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## COMPARATIVE EFFECT OF RHIZOSPHERIC AND ENDOPHYTIC BACTERIA ON TCP AND CaCO<sub>3</sub> SOLUBILIZATION AND GROWTH PROMOTION OF WHEAT (*TRITICUM AESTIVUM* L.)

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**Abstract.** Phosphate is the second most essential nutrient for plant growth and development. Although the soil contains abundant phosphorus, only 0.1% is used by plant roots due to the significant amount of calcium carbonate (CaCO<sub>3</sub>) present in the calcareous soil, which limits the availability of P by reacting calcium ions with phosphate ions. It leads to alkaline reactions by generating insoluble phosphate compounds such as tricalcium phosphate. Here we study to boost phosphate solubilization in PSMs as an alternate strategy. Twenty-one bacterial isolates were selected from two different crops and investigated in vitro for their plant growth potential. All bacteria show capacity for phosphate solubility by producing a clear halo zone beneath and much more around bacterial colonies on Pikovskaya agar. In contrast, on modified Pikovskaya and only seven isolates with 60% reduction in zone formation were revealed to be positive for phosphate solubilization in which SWC5 was the best phosphate solubilizers with 1.25 phosphate solubilization index, respectively. All phosphate solubilizing bacteria showed irregular pattern in lowering the pH ranging from 4.1- 6.27, where maximum decreased in pH was shown by SWS10. All bacteria showed phosphate solubilization ability in Pikovskaya broth phosphomolybdate assay ranging from 6.31 to 20.9 µg ml<sup>-1</sup> in which the highest efficiency was demonstrated by SWS10. Phosphate solubilization efficiency in the potential isolates was confirmed by the presence of PqqE gene. The presence of the pqqE gene which is

involved in phosphate solubilization was found in SWC4, SWC5, SWS10, and SW14 and it was absent in SWS9, SW18, and SW21. All the bacterial isolates were phylogenetically identified by sequencing and analyzing the 16S rRNA gene. The potential isolates *Pseudomonas Koreensis*, *Pantoea dispersa*, *Pseudomonas* sp., *Agrobacterium* sp. and consortia were selected for controlled condition experiment on *Triticum aestivum*. The highest plant dry weight was achieved by application of consortia and *Pseudomonas Koreensis* by increasing 65% and 59%, respectively, compared to control.

**Keywords:** PGPR, biofertilizers, PSB, gluconic acid production, PQQ gene, phosphate solubilization

## Introduction

Cereal crops are essential in agriculture because they play a crucial role in the human diet. Among these wheat (*Triticum aestivum* L.) is one of the major food crops in the world; about 60 percent of the world's population consumes wheat as a staple food (Zhang et al., 2022; Wang et al., 2020). A noticeable increment of approximately 2.5% occurred in Pakistan's wheat production from 24.349 million tons to 24.946 million tons compared to previous years (Iqbal et al., 2022). A noticeable increment in wheat productivity is achieved by phosphorus as a vital plant nutrient. Soil contains of 400-1000 mg kg<sup>-1</sup> of total phosphorus, of which only (1.00-2.50%) is accessible for plant absorption (Riczu et al., 2019). Despite the abundance of organic and inorganic P in soils, accessibility of P is limited to plants because the more significant proportion of P in soils is fixed. P availability is influenced by various parameters such as soil pH, texture, calcium and iron compounds, the existence or absence of microorganisms, and soil structure (Nishigaki et al., 2019). In Pakistani soil, enough phosphorus is present but undergoes 90% to severe phosphorus deficiency. In Pakistan, 250,500 tons of Triple Super Phosphate (TSP) and Di-ammonium Phosphate (DAP) fertilizer are used for wheat production, while only (15-20%) of the applied P convert into accessible form for plant uptake (Adnan et al., 2017). Chemical fertilizers are used to obtain the highest efficiency and best quality product to meet growing food demand, but their use can harm plants and our environment in several ways. Plant growth-promoting bacteria (PGPRs) have the potential to act as biofertilizers, contribute to soil fertility maintenance (Bhardwaj et al., 2014) and could be used alternative to conventional fertilizers for feasible agriculture (Itelima et al., 2018). The final goal was to create environmentally friendly phosphate biofertilizers with minimum input for long-term cereal crop development and good yield. Several soil-borne microbes termed PSM (phosphate-solubilizing microorganisms) are crucial in the soil phosphorus cycle because they convert the insoluble form of phosphorus into a soluble form (Hu et al., 2022; Yi et al., 2022). Hence, plants use it efficiently (Rawat et al., 2021). Numerous phosphate-solubilizing rhizospheric microsbionts colonized the plant root surface and soil, adhering to the roots and forming a symbiotic connection with the plant to help it grow by making nutrients available, creating phytohormones (Elhaisoufi et al., 2020). Microorganisms are endophytic microsbionts that live inside plant tissue and help them to grow. Several direct and indirect methods are involved in organic and inorganic phosphate solubilization by PSMs, which include the release of low molecular organic acids like gluconic acid, citric acid, oxalic acid, and tartaric acid into the surroundings that chelate the phosphate-bound cations, lowering the soil pH via gaseous exchange (Rawat et al., 2021; Huang et al., 2019). Another mechanism for inorganic phosphate solubilization is the production of inorganic acids like Sulfuric acid, carbonic acid, hydrochloric acid, and nitric acid by reducing the same soil pH level as organic acids but with less efficiency (Elhaisoufi et al., 2020) siderophores excretion, Proton extrusion, proton H<sup>+</sup> is discharged, releases

Exopolysaccharides, secretion of alkaline and acid phosphatases. Although PSMs use a variety of approaches to dissolving phosphorus, acid production was found to be the most effective (Yang et al., 2024; Wang et al., 2022; Safdar et al., 2019). Phosphate solubilizing microbial culture illustrated the presence of various organic acids, like malic glyoxylic, fumaric, tartaric, succinic, keto butyric, citrus, oxalic, 2-keto gluconic, and gluconic that play a significant role in phosphorus solubility (Faria et al., 2022). Inorganic phosphate bound to Fe<sup>2+</sup> and Ca<sup>2+</sup> releases during direct oxidation, in which glucose dehydrogenase enzyme converts glucose into gluconic acid. Gluconate dehydrogenase further oxidized gluconic acid into 2-keto gluconic acid. Among these, gluconic acid and 2-keto gluconic acids were found to be more effective in the solubilization of phosphate (Kalayu, 2019). The 2-keto gluconic acid releases phosphorus by acting as a chelator of Ca<sup>2+</sup> and Fe<sup>2+</sup>. Oxalic acid was the most effective in releasing P in fertilized soil, whereas citrate was the most effective in uncultivated soil. Citrate has a higher affinity for Al<sup>3+</sup> and Fe<sup>3+</sup>, while oxalic acid is efficient by chelating with Ca<sup>2+</sup> (Konate et al., 2018).

