# PGPR (Plant Growth-Promoting Rhizobacteria) in Mulberry Rhizosphere with the Potential of Soil Fertilizer Pollution Control

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**Abstract:** PGPR in mulberry rhizosphere were investigated in order to provide a theoretical basis for improving the soil fertility by biological effect and reducing the chemical fertilizer pollution. The three-point sampling methods were carried out in mulberry soil with different fertilities in Silkworm and Mulberry Scientific Research Station of Shandong Agricultural University, where the mulberry species *Husang 32* were planted. Some PGPR were isolated from the samples and the repetitive-element PCR (Rep-PCR) genomic fingerprinting and biological activities were analyzed. The strains with positive biological activities were identified by the analysis of 16S rDNA sequence. The results showed that, based on the analysis of Rep-PCR, 18 kinds of azotobacteria, 18 kinds of phosphor-bacteria and 23 kinds of silicate bacteria were obtained from the samples, respectively. PA19 and PA2 have the highest nitrogenase activities among the azotobacteria. PYP2, PYP4, PYP6, PYP3 have higher organic phosphate solubilizing activities than that of others. FK14, PWP4 have higher inorganic phosphate solubilizing activities than that of others. The potassium-releasing activity of FK2 and FK3 were higher than that of other silicate bacteria. According to identification, 10 isolates with high biological activities were identified as follows: PA19 belonged to genus *Mesorhizobium*, PA2, PYP2, PYP6, PYP3 and FK2 belonged to genus *Pseudomonas*, PWP4 and FK14 belonged to genus *Rhizobium*, FK3 belonged to genus *Sinorhizobium*, and PYP4 belonged to genus *Inquilinum*.

**Keywords:** mulberry rhizosphere PGPR; biological activity; pollution control

## 1 Introduction

The rhizosphere is the volume of soil environment under the influence of plant roots, and the rhizoplane is the plant root surfaces strongly adhering to the soil particles [1]. Many microorganisms are attracted by nutrients exuded from plant roots and this "rhizosphere effect" was first described by Hiltner [2]. The rhizosphere and rhizoplane are colonized more intensively by microorganisms than the other regions of the soil. In the rhizosphere, important and intensive interactions occur among the plant, soil, microorganisms and soil microfauna. In fact, biochemical interactions and exchanges of signal molecules between plants and soil microorganisms have been described and reviewed [3].These interactions could impact significantly on plant growth and crop yields [4]. Among the rhizosphere microorganisms, most attentions were focused on Plant Growth Promoting Rhizobacteria (PGPR) in recent years [5]. PGPR are free-living bacteria [6], some of which can invade the tissues of living plants and cause unapparent and asymptomatic infections [7]. PGPR are not only beneficial to the nutrients releasing from the roots, but also have positive effect on the plants, resulting in a stimulation of the growth.

Rhizosphere bacteria that promote plant growth are considered as an alternative to take the place of chemical fertilizer in agriculture [8]. The production and use of chemical fertilizer not only consume large quantities of energy, but also cause serious environment pollution and damage ecological balance. Besides, long-term application of chemical fertilizer is likely to destroy the aggregation of soil, leading to many adverse consequences. There is overwhelming reported evidence indicating that PGPR utility can be an effective way in sustainable agriculture. PGPR can also be utilized to develop "biofertilizers" so as to improve the productivity of arable land and to reduce the pollution caused by chemicals. The rhizospheres of wild plant species seem to be the best sources from which to isolate PGPR [9]. Plants are selecting bacteria which are beneficial to their health by releasing organic compounds exudates [10], and a kind of selective environment with low diversity are constructed [11]. A large number of PGPR genera have been reported nowadays, such as *Atumefaciens*, *Alcaligenes*, *Arthro-*

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*bacter*, *Azotobacter*, *Bacillus*, *Erwinia*, *Pseudomonas* sp., and *Xanthomonas* [12-14].

Therefore, development of PGPR has attracted a great deal of attention in the area of biological control of environmental pollution. The aims of this work were, on one hand, to isolate PGPR from mulberry rhizosphere, study its biological activities and determine the diversity of PGPR strains, on the other, to investigate the improvement of soil fertility by PGPR in order to reduce the soil fertilizer pollution in mulberry rhizosphere. We isolated PGPR from rhizosphere of mulberry, and some strains with high biological activities were obtained and identified.

