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Phosphate Solubilization by *Bacillus subtilis* and *Serratia marcescens* Isolated from Tomato Plant Rhizosphere

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Abstract

Plants need phosphorus for many physiological activities in a form of phosphate anions. Three different bacterial strains (*Bacillus subtilis* PH, *Serratia marcescens* PH1, and *Serratia marcescens* PH2), recently isolated from tomato plant rhizosphere, have high phosphate solubilization index (SI from 2.8 to 3.2) on Pikovskaya agar medium (which contains calcium phosphate). Moreover, phosphate release from calcium in Pikovskaya broth over 5 days is increasing with cell growth for the different isolates. The most efficient phosphate solubilization case is the mixed culture of the 3 strains (OD_{475} is almost 1). On the other hand, pH values decreased dramatically with time due to organic acids secretion and the maximum acidification level is recoded for *Serratia marcescens* PH2 (pH = 1.94). Interestingly, the isolates are resistance to important pesticides (oxamyl, thiophanate methyl, and captan) that are commonly used in the sampling area. This resistance is very favorable and increases the persistence of the phosphate solubilizing bacteria in contaminated soils. The isolates are therefore plant symbionts and growth promoting agents.

Keywords

Phosphate Solubilization, Pikovskaya Medium, *Bacillus subtilis*, *Serratia marcescens*

1. Introduction

Phosphorus plays a key role for all life forms [1]. It is essential for several plant physiological activities like photosynthesis, cell division, and others [2]. Its deficiency leads to brown and small leaves, weak stem and slow development [3] [4].

Accordingly, phosphorus is one of the major plant nutrients that limits plant growth [5]. It remains insoluble in the soil like other essential nutrients [6]. Phosphate chemical fertilizers are commonly applied to the soil to increase phosphate availability to plants. Unfortunately, soluble inorganic phosphate in such fertilizers is rapidly immobilized and becomes unavailable to plants [3]. To overcome this serious problem, farmers apply phosphate fertilizers in many fold excess [7]. On the other hand, excessive fertilizers use results in contamination of soil with heavy metals [8]. Therefore, an environmentally friend release of fixed and insoluble phosphorus forms is a necessary for increasing the availability of soil phosphorus to plants [9] [10] [11].

Natural phosphate solubilization by different microorganisms [12] [13] [14] [15] is therefore an important phenomenon [16]. The predominant microorganisms that naturally solubilize mineral phosphates are bacteria [17] [18]. Phosphate solubilizing bacteria (PSB) which is associated with plant roots is one of the most significant alternatives for inorganic phosphate fertilizers [19] [20] [21] [22]. This group of bacteria is termed plant growth promoting rhizobacteria [23] [24] which includes many genera such as *Serratia, Rhizobium, Pseudomonas, Paenibacillus, Flavobacterium, Erwinia, Enterobacter, Burkholderia, Bacillus, Azospirillum, Arthrobacter, Acinetobacter*, and *Alcaligenes* [25] [26] [27]. These bacterial genera are then significant biofertilizers for agricultural improvement. Besides, PSB play a key role in cycling of biogeochemical phosphorus in aquatic and terrestrial environment [28]. PSB transform phosphates into soluble forms by secreting enzymes such as phosphatases and/or organic acids [28] [29].

In this study, three different bacterial strains, isolated from tomato plant rhizosphere, were selected for their high phosphate solubilization index and molecularly identified using the 16S rDNA sequencing. The strains ability to release phosphate in Pikovskaya broth was also tested as well as resistance against some commonly used pesticides.

2. Materials and Methods

2.1. Bacterial Isolation and Purification

Roots of tomato plant (found in a farm located at Nubarya, Beheera governorate, Egypt, in May-2017) were washed with sterile distilled water. Washing water was diluted and plated in nutrient agar (Oxoid, England) plates. The plates were incubated for 24 h at 30˚C. Bacterial colonies were carefully examined and predominant phenotypes were randomly selected and purified.