Mineral phosphate solubilization (MPS) in PGPB occurs through an enzyme quinoprotein glucose dehydrogenase which is involved in the biosynthesis of gluconic acid (GA); Pyrroloquinolinequinone (PQQ) is the co-factor of glucose dehydrogenase (GDH) (Li et al., 2022). PQQ is a redox-active low molecular weight co-factor for numerous glucose dehydrogenases in bacteria and also plays a role in methylotrophic metabolism see in *Fig. 1*. PQQ operon comprises six genes, pqqA, pqqB, pqqC, pqqD, pqqE, and pqqF, which are required for PQQ biosynthesis. Different organisms vary in PQQ operon gene arrangement (Yahya et al., 2022). Two distinct PQQ-dependent soluble GDH (sGDH) and inner membrane-bound GDH enzymes have been discovered to be active in Gram-negative bacteria periplasm. Periplasmic gluconic acid has several functions, such as an antifungal, a mineral phosphate solubilizer, and a protist grazing reduction. This acid may be imported into the cytoplasm, catabolized, or secreted into the extracellular space. In the periplasm, the PQQ co-factor and levels of both GDH enzymes can influence the GDH enzyme's activity and phosphate solubility (An and Moe, 2016). This study aimed to evaluate the potential of rhizospheric and endophytic bacteria to solubilize inorganic phosphate in vitro using biochemical and genetic techniques. Efficient P solubilizers were identified taxonomically and evaluated for P uptake ability and PGP under controlled condition experiment.

## Materials and Methods

### *Sample collection*

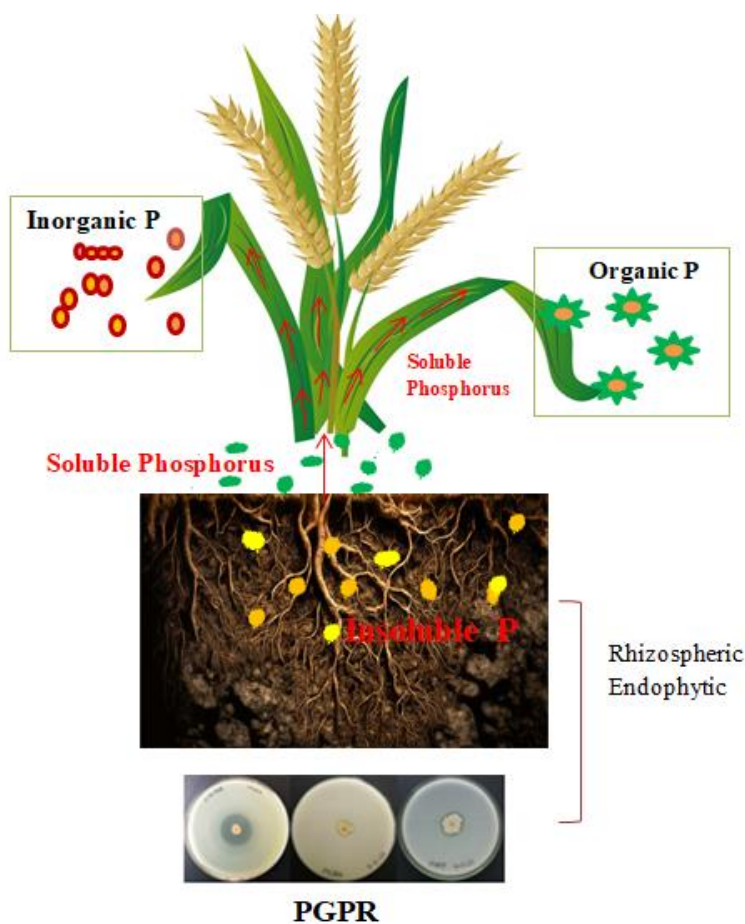
Rhizospheric and endophytic bacteria were collected from the culture bank of the Plant Biotechnology Laboratory, Government College University Faisalabad, Pakistan. These bacteria were isolated from two crops namely chickpea and wheat.

### *Solubilization of phosphate in a Pikovskaya agar media*

The ability to solubilize phosphate was measured according to Nacoon et al. (2020), with some modifications. Pikovskaya agar media containing insoluble tricalcium phosphate (TCP) was used for the selection (Pradhan and Sukla, 2006). The final pH was set at 7.00. Autoclaved media was poured in the plates and solidified under sterile conditions. Plates were incubated over-night at room temperature to confirm the sterility. Bacteria were spot inoculated in the center of each plate and placed in incubator at 28 ±

2°C for seven days. After an incubation period, bacterial colony diameter and halozone diameter were recorded, and the experiment was conducted with three replicates. Phosphate solubilization index (SI) was measured by the given below equation.

$$PSI = \frac{\text{Total diameter of halzone}}{\text{colony diameter}} \quad (\text{Eq.1})$$



**Figure 1.** Mobility of soluble phosphorus from soil microbiome to plants

### ***Solubilization of phosphorus in Pikovskaya (CaCO<sub>3</sub>) agar media***

To examine the potential bacterial isolates for phosphate solubility was measured by Nacoon et al. (2020) with some modifications. Pikovskaya agar media containing calcium carbonate was used for the selection (Pradhan and Sukla, 2006). The media was modified by adding CaCO<sub>3</sub> (3 g/l) to match the soil conditions. Autoclaved media was poured in the plates and solidified under sterile conditions. Plates were incubated over-night at room temperature to confirm the sterility. Bacteria were spot inoculated in the center of each plate and placed in incubator at 28±2°C for seven days. After incubation period, bacterial colony diameter and halozone diameter was recorded with three replicates. Phosphate solubilization index (SI) was measured by the given below.

