Research Journal of Agricultural Sciences
An International Journal

Res. Jr. of Agril. Sci. **12**(1): 218–220

P- ISSN: 0976-1675 | **E**- ISSN: 2249-4538

www.rjas.org

Short Communication

Isolation and Characterization of Phosphorus Solubilizing Microorganisms from Wheat Rhizosphere by Cultivation Dependent Procedures

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Received: 06 Nov 2020 | Revised accepted: 12 Jan 2021 | Published online: 18 Jan 2021

***Key words*:** Phosphate solubilizing bacteria, Wheat, Rhizosphere, Sustainable agriculture, Rhizobacteria

Wheat is an important food supplement in heavily populated geographical areas such as Asia, Africa and the Middle East. In recent years, it has been observed that the yield of wheat has declined due to excessive use of chemical fertilizers leading to its ill effects on plants health in particular and economical loss to the cultivators in general [1]. Phosphate solubilizing rhizobacteria play a very important role for the overall development of wheat plant. Phosphorous even when applied as a biofertilizers soon becomes immobilized through its formation of ions with the soil components. However, the average phosphorous content of the Indian soil is 0.05% phosphorus that constitutes 0.2% of plant dry weight. This in turn becomes fixed and is unavailable for the plant nutrition [2], [3]. Phosphate solubilizing microorganisms have been found to play a very potent role in overcoming this problem [4]. In many studies deficiency of Phosphorous has been found to be a major constraint for increased crop production [5]. As a result, farmers resort to the excessive use of Phosphorous fertilizers, which has detrimental effect on the plant health as well as the overall environment, thus adversely affecting both the economy.There are many known PSB such as members of the genus *Pseudomonas*, *Bacillus*, *Micrococcus*, *Flavobacterium*, *Burkholderia*, *Achromobacter*, *Erwinia*, *Pantoea*, *Streptomyces*, *Acinetobacter*, *Agrobacterium Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Beijerinckia*, *Enterobacter*, *Flavobacterium*, *Methylobacterium*, *Pseudomonas*, *Rhizobium*, *Paenibacillus and Pantoea* [6]. For sustainable agriculture, more study about the potential phosphate solubilizing microorganisms is an absolute necessity.

Soil samples were collected from the wheat crop grown at Wheat Research Station, Baben, Bardoli, Gujarat, India; and also, from the wheat farm located at Abrama region, Surat, Gujarat, India. The roots harbouring soil was dug out and were carefully taken in sterile plastic bags and stored at 4°C before further investigation. In total 2 samples were collected for the isolation of rhizospheric phosphate solubilizing microorganisms. The details of the sampling site are given in the following (Table 1).

Table 1 Details of sampling site

Sr. No.	Collection site	Longitude	Latitude	Sample type
1.	Wheat Research Station, Bardoli	21.124857	73.112610	Rhizosphere
2.	Wheat Farm, Abrama, Surat	21.2755	72.9128	Rhizosphere

Physico-chemical characteristic of soil

pH of the soil has been checked using a digital pH meter and moisture analysis was carried out by oven drying method [2]. Chemical analysis of soil sample includes the following factors/tests:

i. Analysis of total phosphorous by digestion method,
ii. Total nitrogen content by Kjeldalh's method,
iii. Total potassium by Flame photometric method, and
iv. Total organic carbon by physical method [7].

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Isolation of PSMs

For the isolation of phosphate solubilizer, serial dilution technique was followed. From each serial dilution tube, aliquots were spread on Pikovaskaya's agar plate containing insoluble Tricalcium phosphate – $Ca_3(PO_4)^2$. Plates were incubated at 30 ± 0.1°C for 24–48 hours. The colonies showing halo zone were considered as Phosphorous solubilizer. The colonies were purified by repeated streaking and stored at 4°C [4].

Qualitative analysis of phosphate solubilization

For the qualitative estimation, the methodology [8] was followed. The isolated colonies were inoculated on Pikovaskaya's agar media containing insoluble tri calcium phosphate. Phosphate solubilizing efficiency was calculated by measuring the colony diameter and the Halo zone diameter using the following formula:

$$\text{Phosphate solubilization index (SI)} = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

Quantitative analysis of phosphate solubilization

Quantitative estimation is carried out as per the protocol developed by [9] using:

25 ml of NBRIP broth consisting of	
Glucose	10.0 grams
Tri calcium phosphate	5.0 grams
Magnesium chloride hexahydrate	5.0 grams
Magnesium sulphate heptahydrate	0.25 grams
Potassium chloride	0.2 grams
Ammonium sulphate	0.1 grams
Distilled water	1000 ml (final volume)

The broth was inoculated with $3*10^8$ CFU/ml and incubated on a rotatory shaker at 30°C at 120 RPM for 3 days. The cultures were harvested by centrifugation at 10,000 rpm for 10 minutes. Supernatant is then taken and the phosphorus content is estimated by Vanado- molybdate colorimetric method. The absorbance is measured at 430 nm. Each treatment was replicated 3 times and data were expressed as the mean value ± standard error.

Biochemical characterization of selected bacterial isolates

Four isolates which showed maximum phosphate solubilizing activity were further characterized by Gram staining and biochemical tests such as catalase, oxidase, MR-VP, indole, citrate, urease, nitrate reduction and sugar fermentation were carried out as per methodology described by Kreig and Holf [10].

Strain identification

DNA was isolated from the provided culture. Its quality was evaluated on 1.0% Agarose Gel, wherein a single band of high-molecular weight DNA has been observed. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with primer 27F using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Statistical analysis

All the results are statistically analyzed and the results are expressed as mean and standard deviation is calculated.

