applied microbiology

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Isolation of Diverse Phosphate- and Zinc-Solubilizing Microorganisms from Different Environments

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Abstract: This study addresses the challenge of finding novel ways to solubilize phosphorus and zinc for agricultural purposes. The aim was to isolate PSMs (phosphorous-solubilizing microbes) and ZnSMs (zinc-solubilizing microbes) from different environments (e.g., soil amendments, land uses, and crop rotation systems) and evaluate their ability to solubilize different insoluble P sources (e.g., β-tricalcium phosphate (β-TCP), calcium-phytate (CaP), and rock phosphate (RP)) and Zn sources (e.g., zinc carbonate (ZnC), zinc oxide (ZnO), and zinc phosphate (ZnP)). Here, 25 isolates capable of solubilizing either P or Zn sources were isolated and classified by species using 16S rRNA and ITS-region sequencing. Notably, *Aspergillus awamori*, *Fusarium circinatum*, *Fusarium longifundum*, and *Mucor circinelloides*, isolated from cultivated soils and soil amendments, emerged as the most efficient PSMs and ZnSMs. *Mucor circinelloides* exhibited the highest solubilization ability for broths containing β-TCP, CaP, RP, ZnO, and ZnP, with log2-fold changes of 3.7, 1.8, 8.9, 7.8, and 2.4, respectively, compared to the control. For ZnC and ZnO, *Aspergillus awamori* displayed the highest Zn solubilization, with a 2.1 and 3.0 log2-fold change. The study highlights the potential of these strains as biofertilizers and underscores the role of *Mucor* and *Fusarium* genera in zinc solubilization.

Keywords: P-solubilizing microorganisms; Zn-solubilizing microorganisms; soil amendments; cultivated soils; bacteria; fungi

Citation: Islas-Valdez, S.; Afkairin, A.; Rovner, B.; Vivanco, J.M. Isolation of Diverse Phosphate- and Zinc-Solubilizing Microorganisms from Different Environments. *Appl. Microbiol.* **2024**, *4*, 1042–1056. https://doi.org/10.3390/applmicrobiol4030071

Academic Editor: Elena Tamburini

Received: 7 June 2024
Revised: 2 July 2024
Accepted: 4 July 2024
Published: 7 July 2024

1. Introduction

Phosphorus (P) and zinc (Zn) are essential nutrients needed for plant development, crop yield, and grain quality [1]. Yet over 30% of cultivated soils worldwide are alkaline with high calcium carbonate content, hindering nutrient uptake [2–4]. Although P and Zn fertilizers address these deficiencies, >80% of applications result in sparingly soluble nutrients due to the formation of insoluble forms of P and Zn [5,6]. These include tri-calcium phosphate (TCP) and calcium phytate (CaP) for P [5,7] and for Zn compounds such as smithsonite (ZnC, zinc carbonate), zincite (ZnO, zinc oxide), and hopeite (ZnP, zinc phosphate) [3]. These insoluble compounds are known as the P and Zn legacy in the soil [8–14].

Limited and unevenly distributed rock phosphate (RP) reserves a further increase in P fertilizer prices [15,16]. Additionally, over 50% of cereal-cultivated soils globally are deficient in plant-available Zn [2,4,5], affecting 30% of the world's population [17,18]. Efforts to address Zn deficiency through agronomic biofortification in cereals (e.g., wheat and corn) face challenges due to high Zn fertilizer costs [2,4].

Utilizing P- and Zn-solubilizing microorganisms (PSMs and ZnSMs) is an eco-friendly and cost-effective strategy for recycling legacy P and Zn nutrients in the soil. This diverse group includes various bacteria (e.g., *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Burkholderia*) and fungi (e.g., *Penicillium*, *Fusarium*, and *Aspergillus*) [5,19]. A primary issue in PSM

