

Essential Nutrients Solubilization Ability of Fluorescent Pseudomonads and their Multinutrient Management

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Abstract: Soil microorganisms play a significant role in maintaining the ecological balance by active contribution in Carbon, Nitrogen, Sulphur and Phosphorus cycles in nature. Phosphate (P), Potassium (K) and Zinc (Zn) essential for optimum plant growth. Fluorescent pseudomonads are a group of Plant Growth Promoting Rhizobacteria (PGPR) naturally occurring soil microorganisms that aggressively colonize plant root and enhance the growth of plant and yield by direct and indirect mechanisms. In search for efficient nutrient solubilizing strains associated to *Arachis hypogaea*, a total of 55 isolates (JS-1...JS-55) were obtained from the rhizospheric soil of Rayalaseema region of Andhra Pradesh, India. Among 55, eleven isolates exhibited multiple plant growth promoting traits viz., direct mechanisms: Solubilization of Phosphate, Zinc and Potassium. 42 isolates exhibited positively for Tri-calcium phosphate solubilization on pikovskaya agar. 25 isolates were able to solubilizing zinc. JS-17 produced 22.0 mm clear haloes on solid medium amended with ZnCO₃. Similarly, JS-17 and JS-7 showed 31.0 mm zone in ZnO incorporated medium, 24 isolates were able to solubilizing Potassium. Maximum K solubilization occurred when KCl was used as a potassium source followed by K₂SO₄ and mica. These results recommended that the environmental conditions could be optimized for growth of indispensable mineral solubilizing bacteria and these bacterial cultures could be exploited for plant growth improvement under field conditions. Such multiple positive PGP traits and their isolates can be further explored as competent bioinoculants to improving plant growth and yield.

Keywords: Rhizosphere Bacteria, phosphate (P), Potassium (K), Zinc Carbonate, Zinc Oxide, *Pseudomonas* and PGPR.

1. Introduction

Plants need several macro and micro nutrients for their growth and reproduction. These nutrients supplemented through inorganic or organic forms are taken up by the plant roots along with water. Bacteria also play an important role in mobilizing nutrients requisite by the plants to some extent. Phosphorus (P) is a major nutrient after nitrogen (N) that limits plant growth despite being abundant in soils in both organic and inorganic forms [1]. 'P' plays an important role in root development, flower, seed formation, N-fixation in legumes, resistance to plant diseases. Thus to achieve optimum crop yields, soluble phosphate fertilizers have to be applied at high rates [2]. Such soluble inorganic fertilizer in soil is immobilized rapidly and become unavailable to plants [3]. Use of chemical fertilizers causes soil erosion and lower crop yield [4]. Phosphate solubilizing microbes plays an important role in plant nutrition through increase in phosphate uptake by plants and used as biofertilizers of agricultural crops. Soil micro biota enhances 'P' availability to plants by mineralizing organic phosphorus in soil and by solubilizing precipitated phosphates. The solubilization of 'P' in the rhizosphere is the most prevalent mode of action concerned in plant growth promoting rhizobacteria (PGPR) that increase nutrient availability to host plants [5].

Potassium (K) is the third major essential macronutrient most profusely absorbed cation essential for the growth, metabolism and development of plants. The concentration of soluble potassium in the soil is very low and more than 90% of potassium in the soil exists in the form of rocks and silicate minerals which is insoluble. Without sufficient potassium, the plants will have poorly developed roots, grow

slowly, produce small seeds and have lower yields. Due to imbalanced fertilizer application, potassium discrepancy is becoming one of the major constraints in crop production. This emphasized the search to find an alternative native source of K for plant uptake and to preserve K status in soils for sustaining crop production [6, 7]. Rhizosphere bacteria have been found to dissolve potassium from insoluble K-bearing minerals such as micas, illite and orthoclases, by excreting organic acids which also directly dissolved rock K or chelated silicon ions to transport K into the solution [8-10]. Potassium solubilizing microorganisms present in the soil could provide an alternative method to make potassium available for uptake by plants [11, 12].