2.2. Detection and Efficiency Estimation of Phosphate Solubilizing Bacteria

Predominant colonies were selected and tested for production of halozones on Pikovskaya agar (Techno Pharm Chem, Haryana, India) plates to detect phosphate solubilizing bacteria [25]. In 48 h of incubation at 30˚C, colonies showing halozones were selected to test their solubilization index (SI). However, colonies with the highest SI values were chosen for further experiments. Phosphate SI was

determined by measuring both colony and halozone diameters using Edipremono *et al.* [30] formula:

Phosphate SI = (colony diameter + halozone diameter)/colony diameter

Quantitative analysis of phosphate solubilization by bacterial isolates was performed *in vitro* using Pikovskaya broth. Conical flasks containing that broth medium were inoculated with separate and mixed bacterial cultures in triplicates at 30˚C for 5 days on a rotary shaker at 150 rpm. After regular intervals, cultures were centrifuged at 5000 rpm for 10 min and available soluble phosphate was measured in supernatants spectrophotometrically at 475 nm using phosphomolybdate method [31]. In this method, quantification of phosphorous requires the conversion of the phosphorus to dissolved orthophosphate followed by colorimetric determination of dissolved orthophosphate. Ammonium molybdate and antimony potassium tartrate react in an acid medium with diluted solutions of orthophosphate to form an intensely colored antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.

Besides, cultures optical densities at 550 nm were measured before centrifugation and pH values were also recorded.

2.3. Morphological Characterization and Pesticides Resistance

Some morphological features of the isolates were determined such as colony color, cell shape, and Gram stain reaction. Besides, isolates resistance to some commonly used pesticides (oxamyle, thiophonate methyl, and captan) was also tested. Oxamyl is a nematocide (Medmac, Jordon), thiophonate methyl is a fungicide (Wuxi xinan pesticides Co. Ltd., China), and captan is also a fungicide (Arysta Life Science, France). The resistance test was performed using nutrient agar plates supplemented with different pesticides concentrations. Plates were then incubated for 48 h at 30˚C.

2.4. 16S rDNA Sequencing and Analysis

For bacterial identification, the 16S rDNA partial sequencing (approx 900 pb) was performed using the universal and specific primers listed in Table 1 at Macrogen incorporation, Soul, Korea. Sequencing was performed using Big dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were then analyzed on an Applied Biosytems model 3730XL Automated DNA Sequencing System (Applied Biosystems, USA). Finally, sequences were analyzed at DDBJ (DNA Data Bank of Japan) using BLASTN program [32]. After deposition in the GenBank, sequences accession numbers were obtained and listed in Results.

3. Results and Discussion

Predominant colonies in soil that is directly attached to tomato roots were selected and tested for production of halozones on Pikovskaya agar plates to detect

phosphate solubilizing bacteria. Accordingly, solubilization index (SI) values were measured for all of them (data not shown). However, three isolates (C2, T1, and T5) were found to have the highest SI values and therefore were selected for further experiments. Firstly, the three unique isolates were subjected to molecular identification by partial sequencing of the 16S rDNA gene and the accession numbers of the sequences were listed in Table 2. The strains are found to be *Bacillus subtilis* strain PH (C2), *Serratia marcescens* strain PH1 (T1), and *Serratia marcescens* strain PH2 (T5). Secondly, the phosphate solubilization ability of the strains was qualified on Pikovskaya plates. The three strains are potent phosphate solubilizers and the halozone diameter is almost double of the colony diameter (Table 3). Values of solubilization index, SI, are relatively close to each other among the isolates. Similar results have been obtained by Paul and Sinha, 2017 [4]. They recorded a SI of 2.85 for their bacterium, *Pseudomonas aeroginosa* KUPSB12, using Pikovskaya agar plates. These clear zones around colonies are due to the solubilization of phosphate found in Pikovskaya medium. Phosphate solubilization may be due to the production of organic acids, polysaccharides or phosphatases [33] [34]. Uma Maheswar and Sathiyavani, 2012, [35] reported that *B. subtilis* and *B. cereus* are forming halozones in Pikoviskaya agar medium due to solubilization of calcium phosphate. In addition, Widiastuti, 2008, [36] stated that the ratio of clearing zone to colony diameter for two different *Serratia marcescens* isolates were approximately 2.1 and 1.9.

Table 1. Primers used in amplification and sequencing.

Primer	Primer sequence (5'-3')	Amplification	Sequencing
27F	AGAGTTTGATCMTGGCTCAG	•	
1492R	TACGGYTACCTTGTTACGACTT	•	
518F	CCAGCAGCCGCGGTAATACG		•
800R	TACCAGGGTATCTAATCC		•

Table 2. Accession numbers of the new bacterial isolates.

code	strain	Accession number
T1	*Serratia marcescens* PH1	LC335898
C2	*Bacillus subtilis* PH	LC335897
T5	*Serratia marcescens* PH2	LC335899

Table 3. Phosphate solubilization index (SI) for the bacterial isolates.

