Isolation of PGPR strains with phosphate solubilizing activity from Erzurum and Their Molecular Evaluation by Using Newly Designed Specific primer for *pqqB* Gene

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Abstract— Plant growth-promoting rhizobacteria (PGPR) have ability to colonize plant roots and increase plant growth by wide range of mechanisms. These mechanisms include biological nitrogen fixation, phosphate solubilization, production of 1-Aminocyloproporane-1-carboxylate deaminase (ACC) and phytohormones. The phosphate solubilization is one of the most valuable potential for the usage of agricultural fields as a eco-friendly fertilizers. In this context, there are various studies for the correct selection of phosphate solubilization strains with conventional methods. Molecular techniques can be an alternative method instead of conventional methods because of their short time results and low cost. In this context, the aim of present study was to evaluate the usability of *pqqB1* primer set for the selection of local phosphate solubilization strains. Soil samples for PGPR isolation studies were collected from agricultural fields in Erzurum. Dilution series (10^{-1}-10^{-7}) were spread on Luria-Bertani (LB) agar plates and single colonies were selected further purification studies. Then, each of isolates was inoculated onto Pikovskaya's agar plates for phosphate solubilizing capability. From molecular perspective, presence of *pqqB* gene region, closely related to bacterial phosphate solubilizing were monitored by using PCR with designed specific primers (5'-AATCCAAGCCAAAGCCCGTA-3' and 5'- ATTGTCGCCATCATGTGGGT -3'). Additionally, the molecular characterization of the active isolates was done based on the 16S ribosomal RNA gene region sequence analysis method. When the results were evaluated, four isolates (TAGEM15-70-B8, TAGEM15-70-B22, TAGEM15-70-B24, and TAGEM15-70-B29) were determined as active PGPR strains, which have phosphate solubilizing activities. It also gave a specific amplification band on gel electrophoresis, showing presence *pqqB* gene. These were identified as *Rhizobium* sp., *Enterococcus* sp., *Bacillus cereus* and *Acinetobacter* sp.

Index Terms — PGPR, Phosphate solubilization, *pqqB* gene, 16S rRNA

1. INTRODUCTION

Global food demand is rapidly increasing due to human-induced factors all over the world. After discovering green revolution technologies, alternative agricultural practices were used for more crop production such as chemical fertilizers, pesticides and high yielding varieties. However overutilization of chemical fertilizers and plant protecting chemicals caused degradation of ecosystem and environmental problems. As a result, it is a great challenge to explore sustainable strategies to mitigate the detrimental effects of intensive farming practices [1]. Plant growth-promoting rhizobacteria (PGPR) have ability to colonize plant roots and increase plant growth by wide range of mechanisms. These mechanisms include biological nitrogen fixation, phosphate solubilization, production of 1-Aminocyloproporane-1-carboxylate deaminase (ACC) and phytohormones [2]. In this context; the solubilization of phosphate in the rhizosphere is one of the most common mode of action implicated in PGPR that increase nutrient availability and positive effects for plant growth. Phosphorus is the second most abundant metallic

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element after the nitrogen and both organic and inorganic forms is found in the soil. It has play crucial roles in metabolic processes (photosynthesis, energy transfer, nitrogen fixations in legumes, quality of agricultural products, resistance to plant diseases and pathogens) [3]. Phosphate solubulizing microorganisms (PSM) are naturally present in rhizosphere. Bacteria, fungi, actiomycetes and even algae can help in enhancing the availability of phosphorus to plants in many different ways. Bacteria are predominant amongst them and well-known agricultural applications were implemented with them. The most efficient phosphate solubilizing microorganisms pertain to genera *Bacillus* and *Pseudomonas* amongst bacteria and *Aspergillus* and *Penicillium* amongst the fungi. In addition to *Pseudomonas* and *Bacillus*, many different bacteria identified as phosphate solubilizers. For instance; *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Phyllobacterium*, *Azotobacter*, *Xanthomonas*, *Enterobacter*, *Pantoea,* and *Klebsiella* [4]. Numerous studies have been aimed to identification for correct selection of phosphate solubilization strains with conventional methods and the correct results have been yielded with these methods. As an alternative method, molecular techniques can be used instead of conventional methods because of their low cost and short time results [5]. In this regard, phosphate solubilizing bacteria were detected with conventional method and from molecular perspective; a few genes have taken part in literature. Among these, Pyrroloquinoline Quinone genes (PQQ) plays crucial role in the growth and development of almost whole organisms. It is believed that the main source of PQQ in these organisms is microbial resources. PQQ has been regarded as fundamental plant growth promoting factor, supporting cell growth, and stress tolerance in bacteria and plants. PQQ can be appeared in many plants, animals and microorganisms but bacteria can solely synthesize it. PQQ producing bacteria have been characterised in bacteria of variety genera such as; *Acinetobacter, Ancylobacte, Gluconobacter, Hyphomicrobium, Klebsiella, Paracoccus, Polyporus, Pseudomonas, Methylobacillus, Methylophilus, Methylovorus, Methylobacterium, Mycobacterium, Thiobacillus,* and *Xanthobacter.* PQQ is a significant phosphate solubilizing factor. Many of these PQQ producing bacteria are capable of inorganic phosphate solubilization and they can be used for inorganic phosphate fertilizers in agricultural fields. PQQ biosynthetic genes are clustered in the different operons such as; *pqqABCDEF* and *pqqABCDE and* located in *Klebsiella pneumoniae* and *Pseudomonas auruginosa* respectively [6]. In this present study; phosphate solubilizing bacteria were detected with conventional method and also from molecular perspective *pqqB* gene region, closely related to bacterial phosphate solubilizing were monitored by using PCR with designed specific primers for local strains from Erzurum.