Zinc (Zn) is one of the vital micronutrient required relatively in small concentrations (5–100mg kg⁻¹) for optimum plant growth and plays an important role in metabolism [12]. Excessive use of zinc fertilizers causing the impaired absorption of iron and copper in humans. It is also known to suppress male sexuality [13]. Zn solubility is highly dependent upon soil pH and moisture. Hence arid and semiarid areas of Indian agro ecosystems are often zinc-deficient. Microbes are potential alternate that could provide plant zinc requirement by solubilising the complex zinc in soil. Microbes solubilize the metal forms by protons, chelated ligands, and oxido reductive systems present on the cell surface and membranes [13–15]. Several genera of rhizobacteria belonging to *Pseudomonas* spp. and *Bacillus* spp. are reported to solubilize zinc. Thus, identification of efficient microbial strains capable of solubilizing minerals quickly can conserve our existing resources and avoid environmental pollution hazards caused by heavy application of chemical fertilizers. In this study we reported

the *in vitro* mineral solubilization ability of selected strains and their efficiency was calculated.

2. Materials and Methods

2.1. Soil Sample Collection

The rhizosphere soil collected from different ground nut growing areas of Rayalaseema region. Collected soil samples were stored in polythene bags aseptically and maintained at the laboratory for further study. Bacteria were isolated by serial dilution plate method [16]. From the final dilutions of 10^{-5} and 10^{-6} , one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing King's B medium and they were gently rotated clockwise and anti clockwise for uniform distribution and incubated at room temperature ($28\pm 2^{\circ}\text{C}$) for 24 hours. The colonies were viewed under UV light at 360 nm. Colonies with characteristics fluorescent *Pseudomonas* spp. were isolated individually and purified by streak plate method [17] on King's B medium. The pure cultures were maintained on king's B agar slants at 4°C . Fluorescent *Pseudomonas* spp. was characterized on the basis of morphological biochemical and physiological tests as prescribed in Bergey's manual of systematic bacteriology [18].

2.2 Mineral Solubilization

2.2. (1). Screening of phosphate solubilization activity

2.2. (1a). Qualitative method

Isolates were screened for phosphate solubilization on Pikovskaya's medium [19]. Isolates showing phosphate solubilizing ability were spot inoculated at the centre of Pikovskaya's plate and incubated at 28°C . Diameter of clearance zone was measured after 7 days.

Analysis of phosphate solubilizing activity

From the isolates, larger halo zone producing strains were selected for further study. All the observations were recorded in triplicate. The Phosphate Solubilization Efficiency (PSE) is the ratio of total diameter, i.e., clearance zone including bacterial growth and the colony diameter.

$$\text{PSI} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

2.2. (1b). Quantitative method

Pikovskaya's broth medium with Tricalcium phosphate (0.3g/100ml) was prepared and sterilized; 1ml of each isolate was inoculated into the broth medium. Then the inoculated sample was incubated for 7 days on rotatory shaker 28°C after incubation, culture broth was centrifuged at 10,000 rpm for 30min. Uninoculated broth served as control. For quantification of phosphate solubilization, the method described by Olsen and Sommers was adapted [20]. One (1) ml aliquot from the supernatant was taken to which 5 ml of sulphomolybdic acid containing ascorbic acid was added. Final volume of the solution was made up to 50 ml. The absorbance of the reaction mixture was measured at 660 nm and amount of soluble phosphorus was determined from the standard curve of KH_2PO_4 .

2.2. (1c). Optimization of Growth Conditions for Efficient P Solubilization

Solubilization of phosphorus was determined in medium Pikovskaya's broth at neutral pH and $28\pm 2^{\circ}\text{C}$ temperature. The amendments of different sugars and variation in temperature as well as pH were made to find out optimum conditions for efficient solubilization. Six best efficient phosphorus solubilizing bacterial strains were used for P solubilization studies. For measuring the effect of carbon sources, rhizobacterial isolate was inoculated into 25 ml of Pikovskaya's medium broth [19] in which glucose was replaced with either of three different sugars i.e., galactose and xylose respectively. All the inoculated flasks were incubated at $28\pm 2^{\circ}\text{C}$ for 10 days. The amount of P released in broths was estimated after incubation in comparison with a set of uninoculated controls.