Isolate code	Strain	Colony diameter (cm)	Halozone diameter (cm)	SI
T1	*Serratia marcescens* PH1	0.5 ± 0.03	1.1 ± 0.05	3.2
C2	*Bacillus subtilis* PH	0.5 ± 0.02	1 ± 0.1	3
T5	*Serratia marcescens* PH2	0.5 ± 0.05	0.9 ± 0.05	2.8

Thirdly, some morphological traits for the recent isolates are illustrated in Table 4. Interestingly, *Serratia marcescens* has a red color in nutrient agar medium and loses the pigment in Pikovskaya plates. It is obvious that medium composition affects pigment production by *Serratia* marcescens [37]. This is may be due to the difference in nitrogen source. Pikovskaya medium contains inorganic nitrogen (ammonium sulphate) while the nutrient agar contains organic nitrogen (beef extract and peptone) [36]. However, the red pigment, prodigiosin, results from secondary metabolism of *Serratia marcescens* [37].

For more characterization of the recent isolates, resistance against some pesticides (captan, Thiophonate methyl, and oxamyl) is also tested and the results are recorded in Table 5. Generally, the isolates showed relatively high resistance against captan and Thiophonate methyl. Lower resistance was detected against oxamyl. However, both of *Serratia marcescens* strains scored the highest resistance levels against captan. On the other hand, *Bacillus subtilis* PH is the most resistant against thiophonate methyl. Resistance against pesticides, which are commonly used at the sampling area, is an advantage for phosphate solubilizing bacteria because it means more persistence in that harsh environment.

Finally, cell growth patterns and phosphate release of the three newly isolated strains as well as pH changes with time are clearly shown in Figures 1(a)-(d). Obviously, phosphate release which is in a direct proportion with OD475 and cell growth are increasing with days for all of the isolates. However, the mixed culture of the 3 isolates is the most efficient case in phosphate release (Figure 1(d)). On the other hand, pH is decreasing with time due to the secretion of organic acids into the medium for solubilization of calcium phosphate found in Pikovskaya broth [3]. The maximum drop in pH values was parallel with increased

Table 4. Morphological characterization of the new isolates.

Characteristic	Isolates		
	S. marcescens PH1	*B. subtilis* PH	*S. marcescens* PH2
Colony color in nutrient agar	Dark red	White	Slight red
Colony color in Pikovskaya agar	White	White	White
Gram reaction	Negative	Positive	Negative
Cell shape	Rods	Rods	Rods

Table 5. Resistance of the isolates against some commonly used pesticides.

Isolate	Pesticide concentration in ppm		
	Captan	Thiophonate methyl	Oxamyl
Serratia marcescens PH1	2000	1000	150
Bacillus subtilis PH	200	2000	150
Serratia marcescens PH2	2000	500	150

