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Exploring the Uncharted: Zinc and Phosphate Solubilization in Zn-P Isolates from Wheat Rhizosphere Inceptisols

Renu Gupta[1]*, Ravi Kumar[1], Wahidah H. Al-Qahtani[2], Mostafa A. Abdel-Maksoud[3],

[1]Division of Soil Science and Agricultural Chemistry, SKUAST-J, Jammu, J&K (India)

[2]Department of Food Sciences & Nutrition, College of Food and Agricultural Sciences, King Saud University, P.O. Box 270677, Riyadh 11352, Saudi Arabia

[3]Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia

*Correspondence: renugupta2781975@gmail.com ; renuguptaju96@gmail.com (R.G.)

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Exploring the Uncharted: Zinc and Phosphate Solubilization in Zn-P Isolates from Wheat Rhizosphere Inceptisols

Abstract

This research delves into the untapped potential of phosphorous and zinc solubilizing rhizobacteria, known as Zn-P isolates, from wheat rhizosphere inceptisols. A total of thirty rhizosphere soil samples were collected, resulting in recovery of forty unique bacterial isolates. After initial screening, out of forty isolates, recovered on the basis of halozone formation on the nutrient agar medium. four were subjected to biochemical and further molecular identification. Four isolates, identified as *Bacillus subtilis* (Zn-P-1), *Pseudomonas aureginosa* (Zn-P-2), *Staphylococcus aureus* (Zn-P-3), and *Methylobacterium organophyllum* (Zn-P-4) through a set of 16S rRNA primers, forward (5′- GGATGAGCCCGCGGCCTA-3′) and reverse (5′-CGGTGTGTACAAGGCCCGG-3′), exhibited superior solubilization efficiency of phosphorous and zinc These strains were evaluated via in vitro and pot culture assays. The study found that Zn-P-1 demonstrated the highest zinc solubilization (134.87 mg/l) when ZnO was used as the zinc source as compared to $ZnCO_3$ and Zn-EDTA as also highest at 72.32 mg/l in CaH_2PO_4 and lowest at 14.44 mg/l with KH_2PO_4 using P sources, thus highlighting the role of *Bacillus* in zinc and phosphorous activity with substrate type. The inoculation of tri-calcium phosphate (TCP) and $ZnCO_3$, along with *Bacillus* and *Methylobacterium*, led to increased phosphorous and zinc solubilization, uptake, and use efficiency, marking these rhizobacteria as potentially beneficial for nutrient enhancement and PGPR activities in wheat crops grown in inceptisols.

Key words: Wheat; PGPR; inceptisols; Phosphorous; Zinc; rhizobacteria; solubilization

1. Introduction

Next to nitrogen, phosphorus (P) stands out as the second most crucial macronutrient for promoting plant development. It plays a pivotal role in supplying, transferring, and storing energy for all

biochemical processes within plants, as highlighted by Khan et al. (2009). Despite the pressing need for increased crop yields, a mere 0.1 percent of total soil phosphorus is soluble and accessible to plants. This scarcity stems from phosphate fixation and the limited solubility of phosphorus in soil, as elucidated by Pereira and Castro (2014). Indeed, only soluble ionic phosphate (Pi) proves efficient as mineral nutrition for plants. Microbial-mediated phosphorus management plays a crucial role in various biological processes, including the transformation of unavailable and insoluble soil nutrients, as highlighted by Babalola and Glick (2012). The ability of phosphate-solubilizing rhizobacteria (PSRB) to convert insoluble forms of phosphorus into accessible forms, such as orthophosphate, stands as a key characteristic in enhancing crop plant development and output. This process facilitates the utilization of phosphorus by plants, contributing significantly to their growth and productivity.

The ability of phosphate-solubilizing bacteria to convert insoluble phosphorus (P) into soluble P through mechanisms such as the release of organic acids, chelation, and ion exchange contributes to soil fertility enhancement (Sharma et al., 2013). A lot of bacterial strains have been found to be involved in the process of phosphate solubilization. These belong to a diverse range of genera, including *Mesorhizobium*, *Arthrobacter*, *Chryseobacterium*, *Bacillus*, *Gordonia*, *Delftia*, *Enterobacter*, *Mycobacterium*, *Pantoea*, *Klebsiella*, *Micrococcus*, *Flavobacterium*, *Phyllobacterium*, *Rhizobium*, *Serratia*, *Sinorhizobium* and *Pseudomonas*, as highlighted by Chen et al. (2006).Tricalcium phosphate (TCP) is a mineral apatite known to be more easily degradable compared to single superphosphate (SSP). Thus, its use alongside phosphate-solubilizing rhizobacteria (PSRB) has been found to enhance phosphate solubilization by releasing organic acids, thereby rendering native and added phosphorus soluble. influences plant growth positively, as demonstrated by Kshetri et al. (2018).

Zinc (Zn) serves as a critical trace element essential for plant growth and reproduction, participating in numerous biological processes. Low solubility of zinc rather than its scarcity is the main reason for zinc deficiency.

Despite being required in trace amounts, zinc deficiency remains common in wheat during different crop growth phases. The zinc availability is influenced by several factors, including soil texture, pH, phosphorus content, soil type, and prevailing weather conditions. The complex

interplay of these factors affects the solubility and accessibility of zinc in the soil, consequently impacting its uptake by plants and their overall health and development.

Soil moisture and pH levels influence the solubility of zinc which is why semi-arid and arid parts of Indian agro-ecosystems often experience deficiency of zinc. Addressing this deficiency usually requires the external application of soluble zinc sources like $ZnCO_3$, ZnO, and $ZnSO_4$. However, it's crucial to acknowledge that only approximately 20% of the administered zinc is readily accessible for plant absorption, with the rest transforming into different inaccessible forms.

Certain bacteria, including *Burkholderia cenocapacia*, *Bacillus* sp. and *Pseudomonas* sp. have been found to play a crucial role in converting zinc to soluble forms, thereby making zinc available for uptake by plants. This transformative process has been documented by studies conducted by Khande et al. (2017).