To determine the effect of incubation temperature, medium Pikovskaya's broths were inoculated with six selected P solubilizing bacterial strains. Cultures were incubated at different temperatures i.e., 25, 35 and 45°C along with 30°C for 10 days. To study the effect of pH on P solubilization, the Pikovskaya's medium broths were prepared in different pH range i.e., 6.5, 7.5 and 8.5 using N/10 HCl or N/10 NaOH and the broth was buffered with phosphate buffer. After inoculation of bacterial strains, medium broths were incubated at $28\pm 2^{\circ}\text{C}$ for 10 days.

2.2. (2). Potassium solubilization assay: All the isolates were spot inoculated on to the modified Aleksandrov medium [21] plates. Plates of modified Aleksandrov medium (A) having mica powder (insoluble form of potassium) and medium (B) having soluble form of potassium i.e., K_2HPO_4 and (C) KCl were prepared. A loopful of 48-hour old growth of the rhizobacterial strains was spotted on above prepared plates. The bacterial cultures were spotted on each plate and cultures were spotted in same sequence on both types of medium plates. Plates were incubated at $28\pm 2^{\circ}\text{C}$ for 7 days. Detection of potassium solubilization by different rhizobacterial isolates was based upon the ability of solubilization zone formation.

2.2. (3). Zinc solubilization assay: All the isolates were inoculated on to the modified Pikovskaya medium [19] (Glucose, 10 g; $\text{Ca}_3(\text{PO}_4)_2$, 5.0 g; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; NaCl, 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1g; KCl, 0.2 g; Yeast extract, 0.5 g; MnSO_4 , Trace; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, Trace; Agar, 15 g; Water, 1000 ml; pH, 7.0 ± 0.2) containing 1% insoluble zinc compounds (A)ZnO and (B)ZnCO₃. A loopful of 48 hour old growth of the rhizobacterial strains was inoculated on above prepared plates. The bacterial cultures were spotted on each plate and cultures were inoculated in same sequence on insoluble zinc compound i.e ZnO and ZnCO₃ containing medium plates. All the plates were incubated for 48 h at 28°C for 5days. The halo zone around the colony was measured and considered as zinc solubilizing bacteria.

3. Results and Discussion

The occurrence of bacteria in rhizosphere is based on the concentration of nutrient available. Due to constant release of nutrients from plant roots, soil microbes are found to

dominate the niche and thereby help in plant growth promotion under different mechanisms. PGPR are mainly used as inoculants for enhancing the growth and yield of agriculture crops, however screening for the efficient PGPR strains selection needs to be very critical. In this preliminary study, to identify capable microorganisms with a potential to solubilize more than one nutrient was successful.

Phosphorus plays a crucial biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant. It helps plants to survive winter rigors and also contributes to infection resistance in some plants [22]. P availability is low in soils because of its fixation as insoluble phosphates of iron, aluminium and calcium. Phosphate solubilizing microbes plays an important role in plant nutrition through increase in phosphate uptake by plants. The isolates were evaluated for Phosphate solubilizing microbe in Pikovskayas agar medium. Some of the bacteria in addition solubilize inorganic phosphate, making soil phosphorus otherwise remaining fixed available to the plants [23] due to excretion of organic acids [24] and through carbon and nitrogen sources, salt, pH, temperature [25]. Phosphate solubilizing stimulates plant growth through P nutrition, increasing the uptake of N, P, K and Fe. Some of these isolates also oxidize sulphur which is unavailable to plants and make it available to plant uptake [26]. Phosphorus biofertilizers could help increase the availability of accumulated phosphates for plant growth by the increasing the efficiency of biological nitrogen fixation and the availability of Fe, Zn through production of plant growth promoting substances [27] and solubilize phosphate thereby making it available for plants [28]. The ability of a few soil microorganisms to convert insoluble form of phosphorus to an accessible form is an important trait in plant growth promoting bacteria for increasing plant yields [29].