2 MATERIALS AND METHODS

2.1 Collection of rhizospheric soil samples

Soil samples were collected from agricultural areas in Erzurum province, Turkey. The samples were brought to the laboratory in sterile tubes. Dilution series (10^{-1}-10^{-7}) were spread on Luria-Bertani (LB) agar plates and single colonies were selected for purification studies.

2.2 Isolation of phosphate solubilizing bacteria (PSB) with conventional method

Collected the rhizospheric soil samples were used for isolation phosphate solubilizing bacteria on Pikovskaya's (PKV) agar medium containing the following (g/L): 10.0 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0,2 g KCl; 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$. 0.002 g $FeSO_4 \cdot 7H_2O$ and 1000 ml distilled water. The medium was autoclaved at 121 °C for 15 minutes and then the medium was mixed well and poured to petri dishes. Phosphate solubilization was scrutinized on the PKV agar medium after 2-7 days incubation at 28 °C [7].

2.3 Assessment of newly designed *pqqB* primer set for detection of phosphate solubilisation DNA isolation from rhizospheric bacterial samples

Genomic DNA isolation of bacterial isolates were performed according to the modified method described by Sezen 2015 [8].

Amplification of 16S rRNA region from rhizospheric bacterial samples

The 16S rRNA gene regions were amplified using polymerase chain reaction for molecular identification of the bacterial isolates. In this reaction, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) were used as forward and reverse primers, respectively. The reaction was carried out in a 30 µl reaction mixture containing; 1.2 µl of dimethyl sulfoxide (DMSO), 0.6 µl $MgCl_2$, 0.6 µl dNTP, 3 µl forward primer and 3µl reverse primer, 0.3 Taq DNA, 3 µl 10XPCR buffer, 15.3 distilled water, 3 µl DNA template. The reaction was performed with

an initial step at 95 ºC for 2 min, and 36 cycles of 1 min at 94 ºC, 1 min at 54 ºC, 2 min at 72 ºC, followed by a final 5 min extension step at 72 ºC, then brought down to 4 ºC.

Analysis of PCR products

Amplified PCR products were analysed by QIAxcel® advanced analysis system.

Sequence analysis of 16S rRNA gene region

The amplified gene products were sequenced by Macrogen Inc. (Netherlands). The nucleotide BLAST (Basic Local Alignment Search Tool) search program of NCBI was used to determine the nucleotide sequence homology and compared to other bacterial sequences available in the GenBank® (http://blast.ncbi.nlm.nih.gov/blast.cgi). The gene sequences were also submitted to GenBank® and accession numbers were assigned [9].

Specific primer designing

Specific primer of selected gene region (*pqqB*) is designed with NCBI primer tool database and obtained primer from this database was checked *in silico* conditions by using computer [5].

3 RESULTS AND DISCUSSION

This study aimed to isolate and identify phosphate solubilizing bacteria from agricultural fields from Erzurum with conventional and molecular methods. Microorganisms play key roles transforming growth factors and nutrients. Phosphorus is one of the most pivotal element for all living organisms on the earth. Hence, importance of studies on phosphate solubilizing bacteria have been increasing nowadays on the different fields such as; agricultural and farming areas. Recently, the researchers have given great attention to plant growth-promoting rhizobacteria (PGPR) because of their positive effect of soil health and improve of crop production instead of detrimental effect of chemical fertilizers. Until today, Identification of the correct selection of PGPR strains is done by conventional methods. Beside this method, from molecular perspective, specific genes are defined. For instance, *nif* genes for nitrogen fixation, *acdS* gene for ACC deamination, *pqq* genes for phosphate solubilization have been identified. From molecular perspective, presence of *pqqB* gene region, closely related to bacterial phosphate solubilizing were monitored by using PCR with designed specific primers (5'-AATCCAAGCCAAAGCCCGTA-3' and 5'-ATTGTCGCCATCATGTGGGT -3'). Additionally, the molecular characterization of the active isolates was done based on the 16S ribosomal RNA gene region sequence analysis method. When the results were evaluated, four isolates (TAGEM15-70-B8, TAGEM15-70-B22, TAGEM15-70-B24, and TAGEM15-70-B29) were determined as active PGPR strains, which have phosphate solubilizing activities (Table 1). It also gave a specific amplification band on gel electrophoresis, showing presence *pqqB* gene. These were identified as *Rhizobium* sp., *Enterococcus* sp., *Bacillus cereus* and *Acinetobacter* sp.

TABLE I

SEQUENCES RESULTS

SEQUENCE			
Codes Numbers of Test Microorganisms	**Gen Bank Accession number**	**Percent identify***	**Closest phylogenetic relative (GeneBank accession number)**
TAGEM15-70-B8	MG584451	%100	*Rhizobium* sp.
TAGEM15-70-B22	MG685815	%100	*Enterococcus* sp.
TAGEM15-70-B24	MG685817	%100	*Bacillus cereus*
TAGEM15-70-B29	MG685822	%99	*Acinetobacter* sp.

4 CONCLUSION

Identification of nitrogen fixation, indole acetic acid production, phosphate solubilizing bacteria from soil samples with conventional method is time consuming and laborious. Today, numerous genes have been

reported that have related with nitrogen fixation, ACC deamination and phosphate solubilization etc. [10].
Designed specific primers for these genes will be more convenient and useful because of their low cost and short time results. These molecular techniques target conserved DNA sequences with well-defined gene clusters. The sensitivity and specificity of detection depend on the selection or design of appropriate targets, with primers and on control of the stringency of PCR amplification. Consequently, the specific primer sets should design according to the local samples because of physiology and genetic adaptation of PGPR strains. This method opens up new horizon for better selection of PGPR strains and usage for greater agricultural product performance.

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