## 2 Materials and Methods

### 2.1 Samples

The field soil was located in the mulberry forest with different fertilities in Silkworm and Mulberry Scientific Research Station of Shandong Agricultural University, where the monoculture of *Husang 32* lasted for more than five years. The rhizosphere soil samples were obtained by three point sampling method from mulberry soils, each of which 1kg of rhizosphere soil sample was collected from the upper layer of soil around 15 cm depth, according to literature [15]. It was carried out during November. The maximum and minimum temperature were 25℃ and 20℃, respectively, and the average relative air humidity was about 65%.

### 2.2 PGPR Isolation from Mulberry Rhizosphere

In the lab, the soil loosely adhering to the roots was removed aseptically, and the firmly adhered soil was suspended in flasks with 90ml sterilized water. The flasks were incubated in a rotary shaker at 120 rpm under 28℃ for about an hour. After that, roots were taken out from these suspensions and washed by deionized water to remove the soil particles. Then the surface of roots was sterilized by ethanol and the sample was macerated with mortar and pestle in sterilized water. Ten-fold diluent of the washing liquid were prepared and spread agar plate containing different culture media followed with incubation at 28℃. The azotobacteria, organic phosphate solubilizing bacteria and silicate bacteria were screened 3 days later and the inorganic phosphate solubilizing bacteria were selected after 7 days of incubation.

### 2.3 Repetitive-element PCR Genomic Fingerprinting (Rep-PCR)

The isolates of the four types of PGPR were analyzed by repetitive-element PCR genomic fingerprinting. The primer of Rep-PCR was BOX-AIR (sequence: 5'-CTACGGCAAGGCGACGCTGACG-3') and the bacterial genomic DNA were extracted as the PCR templates. The reaction mixture contained 2.5μl 10 × PCR Buffer, 2 μl 10 mmol/l dNTP, 1.5 μl 25 mmol/l $MgCl_2$, 2 μl 10umol/l primer solution, 1 μl DNA template, 0.15 μl taq polymerase, and the volume was complement by double distilled water to 30 μl. The PCR procedures were as follows: step 1, 95℃ for 7min; 35 cycles of step 2, 94℃ for 1min，53℃ for 1min and 65℃ for 8min; step 3, 16 min incubation at 65℃. The analysis of PCR products was performed by Cross Checker Software and the clustering scheme of the isolates was constructed by the program of SAS 8.0.

### 2.4 Biological Activities of the Isolates

Nitrogenase activities of azotobacteria were determined by acetylene reduction methodology (ARM). The phosphate solubilizing activities of phosphor bacteria were determined by the method of H.A. Alikhani [16].The potassium-releasing activities of silicate bacteria were estimated by determination of potassium content with Flame Spectrophotometer.

### 2.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The genotypes of representative isolates were determined by ARDRA. Full-length of 16S rRNA gene fragments were obtained by PCR amplification with forward primer "ACGGCTACCTTGTTACGACT" and reverse primer "AGAGTTTGATCCTGGCTCAG". The reaction mixture contained 2.5 μl 10 × PCR Buffer (200 mM Tris-HCl, 500 mM KCl, pH adjusted to 8.4), 2.5 μl 10 mmol/l dNTP, 2.0 μl 25 mmol/l $MgCl_2$, 1μl primers solution (10μmol/l each); 0.5μl Taq polymerase, 1μl template, and the volume was complement by double distilled water to 30 μl. The PCR procedures were as follows: step 1, 94℃ for 3 min; 35 cycles of step 2, 94℃ for 1 min, 54℃ for 1min and 72℃ for 1min; step 3, 10 min incubation at 72℃.