Zinc-solubilizing bacteria utilize diverse mechanisms, including acidification, siderophore production, and oxidoreductive systems on cell membranes, to solubilize zinc. They release organic acids into the soil, which sequester zinc cations and can also chelate zinc, thus improving zinc availability for plant uptake. The activities of these bacteria play a crucial role in mitigating zinc deficiency and fostering robust plant growth in agricultural environments.

Furthermore, PSRB (phosphate-solubilizing rhizobacteria) and ZnSRB (zinc-solubilizing rhizobacteria) isolates obtained from indigenous wheat crops offer a promising alternative technique to address soluble phosphorus, zinc, and macronutrient deficiencies in the wheat rhizosphere, as highlighted by Khan et al. (2006). These isolates encompass a diverse range of unrelated bacteria, suggesting their potential to address multiple nutrient deficiencies simultaneously.

Hence, research on zinc and phosphorus solubilization by bacteria carries significant importance in enriching zinc nutrition in plants and tackling nutrient deficiencies in agricultural systems. It offers valuable insights into the potential of microbial interventions to enhance nutrient availability, soil fertility, and ultimately crop productivity. With this background the present study was conducted to untap the potential of phosphorous and zinc solubilizing bacterial isolates from wheat rhizosphere from inceptisols of two wheat growing districts of Jammu province of J&K (India)

2. Materials and Methods

2.1 Isolation of bacteria

In Udhampur and Jammu districts of Jammu and Kashmir, India, thirty rhizosphere soil samples were collected from the wheat rhizosphere using GPS coordinates. These samples were subjected to analysis, resulting in the recovery of forty bacterial isolates based on formation of halozone of solublization on nutrient agar medium. To commence the isolation process, 0.5 mm of the diluted sample suspension from a 10-5 dilution was evenly spread on nutrient agar plates followed by their incubation for a week at 28±2°C in a biochemical oxygen demand (BOD) incubator. Following the incubation period, isolates showing halozone formation were identified and selected for further analysis.

Subsequently, these selected isolates were subcultured on modified Pikovskaya medium (PVK medium) (Pikovskaya, , 1948) and zinc-solubilizing medium. This step allowed for the characterization and evaluation of the isolates' abilities to solubilize zinc and phosphorus, respectively, two very essential plant growth and development nutrients. The subculturing process served to isolate and identify bacterial strains with potential applications in agriculture, particularly in enhancing nutrient availability in the soil for improved crop productivity.

2.2 Measurement of pH

The pH levels of the Zn-P culture filtrates and the uninoculated samples were checked by filtering the culture using an Elico pH meter.

2.3 Zn-Solubilization (Plate Assay)

For zinc assay, all isolates were inoculated into zinc agar medium containing 0.1% insoluble zinc compounds viz. ZnO, $ZnCO_3$ and Zn-EDTA followed by their incubation for 48 hours at 30ºC. Further the clearing zone diameters around each colony was measured. The screening of isolates was done on the basis of solubilization potential and the solubilization criteria was taken as a preliminary tool for assessing potential of Zn-P isolates. The solubilization criteria was studied as

benchmark test for further biochemical (using chemical kit-KB002 [R]-Himedia) and molecular identification , and its evaluation under pot culture assays.

2.4 Isolation of Genomic DNA Methodology

Genomic DNA of the screened isolates was isolated following (Wilson, 2001).

2.5 Electrophoresis of Genomic DNA

A 0.8% agarose gel was prepared by dissolving agarose powder in 1× TAE (Tris-Acetate-EDTA) buffer. Ethidium bromide, a fluorescent dye, was added to the agarose gel to enable visualization of DNA bands under UV (ultraviolet) light. The DNA samples, along with a DNA ladder marker ranging from 100 to 1500 base pairs (bp), were carefully loaded into the wells of the agarose gel. The gel was submerged in a tank containing 1× TAE buffer. An electric current is applied across the gel, causing the negatively charged DNA molecules to migrate towards the positive electrode. The gel is typically run at a constant voltage, such as 100 V and it was was visualized on a UV transilluminator.

2.6 Amplification of Genomic DNA

- The amplification of genomic DNA was conducted through the polymerase chain reaction (PCR) using using a set of forward (5′-GGATGAGCCCGCGGCCTA-3′) and reverse primers (5′-CGGTGTGTACAAGGCCCGG-3′) for 16S rRNA. The PCR reaction mixture, totalling 25 µl, comprised 50 ng of genomic DNA, along with the following components: 15 mM Tris/HCl pH 8.5, 10 mM KCl, 0.1% (v/v) Triton X, 3 mM $MgCl_2$, 0.25 mM each deoxyribonucleotide triphosphate (dNTP), 2 units of Taq DNA polymerase, and 0.5 µM each forward and reverse primer. The amplification process was conducted in a thermal cycler, with a total of 35 cycles. The cycling conditions involved an initial denaturation step at 94°C for 5 minutes, followed by 33 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds. After amplification, the PCR products were subjected to electrophoresis in a 1.0% agarose gel. The gel, stained with a DNA-intercalating dye such as ethidium bromide, was then placed under UV transillumination to visualize the amplified DNA bands. The DNA bands were captured using a gel documentation system, allowing for further analysis and documentation of the PCR results.