isolation is the conventional use of beta-tricalcium phosphate (β-TCP) as the sole source of P to identify PSMs [20,21]. This method is debated because beta-tricalcium phosphate does not accurately represent the diverse and complex forms of unavailable phosphorus found in natural soils, which can lead to discrepancies and overestimations of PSM efficiency in laboratory conditions compared to field conditions. Thus, it is recommended to use a combination of insoluble phosphorus sources, such as phytin, rock phosphate (RP), and zinc phosphate (ZnP), for the isolation of phosphate-solubilizing microorganisms (PSMs) [22,23]. This is because relying on a single source, like tri-calcium phosphate (TCP), has shown limitations, as many strains selected this way are ineffective on other insoluble P compounds and in farm conditions. Using multiple unavailable P sources increases the likelihood of isolating a diverse and effective range of PSMs, which are better suited for varying soil conditions [24–26]. Furthermore, although single-inoculum approaches that integrate PSMs and ZnSMs have been widely studied, these approaches typically test only one type of insoluble P and Zn source [27–30]. Limited information is available on PSM and ZnSM consortia, which have also been tested, although with a single insoluble P and Zn source [31]. Therefore, using PSM and ZnSM consortia or single strains with the ability to solubilize multiple insoluble P and Zn sources may prove to be a more effective strategy for addressing P and Zn deficiency under agricultural conditions [31].

Understanding the population density of P- and Zn-solubilizing microorganisms (PSMs and ZnSMs) in different environments is crucial for the effective screening of these microbes. For instance, soil management practices, including soil amendment application, intercropping, or crop rotation, have been shown to increase PSM and ZnSM diversity and abundance [32,33]. Li et al. [34] reported that PSMs and ZnSMs are more densely populated in the rhizosphere, bulk soils, compost, and plant roots than in sediments or water bodies. Land use also plays a role, with uncultivated soils (e.g., forest soils) harboring a richer diversity of microbes compared to agricultural, grassland, and mined soils [20,34]. Conversely, Fernández et al. [35] reported that PSM community structure was 81% higher in cultivated soil than in natural soils (e.g., grasslands), and noted differences among distinct cultivated soils. This difference was particularly pronounced in cultivated soils under crop rotation systems (legume–wheat) compared to monocropping systems [3]. In contrast to chemical fertilizers that can negatively impact soil health, waste-based fertilizers such as vermicompost and compost are gaining popularity [36]. These waste-based fertilizers are known for their rich microbial diversity, including genera capable of solubilizing P and Zn [37–39]. Therefore, the present research hypothesized that screening microbes from different environments will aid in selecting elite PSMs and ZnSMs.

The present study introduces a novel approach for the isolation of PSMs and ZnSMs. To achieve this, our objectives were to (1) isolate PSMs and ZnSMs from different environments, including those with different land uses, crop rotation systems, and soil amendments, and (2) quantitatively and qualitatively screen PSMs and ZnSMs capable of solubilizing different sparingly soluble P sources (i.e., TCP, CaP, and RP) and Zn sources (i.e., ZnC, ZnO, and ZnP). These beneficial microbes hold potential use in agricultural biofertilization.

2. Materials and Methods

2.1. Soil Sample Collection and Soil Amendments

In May 2023, topsoil samples (5–15 cm deep) were collected from the Agricultural Research, Development, and Education Center (ARDEC) at Colorado State University in Fort Collins, CO (40°36′36.9″ N and 104°59′38.2″ W). According to the World Reference Base for Soil Resources (WRB), forest soil (FS) is classified as luvisol, Agricultural soils, including wheat–barley soil (WBS), corn–sorghum soil (CSS), and pinto bean–cowpea soil (PCS), are classified as chernozems. These samples were obtained from agricultural soils under different crop rotation systems, including wheat–barley (WBS), corn–sorghum (CSS), and pinto bean–cowpea (PCS). Additionally, topsoil samples (5–15 cm deep) were gathered from an uncultivated forest soil (FS) in Poudre Canyon, located at Greyrock Mountain in Fort Collins, CO. A single composite sample from each site (i.e., agricultural

and uncultivated soils) was obtained from six samples that were randomly collected and then homogenized. The study also encompassed an investigation of two soil amendments: pure worm casting organic fertilizer (WSA, UNCO Industries, Inc., Union Grove, WI, USA) and pro sheep and peat compost (CSA, Permagreen Organics, Co., Arvada, CO, USA), both purchased from commercial suppliers. All collected samples were stored at 4 °C until further biological analysis to assess their impact on the isolation of potential PSMs and ZnSMs, considering varying soil management practices. The physicochemical characterization of both soil samples and soil amendments (Table 1) was conducted using the following methods: a 1:1 soil-to-water ratio for pH measurement, the DTPA-extractable Zn method for zinc analysis, the loss-on-ignition method for organic matter determination, and the Menlich 3 method for testing phosphorus. These analyses were performed by Ward Laboratories, Inc. (Kearney, NE, USA). The conversion of phosphorus into a form accessible to plants is significantly influenced by soil properties, notably pH and iron content. These factors affect the availability of phosphorus by forming soluble or insoluble complexes. For the isolation of effective phosphorus-solubilizing microorganisms (PSMs), it is critical to choose substrates that closely mimic these natural soil conditions. This approach ensures that the isolated microorganisms are not only effective under laboratory conditions but are also capable of enhancing phosphorus availability in a variety of agricultural settings. Thus, our substrate selection was guided by these considerations to optimize the practical applicability of the isolated PSMs.

