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Isolation of bacteria from agricultural soil and screening it for PGPR traits

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ABSTRACT

Rhizobacteria owning multiple plant growth-promoting activities were isolated from the rhizospheric soils of plants flourishing in a semi-arid region. Plant Growth Promoting Rhizobacterial (PGPR) strains were segregated and screened for their plant growth-promoting activities like phosphate solubilization, production of indole- acetic acid, ammonia, hydrogen cyanide (HCN). Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). PGPR is highly assorted and in this review, we focus on rhizobacteria as biocontrol agents. PGPR can affect growth directly or indirectly. Direct promotion of plant growth by PGPR involves both providing plants with a compound synthesized by the bacterium or helping the uptake of certain nutrients from the environment; while mechanisms of biological control by which rhizobacteria can support plant growth indirectly, i.e., by decreasing the level of disease, include antibiosis, induction of systemic resistance, and struggle for nutrients and niches.

Keywords— *PGPR, Nutrients, Biological fertilizers, Phosphate, Solubilization*

1. INTRODUCTION

Quality and quantity of food are going to be important challenges in coming time Continuous population growth requires production of more agricultural products and to inevitably move towards increased production per unit area. This cannot be achieved without the application of either chemical or bio-based fertilizers. Since fertilizer management is considered as one of the main factors of sustainable agriculture, gradual replacement of chemical fertilizers with biological fertilizers is quite inevitable due to their advantages and cost-effectiveness. The history of plant inoculation with useful bacteria goes back to many centuries ago. For instance, by experience, farmers knew that if the soil in which legumes were planted was mixed with the soil for non-legume crops, it resulted in an increased crop yield. In late 19th century, the first license for producing a biological fertilizer known as Nitragin was issued for the production of rhizobium inoculants and after that, inoculation of legumes started to be practiced in many countries using rhizobium fertilizers [1]. The rhizosphere, the narrow zone of soil that surrounds and influences the plant roots, is home to a large number of microorganisms and is considered to be one that can have profound effects on the growth, nutrition and health of plants in agro-ecosystems [2]. The rhizosphere, microbiota can contain up to 1011 microbial cells per gram of root [3] and more than 30,000 prokaryotic species [4]. Bacteria able to colonize plant root systems and promote plant growth are referred to as plant Growth Promoting Rhizobacteria (PGPR) [5].

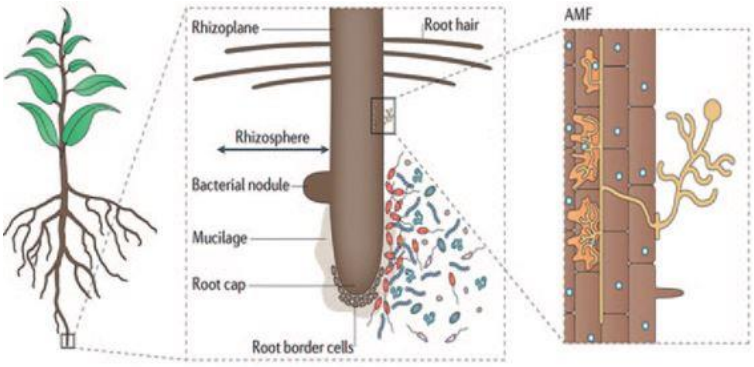

Fig. 1: Representation of a Rhizospheric Zone [6]

Table 1: PGPR and their effect on growth parameters/ yields of crop/fruit plants

PGPR	Crop parameters
Rhizobium leguminosarum	Direct growth promotion of canola and lettuce
Pseudomonas putida	Early developments of canola seedlings, growth stimulation of tomato plant
Azospirillum brasilense and *A. irakense*	Growth of wheat and maize plants
P. flurescens	Growth of pearl millet, increase in growth, leaf nutrient contents and yield of banana (*Musa*)
Azotobacter and *Azospirillum* spp.	Growth and productivity of canola
P. alcaligenes, *Bacillus polymyxa*, and *Mycobacterium phlei*	Enhances uptake of N, P and K by maize crop
Pseudomonas, *Azotobacter* and *Azospirillum* spp.	Stumulates growth and yield of chick pea (*Cicer arietinum*)
R. leguminismarum and *Pseudomonas* spp.	Improves the yield and phosphorus uptake in wheat
P. putida, *P. flurescens*, *A. brasilense* and *A. lipoferum*	Improves seed germination, seedling growth and yield of maize
P. putida, *P. fluorescens*, *P. fluorescens*, *P. putida*, *A. lipoferum*, *A. brasilense*	Improves seed germination, growth parameters of maize seedling in greenhouse and also grain yield of field grown maize