2.7 DNA sequencing and sequence analysis

The gel aliquots with amplified products of biochemically screened isolates were subjected to sequencing and further analysis and phylogenetic tree was constructed following procedure as outlined by Williams et al. (2000).The result of most potent Zn-P-1 solubilizer (*Bacillus subtilis*) is presented in Fig.5

2.8 Effect of P and Zn Sources on P-Solubilisation and Zn –Solubilisation (Broth assay)

2.8.1 P-solubilization

The effect of different soluble and insoluble phosphorous sources was studied with the addition of 1% of respective phosphorous salts viz. TCP, CaH_2PO_4 and KH_2PO_4 and zinc sources, by 1% ZnO, 2% $ZnCO_3$ and 1% Zn EDTA in respective PVK medium and zinc solubilizing medium inoculated with selected microbial culture of each strain and incubated and added with 25 ml Barton's reagent, final volume being 50 ml each and respective OD was measured using spectrophotometer

The bacterial isolates were individually inoculated into PVK medium supplemented with0.1% CaH_2PO_4 and KH_2PO_4, as well as zinc solubilizing medium containing 1% ZnO, 2% $ZnCO_3$, and 1%Zn-EDTA. Each flask was inoculated with 1 ml suspension of the test culture, and centrifuged to remove cellular debris and cells and subsequently, analysed using Atomic Absorption Spectrophotometry

2.8.2 Zn -solubilisation

2.8.3 Zinc solubilization in basal (PVK and ZnS) medium

The experimental procedure involved inoculating bacterial isolates into basal medium for comparison at 7th and 10th days in PVK medium, and the 10th and 13th days in the zinc medium. The assessment of available zinc content was based on a turbidity test, which provides a qualitative measure of the solubilization process, using Atomic Absorption Spectrophotometry (AAS). The results of zinc solubilization were tabulated on the 13th day, coinciding with the point of maximum growth observed in the bacterial cultures.

2.8.4 Zinc tolerance, solubilization and screening of Zn-P isolates

The ability of the selected isolate to tolerate solubilized zinc was assessed under in vitro conditions using nutrient broth supplemented with different concentrations of soluble zinc ($ZnSO_4$). Initially, the nutrient broth was prepared and divided into 10 ml aliquots in test tubes to facilitate the growth assessment of the zinc-phosphate (Zn-P) isolates. To evaluate zinc tolerance, the isolated phosphate-solubilizing rhizobacteria (PSRB) were streaked onto nutrient agar supplemented with 1 mg/l zinc sulphate heptahydrate were then incubated to allow for bacterial growth.at 37˚C. Subsequently, (MIC) of the isolates was determined using the agar plate dilution method, as described by Luli et al. (1983). and concentrations of zinc sulphate heptahydrate, ranged from 1 mM to 15 mM. Following the incubation period, the MIC of the bacterial isolates was determined by observing their growth on after they failed to develop on the plates.

2.8.5 Determination of interaction of PGPR activities in wheat

The pH of the soil was determined using the method outlined by Jackson et al. (1973).

For estimating available phosphorus, Olsen's extractant method was employed. Available zinc in the soil was determined using the DTPA extraction method (Lindsay and Norvell, 1978).

Soil Microbial biomass carbon (SMBC) was quantified using the chloroform fumigation extraction method developed by Vance (1987). Phosphatase activity was determined by colorimetric estimation of p-nitrophenol released by phosphomonoesterase activity, following the method described by Tabatabai and Bremner (1969). Phytic acid content was assessed using the method outlined by Haung and Lantzsch (1983). Various efficiencies were calculated using specific formulas. Agronomic efficiency (AE) was computed using the formulas provided by Fageria and Baligar (2003) and Ladha et al. (2005). Recovery efficiency (RE) was determined using the formulas by Doberman (2005) and Ladha et al. (2005). Physiological efficiency (PE) calculations were based on formulas provided by Fageria and Baligar (2003) and Doberman (2005). The Zinc Harvest Index (ZHI) was calculated using the formula provided by Fageria and Baligar (2003). Total phosphorus (Grain + straw) from plant samples was estimated using the Vandomolybdate yellow color method from diacid extract, measured using a spectrophotometer following the method by Piper (1966). The zinc content in the dry matter of wheat grain and straw was determined using an Atomic Absorption Spectrophotometer. Grain and straw yield were estimated at harvest to complete the analysis.

3 Results and Discussion

and identified as *Bacillus subtilis* strain (Zn-P-1), *Pseudomonas aureginosa* (Zn-P-2), *Staphylococcus aureus* (Zn-P-3) and *Methylobacterium organophyllum* (Zn-P-4). The nomenclature was assigned to the isolates on the basis of P and Zn solubilization, first three isolates were P- solubilizers and also exhibited Zn- solubilization ability, whereas fourth isolate was Zn-solubilizer and exhibited P- solubilization ability.

3.1 Solubilization capability of isolates

The solubilization capability of the isolates was assessed by observing the clear zones formed around the wells after 7th and 9th days of incubation on PVK agar and ZnS agar at 28°C. All bacterial isolates exhibited clear zones on ZnS agar, with four selected isolates showing maximum clearance zones of varying diameters. The halo zone diameters ranged from 0.12 to 0.55 cm among the four isolates. The Solubilization Index (SI) of Zn-P-4 was found to be the highest, reaching 0.35 and 0.55 at 10th and 13th days, respectively, among all the isolates. The phosphate solubilizing efficiency (SE %) was calculated for the selected isolates, ranging from 112 to 155, with the highest value of 155 observed for Zn-P-4. Maximum solubilization was observed on the 13th day (Fig.1). The notable SI and SE (%) observed with the Zn-P-4 isolate may be attributed to the release of IAA and gluconic acid, as suggested by Potshangbam et al. (2018). Zn-P-1 isolate exhibited the highest values of SI and SE (%) following Zn-P-4 isolate. The observed variation in solubilization ability with different zinc sources could be attributed to the metabolic activity of the given strain, aligning with the findings of Rehman et al. (2021).

3.2 Effect of P and Zn source on P Solubilisation:

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The solubilization potential was evaluated through qualitative and quantitative methods under in vitro conditions, revealing that Zn-P-1 and Zn-P-4 were among the most potent solubilizers.