For the proper growth of the plants, the analysis of physical and chemical characteristics of the soil is utmost necessary. In the present study, soil parameters such as pH, moisture content, organic carbon, total nitrogen, total potassium and total phosphorous level were checked and the results are shown in the (Table 2). Through this study it will be helpful to know the kind of nutrition that is needed for addition in the soil, thereby leading to an increased production of wheat crop in terms of both quality and quantity.

Table 2 Physico-chemical characteristics of soil

Sample	pH	Moisture (%)	Nitrogen (mg/L)	Organic carbon	Organic matter	Potassium (mg/g)	Phosphorus (mg/g)
Farm A	7.38±0.05	18.41±0.7	15.35±0.28	9.53±0.22	16.56±0.27	0.13±0.04	0.28±0.002
Farm B	7.58±0.02	9.00±0.09	2.33±0.06	10.52±0.05	17.83±0.20	1.33±0.04	0.16±0.0007
Farm C	7.62±0.02	6.67±1.5	81.27±0.05	1.78±0.0004	3.28±0.19	2.51±0.02	0.0817±0.008

The presence of the phosphate solubilizing bacteria ranged from $3.6*10^4$ to $4.9*10^4$ CFU/g of rhizospheric soil as indicated in (Table 2). The occurrence of PSB was greater than $3.1*10^4$ CFU/g indicating that their abundance is greater due to variations in different rhizospheric regions [11]. Several studies also suggests the presence of a large number of phosphate solubilizing microorganisms present in the rhizosphere of important crops such as maize, sorghum, rice, barley, wheat and chickpea [12].

Table 3 Details of sampling sites and isolated phosphate solubilizing microorganisms

Sample No.	Field site	CFU/g	Total No. of isolates showing phosphate solubilization	Isolate code	Highest solubilizer
Farm A	Wheat research station, Bardoli	$4.9*10^4$	11	FA1, FA2, FA3, FA4, FA8, FA11, FA14, FA16, FA18, FA21, FA22	FA3, FA22
Farm C	Wheat farm, Abrama, Surat	$3.6*10^4$	10	FC1(1), FC1(2), FC3, FC5, FC7, FC8, FC9, FC10, FC11, FC13	FC9, FC10

Table 4 Identification of highest phosphate solubilizer

Isolate code	Quantitative (Solubilization index)	Quantitative (mg/ml)	Identified as
FA22	2.48±0.09	0.370±0.01	*Micrococcus luteus*
FA3	2.69±0.11	0.319±0.017	*Stenotrophomonas maltophilia*
FC9	2.70±0.26	0.613±0.09	*Bacillus subtilis*
FC10	2.91±0.14	0.513±0.12	*Exiguobacterium indicum*

In the present study, 21 bacteria were isolated from 2 farms which are labelled as Farm A and Farm C respectively as shown in (Table 3). All of these isolates were screened for their phosphate solubilization both qualitatively and quantitatively. Out of these 21 isolates, four isolates showed maximum phosphate solubilization. The results of the qualitative and quantitative tests are shown in (Table 4). For the qualitative tests the isolates which produced phosphate

solubilization index greater than 2 were selected. Similar results were observed by [13] in which the SI of the isolates ranged from 1.40 to 3.06. The quantitative estimation showed phosphate solubilization ranging from 0.213 to 0.613 mg/ml.

Table 5 Results of biochemical test of bacterial isolates

Biochemical teste	Cat	Oxi	I	M-R	V-P	C	Lac	Glu	Man	Suc	Mal	Mo	Nitrate	U
FA22	+	+	-	-	+	-	-	-	-	+	+	-	-	-
FA3	+	-	-	-	-	-	-	+	-	+	-	+	+	-
FC9	-	-	-	-	-	-	-	+	-	+	-	+	-	-
FC10	-	-	-	+	+	+	-	+	-	-	-	-	-	-

As shown in (Table 5) following conclusions can be derived from the results of biochemical tests:

1. Isolate No. FA22 was identified as *Micrococcus luteus* from its biochemical characteristics.
2. The results of 16srRNA sequencing suggests that
   a. Isolate No. FA3 showed 100% identity to *Stenotrophomonas maltophilia*
   b. Isolate no.FC9 showed 99.14% similarity to *Bacillus subtilis* and
   c. Isolate FC10 showed 96.99% similarity to *Exiguobacterium indicum*

This result suggests that such phosphorous solubilizing microorganisms can be efficiently used as bioinoculants and also can be used as a replacement of the chemical fertilizers. Studies have shown that there are large numbers of phosphate solubilizing microorganisms present in the rhizospheric soil as compared to the non rhizospheric soil [13]. Detail investigation of such areas can provide us an opportunity to replace tooth and nail the ill effect causing chemical fertilizers.

SUMMARY

Phosphate solubilizing microorganisms found in the rhizosphere of wheat are responsible for providing phosphorus essential for the growth and development of the plants. In the present study phosphate solubilizing microorganisms were isolated, screened and characterized for their phosphate solubilization efficiency ultimately providing nutrition in wheat. 21 isolates showing different levels of phosphate solubilizing activity in both agar plate and broth assays using Pikovaskaya's medium were obtained through screening. Among all, four isolates showed maximum phosphate solubilization and were hence employed for 16S rRNA sequencing. The PSB labelled as FA22, FA3, FC9 and FC10 were tentatively identified as *Micrococcus species*, *Stenotrophomonas maltophilia*, *Bacillus subtilis* and *Exiguobacterium indicum* respectively through 16s rRNA gene sequencing. Our results suggest that these isolates can be efficiently used as bioinoculants for better production of wheat in terms of quantity as well as quality even in low fertility soils.

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