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EVALUATION OF BACILLUS SPP. FOR PHOSPHATE SOLUBILIZATION AND PLANT GROWTH PROMOTION PRATIBHA

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ABSTRACT
Plant growth-promoting rhizobacteria (PGPR) represent a group of free-living bacteria inhabiting the rhizospheric soil and improving plant growth. These PGPRs have a dynamic role in plant growth by serving as bio-fertilizers. Therefore, we focused the present research investigation on isolating and characterizing phosphate solubilization and PGPR-producing rhizobacteria. Twenty-two bacteria were isolated, from which three bacteria belong to *Bacillus* spp. and were shown PGPR activity. We examined phosphate solubilizing efficacy, ammonia secretion, hydrogen cyanide production, and IAA production to evaluate the PGPR efficacy of bacterial isolates. *Bacillus* sp. PGPR-1 exhibited significant phosphate solubilization (2.4 ±0.039 mm ±SD zone diameter) and IAA (16.2 ±0.12 µg/mL ±SD) production along with ammonia and hydrogen cyanide secretion. *Bacillus* sp. PGPR-1 has potentially been used as an additives to fortify bio-fertilizer for sustainable agricultural management.

Keywords: *PGPR, rhizospheric soil, phosphate solubilizing efficacy, IAA, ammonia production, Hydrogen cyanide, Bacillus sp. PGPR-1*

1. INTRODUCTION
The ecological integrity of the soil is a primary factor that needs to be considered. The effective use of fertilizer and pesticides enhances plant growth (Gouda *et al*., 2018; Molnár *et al*., 2020). Plants often use 50% of the chemical fertilizer and the leftovers mostly remain in the soil and later interfere with water pollution during the rainy season (Sharma and Singhvi, 2017). Several literatures have stated the undesirable effects of synthetic inorganic chemical fertilizers and biocides including pesticides, insecticides, weedicides, herbicides and so forth (Rani *et al*., 2021; Sabarwal *et al*., 2018). However, the harmful effect of fertilizers on the soil is not reflected immediately due to the buffering capability of the soil. Long-term exposure to pollutant fertilizers is responsible for soil degradation and poor soil profile that further induces soil acidity and severely damages the ecological balance of the soil microbiota (Sharma and Sanghvi, 2017). Besides, the effective use of biofertilizers has the potential to cover up the requirement of chemically derived fertilizers and biocides.
PGPRs promote plant growth and development through nutrient acquisition, phytohormone synthesis, and siderophore production by direct mode (Rai *et al*., 2020; Nazir *et al*., 2018). Adnan *et al*. (2020) mentioned that PGPRs have emerged as sustainable agricultural practices that could enhance plant growth to safeguard the soil system's ecological integrity. Moreover, microbial-derived PGPRs

stimulate plant growth which also have the potential to mitigate the pollution level in the surrounding environment, increase crop yields, control pests, and be considered an ecofriendly approach for agriculture practices. Mustafa *et al*. (2019) mentioned that the PGPRs indirectly activate the immune system of plants against pathogens and simultaneously induce plant growth. Abiotic stress generally triggers the interaction between soil and plant which reduces crop yield. PGPR stimulate abiotic stress response and encourages plant growth by solubilizing phosphorus, nitrogen fixation, phytohormone synthesis, suppressing pathogens and so on (Abdelwahed *et al*., 2022; Rai *et al*., 2020; Prasad *et al*., 2019).

The bacteria and fungi have been predominantly associated with ensuring nutrient availability to the plant and producing PGPRs to induce plant growth and development (Aeron *et al*., 2020; Péterfi and Domokos, 2018). PGPRs group of bacteria have been widely reported for nitrogen fixation, phosphate solubilization, phytohormones e.g., indole-3-acetic acid (IAA), cytokinin, and siderophores production (Basu *et al*., 2021). Bacteria genera viz., *Bacillus*, *Azospirillium*, *Pseudomonas*, *Burkholderia*, and *Klebsiella* have been divulged for PGPR synthesis (Kaymak, 2010). Besides, *Bacillus* spp. has been extensively studied for PGPR production (Sansinenea, 2019). *Bacillus* spp. participate in phosphate solubilization, facilitate nitrogen acquisition, phytohormone synthesis (such as Gibberellin and IAA), siderophore production, and suppress phytopathogens alone or with other PGPR species (Kaymak, 2010; Joo *et al*., 2004; Gutierrez-Manero *et al*., 2001). Moreover, *Bacillus* spp. has been reported to form endospores, which enables them to survive under a broad range of environmental conditions viz., high temperatures and pH (Kaloterakis *et al*., 2021). Therefore, these properties enforce them as a suitable candidate for a green agricultural revolution with enhanced crop yield (Saxena *et al*., 2020). Thus, we have done a systematic evaluation towards the development of biofertilizer using *Bacillus* spp. for phosphate solubilization and plant growth promotion.