3.1 Qualitative method

A total of 42 Phosphate solubilizing microbial colonies were isolated on the Pikovskayas agar medium, containing insoluble Tri-calcium phosphate (TCP) from agricultural soil. Out of 42 microbial isolates 6 isolates showed highest Phosphate Solubilization Index (PSI) ranged from 1.55 - 2.88 were selected for further studies. The colonies showing clear halo zones around the microbial growth were considered as phosphate solubilization were shown in the figure 1.

Table 1: Phosphate solubilizing activities of six most P solubilizing activities

Bacterial isolate	Colony measurement (cm)	Zone measurement (cm)	Solubilization index(SI)*
JS-17	2.2	1.5	2.88
JS-7	1.8	1.6	2.68
JS-31	1.7	1.3	2.46
JS-16	1.5	1.3	2.36
JS-24	1.3	0.9	1.99
JS-44	0.8	0.6	1.55

*SI= (Colony diameter+ Halo zone)/colony diameter

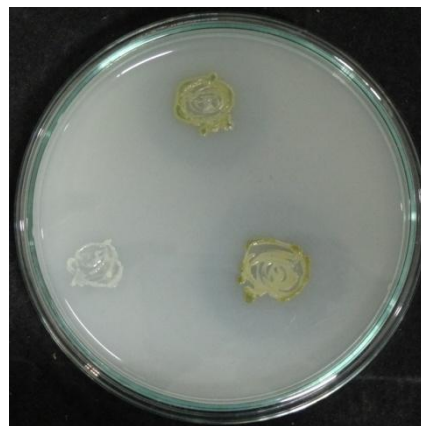


Figure 1: Formation of solubilization zone on PVK media.

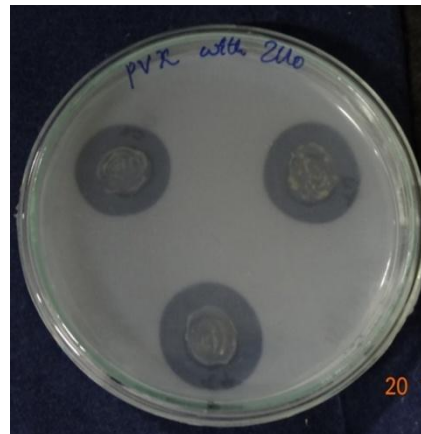
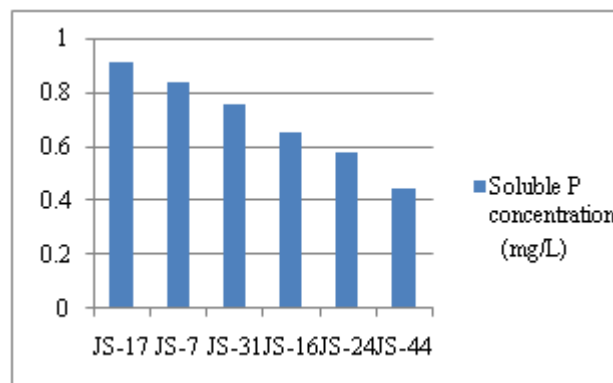


Figure 2: Formation of solubilization zone on modified PVK amended with ZnO.

3.2 Quantitative Method

Among these 6 potent isolates, 3 strains showed maximum PSI of JS-17, JS-7 and JS-31 in agar plates along with high soluble phosphate production of 0.92mgL⁻¹, 0.84mgL⁻¹ and 0.76 mgL⁻¹ in broth culture.