3.3 Phosphate solubilisation

3.3.1 Plate assay

The halo zone of solubilization ranged from 11.00 mm to 15.5 mm, with Zn-P-1 exhibiting the maximum zone of solubilization. A correlation was observed between the incubation time and the size of the zone, indicating that an increase in the incubation period led to larger zones for each isolate. The percent solubilization efficiency ranged from 112% to 155% on the 10^{th} and 13^{th} day of the incubation period, with the SE% increasing over time. The solubilization index, based on colony diameter and halo zone for each PSB isolate, varied from 0.12 to 0.55 among the four efficient isolates, with the index enhancing with the incubation period. The diameter of the clear halo zone formed by the bacterial isolates directly correlated with phosphate solubilization efficiency, and an increase in the incubation time led to larger zones for each isolate as reported by Lavakush et al. (2012) using Pikovskaya's media, and are consistent with the findings of Cavite et al. (2018), who suggested that the zone formation could be attributed to the activity of phosphate enzymes by Zn-P-1 and Zn-P-4 isolates. A high PSI index (2.82) recorded by the Zn-P-1 isolate indicates that this strain isolated from the wheat rhizosphere is an effective phosphate solubilizer, which is in consistency with the findings of Aarab et al. (2019).

3.3.2 Broth assay

All the isolated strains effectively solubilized the insoluble zinc (Zn) sources, including $ZnCO_3$, ZnO, and Zn-EDTA, as well as the phosphorus (P) sources, namely KH_2PO_4 and CaH_2PO_4, under the broth assay conditions. The release of phosphorus was comparatively low in the CaH_2PO_4-amended medium compared to the KH_2PO4 medium among the P sources, whereas the release was lowest for ZnO compared to $ZnCO_3$ among the Zn sources, and these differences were statistically significant (Table 1). Among the P sources, P release ranged from 8.39 to 10.12 µg/ml in CaH_2PO_4, with the lowest release for *Staphylococcus* (Zn-P-3) and the highest for Bacillus (Zn-P-1), and from 4.86 to 11.24 µg/ml in KH_2PO_4-amended medium. Among the Zn sources, P release ranged from 5.02 to 8.34 µg/50 ml in ZnO (Zn-P-3), with the lowest for *Staphylococcus* and the highest for *Bacillus* (Zn-P-1), and from 4.00 to 15.17 µg/50 ml in Zn-EDTA, with the lowest for Pseudomonas (Zn-P-2) and the highest for Bacillus (Zn-P-1). In $ZnCO_3$-amended medium, P release ranged from 5.31 to17.20 µg/ ml, with the lowest for *Methylobacterium* and the highest for *Bacillus* (Zn-P-1). Phosphorus release varied from 3.08 to 17.20 regardless of the wavelengths measured at 660 nm and 420 nm, with the highest values noted at the 660 nm wavelength on the 13th day (Table 1). The greatest phosphorus solubilization, specifically 0.684 mg/ mm, was observed for Zn-P-1 (*Bacillus*) on day 13^{th}, followed by a subsequent decline in solubilization. The

highest phosphorus release was recorded for Bacillus (Zn-P-1), which could be attributed to various mechanisms of phosphate solubilization by all three strains, with solubilization notably decreasing alongside a decline in pH, reaching its lowest point on the 13th day (Fig. 2). Similar findings were reported by Eramma et al. (2020).

3.4 Zinc Solubilization (release)

3.4.1 Plate Assay

All the isolated strains effectively solubilized the insoluble zinc (Zn) sources used, namely $ZnCO_3$, ZnO, and Zn-EDTA, as well as the phosphorus (P) sources, namely KH_2PO_4 and CaH_2PO_4, under the plate assay conditions. The zone of solubilization was comparatively higher in ZnO-amended medium compared to $ZnCO_3$ and Zn-EDTA in the plate assay. These results align with earlier studies that reported improved solubilization of Zn in ZnO-enhanced medium (Rehman et al., 2021). The size of the zone of solubilization ranged from 12.5 mm to 14.0 mm in $ZnCO_3$ and from 13.00 mm to 15.5 mm in ZnO-incorporated medium, and from 13.5 mm to 14.5 mm in Zn-EDTA. Furthermore, the zone of solubilization was higher in CaH_2PO_4-amended medium compared to KH_2PO_4 medium. The size of the solubilization zone ranged from 8.0 mm to 9.50 mm in CaH_2PO_4 and from 6.5 mm to 8.0 mm in KH_2PO_4-incorporated medium. It could be presumed that solubilization of Zn by the isolates might be attributed to organic acid production of Bapiri et al. (2012).

3.4.2 Broth assay (Selective medium)

The quantitative estimation of Zinc content in PVK broth was measured through AAS at 660 nm wavelength. The highest zinc solubilization was found to be 10.5 mg/l in Zn-P-1 isolate and lowest 2.50 mg/l in Zn-P-3, whereas highest zinc solubilization was found to be 11.80 mg/l in Zn-P-1 isolate and lowest 3.90 mg/l in Zn-P-3in PVK medium, at 7th and 10th days respectively (Table 2). A significant difference was observed as compared to control at 7$_{th}$ and 10th days in PVK medium. The estimation of Zinc content in zinc solubilizing broth was determined through AAS at 660nm wavelength and ranged from 37.75 in Zn-P-3 to 43.00 mg/l in Zn-P-1 isolate and also 39.87 mg/ml in Zn-P-3 to 44.25mg/l in Zn-P-1 Bacillus isolate at 10th and 13th day respectively. A significant difference was estimated as compared to control at 10th and 13th day in ZnS medium. This might be due to the fact that *Bacillus* sp. exhibited more solubilization as compared to other

strains and activity is pronounced with substrate type. Similar results were reported by (Ahmad et al., 2021).

