	RIG-146	++	++	+++++	+	++	++	54	39	12

Table 3. Effect of rhizobacterial isolates on various agronomical and germination parameters

S. No.	Isolates	Per cent germination (%)*	Shoot length (cm)*	Root length (cm)*	Fresh weight (g)	Dry weight (g)	Vigor index-I	Vigor index-II
1.	Control	53	9.1	3.4	3.1	0.4	662.5	19.6
2.	RIGA-65	97	11.2	5.5	5.7	0.9	1619.9	87.8
3.	RIGB-93	83	9.4	7.0	4.4	0.7	1361.2	54.8
4.	RIGB-130	81	7.5	4.0	3.8	0.6	931.5	49.7
5.	RIGB-146	22	4.5	4.2	0.2	0.0	191.4	0.7

showed that the *B. subtilis* strain RIGA-65 was observed as a very potential rhizobacterial strain with the best PGP traits. Research studies have reported the use of *B. subtilis* in the form of bioformulation for improving crop productivity (Egamberdieva and Adesemove, 2016; Zahir *et al.*, 2018). Among all the tested rhizobacterial isolates evaluated based on parameters like biochemical, qualitative and quantitative PGPR traits, *B. subtilis* strain (RIGA-65) was the most potent strain (Fig. 2). The impact of this strain on germination%, root length, shoot length, fresh and dry weight, and seed vigour index had been the maximum of all. Similar growth promotional activities by PGPRs had been reported by various researchers along with their ability to provide protection against biotic and abiotic stresses (Mahmood *et al.*, 2016; Kumari *et al.*, 2018; Nihayati *et al.*, 2019; Tripathi *et al.*, 2022; Pallavi *et al.*, 2023).

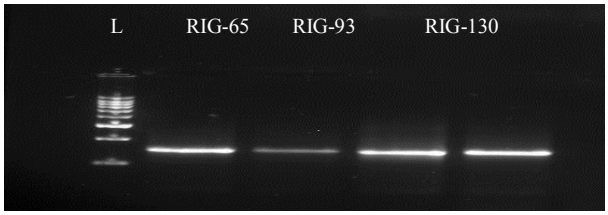

Fig. 2. PCR amplification of 16S rRNA of bacterial isolates on 1.2% agarose gel.

Note: Lane 1-4: 16S rRNA PCR product (3 µl) of different rhizobacterial isolates, L: 1 kb of DNA ladder (5 µl) (Bangalore Genei).

CONCLUSION

PGPRs can affect plant growth through various mechanisms, either indirectly or directly and these can be used for sustainable agriculture. The most potent strain of *Bacillus subtilis* strain RIGA-65 was reported to show a significantly higher germination percentage. The most important part of this study was that the seeds were coated with talc-based bioformulation. The coated seeds increased germination efficiency and enhanced seed vigour and yield vigour in mung bean crop. Inoculation with PGPR showed beneficial effects on plant growth and development and thus may be used as a biofertilizer for agriculture as a potential way to reduce negative environmental impacts caused by the continued use of chemical fertilizers.

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