2. MATERIALS AND METHODS

Samples were collected from the Agricultural land of Bahtarai Village under the Bilaspur region (22.11, 82.18) in July 2024. The soil samples were collected from 5.0 cm of depth, in a clean and sterile polythene bag and brought to the laboratory for the isolation of PGPR bacteria.

2.1. Isolation of Bacteria

The collected samples were passed through a 0.4 mm mesh sieve to remove soil lumps and processed for serial dilution. Serially diluted samples (10^{-5}) were inoculated on Nutrient Agar Media (NAM) plates. Inoculated plates were incubated at 37°C for 24 hours.

2.2. Identification of Bacterial Isolates

The potent PGPR-producing bacterial isolates were identified as per the key provided by Bergey's Manual (Garrity *et al*., 2005). Pure colonies of PGPR-producing bacterial isolates were identified on the observations noted from Gram stain, spore formation, colony characters, and biochemical characteristics.

2.3. Screening of PGPR bacteria

The bacterial isolates were examined for Phosphate solubilizing, IAA, and ammonia production.

2.3.1. Phosphate solubilization

Phosphate solubilizing bacteria were screened using Pikovskaya's Agar Medium (PAM) as mentioned by Rai *et al*. (2020). The bacterial isolates were inoculated in PAM and incubated for 5 days at 37°C. The appearance of clear zones around the bacterial colonies confirms the phosphate solubilizing property of bacterial strains.

2.3.2. IAA Production

The efficacy of bacterial strains to produce IAA was assessed using UV-VIS spectrophotometerbased assay as stated by (Ehmann, 1977) Salkowski reagent using the Salkowski's method (Ehmann, 1977)

with slight modification. The bacterial isolates were inoculated in yeast malt dextrose (YMD) broth with tryptophan and incubated at 28°C for 5 days. The broth was centrifuged at 10000 rpm for 15 min. A 1.0 ml of supernatant was collected and 2.0 ml of Salkowski's reagent was added. The reaction mixture was kept in the dark. The optical density (OD) of the reaction mixture was recorded at 530 nm using UV-VIS Spectrophotometer.

2.3.3. Ammonia production

The bacterial isolates were examined for ammonia production using Nessler's reagent (Mohite, 2013). Fresh bacterial culture was mixed with 10 ml of peptone water and incubated at 30°C for 72 hours. Nessler's reagent (0.5 ml) was mixed with the reaction mixture. The colour change from pale yellow to dark brown confirms the ammonia production by bacterial isolate.

2.3.4. Hydrogen Cyanide Production

Hydrogen cyanide production in bacterial isolates was inspected using the method described by Sehrawat *et al*. (2022) Fresh bacterial cultures were streaked in NAM supplemented with Glycine. Whatman filter paper-soaked reagent consisting of 0.5 % picric acid and 2 % sodium carbonate, was covered over the inoculated NAM plates and incubated at 37°C for 5 days. The development of orange-red colour indicates the presence of hydrogen cyanide.

All the experimental analysis was done in triplicates to minimize the error rate. Observed data were processed and graphs were prepared using MS Office Excel 2021 with an error bar.

3. RESULTS AND DISCUSSIONS

Twenty-two bacterial strains were isolated from the samples. We have scientifically evaluated bacteria isolates for PGPR production efficacy in terms of Ammonia, Hydrogen Cyanide, IAA production. A total of nine bacterial isolates have shown PGPR activity. Among them, three bacterial isolates belong to *Bacillus* spp. The PGPR efficacy of *Bacillus* spp. is shown in Table 1. *Bacillus* sp. PGPR-1 isolate showed significant PGPR efficacy with maximum Phosphate solubilization of 2.4 mm zone diameter (Fig. 1) and IAA production of 16.2 ±0.12 µg/mL ±SD (Fig. 2).