3.4.3 Broth assay (P and Zn-sources):

All the isolated strains effectively solubilized the insoluble zinc (Zn) sources used, namely $ZnCO_3$, ZnO, and Zn-EDTA, as well as the phosphorus (P) sources, namely KH_2PO_4 and CaH_2PO_4, under the broth assay conditions (Table 3, Fig. 3). Zn-P-4 (*Methylobacterium*) was among the most potent solubilizers to release zinc when using $ZnCO_3$ and Zn-EDTA, whereas Zn-P-1 released the highest zinc when using ZnO as zinc sources. Zinc release was highest at 149.4 mg/l in Zn-EDTA and lowest at 6.64 mg/l in $ZnCO_3$ in the selective medium using Zn sources. as also reported by Borah et al. (2018). The modified ZnO media provided instant Zn release compared to other Zn sources used for Zn-P-4 organisms as zinc sources (Gandhi and Muralidharan, 2016). The pH ranged from 1.9 to 4.7 using P sources and 4.2 to 6.9 using Zn sources and was statistically significant. The lowest pH value was observed with CaH_2PO_4 and the highest was observed in ZnO and Zn-EDTA at the 13th day of the incubation period.The least pH value (4.8) was shown by Zn-P-1 culture on the 13th day after inoculation, indicating higher acidity due to growth (Fig. 3b). The decrease in pH of the medium containing the insoluble salt confirms the solubilization effect, which may be attributed to the production of organic acids, as reported by Rehman et al. (2021). The pH of isolates was observed on successive days in PVK and ZnS broth against control media and ranged from 2.9 to 4.6. The lowest pH value (3.0) in isolate Zn-P-3 at day 13th and the highest pH was observed as 4.7 in isolate Zn-P-4.9. The remarkable decline in pH was observed till day 13, after which pH attained a constant value. Keeping the solubilization criteria as a preliminary tool for assessing the potential of Zn-P isolates, the evaluation of isolates and its interaction effect on PGPR activities and P & Zn solubilization in wheat rhizosphere were studied under pot culture assays. Following initial screening, twelve bacterial isolates were obtained on nutrient agar supplemented with a 1 mM concentration of zinc, out of which four were selected based on their solubilization abilities. Nutrient agar containing varying concentrations of zinc sulfate heptahydrate (ranging from 1 to 15 mM) was employed to determine the minimum inhibitory concentration (MIC) for zinc sulfhate heptahydrate on the 6th, 10th, and 24th days, respectively. The minimum inhibitory concentration (MIC) of the selected bacterial strains was assessed, revealing that strains Zn-P-1 and Zn-P-4 exhibited the highest levels of growth and were

submitted to NCBI GenBank. Halozone of solubilization ranged from 7 mm to 16 mm. The highest SI and SE (%) value was found to be in the 10 mM concentration of zinc sulphate heptahydrate. The lowest SI and SE (%) value was found to be in 1 mM as well as 15 mM concentration of $ZnSO_4.7H_2O$ by Zn-P-1 and Zn-P-4 isolates respectively. (Fig. 4). MIC value up to 11.5 mmol/L and 24 mg/ml was also reported by Sen and Joshi (2017) and is in consonance with our observations. Halozone diameter ranged from 7.0 mm to 11 mm, 8 mm to 14 mm, and 8 mm to 16 mm at 6th day, 10th day, and 24th day respectively. High zinc tolerance ability in 10 mM concentration suggests their potential for detoxifying mechanism as reported by Mazalan et al. (2020).

3.5 Cultural and molecular characterization of potent isolate(Zn-P-1)

The most potent Zn-solubilizing rhizobacteria isolated from the rhizosphere wheat cultivating soil from different locations were subjected to biochemical identification viz, Citrate utilization, Urease, Nitrate reduction and Catalase. All biochemical tests exhibited positive results except gram staining which exhibited negative results and was creamish yellow in colour. The most potent Zn Solubilizing bacteria was ***Bacillus* strain** (Zn-P-1) identified as (*Bacillus subtilis* subsp. subtilis strain- 3619) following molecular identification which was further submitted to NCBI genbank and coded as **ON024399**.1. The highest % similarity with other accessions was OK135798-100% and MT538491- 99.84%

3.6 Effect of P & Zn sources on solubilization and PGPR activities in wheat.

All the isolated strains effectively solubilized the insoluble phosphorus (P) and zinc (Zn) sources. Among them, Zn-P-1 (*Bacillus*) isolate showed maximum potential of P and Zn solubilization (Table 4), resulting in an elevation in soil-available phosphorus, phosphatase activity, soil microbial biomass carbon, and available zinc, as outlined in Table 4. along with a parallel decline in soil pH and phytic acid in the grain of wheat crop as compared to control. Similar results were also reported by Raghuveer et al. (2017). This might be due to the release of organic substances from the roots to the rhizosphere soil, thereby elevating the microbial biomass in the rhizosphere, as well as the supply of mineralization of carbon (C) and nitrogen (N), resulting in the enhancement of indigenous microflora (Kumar et al., 2021). The increase in Zn availability and decline in pH might be attributed to the production of organic acids, which serve as key mechanisms to solubilize the complex Zn into a soluble form by lowering the pH of the microbial surrounding (Kushwaha

et al., 2021), thus increasing Zn availability and assimilation in plants as reported by Kusale et. al,.2021

3.7 Interaction effect of P (TCP) &Zn (ZnCO3) sources along with isolates at harvest stage.

The data from (Table 5) revealed significant differences in various types of phosphorus (P) and zinc (Zn) efficiencies, including agronomic efficiency (AE), physiological efficiency, recovery efficiency, P and Zn use efficiency, as well as P and Zn harvest index, P and Zn uptake, and yield. The highest values were recorded in treatment combinations involving TCP along with *Bacillus* isolate (P1B1), as well as $ZnCO_3$ along with *Bacillus* isolate (P2B1) as compared to control(B_0) . This increase in efficiency might be attributed to the enhanced P release and increased P solubilization capacity of the *Bacillus* isolate, resulting in an upsurge in nutrient uptake by the plant, which aligns with findings by Kshetri et al. (2018). The combined inoculation of $ZnCO_3$ and the *Bacillus* isolate demonstrated a synergistic relationship, leading to increased Zn solubilization and Zn uptake, ultimately enhancing plant growth parameters. This finding is consistent with studies by Akhtar et al. (2013). In *Capsicum annum* also it has been observed that Zn solubilizing *Bacillus* sp. promoted different plant growth parameters (Khan et al. 2021).