1.1 Location of study area

Unnao district represents flat topography with a general elevation of 98 m (322 ft.) covering an area of 4558 km^2 . By virtue of its geographic setting in the great (Ganga) plains, the land is highly fertile. The soil is mostly alluvial. The district is mainly drained by the river Ganga and its tributaries Kalyani, Khar, Loni and Marahai in the western part of the district and by Sai river in the eastern part of the district. All these rivers are perennial in nature. About 87% area of the net sown area (3,00,000 hectares) is irrigated both by surface water (Sharda Canal network system) and ground water through shallow and moderately deep tubewells. The share of surface water irrigation is 48% while that of ground water is 52%.

Soil found in Unnao industrial and surroundings village of Unnao district exhibit a wide variance in composition and appearance. The major part of area consists of ordinary soils known locally as Bhur or sand on the ridges, Matiar or clay in the topographic lows and Dumat or loam on the plains. Clay is dominant in the areas where "Reh" or USAR prevails. Alluvial soils of river valleys notable the "Kachhar" of the Ganga formed by repeated deposition of silt brought down by the existing river system during floods. [7]

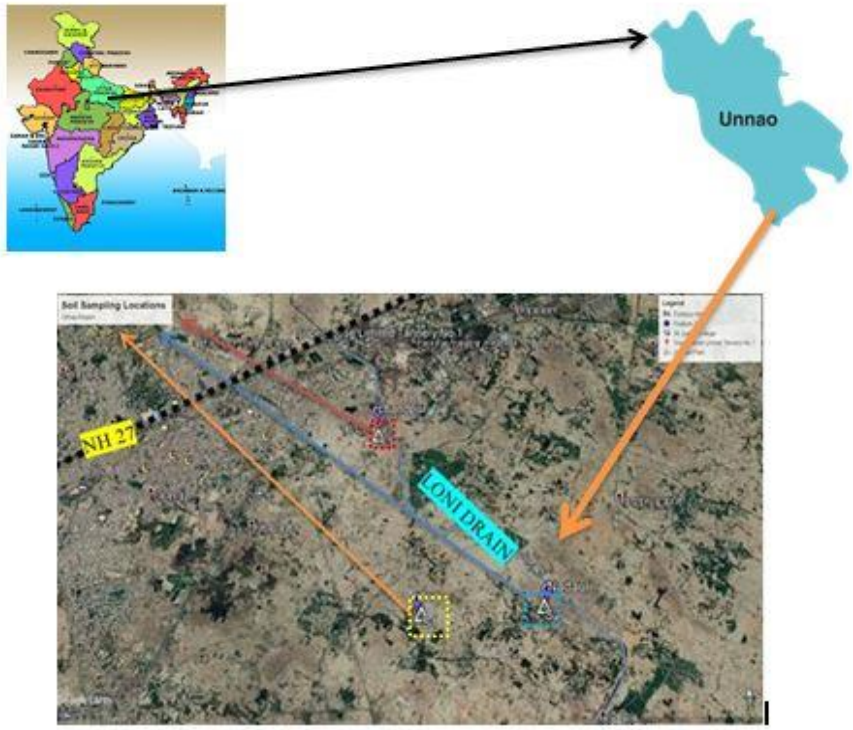

Fig. 2: Satellite Imagery of the Soil Sampling Location

2. MATERIAL AND METHOD

2.1 Sample Selection

The plant growth promoting rhizobacteria were isolated from the rhizosphere of following plants from the agricultural soils of Unnao Region:

2.1.1 Common wheat (*Triticum aestivum*): Loosely attached soil was separated from the roots. The roots were shaken tenderly to remove excess soil. After extracting the soil loosely adhering to root, the soil adhering firmly to the root of each plant was accumulated through brushing (termed rhizosphere soil sample, RSS).