$$SI = \text{total diameter of halozon/colony diameter} \quad (\text{Eq.2})$$

### ***pH determination***

To determine pH in liquid media under sterile conditions, two loops of phosphate-solubilizing bacteria were inoculated in each 25 ml volumetric flask having 10 ml Pikoviskaya's broth (pH 7). The 10 ml culture in the 25 ml flask was incubated at 120 rpm at  $28 \pm 2$  °C in a rotary shaker for seven days. Control was sterile, un-inoculated media. A digital pH meter was used to record the initial pH and the pH change after seven days with three replicates (Danaraj et al., 2022)

### ***Solubilization of phosphate in liquid medium by isolated bacteria***

The ability to solubilize inorganic phosphate in liquid media was measured by phosphomolybdate blue color method (Murphy and Riley, 1962). Four solutions, namely solution-A and solution-B, solution-C, and solution-D, were made. Taking (25 ml) sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) (5N), (7.5 ml) Ammonium molybdate (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> · 4H<sub>2</sub>O, (15 ml) Ascorbic acid (C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>) (0.1M), and (2.5 ml) Antimony potassium tartrate K<sub>2</sub>Sb<sub>2</sub>(C<sub>4</sub>H<sub>2</sub>O<sub>6</sub>)<sub>2</sub>·3H<sub>2</sub>O made mixed reagents. Under sterile conditions, two loops of phosphate-solubilizing bacteria were inoculated in each 25 ml volumetric flask having 10 ml Pikoviskaya's broth (pH 7). The 10 ml culture in the 25 ml flask was incubated at 120 rpm at  $28 \pm 2$  °C in a rotary shaker for seven days. After incubation period, the cultures were collected by centrifugation at 8000 rpm for 10 minutes. Eppendorf tubes were filled with 1 ml of supernatant. In 1 ml supernatant, add 0.1 ml mixed reagents. The control was a sterile, non-inoculated media. The quantity of soluble inorganic phosphate in the culture supernatant was determined by using the phosphomolybdate blue color method (Fitriatin et al., 2022). Using the standard phosphorus KH<sub>2</sub>PO<sub>4</sub> curve, available phosphorus concentration (µg/ml) was estimated by Murphy and Riley (1962). Bacterial solubilization of inorganic phosphate in culture media was calculated using standard curve trend line (Võsa et al., 2021) and the quantitative based experiment was conducted in three replications.

### ***PQQ gene amplification***

The GeneJET™ Genomic DNA Purification Kit was used to obtain genomic DNA from bacteria by following instructor instructions. PQQ gene sequence of 900bp amplification was performed using set of primers PQQR1-(‘5-GARCTGACYTAYCGCTGYCC-3’) and PQQR2-(‘5-TSAGSAKRARSGCCTGRCA-3’). 25 µL of PCR reaction mixture was prepared. The reaction mixture was placed in thermo cycler machine by following conditions (Ben Farhat et al., 2009). To determine the presence of the amplified gene and determine its level of integrity, an agarose gel containing 1.0 percent agarose in 0.5X TAE buffer was prepared and the experiment were performed in series of trials.

### ***16S rRNA gene amplification***

Based on the positive responses to all of the experiments, two bacterial strains from Chickpea and Soybean, SWC4 and SWS10 were selected for further molecular identification. These strains showed efficient phosphate solubilization by qualitative and quantitative assays, as well as excellent performance in all aspects of plant growth. Screened bacterial isolates were identified by 16S rRNA gene sequence analysis based on their plant growth-promoting features. The GeneJET™ Genomic DNA Purification Kit was used to obtain genomic DNA from bacteria by following instructor instructions.

The sequence of primers fD1 (5'- AGAGTTTGATCCTGGCTCAG- 3') and rD1 (5'- AAGGAGGTGATCCAGCC- 3), binds at 8-27 bp, and 1541-1525 bp, respectively and amplify 1500 bp fragment (Weisburg et al., 1991). 25 µL of PCR reaction mixture prepared. The reaction mixture was placed in thermo cycler machine. To determine the presence of the amplified gene and determine its level of integrity, an agarose gel containing 1.0 percent agarose in 0.5X TAE buffer was prepared.

### ***Pot experiment: Co-inoculation of endophytic and rhizospheric bacteria***

Potential isolates were tested under controlled condition experiment. Seeds of wheat cultivar Akbar 2019 were surface-sterilized with 70% ethanol for 10 seconds, washed with sterile water to remove any remaining ethanol, then washed again with 5% bleach for thirty seconds and finally washed in series with autoclaved sterilized water. Surface sterilized seeds were allowed to germinate in dark at  $25 \pm 2^\circ\text{C}$  on water agar plate. One germinated seedlings of equal size were put in per pots contain sterilized sand containing 1 g/kg TCP. Selected bacteria were cultured in YEM broth media and maintained the OD<sub>600</sub> at 0.2-0.5.100 µl of seven treatments, including five potential bacterial isolates, consortia containing (SWC4, SWC5, SWS9, SWS10, SW14) and DAP were applied in four replicates. Pots were placed in growth chamber at  $30 \pm 2^\circ\text{C}$  (day) and  $20 \pm 2^\circ\text{C}$  (night) for long day photoperiod.10 ml Hoagland solution and distilled water were supplied on alternate days. Experiment was performed in completely randomized design with four replicates and plants were harvested after 6 weeks and agronomical parameters such as shoot and root length, root and shoot fresh weight, root, and shoot dry weight, P available in sand, plant P uptake was measured and statistically analyzed.

### ***Phosphorus availability in the sand after bacterial inoculation***

#### ***Soil Phosphorus analysis***

The phosphomolybdate blue color method was used to calculate the number of accessible phosphates solubilized by bacteria. Sand sample taken from Thirty-six pots after 60 days was used to determine the presence of phosphate in the soil. The sand sample was allowed to air dry. To determine the solubility of P in the sand under sterile conditions 1 gram of soil sample was measured and dissolved in 7 ml of extraction solution (Amadou et al., 2022) with some modifications. After centrifugation 0.1 ml mixed reagent was added in 1 ml of supernatant. Un-inoculated media was used as control. The phosphorus content of a sand sample was determined by the phosphomolybdate blue colorimetric technique (Jones et al., 2019).