4. Conclusions

Our study highlights the significant potential of Zn-P-1 (*Bacillus* isolate) in solubilizing phosphorus (P) and zinc (Zn), enhancing soil availability of P and Zn, promoting phosphatase activity and soil microbial biomass carbon, and increasing available Zn in the soil. Additionally, it underscores the concurrent decline in soil pH and phytic acid levels in wheat crop grains. Furthermore, our findings suggest that treatment combinations involving TCP with *Bacillus* isolate (P_1B1) and $ZnCO_3$ with *Bacillus* isolate (P_2B_1) demonstrated the highest levels of various phosphorus (P) and zinc (Zn) efficiencies, encompassing agronomic efficiency, physiological efficiency, recovery efficiency, as well as P and Zn use efficiency. Additionally, they exhibited superior P and Zn harvest index, uptake, and yield. The combined inoculation of $ZnCO_3$ and the

Bacillus isolate demonstrated a synergistic relationship, leading to increased P and Zn solubilization, uptake, and ultimately, improvements in plant growth parameters. The identified isolate, Zn-P-1, holds promise for further utilization as a component in consortium mixtures and biofertilizers aimed at enhancing sustainable soil and plant health. This suggests a practical application for agricultural improvement strategies using *bacillus* species and exploring more substrates type.

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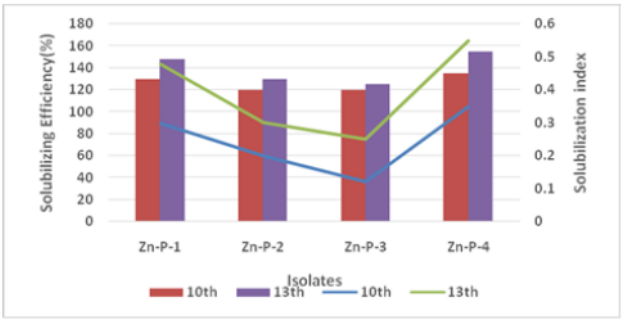


Figure 1. Solubilization index and efficiency of Zn-P isolates

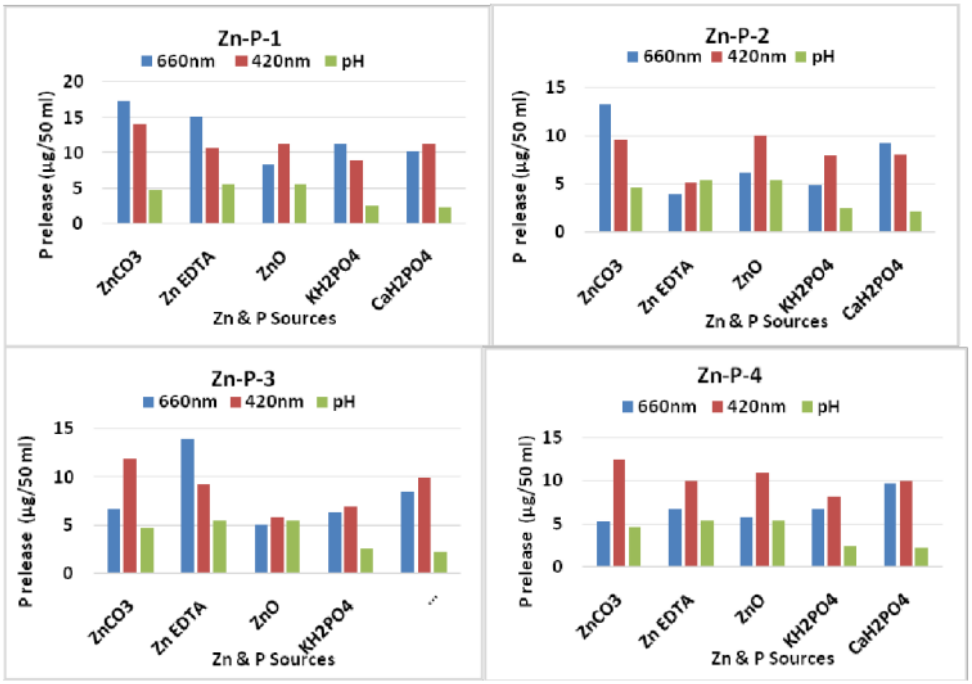


Figure 2. P-solubilisation (µg /50 ml-Barton's) of Zn-P isolates at 13th day of incubation.

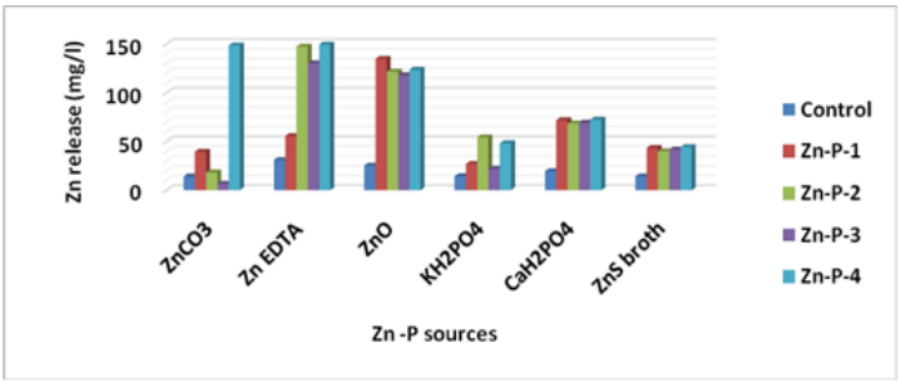
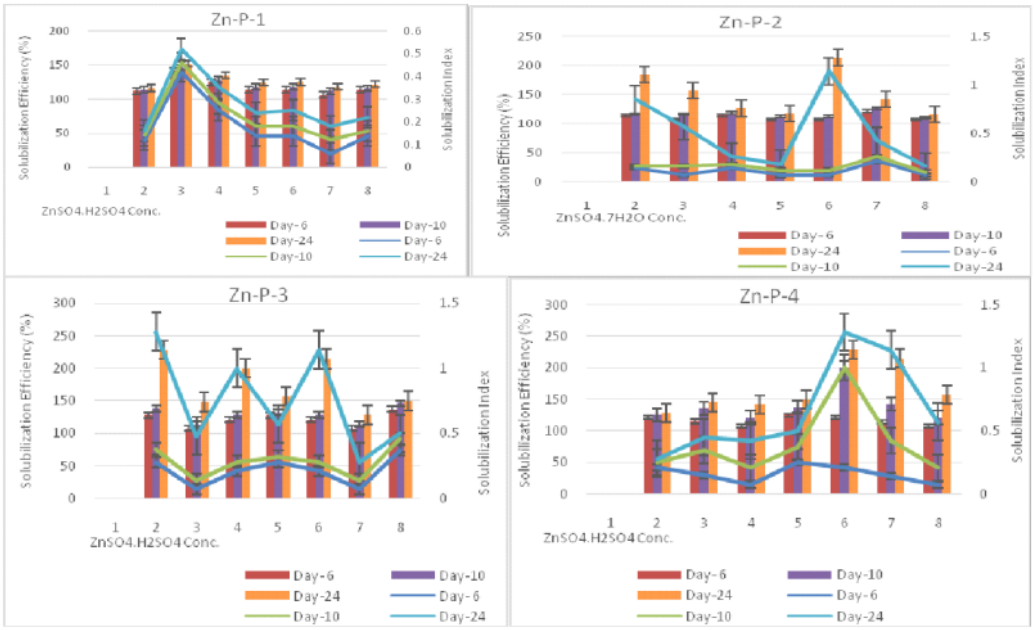