## 3 Results and Discussion

### 3.1 Rep-PCR Genomic Fingerprinting Analysis

Rep-PCR analysis of 85 PGPR isolates was performed, and the clustering scheme was constructed by SAS 8.0 (Fig.1). It is showed that 20 isolates (the fifth, ninth, fourteenth, sixteenth, seventeenth, nineteenth, twenty-second, twenty-fourth, twenty-ninth, thirtieth, thirty-first, thirty-second, thirty-fourth, thirty-fifth, thirty-sixth, thirty-seventh, thirty-ninth, fortieth, forty-fourth and the forty-ninth) had the same repetitive-element PCR genomic fingerprinting, and other isolates had the particular repetitive-element PCR genomic fingerprinting of their own. Therefore, the 85 PGPR isolates were divided into 49 clusters at the species level. By the analysis of Rep-PCR, 18 kinds of azotobacteria, 18 kinds of phosphor bacteria and 23 kinds of silicate bacteria were obtained from mulberry root. It is also revealed that the patterns of Rep-PCR were specific for different bacterial species and strains.


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**Fig.1 Cluster analysis of PGPR Rep-PCR fingerprints**

## 3.2 Nitrogenase Activities of Azotobacteria

The codes for the 18 kinds of azotobacteria were PK1, PA2, PA3, FA20, PA19, PA23, FA17, FA8, PA4, FA1, FA10, PA13, FA15, FA4, PK5, PA20, FA9 and FA2, respectively. The measurement of nitrogenase activities showed that different strains of azotobacteria had different nitrogenase activities, ranging from 11.07 to 388.37 nmol $C_2H_4$/ hml. PA16 showed the highest activity of 388.37 nmol $C_2H_4$/hml, while FA4 had the lowest activity of 11.07 nmol $C_2H_4$/hml. PA19 and PA2 had higher nitrogenase activities than that of others. By ANOVA analysis of SAS 8.0, the activity values of FA16 and PA2 were notable different from others. The *P*-value was smaller than 0.0001, which meant the model was highly significant.

## 3.3 Phosphate Solubilization Activities of Phosphor Bacteria

The codes for 18 kinds of phosphor bacteria were FWP8, FWP1, FWP9, FWP6, PA4, FK14, PK1, FWP11, PA23, PYP3, PWP3, PWP6, FK11, PYP2, PA13, PWP1, PYP6 and FA2, respectively. The organic and inorganic phosphate solubilization activities were determined with lecithin plates and phosphor plates, respectively.

For organic phosphate ones, turbid circle formed except bacteria FYP5 in lecithin plate 24 h after inoculation. The diameter of circle size varies from 0.92 to 1.86 cm. After incubated for 72 h, palpable turbid circle were formed with the diameter ranging from 1.44 to 2.89 cm. The values of the organic phosphate solubilization activities of phosphor bacteria were analyzed by the method of ANOVA using SAS 8.0 software, and the *P*-value was 0.0029. PYP2, PA13, PYP6, PYP3 had higher organic phosphate solubilization activities than that of others.

For inorganic phosphate ones, transparent circle formed in phosphor plate 24h after inoculation and the diameter of circle size varies from 0.82cm to 1.97cm. After incubated for 72h, palpable transparent circle were formed with the diameter ranging from 1.41 to 2.89cm. The values of the inorganic phosphate solubilization activities of phosphor bacteria were analyzed by the method of ANOVA using SAS 8.0 software and the *P*-value was less than 0.0001. FK14, FK11 had higher inorganic phosphate solubilization activities than that of others.

Strains of FWP8, FWP6, PA4, FK14, PK1, PWP3, FK11 and PWP1 had no organic phosphate solubilization activities but had inorganic phosphate solubilization activities. Strains PA23, PYP3, PYP2, PA13 and PYP6 had no inorganic phosphate solubilization activities but had organic phosphate solubilization activities. Strains FWP1, FWP9, FWP11, PWP6 and FA2 had both organic and inorganic phosphate solubilization activities.

## 3.4 Potassium-releasing Activities of Silicate bacteria

The codes for 24 kinds of silicate bacteria were FK7, FK14, FK8, FK4, PA6, PK1, FK3, FK1, FA17, FK2, FK6, FK4, FK11, FK4, PK23, PK17, FA6, PK12, FA1, PA13, FK23, PK7, PK5 and FK5, respectively. The potassium-releasing activities of silicate bacteria were investigated by Flame Spectrophotometer. The concentration of $K^+$ in each culture was higher than control. $K^+$ concentration of FK2 and FK3 were higher than that of others, which achieved 190mg/l and 187 mg/l. 72 h after inoculation, and increased 40.79％ and 40.60％ than control.