2.2 Preparation of Nutrient Broth

Nutrient Broth is used for the general cultivation of less fastidious microorganisms, can be enriched with blood or other biological fluids.

Table 2: Composition of Nutrient broth Media

S no.	Ingredients	g/L
1	Peptic digest of animal tissue	5.00
2	Sodium chloride	5.00
3	Beef extract	1.50
4	Yeast extract	1.50
5	Final pH (at 25°C)	7.1±0.2

2.3 Serial Dilution for isolation of Bacteria from the soil

The Purpose of serial dilution was to determine the number of bacteria per unit volume in the original culture, determination of the culture density in cells per ml. Once the culture had been diluted it could be spread on agar plates. Agar plates allow for individual bacterial cells to be separated spatially. If done correctly, there is a low probability of having two cells very close to each other. When each of these spatially separated cells multiplies, spatially separated colonies were formed.

2.3.1 Procedure: The soil samples were serially diluted from 10^{-3} to 10^{-7} dilutions using sterile distilled water as a blank and they were inoculated on the nutrient agar medium by pour plate technique. After 24 hours of incubation at 37°C the colonies were counted.

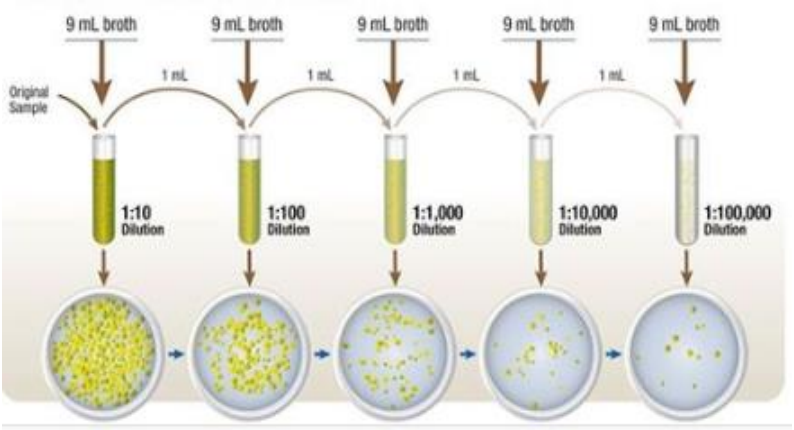

Fig. 3: Pour Plate Method

2.4 Streak Plate Method

2.4.1 Streak plate technique: is used for the isolation into pure culture of the organisms (mostly bacteria), from mixed population. The inoculum is streaked over the agar surface in such a way that it "thins out" the bacteria. Some individual bacterial cells are separated and well spaced from each other.

2.4.2 Procedure: This technique was done using an inoculating loop. 0.1 mL of the bacterial suspension was placed in the center of the plate using it.The glass rod was sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame. The burning alcohol sterilizes the loop at a cooler temperature than holding the rod in the burner flame thus reducing the chance of burning fingers.

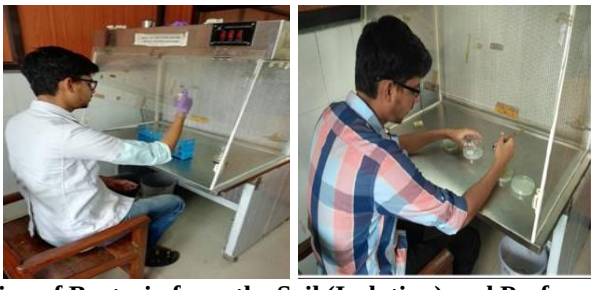

Fig. 4: Performing Serial Dilution of Bacteria from the Soil (Isolation) and Performing the Streak Plate Method in Laminar Air Flow Chamber