Figure3. Zinc release (mg/l) using Zn and P sources at 13th day



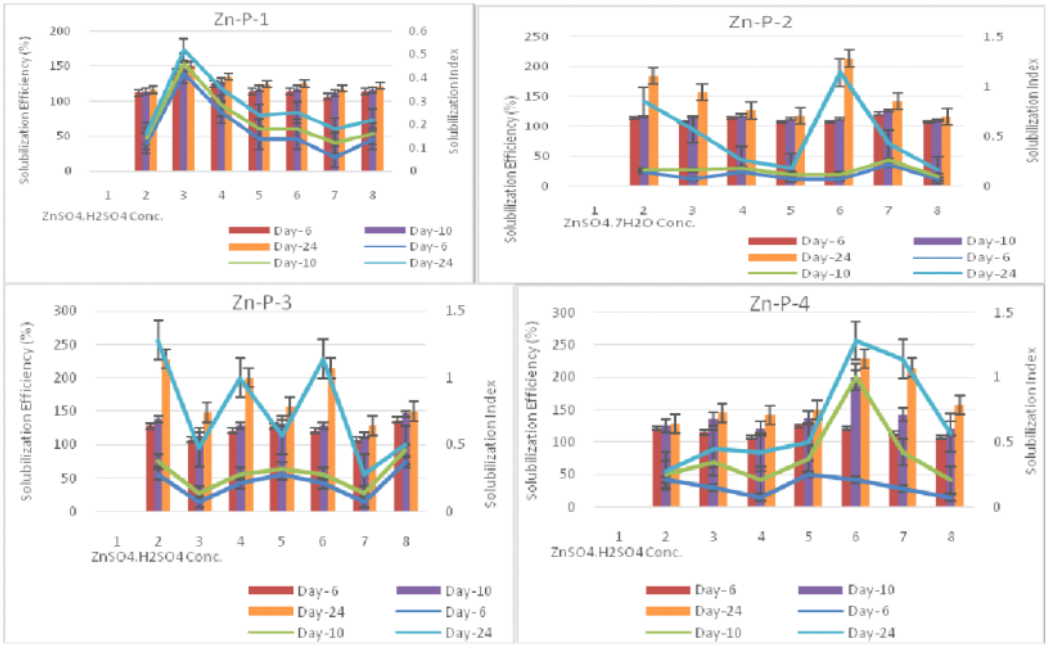


Figure 4. Zinc Tolerance ability*Horizontal axis entries = 2=2mM, 3=2.5mM, 4=5mM, 5=7.5mM, 6=10mM, 7=12.5mM and 8= 15mM

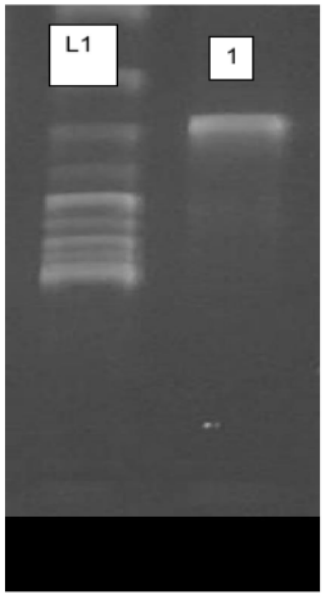


Fig.5.a.PCR amplified 16s rRNA,.Lane Description : L- Ladder(500bp);1-M1

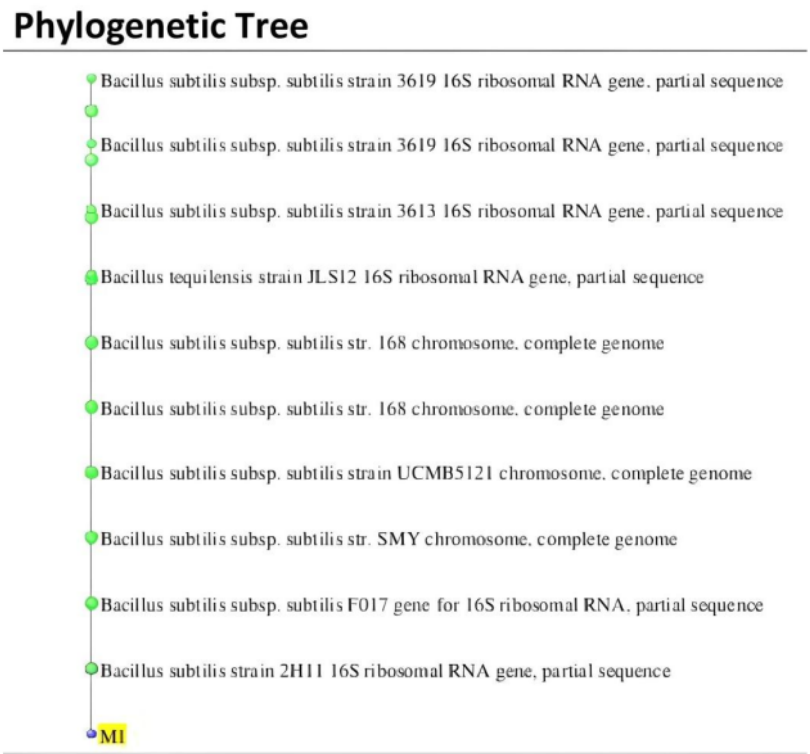


Fig.5b.Phylogenetic tree

Table 1. Quantitative estimation of P solubilization (µg /ml) using Zn and P sources.