#### ***Plant Phosphorus uptake analysis***

Thirty-six plant samples were collected and cleaned with deionized water and oven-drying at  $70^\circ\text{C}$ . after oven drying the plants were converted into the fine powder by using mortar and pestle. After grinding uniformly, 1 g of the plant sample was mixed in 0.1 ml mixed reagent with some modifications. Plant phosphate content was calculated by using molybdate blue method (Jones et al., 2019).

### ***Statistical analysis***

Solubilize inorganic phosphate in liquid media was visualized under UV- 1200 spectrophotometer, S/N: UEC1401039 by phosphomolybdate blue color method as previously described (Murphy and Riley, 1962). All the experiment was conducted in

triplicates and significance difference was identified by the least significant difference (LSD) and ANOVA Statistix 10.0 software.

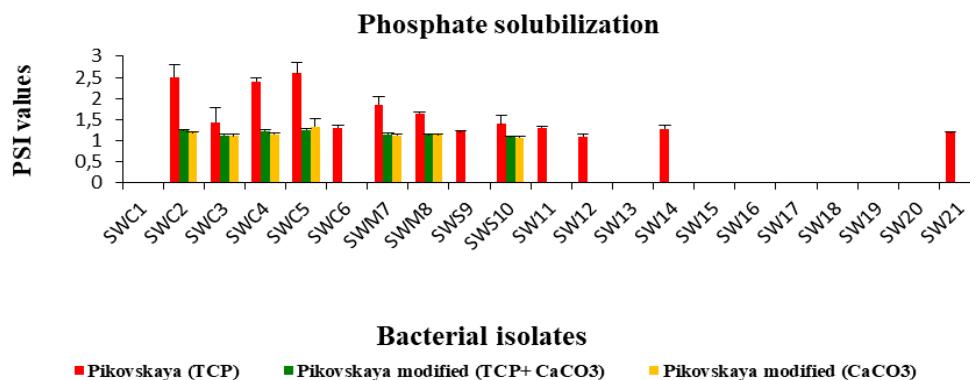
## Results

### *Phosphate solubilization on Pikovskaya agar media*

The selected 21 bacterial isolates were inoculated on a Pikovskaya media for phosphate solubilization. Out of twenty-one, thirteen isolates show capacity for phosphate solubility by producing a clear halo zone around bacterial colonies. SWC2, SWC4, SWC5, and SWM7 strains exhibited clear Pikovskaya beneath and much more in the surrounding bacteria, producing a countable halo zone in the surrounding. However, SWC3, SWC6, SWM8, SWS9, SWS10, SW11, SW12, SW14, and SW21 displayed moderate solubilization clear Pikovskaya media only beneath the bacterial colony and can produce a crescent-like halo zone in the colony surrounding. On the other hand, SWC1, SW13, SW15, SW16, SW17, SW18, SW19, and SW20 were found as non-solubilizers that do not clear Pikovskaya media, so they produce no halo zone presented in *Table 1, Fig. 2*.

**Table 1.** Bacterial phosphate solubilization zone size was presented on Pikovskaya, Pikovskaya modified (TCP+CaCO<sub>3</sub>) and Pikovskaya modified (CaCO<sub>3</sub>) after seven days values are mean ± standard error (n=3)

Isolates	Pikovskaya (TCP) (mm)	Pikovskaya (TCP+CaCO <sub>3</sub> ) (mm)	Pikovskaya (CaCO <sub>3</sub> ) (mm)
SWC1	0	0	0
SWC2	2.51 ± 0.3	1.24 ± 0.03	1.18 ± 0.03
SWC3	1.44 ± 0.35	1.12 ± 0.02	1.11 ± 0.04
SWC4	2.4 ± 0.08	1.23 ± 0.03	1.15 ± 0.03
SWC5	2.6 ± 0.25	1.25 ± 0.04	1.33 ± 0.2
SWC6	1.3 ± 0.07	0	0
SWM7	1.85 ± 0.18	1.15 ± 0.03	1.12 ± 0.03
SWM8	1.64 ± 0.04	1.13 ± 0.02	1.13 ± 0.02
SWS9	1.21 ± 0.03	0	0
SWS10	1.4 ± 0.21	1.08 ± 0.01	1.07 ± 0.02
SW11	1.3 ± 0.04	0	0
SW12	1.1 ± 0.04	0	0
SW13	0	0	0
SW14	1.28 ± 0.07	0	0
SW15	0	0	0
SW17	0	0	0
SW18	0	0	0
SW19	0	0	0
SW20	0	0	0
SW21	1.18 ± 0.03	0	0



**Figure 2.** SWC2, SWC4, SWC5, and SWM7 isolates dissolve maximum phosphorus in Pikovskaya (TCP), modified Pikovskaya (TCP+CaCO<sub>3</sub>) agar and modified Pikovskaya (CaCO<sub>3</sub>) agar

### ***Phosphate solubilization on modified Pikovskaya agar media***

The selected 21 bacterial isolates were inoculated on a modified Pikovskaya agar media (CaCO<sub>3</sub>) for phosphate solubilization in which TCP and CaCO<sub>3</sub> was added in equal amounts according to our soil condition. Out of twenty-one bacteria, seven isolates showed capacity for phosphate solubility by producing a clear halo zone around bacterial colonies. SWC2, SWC4, SWC5, and SWM7 isolates exhibited higher phosphate solubilization ability by producing solubility index of 1.24, 1.23, 1.25 and 1.15, respectively. SWC3, SWM8, and SWS10 isolates exhibited moderate phosphate solubilization ability by producing solubility index of 1.12, 1.13 and 1.08 respectively. Besides this, SWC1, SWC6, SWS9, SW11, SW12, SW13, SW14, SW15, SW16, SW17, SW18, SW19, SW20, and SW21 showed no phosphate solubilization ability indicated in Fig. 2.