Screening of Phosphate Solubilizing Bacteria (PSB) .In most bacteria, mineral phosphate-dissolving capacity had been shown to be related to the production of organic acid such as Gluconic Acid (GA) [8], earlier which was reported to produce by direct oxidation of glucose. Mainly the biosynthesis of GA reported to carry out by the Glucose Dehydrogenase (GDH) enzyme and the co-factor, Pyrroloquinoline Quinone (PQQ). The p-sol ability was checked on Pikovskaya's media plate. Mainly this media contains calcium phosphate which acts as a source of phosphate. Moreover, GA producing rhizobacteria could be easily differentiated on the Pikovskaya's media plate by observing a clear halo zone due to the release of phosphorous from the media. Following steps were employed for isolation of PSB:

(a) 1 g dried Rhizospheric Soil Sample (RSS) was added to a 25 ml flask with 9.0 ml sterilized distilled water and incubated for 30 minutes (min) at 30 °C in a shaker at 250 RPM. The resulting suspension was decimally diluted (10^{-2}-10^{-6}) with sterilized distilled water.
(b) All RSS were appropriately diluted and plated on Pikovskaya agar media to get approximately 100 colonies per plate. The plates were incubated at 30 °C for 2 days. Colonies that showed halo zone was considered as PSB.
(c) Reconfirmation of PSB was done on Pikovskaya's media plates. (d) All PSB strains were streaked on nutrient agar media for colony purification.
(d) Purified strains were re-analyzed for p-sol on Pikovskaya's media plates.
(e) Glycerol stocks of all P-solubilizing strains were made in 30% glycerol and stored at -80 °C freezer.

2.5 Phosphate solubilization

Bacterial culture was spot-inoculated on the surface of the plate containing Pikovaskaya's medium and incubated in an incubator at 28 °C for 7 days. P-solubilization was determined by the development of the clearing zone around a bacterial colony [9]

2.6 Production of Ammonia

Bacterial isolate was tested for the production of ammonia in peptone water. The freshly grown culture was inoculated in 10 ml peptone water in a test tube and incubated for 48-72 h at 36±2 °C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to the yellow color indicated a positive test for ammonia production [10].

2.7 Production of Indole acetic acid

Bacterial culture was grown in LB medium amended with 100 mg L-1 tryptophan as the precursor of IAA by incubating in a shaker at 250 rpm at 28 ± 2oC for 7 days. Indole acetic acid (IAA) production was assayed colorimetrically by using Salkowski reagent (1ml of 0.5M FeCl3 in 50 ml of 35% HClO4) and the absorbance of the resultant pink color at 535nm in the colorimeter. The appearance of a pink color in test tubes indicated IAA production. The concentration of IAA was determined by comparison with a standard curve [11].

2.8 HCN Production

Briefly, the strain was streaked on the nutrient agar amended with glycine (4.4 g/L). A Whatman filter paper No.1 soaked in 2% sodium carbonate prepared in 0.5% picric acid solution was placed at the top of the plate. The plated sealed with a parafilm were incubated at 28±2oC for 4 days change of filter paper from orange to red if found, was noted down, hence confirming HCN production.[12]

Table 3: Examples of different phytohormone-producing PGPR

Phytohormones	PGPR
Indole-3-acetic acid (IAA)	*Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*
Zeatin and ethylene	*Azospirillum* sp.
Gibberellic acid (GA_3)	*Azospirillum lipoferum*
Abscisic acid (ABA)	*Azospirillum brasilense*

3. RESULT AND DISCUSSION

Table 4: Plant Growth Promoting Traits of the Isolate

PGP Traits	Response
Ammonia Production	+ve
Phosphate Solubilization	+ve
IAA Production	-ve
HCN	+ve

3.1 Phosphate Solubilization

On Pikovaskaya's agar plates a clear zone was observed around the bacterial colonies. The solubilization ability of rhizosphere microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition. It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids, through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms. PSB has been introduced to the Agricultural community as phosphate Bio fertilizer.