Table 1. Biochemical Characteristics of Bacterial isolates

Isolate	**PGPR-1**	**PGPR-2**	**PGPR-3**
Colony Colour	Cream	Cream	White
Colony Shape	Irregular	Irregular Wrinkled	Convex
Surface Texture	Rough	Rough	Smooth
Gram Stain	+	+	+
Cell diameter	0.93 µm	0.87 µm	1.24 µm
Motility	+	+	+
Spore	+	+	+
6.5 % NaCl	+	+	-
Growth at 55^0C	-	+	-
Catalase	+	+	+
Oxidase	-	-	+
Citrate	+	+	+
Urease	-	-	-
TSI	Alk/Acid Butt	Alk/Acid Butt	Acid/Acid
Indole	-	-	-
VP	+	+	-
Starch Hydrolysis	-	+	+

Table 2. Screening of Bacterial isolates for PGPR

Isolate	***Bacillus* sp. PGPR - 1**	***Bacillus* sp. PGPR -2**	***Bacillus* sp. PGPR – 3**
Phosphate solubilization (mm ±SD zone diameter)	2.4 ±0.039	1.8 ±0.017	2.1 ±0.025
Ammonia Production	++	++	+
Hydrogen Cyanide Production	++	+	-
IAA (µg/mL ±SD)	16.2 ±0.12	13.7 ±0.084	15.9 ±0.1