### ***Solubilization of phosphorus in Pikovskaya (CaCO<sub>3</sub>) agar media***

Out of twenty-one bacteria, seven isolates showed a positive response by the formation of halo zone on a modified Pikovskaya agar media in which CaCO<sub>3</sub> was added in equal amounts according to our soil condition. SWC2, SWC4, SWC5, and SWM7 isolates exhibited higher phosphate solubilization ability by producing solubility index of 1.18, 1.15, 1.33 and 1.12, respectively. SWC3, SWM8, and SWS10 isolates exhibited moderate phosphate solubilization ability by producing solubility index of 1.11, 1.12 and 1.07 respectively. Besides this, SWC1, SWC6, SWS9, SW11, SW12, SW13, SW14, SW15, SW16, SW17, SW18, SW19, SW20, and SW21 showed no phosphate solubilization ability presented in Fig. 2.

### ***pH measurements***

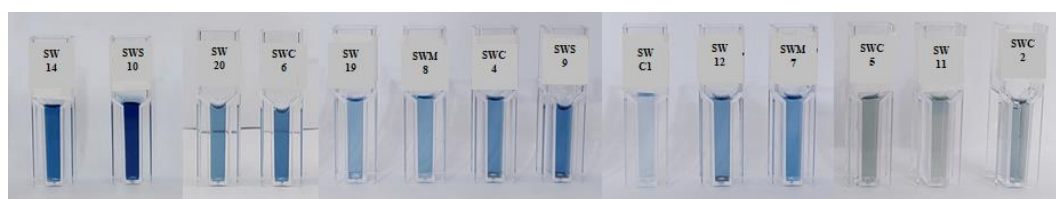
Phosphate-solubilizing bacteria decreased pH of the Pikovskaya broth medium compared to an un-inoculated sterile control that was incubated for seven days. All phosphate solubilizing bacteria showed fluctuations in lowering the pH. Observations of changes in pH showed a decreased from 7.0 (the control) to 4.31 (SWC5). The SW isolates decreased minimum pH as compared to other isolates actively decreased the pH of the broth. SWC4, SWC5, and SWC6 showed pH decrease ranging from (4.4, 4.31, and 4.52). Phosphate solubilization increased by reduction of the pH of broth.

### ***Solubilization of phosphate in liquid medium by bacterial isolates***

A quantitative analysis of modified PVK liquid medium enriched with inorganic P was used to confirm the efficacy of the isolated strains in solubilizing phosphates (TCP). All isolates that showed halo zone formation also have the capability of P solubilization in liquid media. The concentration of soluble phosphorus release (Pr) that was released by the strains in the liquid medium ranged from 6.31 to 20.9  $\mu\text{g ml}^{-1}$  depending on the strain (Table 2). SWC6, SWS9, SWS10 and SW14 had the greatest concentrations of phosphorus release (16.5  $\mu\text{g ml}^{-1}$  and 16  $\mu\text{g ml}^{-1}$ , 20.9  $\mu\text{g ml}^{-1}$ , 18.6, respectively) with a considerable drop in pH from 7 to (4.52, 4.72, 4.1 and 4.64, respectively) in liquid media. On the other hand, the isolates of SWC1 and SWC2, SWC5 and SW11 released the low quantities of phosphorus (9.5  $\mu\text{g ml}^{-1}$  and 8.4  $\mu\text{g ml}^{-1}$ , 6.6  $\mu\text{g ml}^{-1}$ , 6.31  $\mu\text{g ml}^{-1}$ , respectively). The capacity of the bacteria to mobilize phosphate was directly linked to the change in pH that occurred in the medium. During the incubation time, the growth of bacteria rise significantly, the pH of the broth was reduced, and soluble P was also raised. Phosphate solubilizing isolates SWC1, SWC6, SWS9, SW11, SW12, SW14, SW19 and SW20 did not display solubilization index (SI) on modified Pikovskaya. There was considerable phosphate solubilization in a liquid media, demonstrating that the presence of a visible halo zone is not a valid criterion for isolating PSB because many isolates did not produce a halo zone in an agar media had significant phosphate solubilization in a liquid culture medium see in Fig. 3.

**Table 2.** pH decrease and inorganic phosphorus release by bacteria after seven days. pH and inorganic phosphorus ( $\mu\text{g ml}^{-1}$ ) values are mean  $\pm$  standard error (n=3)

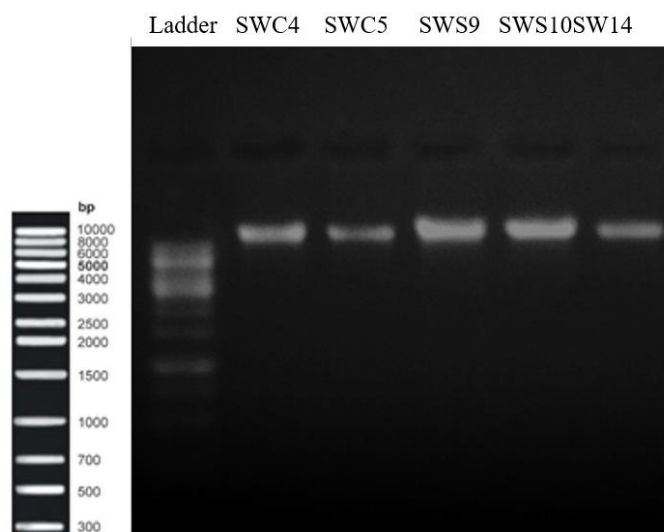
Isolates	Pikovskaya (TCP+CaCO <sub>3</sub> ) (mm)	pH	Molybdate assay ( $\mu\text{g ml}^{-1}$ )
SWC1	0	6.03 $\pm$ 0.18	9.5 $\pm$ 0.05
SWC2	1.24 $\pm$ 0.03	5.01 $\pm$ 0.25	8.44 $\pm$ 0.03
SWC4	1.23 $\pm$ 0.03	4.4 $\pm$ 0.36	13.4 $\pm$ 0.06
SWC5	1.25 $\pm$ 0.04	5.41 $\pm$ 0.33	6.6 $\pm$ 0.03
SWC6	0	4.52 $\pm$ 0.19	16.5 $\pm$ 0.08
SWM7	1.15 $\pm$ 0.03	5.18 $\pm$ 0.16	15.3 $\pm$ 0.05
SWM8	1.13 $\pm$ 0.02	5.66 $\pm$ 0.62	11.7 $\pm$ 0.02
SWS9	0	4.72 $\pm$ 0.52	16 $\pm$ 0.05
SWS10	1.08 $\pm$ 0.01	4.1 $\pm$ 0.1	20.9 $\pm$ 0.08
SW11	0	5.27 $\pm$ 0.28	6.31 $\pm$ 0.07
SW12	0	5.1 $\pm$ 0.3	15.1 $\pm$ 0.1
SW14	0	4.64 $\pm$ 0.19	18.6 $\pm$ 0.06
SW19	0	6.27 $\pm$ 0.4	10.4 $\pm$ 0.04
SW20	0	6.24 $\pm$ 0.28	15.6 $\pm$ 0.1