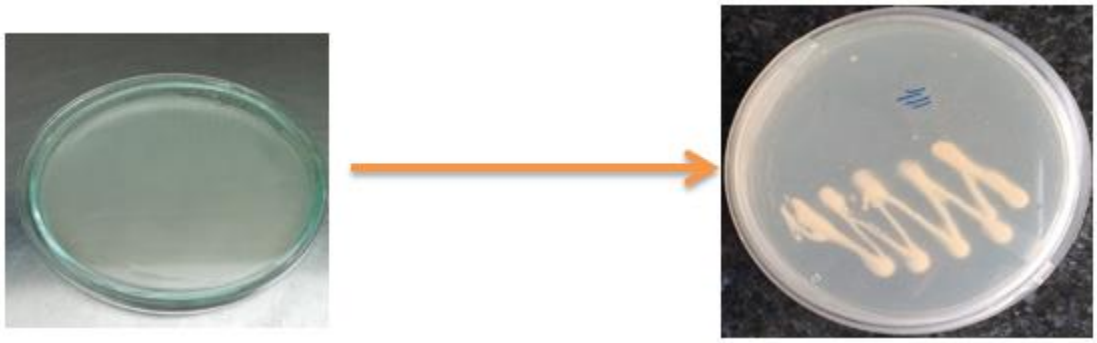

Fig. 5: Phosphate solubilization by the isolates

3.2 HCN Production

A change in color of the filter paper was noticed from light orange to reddish orange, which confirmed the production of HCN by the isolated bacterial strain. Hence the isolating strain might be PGPR. The production of the HCN by the isolates is a healthy sign that the plant will resist activities in the rhizosphere or root zone due to the pathogenic bacteria or fungal activity.

3.3 NH_3 Production

In addition, of Nessler's reagent to put-on water inoculated with bacterial strain, a considerable change in color from brown to yellow was noticed, thus confirming the production of ammonia. There are a number of sources of ammonia secretion of rhizospheric microorganisms. Ammonia and extracellular proteins are the nitrogenous secretions of nitrogen fixers in nitrogen free or deficient medium.

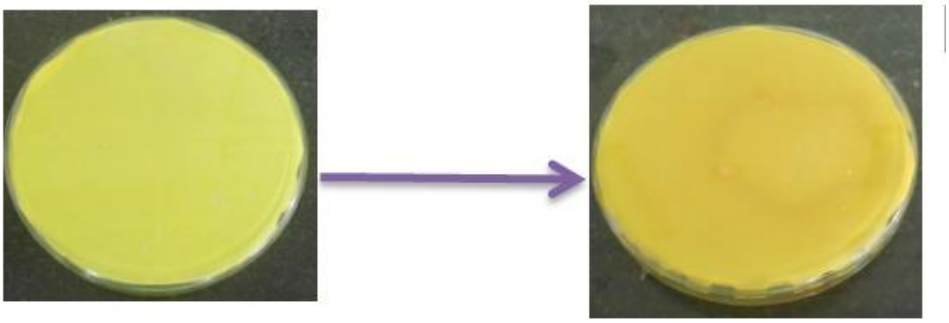

Fig. 6: HCN production by the isolates (orange color)

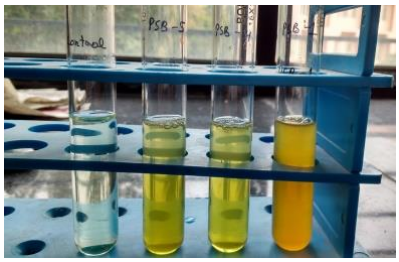

Fig. 7: Ammonia production by the isolates (yellow colour)

4. CONCLUSION AND RECOMMENDATIONS

PGPR are found in plant roots or in the adjacent soil and contribute to the plant's growth and development through multiple direct and indirect mechanisms. PGPR have been investigated in search of efficient ways to use them to improve agricultural production in a low impact ecological way. All the isolated strains in this study showed varying levels of plant growth promoting activities, and all had an overwhelmingly positive effect on the plants for all the investigated bioprocesses: IAA production, Nitrogen fixation and cellulase activity in addition to their antifungal properties. Based on these results, the isolated PGPRs in this study could constitute an efficient and more eco-friendly alternative to chemical fertilizers and fungicides in the processes of biostimulation, bio-fertilization and biological control.

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