## 3.5 Sequences Analysis of 16s rDNA

10 isolates with high biological activities (PA19, PA2, PYP2, PYP4, PYP6, PYP3, FK14, FK2 and FK3) were selected for strain identification. 16S rRNA gene fragments of 10 isolates (about 1.5 kb) were sequenced and analyzed utilizing the tool of BLAST on NCBI website.

The strain PA19 showed high homology (97-99%) with genus *Mesorhizobium*. Phylogenetic tree of PA19

was constructed using DNAman software with sequences of different *Mesorhizobium* and *Brevibacillus laterosporus* species from Genbank database. It showed 97% identity with *Mesorhizobium* sp. 90 (AY225391), which indicates this isolate belongs to genus *Mesorhizobium*.

Isolates PA2, PYP2, PYP6, PYP3 and FK2 showed high homology (95-99%) with genus *Pseudomonas*. Phylogenetic tree of PA2, PYP2, PYP6, PYP3 and FK2 was conducted by DNAman with the sequences of different species of *Pseudomonas* obtained from GenBank database. PA2 showed 98% identity with *Pseudomonas* sp. KBOS03 (AY653220). PYP2 showed 98% identity with *Pseudomonas* sp. c58 (AB167219). PYP6 showed 99% identity with *Pseudomonas* sp.An15 (AJ551153). PYP3 showed 96% identity with *Pseudomonas* sp. P97.26 (AY456707). FK2 showed 99% identity with *P. veronii* strain A1XB2-5 (AY512619). It indicates that these isolates belong to genus *Pseudomonas*.

The isolates FK11 and FK14 showed high homology (97-99%) with *Rhizobium* genus. Phylogenetic tree of FK11 and FK14 was conducted by DNAman with sequences of different species of *Rhizobium* obtained from Genbank database. FK11 showed 98% identity with *Rhizobium* sp. tpud. 40a (AY691399). FK14 showed 97% identity with *Rhizobium* sp. tpud. 40a (AY691399).

The isolate FK3 showed high homology (97-99%) with genus *Sinorhizobium*. Phylogenetic tree of FK3 was conducted by DNAman with the sequences of different species of *Sinorhizobium* obtained from Genbank database. FK3 showed 99% identity with *Sinorhizobium* sp. (AJ012211). It indicates that this isolate belongs to genus *Sinorhizobium*.

Isolate PA13 showed high homology (98-99%) with genus *Inquilinum*. Phylogenetic tree of PYP4 was conducted by DNAman with the sequences of different species of *Inquilinum* obtained from Genbank database. PA13 showed 98% identity with *Inquilinus ginsengisoli* (AB245352), which indicates that this strain belongs to genus *Inquilinum*.

The sequences obtained in this study were registered in GenBank nucleotide sequence database. The accession numbers of strains PA19, PA2, PYP2, PYP4, PYP6, PYP3, FK14, FK2 and FK3 were EF462392, EF462383, EF462379, EF462370, EF462378, EF462368, EF462364, EF462372 and EF462385, respectively.

## 4 Conclusions

PGPR research is becoming more and more popular in the area of biofertilizer in recent years. The characteristic of improving soil fertility is considered as an important measure to control soil chemical pollution. In this study, we investigated the diversity and biological function of PGPR in mulberry soil. The results indicated that there are many kinds of azotobacter, phosphor bacteria and silicate bacteria colonized in mulberry rhizosphere, which showed high biological activity as a type of biofertilizer. Mature technologies for determination of nitrogenase activity, phosphate solubilization activity and potassium-releasing activity were applied in this research, and 16S rDNA gene detection was proved to be an effective way for identification of bacteria in the investigation. It is suggested that the PGPR isolated from mulberry rhizosphere have the potential of controlling of the soil chemical pollution. The research hopes to provide a theoretical basis for improving the soil fertility by biological effect of PGPR and reducing the soil pollution caused by chemical fertilizer.

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