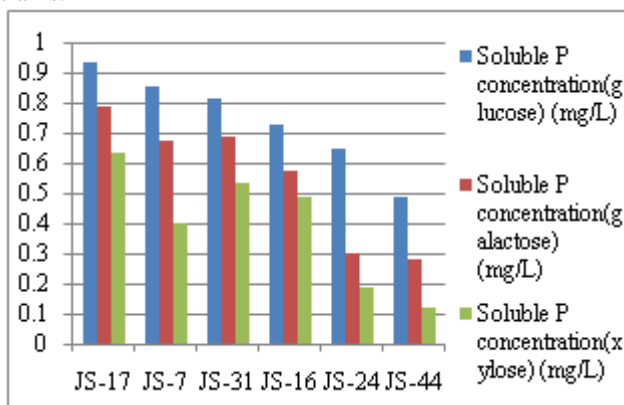
Graph1: Phosphate solubilizing activities of six most P solubilizing isolates

3.3 Optimization of Growth Conditions for Efficient P Solubilization

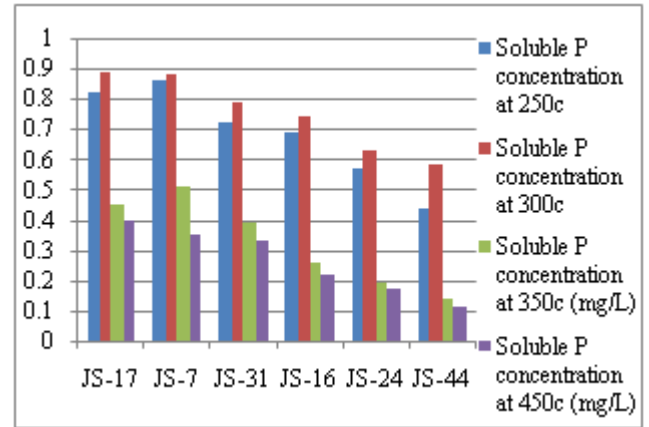
Diverse factors (salts, pH and temperature) can influence the capacity of phosphobacteria to solubilize 'P' [30]. It is assumed that the secretion of organic acids as evidenced by a fall in pH of the culture medium would also help in solubilizing phytates in soil. The ability of rhizosphere bacteria to solubilize insoluble P minerals has been

accredited to their capacity to reduce pH by the excretion of organic acids (e.g. gluconate, citrate, lactate and succinate) and protons (during the assimilation of NH_4^+) [31]. Glucose was replaced with other sugars, it was found that P solubilization was comparatively less in galactose and xylose amended broth. Maximum P solubilization was observed with strain JS-17 in glucose amended broth whereas bacterial isolates JS-31 and JS-16 showed significant P solubilization with all the three sugars. In culture media, 'P' solubilising ability is usually related to the degree of acidification of the medium [32]. Under controlled growth conditions, various studies have confirmed enhanced growth and 'P' nutrition of plants inoculated with phosphate solubilizing microorganisms [33].

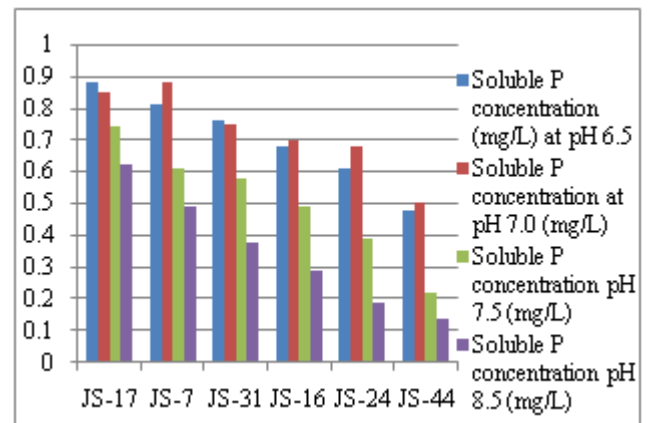
The solubilization of TCP was possible by acidification of the medium and acidity contributed to its solubilization by the release of carboxylic acids [34]. Most of the bacteria prefer neutral pH for their growth. Therefore, to determine the effect of pH on P solubilization, selected bacterial cultures were grown under different pH conditions. It was found that P solubilization was maximum when bacterial strains were grown in a medium with pH 6.5- 7.0. With increase in pH of the medium, P solubilization decreased. Maximum P solubilization was observed with bacterial strain JS-17 at pH 6.5 but JS-7 solubilize P maximum at neutral pH. Application of phosphate solubilizing bacteria to improve plant growth by solubilizing insoluble inorganic phosphates in soil [35]. Phosphate solubilizing PGPR and their plant growth promoting effect on maize was also reported [36]. These isolates have the ability to increase plant growth by producing IAA in previous reports [37]. Different temperatures were used for growth and P solubilization by selected bacterial cultures and it was found that bacterial isolate JS-7 caused maximum solubilization at 25°C and K solubilization by this strain decreased at higher temperatures of incubation. Bacterial isolate JS-17 caused maximum solubilization at 30°C, whereas other bacterial strains showed significant solubilization in the temperature range of 25°C to 35°C. P solubilization decreased at higher temperature of incubation i.e., 45°C with all the bacterial strains.