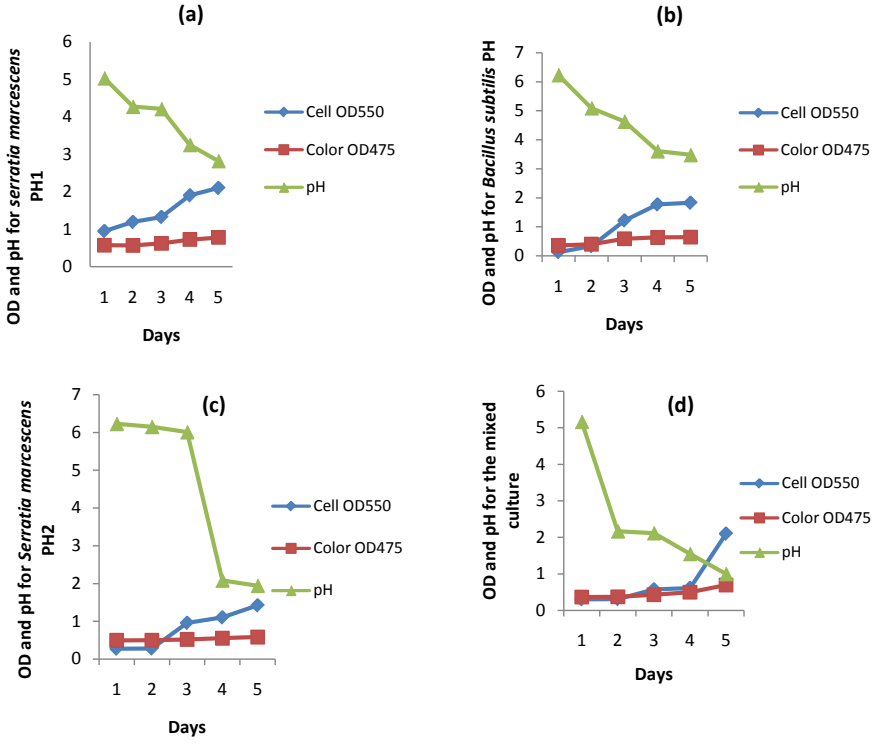

Figure 1. Bacterial growth in Pikovskaya broth medium and phosphate release represented by the optical densities, OD, at 550 and 475, respectively, and culture pH values of the three strains ((a), (b), and (c)) and the mixed culture (d).

phosphate solubilization levels. The maximum acidification level was recorded for *Serratia marcescens* strain PH2 (pH = 1.94). However, in the mixed culture medium, pH dropped dramatically to 1 in 5 days from an initial point of 7. Phosphate solubilizing bacteria are usually produce lactic, gluconic, isobutyric, ketogluconic, oxalic, acetic, and citric acids. Besides, the mechanism of mineral phosphate solubilization is due to production of organic acids and/or phosphatases [38] [39] [40]. However, inorganic phosphate is solubilized by both organic and inorganic acids of phosphate solubilizing bacteria. Carboxyl and hydroxyl groups in these acids chelate Ca, Fe, and Al cations [41]. Usually, calcium phosphates (including rock phosphate ores) are insoluble in soil [41]. Gerretsen [42] reported that when pure cultures of soil bacteria are added to the soil, plant phosphate nutrition is increased throughout increased calcium-phosphate solubility. Soil pH is decreased in parallel and therefore phosphate solubilization is the net result of both pH decrease and acids production [43]. In other words, carboxylic and hydroxylic anions produced by phosphate solubilizing bacteria have high calcium affinity and therefore can solubilize more phosphorus than acidification alone [44]. Accordingly, there is a symbiotic relationship between plants and phosphate solubilizing bacteria [45] [46], as bacteria provide the soluble phosphate and plant roots provide carbon compound such as sugars [47]. The net result of this relationship is crop production enhancement [48] [49]. The most significant solubilizers of phosphate are mainly belonging to *Bacillus*

spp. such as *B. subtilis, B. cereus, B. polymyxa*, *B. circulans, B. circalmous* and *B. megaterium* [50]. Patil, 2014, [51] has reported that *B. subtilis* is a powerful phosphate solubilizer that tolerates soil salinity. Besides, phosphate solubilizing *Bacillus megaterium* mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote Mustard plant growth [52].

In 2008, Widiastuti [36] reported phosphate solubilization in Pikovskaya medium by *Serratia marcescens* and stated the relationship between phosphate solubilization and red pigment production. Previous studies reported the production of organic acids by *Serratia marcescens* [53]. Others proved the presence of genes such as *pqq* and *gdh* which are coding for phosphatase activity in *Serratia marcescen*s [54] [55] as well as *Pseudomonas* [56]. Moreover, Lavania and Nautiyal [57] recorded that the soil isolate *S. marcescens* NBRI1213 is an efficient phosphate solubilizer and a potential plant growth promoting agent. Besides, Behera *et al*., and others [1] [58] [59] [60] [61] stated acid phosphatase production by *Serratia*. In addition to phosphate solubilization, *Serratia* and *Alcaligenes faecalis* have an antagonistic activity against plant pathogens [62] [63] [64] and can produce hydroxyl apatite [65]. In our research, the maximum phosphate solubilization efficiency was recorded in 5 days for the mixed culture followed by *Serratia marcescens* PH1. Previous studies recorded different periods to reach maximum phosphate solubilization. For instance, some researchers have reported 3, 4, 10, and even up to 15 days [66].

4. Conclusions

In the present study, three different tomato rhizosphere bacterial strains are used for phosphate solubilization in Pikovskaya medium. These isolates are characterized by:

1) High phosphate solubilization index.

2) Increasing ability to release mineral phosphate over days with a dramatic decrease in pH values.

3) Resistance to pesticides that are commonly used in the sampling location.

All of these advantages make the bacterial isolates suitable as plant growth promoting symbionts that persist contamination conditions and make free phosphate anions available for plants.

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