**Figure 3.** Phosphate solubilization by bacteria in broth media

### **Bacterial DNA extraction**

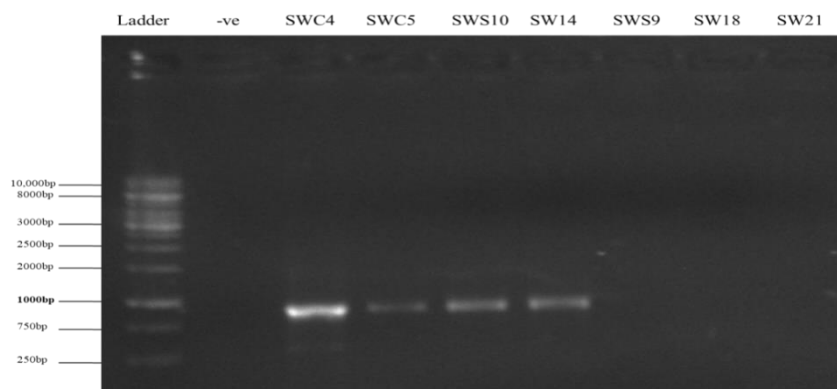
The extraction of genomic DNA of selected isolates (SWC4, SWC5, SWS9, SWS10 and SW14) is shown in Fig. 4. Genomic DNA (10,000 base pairs) was found near the wells.



**Figure 4.** Genomic DNA products obtained with The GeneJET™ Genomic DNA Purification Kit by following instructor instructions and electrophoresed at 100 volts for 30 minutes, viewed under UV illuminator in Gel Documentation system (Biorad, USA)

### **In bacterial genomes pqqE gene detection**

The evaluation was done on the genomes of selected bacterial isolates to determine presence of the genes that code for the cofactor PQQ of the GDH enzyme involved in phosphate solubilization. This enzyme is the one responsible for the synthesis of gluconic acid. Employing two sets of specific primers (PQQR1 and PQQR2), the pqqE gene fragment was amplified corresponding to ~900 bp, respectively. SWC4, SWC5, SWS10 and SW14 represent pqqE gene of (900 base pairs) (Fig. 5). The SWS9, SW18, and SW21 genes did not produce any detectable PCR amplification products.



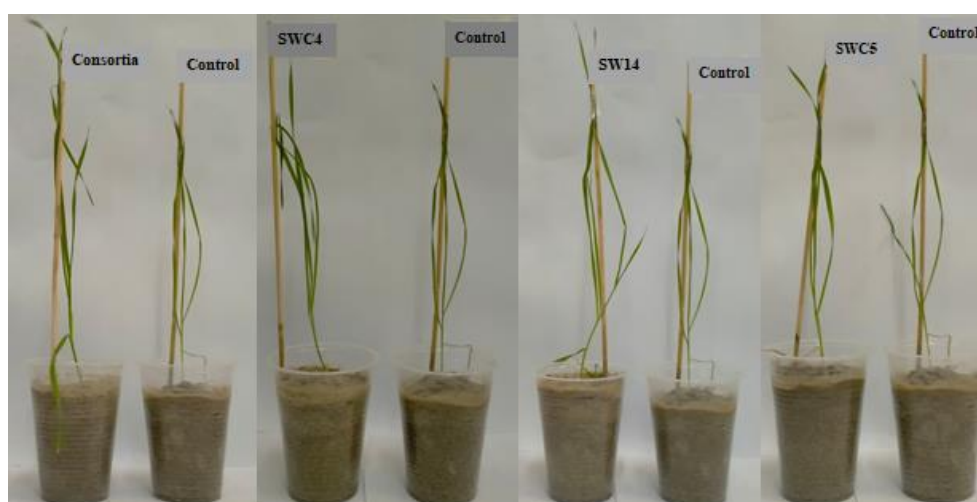
**Figure 5.** Ladder, control, SWC4, SWC5, SWS10, SW14 and SWS9, SW18, SW21 is represented. The amplification products (900 base pairs) obtained with primers PQQR1 and PQQR2 are represented respectively

### Analysis of PSB for wheat yield variables in Pot assay

Our study found that inoculation of bacteria individually or in combination of different strains produced the most significant outcomes in terms of growth characteristics. Plant growth-promoting bacteria-based bio fertilizers used in this study are highly suggested for boosting wheat plant growth. Under controlled conditions, when consortia inoculated in pots the following outcome were shown shoot length (51%), root length (36%), dry weight of shoots (24%), %, dry weight of roots (21%), in wheat plants as compared to control, which did not receive any fertilizers or bacterial isolates. In single inoculation *Pantoea dispersa* showed the greatest rise shoot length (62%) root length (47%), dry weight of shoots (37%), and dry weight of roots (32%). *Agrobacterium sp.* demonstrate less beneficial influence on the plant's height, and even its weight (Table 3, Fig. 6). The strains *Pseudomonas Koreensis*, *Pseudomonas sp.* and consortia also produced a considerable amount of quantitative phosphate solubility and zone formation and have more potential for plant growth promotion.