Graph 2: Phosphate solubilization from different carbon source



Graph 3: P solubilization by fluorescent pseudomonads at different temperatures



Graph 4: P solubilization by fluorescent pseudomonads at different pH

3.4 Potassium Solubilization

Potassium (K) is plays an important role in the growth and development of agricultural crops. There are several processes that provide to the availability of potassium in the soil. Thus, the amount present is inadequate to meet crop requirement. Potassium solubilizing bacteria are capable to solubilize potassium rock through production and secretion of organic acids [38]. Bacterial strain JS-17 caused maximum solubilization. Maximum K solubilization occurred when KCl was used as a potassium source followed by K_2SO_4 . Majority of the silicate solubilizers were identified as *Bacillus* sp. and *Pseudomonas* sp. When amendment of different forms of potassium sources was made to replace mica powder in the medium, it was found that K solubilization by all the bacterial strains was much higher in KCl and K_2SO_4 amended medium broth than mica powder containing samples. Similarly, potassium solubilizing bacteria have been isolated from the roots of cereal crops by use of potassium bearing minerals and soil [39-42]. Among the K bearing silicate minerals, mica was found to endure readily [43]. Silicate dissolving bacteria exhibited inhibitory activity on the growth of Gram negative bacteria *E.coli* [44]. These K solubilizing isolates were antagonistic to pathogenic fungi *Aspergillus niger* which causes collar rot in ground nut [37]. The efficiency of potassium solubilization by different bacteria was found to vary with the structure and chemical composition of the potassium containing minerals [45, 46].

Table 2: Solubilization zone produced on modified Aleksandrov medium amended with KCl, K₂SO₄, mica powder are the potassium sources

Bacterial isolate	KCl Solubilization zone diameter (mm)	K ₂ SO ₄ Solubilization zone diameter (mm)	Mica powder solubilization zone (mm)
JS-17	13.2	9.2	4.0
JS-7	12.6	8.1	3.5
JS-3	12.2	7.2	3.1
JS-16	11.4	6.4	2.6
JS-31	10.5	5.2	2.2
JS-53	10.0	4.1	1.8