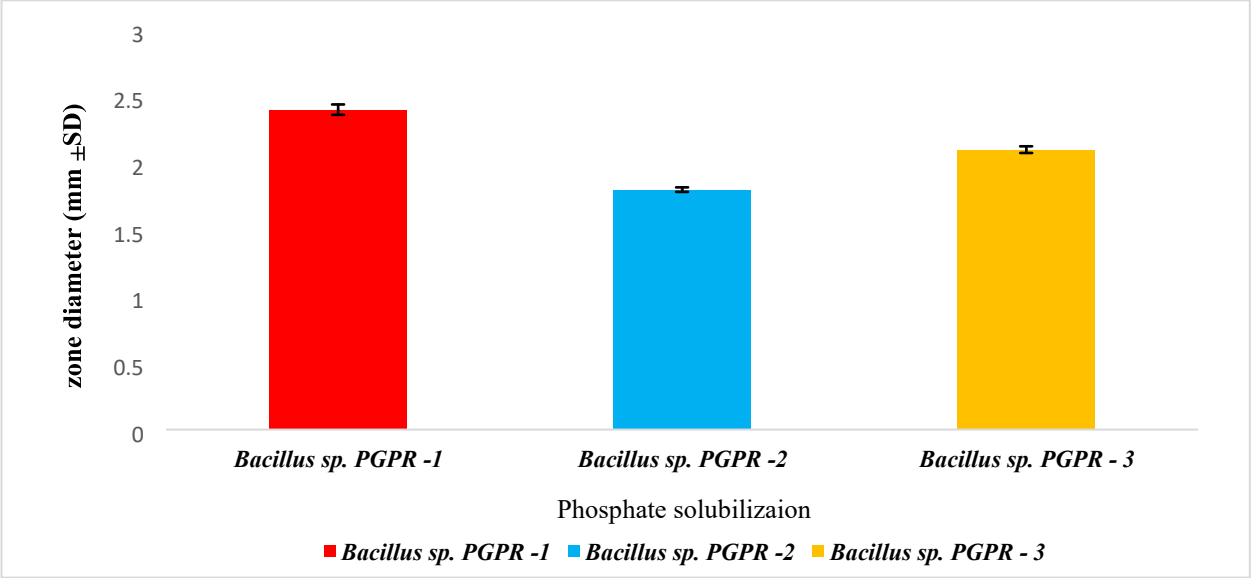

Fig. 1. Phosphate solubilization efficacy of PGPR *Bacillus* species

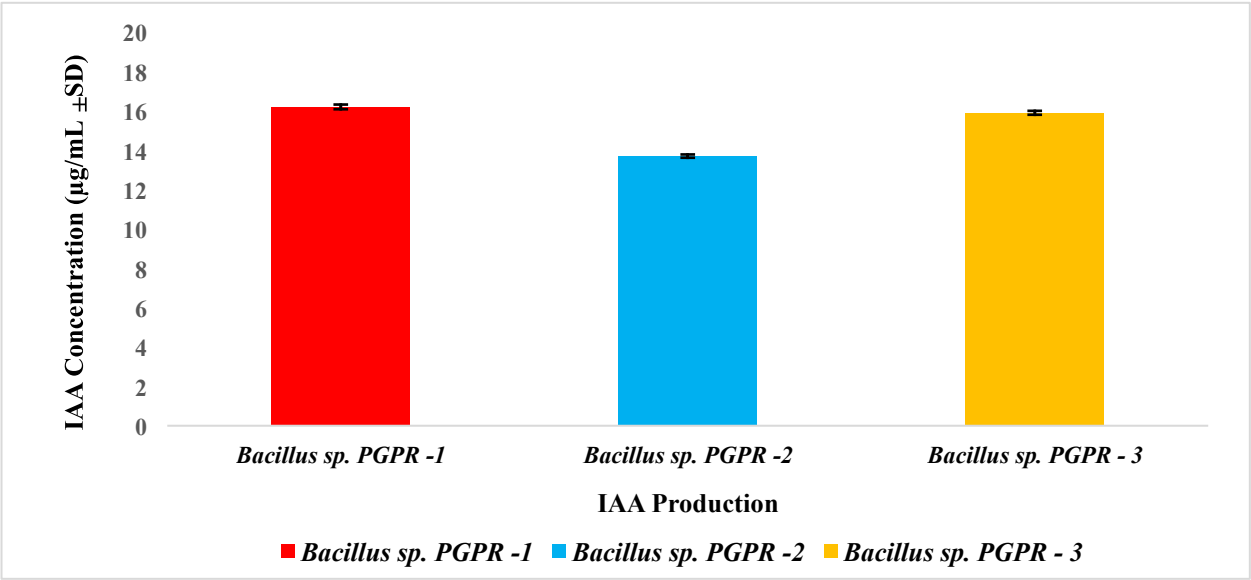

Fig. 2. IAA Production efficacy of PGPR *Bacillus* species

We have observed close similarity of PGPR-1, PGPR-2, PGPR-3 to *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus megaterium* respectively. In support to our findings, Kashyap *et al*. (2019) delineated that the *Bacillus* spp. are dominant PGPR-producing rhizobacteria in tropical regions. Besides, *Bacillus* spp. has also been disclosed to minimize the salinity stress in plants (Kaloterakis *et al*., 2021).

PGPR enhance crop yield by synthesizing ammonia that increases soil pH and combats fungal pathogens by inhibiting mycelial formation (Mohanty *et al*., 2021) in an indirect mode. HCN has been reported for beneficial action in plants by inhibiting the pathogenic organism in rhizosphere (Mazumdar *et al*., 2020). Phosphates often occur as insoluble in acidic soils which are predominant in tropical countries like India (Wang *et al*., 2021). This problem has naturally been compensated by phosphate solubilizing bacteria (PSB) e.g., bacterial genera including *Pseudomonas*, *Bacillus*, *Flavobacterium*, and *Rhizobium*, by secreting organic acid which acidifies soil and hydroxyl and carboxyl groups of organic acids facilitates conversion of insoluble phosphate to it solubilize form (Pathak *et al*., 2019; Saritha and Prasad Tollamadugu, 2019). This bioconversion is enormously important to phosphorus uptake by plants (Tang *et al*., 2020). But, Chen *et al*. (2021) have reported that long-term use of chemical fertilizers reduces phosphorus uptake by plants. The PGPR bacteria secrete IAA in the rhizosphere which promotes plant growth by stimulating cell elongation and proper organ development (Kumar *et al*., 2019). IAA induces root length in plants which enhances the nutrient uptake by plants and encourages the growth rate of plants (Kumar *et al*., 2019). Nevertheless, Antoun and Prevost (2005) mentioned that around 2 to 5% of the rhizosphere bacteria are PGPR and further could be employed for sustainable agriculture.

Conclusions

PGPR bacteria are well-known free-living rhizospheric bacterial communities that induce plant growth by supplying phytohormones and nutrient availability. Nitrogen fixation, phosphate solubilization, suppressing pathogens, and secretion of plant hormones are crucial aspects of PGPR bacteria that attract the scientific group. *Bacillus* spp. have extensively studied for PGPR. However, the genetic engineering tools and techniques integrated with omics studies need to be explored more scientifically for customized applications. Presently, PGPR is considerably applicable in agriculture by means of biofertilizers and the recycling of minerals into soluble form.

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