Isolate	$ZnCO_3$	ZnEDTA	ZnO	KH_2PO_4	CaH_2PO_4
	AtWavelength420nm				

SEm(±)	0.012	0.012	0.047	0.046	0.046
CD at 5%	0.037	0.037	0.142	0.138	0.138
	AtWavelength660nm				
SEm(±)	0.05	0.042	0.047	0.042	0.012
CD at5%	0.15	0.140	0.140	0.142	0.037

Table 2. Zinc solubilization (release-mg/l) in selective media.

S. No.	Isolate	PvK broth (mg/l)		ZnS broth (mg/l)	
		Day 7	Day 10	Day 10	Day 13
1.	Zn-P-1	10.5	11.80	42.87	43.25
2.	Zn-P-2	8.25	9.50	40.75	41.75
3.	Zn-P-3	2.50	3.90	37.75	39.87
4.	Zn-P-4	4.50	5.75	43.00	44.25
5.	Control	1.50	2.85	13.50	14.10
	SEm(±)	0.24	0.47	0.50	0.53
	CD at 5%	0.76	1.57	1.67	1.69

Zn-P-1, Bacillus, Zn-P-2- Pseudomonas, Zn-P- 3-Staphylococcus, Zn-P- 4- Methylobacterium

Table 3. Zinc solubilization (release -mg/l) using Zn and P sources at 13th day

Media	Control (mg/l)	Zn-P-1 (mg/l)	Zn-P-2 (mg/l)	Zn-P-3 (mg/l)	Zn-P-4 (mg/l)
$ZnCO_3$	14.06	39.42	18.26	6.64	148.57
ZnEDTA	30.95	55.61	147.32	130.31	149.4
ZnO	25.31	134.87	121.59	117.86	123.67
KH_2PO_4	14.44	26.97	54.14	21.99	48.14
CaH_2PO_4	19.42	71.79	68.47	69.30	72.32
ZnS broth	14.10	43.25	39.87	41.75	44.25
SEm(±)	0.44	0.52	0.53	0.41	0.57
CD at 5%	1.39	1.68	1.72	1.33	1.83

Table 4. Effect of P & Zn sources on solubilization and PGPR activities in wheat.

Treatments	P Solubilization Quantitative at 10th Day (μg ml^{-1})			Zn Solubilization Quantitative at 13h Day (μg ml^{-1})			Soil av. P(mg kg^{-1})	Soil pH	Phosphatase activity (μg- PNP g^{-1}soil hr^{-1}).	SMBC (μg^{-1}soil)	Soil avail. Zn (mg kg^{-1})	Phytic acid(%)
	TCP	KH_2PO_4	CaH_2PO_4	$ZnCO_3$	ZnO	Zn EDTA						
Control	-	-	-	-	-	-	11.34	7.29	37.90	87.53	1.11	1.29
P_1-TCP	-	-	-	-	-	-	18.25	7.26	48.62	93.33	1.29	1.13
P_2-$ZnCO_3$	-	-	-	-	-	-	16.92	7.11	46.89	93.17	1.67	0.72
Isolate												
B_0 (Control)	2.18	3.21	5.25	14.06	25.31	30.95	12.17	7.31	40.10	89.63	1.19	1.24
B_1	18.73	11.24	10.12	39.42	134.87	55.61	18.06	7.14	48.02	92.81	1.49	1.11
B_4	7.25	5.24	6.69	148.57	123.67	149.4	14.43	7.24	42.27	90.91	1.32	0.87
SEm(±)	0.30	0.39	0.55	0.93	0.41	0.36	0.48	0.08	0.21	0.11	0.41	0.03
P*B(CD at 5%)	1.00	1.13	1.60	2.40	1.63	1.18	1.28	0.21	0.46	0.36	NS	0.09

Table 5. Interaction effect of P & Zn sources along with isolates at harvest stage.

Treatments P_1-TCP	PUE (%)	PHI (%)	PE (g/g)	RE (g/g)	AE (g/g)	P-Uptake(Grain +Straw)(g/pot)	Zn-Uptake(Grain +Straw)(g/pot)	Grain yield(g/pot)	Straw yield(g/pot)
P_1B_0	3.32	0.519	0.90	332	18.03	7.22	86.32	17.05	21.32
P_1B_1	5.93	0.512	0.83	593	20.01	11.44	93.31	20.24	25.31
P_1B_2	4.83	0.514	0.89	483	19.34	9.66	92.10	19.17	23.92
P_2-$ZnCO_3$	ZUE (%)	ZHI (%)							
P_2B_0	1.07	0.289	0.15	107	5.96	6.36	87.06	12.89	16.19
P_2B	2.54	0.298	0.35	334	6.99	9.92	96.61	17.21	21.51
P_2B_2	2.57	0.305	0.31	316	6.78	8.86	95.84	16.33	20.42

PUE- Phosphorus Use Efficiency (%), **PHI**- Phosphorus Harvest Index, **PE**- Physiological Efficiency, **RE** -Recovery Efficiency, **AE**- Agronomic Efficiency, **ZUE**- Zinc Use Efficiency, **ZHI**- Zinc Harvest Index

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