3.5 Zinc Solubilization Activity

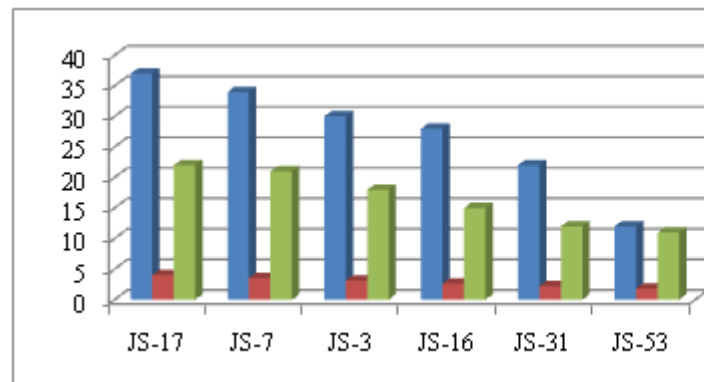
Zinc is one of the eight essential micronutrients required for healthy growth and reproduction of crop plants. From the data, it is obvious that the strains varied in their ability to solubilize different forms of insoluble zinc. Out of 55 strains, we could identify 14 strong Zn solubilizers. In the past also, efforts were made to identify zinc solubilizers with varying abilities. The demonstrated variation in the ability of solubilizing given zinc sources could be due to metabolic activity of a given strain [47]. There are different mechanisms of solubilization which have been identified with proton excretion, production of organic acids and other chelating metabolites [48]. Organic acid production by microbial strains plays a major role in solubilization [49]. The zinc solubilization in our studies could be due to production of organic acids, like gluconic acids that is increased by the fall in pH of culture media noted in all cases. The transformation of glucose to gluconic acid by the glucose oxidative external pathway in *Pseudomonas* spp. and other bacteria has been interpreted as a competitive approach by microorganisms [50]. Zinc phosphate solubilization by a strain of *P. fluorescens* was also reported [51]. The supplementing medium with zinc sulphate resulted in more gluconic acid production. Plant growth parameters of legume crop increase when seedlings were inoculated with zinc solubilizing bacteria compared to uninoculated controls [52]. The potential of rhizobacteria to promote nutrient uptake and plant growth could be due to synergistic action of the growth promoting traits than individual effect [53]. This solubilization property is important in nutrient cycling. Zinc phosphate solubilization by a strain of *Pseudomonas fluorescens* was also investigated [54].

All the selected strains of *Pseudomonas* used could effectively solubilize the insoluble Zn compounds used namely ZnCO₃ and ZnO. It is apparent from the zinc solubilization data that the solubilization potential varied with each isolate. The zone of solubilization was comparatively high in ZnO amended medium as compared to ZnCO₃. Size of the solubilization zone ranged from 7 to 22mm in ZnCO₃ and from 9 to 33mm in ZnO incorporated medium. Among the cultures JS-16, and JS-17 showed the highest solubilization zone in ZnCO₃, where as JS-17 and JS-7 showed 31mm zone in ZnO amended medium (Table 3)

Table 3: Solubilization zones produced on modified PVK medium amended with ZnO, ZnCO₃

Bacterial isolate	ZnO Solubilization zone diameter (mm)	ZnCO ₃ Solubilization zone diameter (mm)
JS-17	31	22
JS-7	31	21
JS-3	26	18
JS-16	22	15
JS-31	13	12
JS-53	12	11

Most of the soils are rich in total Zn, K and P. However, their availability to the plants when needed is very limited. Pseudomonads able to solubilize P, K and Zn and thus, offer best possible nutrient recycling mechanism at a low input cost where expensive inorganic fertilizers are becoming too expensive to small and marginal farmers. In the present study, bacteria when characterized by P, K and Zn solubilization was observed that some strains have high potential to solubilize the said essential nutrients. *Pseudomonas* isolate JS-17 showed TCP solubilization, and were also able to solubilize insoluble Zn sources. Interestingly, isolate JS-16 that could solubilize maximum Zn could not show any Pi solubilization. In this study we could find 6 isolates that could solubilize P, K and Zn. There are some PGPR that can fix nitrogen, solubilize mineral nutrients and mineralize organic compounds [55]. As we could screening for a potential PGPR strains on the basis of direct plant growth promoting traits viz., IAA, solubilization of Phosphate, Zinc solubilization, Potassium solubilization.

**Graph 5:** Comparative solubilization zones of P, K & Zn minerals

4. Conclusion

The experiment performed under *invitro* condition showed that these isolates may promote plant growth promoting potentials. PGPR include facilitating the uptake of certain nutrient like nitrogen, phosphorus, potassium and zinc forms for nutrient availability from the soil and root environment by producing plant hormone indole-3-acetic acid and multinutrient solubilization. Such multiple positive PGP Traits isolates can be further explored as potential biofertilizer for the sustainable agriculture. The direct promotions of plant growth by such situation warrants for compatibility between such strains for formulating consortia and use in the field. However, if strains that possess ability to mobilize multi-nutrients could be a benefit as they could be deployed directly with minimum efforts without going through the formulation.

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