**Table 3.** The influence of PSB on morphological features of the wheat plant each value represents mean  $\pm$  standard deviation (n= 4)

Isolate	Shoot length (cm)	Root length (cm)	Root fresh weight (mg)	Shoot fresh weight (mg)	Root dry weight (mg)	Shoot dry weight (mg)
Control	24.6 $\pm$ 3.8 c	3.3 $\pm$ 0.9 c	169 $\pm$ 2.9 e	300.8 $\pm$ 9.1 d	12.3 $\pm$ 2.2 b	30.5 $\pm$ 3.4 d
SWC4	34.2 $\pm$ 3.0 ab	4.5 $\pm$ 1.1 bc	210 $\pm$ 4.2 cd	410.3 $\pm$ 8.8 c	16.8 $\pm$ 2.1 ab	35.3 $\pm$ 2.8 bcd
SWS9	38 $\pm$ 5.4 a	5.7 $\pm$ 1.1 ab	261.3 $\pm$ 34.4 a	472 $\pm$ 9.4 a	18.2 $\pm$ 2.4 ab	43 $\pm$ 3.2 a
SWS10	34.3 $\pm$ 3.8 ab	3.5 $\pm$ 0.4 c	186.5 $\pm$ 25.0 de	443.5 $\pm$ 28.6 ab	14.3 $\pm$ 7.5 ab	33 $\pm$ 1.8 cd
SWC5	30 $\pm$ 6.7 bc	3.7 $\pm$ 1.0 c	225.5 $\pm$ 23.4 ab	430 $\pm$ 45.3 bc	14.1 $\pm$ 3.7 ab	38 $\pm$ 2.9 abcd
SW14	36.3 $\pm$ 5.1 ab	6.4 $\pm$ 0.8 a	226.5 $\pm$ 6.3 c	462 $\pm$ 2.9 a	21.8 $\pm$ 9.2 a	42 $\pm$ 2.9 ab
Consortia	37.3 $\pm$ 4.8 a	4.5 $\pm$ 0.8 bc	221.5 $\pm$ 8.0 c	330 $\pm$ 8.0 d	16.3 $\pm$ 6.2 ab	37 $\pm$ 6.8 abcd
DAP ANOVA	32 $\pm$ 3.6 ab*	3.4 $\pm$ 0.2 c*	230.8 $\pm$ 7.8 bc*	452 $\pm$ 7.5 ab*	12.9 $\pm$ 2.5 b**	39 $\pm$ 10.9 abc*



**Figure 6.** Effect of inoculating potential isolates on growth under controlled circumstances

Table 4. showed the results of Pearson correlation. As evident from the table, the highest correlation coefficient is 0.978 between Root dry weight (RDW) and Root length (RL) followed by 0.9437 between roots fresh weight (RFW) and shoot dry weight (SDW). Similarly, there is a positive correlation between root RDW and shoot length (SL) 0.7419, followed by 0.7507 between Root lengths (SL).

**Table 4. Results of Pearson correlation**

	RDW	RFW	RL	SDW	SFW
RFW	0.5302 <sup>ns</sup>				
RL	0.978 <sup>**</sup>	0.5975 <sup>ns</sup>			
SDW	0.6905 <sup>ns</sup>	0.9437 <sup>**</sup>	0.7507 <sup>*</sup>		
SFW	0.4327 <sup>ns</sup>	0.6257 <sup>ns</sup>	0.4106 <sup>ns</sup>	0.6919 <sup>ns</sup>	
SL	0.7419 <sup>*</sup>	0.6754 <sup>ns</sup>	0.6716 <sup>ns</sup>	0.6716 <sup>ns</sup>	0.5054 <sup>ns</sup>

\*=significant at 0.1 probability level, \*\*=significant at 0.05 probability level, ns= non-significant

### **Phosphorus availability in the sand after bacterial inoculation**

After harvesting the plants, soil's p content was examined. PSB inoculation enhanced the soil's P availability (Table 5). According to the results; phosphate solubilizing bacteria treated soil had much more accessible P than control soil. The outcomes were more effective in the presence of the bacterial consortium as compared to the addition of individual PSB. The amount of accessible phosphorus in the TCP-amended sand that had been inoculated by the consortium was (139%) more than that in the control. Moderate range of accessible P was seen in the TCP added sand after a single inoculation of and SWS10. In TCP, modified sand treatments with *Pseudomonas Koreensis* and *Pantoea dispersa* strains resulted in the greatest increase in accessible phosphorus (103%, 92%).

**Table 5. Availability of P in sand and plants P uptake**

Treatments	Available P content in soil (µg g <sup>-1</sup> )	P uptake in plants (mg plant <sup>-1</sup> )
Control	8.47 ± 0.40	7.23 ± 0.31
SWC4	17.23 ± 0.34 (103)	13.83 ± 0.48 (92)
SW14	13.0 ± 0.4 (53)	9.6 ± 0.3 (33)
SWS10	16.3 ± 0.2 (92)	13.2 ± 0.4 (79)
SWS9	10.2 ± 0.29 (20)	8.2 ± 0.16 (13.4)
SWC5	14.9 ± 0.59 (75)	11.8 ± 0.48 (63)
Consortia	20.3 ± 0.50 (139)	15.3 ± 0.30 (112)
DAP	14.9 ± 0.48 (75)	11.5 ± 0.27 (59)

### **Plants' P uptake after bacterial inoculation**

Due to an increase in the amount of accessible phosphorus in the soil, plant absorption of phosphorus also increased (Table 5). The plants inoculated with the consortium in the TCP-treated soil showed the greatest increase in P absorption. Plants treated with *Pseudomonas Koreensis* and *Pantoea dispersa* exhibited the maximum increase in P uptake (92%, 79%). *Pseudomonas Koreensis* demonstrated a greater increase in P absorption respectively than the other individual treatments when grown in soil that had been modified with TCP.

## Discussion

Bacteria interacting with the roots are beneficial for plant growth promotion. Understanding the composition and function of the bacteria that are associated with the root is important for sustainable agricultural production so that the agriculture system could be less dependent on chemical fertilizers. Climate change and the unexpected rise in world population made crop production difficult. A sustainable rise in crop production is the dire need to cope with the future food demand (Khatoon et al., 2023; Sun et al., 2023). There must be a solution for pathogens, pests, and environmental pressures which magnanimously affect crop production. Microbes found in plants can enhance growth by providing nourishment and exhibiting stress tolerance. Biofertilizers can help to increase crop production by reducing environmental stresses (Bhardwaj et al., 2014). The microorganisms that live inside and outside of plants can colonize plant roots and enhance plant growth through both direct and indirect methods (Pareek et al., 2023). Rhizobacteria enhance the root system by producing phytohormones that promote lateral root branching and enhance overall plant growth (Vacheron et al., 2013; Verbon and Liberman, 2016; Ihsan et al., 2024). Phosphate is one of the essential nutrients for plant growth production. Even though soils have high amounts of total phosphate, the quantity of phosphate accessible to plants is relatively low because phosphorus may form chelates with several other cations, including Ca<sup>2+</sup>, Fe<sup>3+</sup>, and Al<sup>3+</sup>. As phosphate fertilizer is applied to soil, it becomes inaccessible to plants (Al-Amri et al., 2020).

Alternative approaches are necessary to reduce the severe effects of soil and convert the inorganic or organic form of P into an accessible form so that plants may take it easy. One way to get soluble P is to use microorganisms in the root zone of soil to convert phytate to soluble P (Al-Amri et al., 2020). Phosphate can be made more readily available to plants by bacteria capable of solubilizing phosphate (PSB). Phosphate-solubilizing bacteria can dissolve phosphorus, so plants absorb it easily (Kumar, 2016). More than half of the world's population relies on cereal crops as the primary source of their food supply (Tian, 2016). To fulfill the increasing world population's food demand, it is also necessary to enhance wheat yield worldwide.

Generally, the soil consists of 400-1000 mg kg<sup>-1</sup> of total phosphorus, of which only (1.00-2.50%) is accessible for plant absorption (Aberathna et al., 2022). But most of Pakistan's soil is calcareous (CaCO<sub>3</sub>> 3.0%), and soil pH ranges from 7 to 9, leading to TCP formation (Ashraf et al., 2018). In this study, 21 bacterial isolates from two different crops were inoculated on a modified Pikovskaya media (TCP+CaCO<sub>3</sub>) for phosphate solubilization in which TCP and CaCO<sub>3</sub>> 3.0% were added according to our soil condition (Aimen et al., 2022). Seven isolates were revealed to be positive for phosphate solubilization on this media, in which four isolates, was demonstrated to be efficient phosphate solubilizers. All bacteria show phosphate solubilization ability on Pikovskaya (TCP) agar media. Only those bacteria will be considered effective in solubilizing phosphate on this concentration set according to our soil condition. The significant amount of CaCO<sub>3</sub> present in the calcareous soil limits the availability of P by reacting calcium ions with phosphate ions and other essential nutrients for agricultural land. It leads to alkaline reactions by generating insoluble phosphate compounds such as tricalcium phosphate (Bahadur et al., 2017; Mwafulirwa, 2018).

Phosphate solubilizing isolates SWC1, SWC6, SWS9, SW11, SW12, SW14, SW19 and SW20 did not display solubilization index (SI) on modified Pikovskaya. There was considerable phosphate solubilization in a liquid media, demonstrating that the presence of a visible halo zone is not a valid criterion for isolating PSB because many isolates did

not produce a halo zone in an agar media had significant phosphate solubilization in a liquid culture medium. Previously, Nautiyal (1999) reported similar findings that numerous bacteria can solubilize significant amounts of P in the broth even when they don't display a halo zone in PVK plates. Phosphate solubilization increased by reduction of the pH of broth. A decrease in pH and a significant rise in soluble P in the solution by microorganisms were also mentioned in earlier reports (Hussein et al., 2019).

The *pqqE* gene, which codes for the PQQ, has a role in the solubilization of phosphorus as a co-factor in the extracellular oxidation of glucose to gluconic acid catalyzed by glucose dehydrogenase (Choudhary et al., 2022). The presence of *pqqE* gene in SWC4, SWC5, SWS10, and SW14 shows that these bacteria make PQQ, and, as a result, they produce gluconic acid. The absence of the *pqqE* gene from the DNA of SWS9 isolates revealed that these bacteria could be using a different route to produce gluconic acid (Haroon et al., 2023). SW18 and SW21 did not observe the *pqqE* gene because these isolates were non-phosphate solubilizers.

Phosphate solubilizing bacteria have ability to promote plant growth by increasing phosphate solubilization. According to previous research, Chawla and Sadawarti (2020) demonstrated that various crops can benefit from bio-fertilizers, which have been proven to increase yields by 10–25%. Bacterial treatments *Pseudomonas Koreensis*, *Pantoea dispersa*, *Pseudomonas* sp., *Agrobacterium* sp., and consortia in combination with TCP, resulted in a significant increase in agronomical parameters. In this study, *Pseudomonas Koreensis* and consortia showed highest potential in increasing plant growth parameters. Previously, Arruda et al. (2013) demonstrated that phosphate solubilizing bacteria have the ability to promote wheat growth.

PSB strains had significant potential to release phosphorus (P) from the soil, making readily available P for plant roots. It might be because the action of PSB allows for more efficient utilization of phosphorus from the overall pool of soil nutrients. In this research study, wheat plants showed the most significant rise in P content when treated with PSB *Pseudomonas Koreensis* and *Pantoea dispersa*. These potential bacteria had a more significant effect on effectively release phosphorus (P) from the soil, making P more readily available for plant roots. Our results are in agreement with Swarnalakshmi et al. (2013) that inoculation of wheat with TCP and *Pseudomonas* showed a more significant impact on phosphate content. Alam et al. (2022) also demonstrated that microbial accessible P is equivalent to plant-available P due to the mineralization of complex phosphorus compounds.

After successful field trials these PGPR can be used as biofertilizers in future. For the maintenance of agriculture sustainability usage of biofertilizers should be increased.

## Conclusion

This study demonstrates that rhizospheric and endophytic PSB exhibited good potential for plant-promoting characteristics and could be used as a biofertilizer source and contribute to sustainable agricultural productivity, as phosphorus can be a major limiting factor due to slow diffusion and high fixation into inorganic insoluble TCP formation in high calcium carbonate content soil. These phosphate-solubilizing bacteria provide a sustainable solution to this vicious cycle by colonizing the rhizosphere, enhancing acid phosphatase activities, and boosting resistance against highly calcareous soils. Exploiting the potential of phosphate-solubilizing bacteria is extremely promising. Replacing DAP with PSB to produce organic food can save billions of rupees in country

import expenditures but also assist in generating employment, reducing health issues, and improving the environment. For agriculture productivity maintenance, phosphate solubilizing PGPR-based biofertilizers should be increased to meet future demands, which reduce economic, social, and environmental costs.

**Conflicts of Interest.** The authors declare that they have no conflict of interest.

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