Table 1. Physicochemical properties of environments chosen for isolating PSMs and ZnSMs.

Parameters	FS	CSS	PCS	WBS	CSA	WSA
Soil pH	7.1	8.3	8.6	8.4	8.9	7.2
Available P (mg kg^{-1})	15	91	73	65	1746	786
Available Zn (mg kg^{-1})	1.62	1.46	1.83	1.36	133.5	119.9
Organic matter (%)	4.3	2.7	2.6	3.0	19.2	43.5

FS: forest soil; CCS: corn–corn soil; PCS: pinto bean–cowpea; WBS: wheat–barley soil; CSA: compost soil amendment; WSA: worm casting soil amendment.

2.2. Prescreening and Isolation of PSMs and ZnSMs

For the isolation of PSMs and ZnSMs in May 2023, 1 g of each soil or amendment sample was homogenized in 9 mL of sterile 0.85% saline solution, followed by 10-fold serial dilution (10^{-1} to 10^{-10}) using a modified method [40]. The resulting suspensions were plated in triplicate onto Plate Count Agar (PCA, EM Industries, Inc., Darmstadt, Germany) and incubated at 30 °C overnight to identify the dilution from which it was possible to count colony-forming units (CFUs) for further studies.

For PSMs, a 100 mL aliquot of the serial dilution with the viable colony count of each suspension was spread onto the National Botanical Research Institute's Phosphate (NBRIP) medium agar [41] supplemented with β-tricalcium phosphate (β-TCP) (Sigma-Aldrich, St. Louis, MO, USA), calcium phytate (CaP) (TCI America, Portland, OR, USA), or rock phosphate (RP). The NBRIP medium for RP was modified by adding bromophenol blue dye at the concentration indicated by Li et al. [42] to improve the visualization. After seven days of incubation at 30 °C, PSMs with clear halos (i.e., for β-TCP and CaP) or yellow halos (i.e., for RP) were purified by re-streaking five times on NBRIP media plates to obtain pure strains. The purified strains were preserved at −80 °C in potato dextrose broth (PDB, Difco Laboratories, Sparks, MD, USA) supplemented with 50% (*v*/*v*) glycerol (Sigma-Aldrich, MO) for further analysis.

Similarly, a 100 mL aliquot of the serial dilution with the viable colony count was evaluated for ZnSMs on modified Pikovskaya medium [41] supplemented with 0.1% ZnO (Spectrum Chemical MFG, New Brunswick, NJ, USA), ZnP (Thermo Fisher Scientific, Waltham, MA, USA), or ZnC (MP Biomedicals, LLC, Solon, OH, USA). Modified Pikovskaya medium is regarded as the most efficient medium for screening ZnSMs [29]. The same storage method as for PSMs was applied to ZnSMs.

2.3. Taxonomic Characterization of Isolated Strains

A total of 25 isolates exhibiting either P- or Zn-solubilizing ability were obtained in June 2023 from the different environments during the prescreening. Among these isolates, 7 bacterial strains underwent identification through 16S rDNA analysis, while 18 fungal strains were subjected to ITS rDNA gene sequence analysis. For bacterial and fungal identification, single colonies of each strain were plated on PDA plates and incubated at 30 °C overnight to obtain fresh cultures. Subsequently, the PDA plates were shipped for DNA extraction and analysis using Sanger DNA sequencing services provided by Azenta Life Sciences-Genewiz, Inc. (South Plainfield, NJ, USA).

2.4. Final Screen of PSMs and ZnSMs

The experimental procedure closely followed that of the prescreening, with the exception that only strains consistently performing well in different P and Zn media were selected for further analysis. Out of the initial 25 strains isolated from the prescreening, a total of six strains were retained for further analysis. These six strains underwent additional testing in both solid and liquid NBRIP and modified Pikovskaya media to identify those with the ability to solubilize two or more insoluble P and Zn sources.

Qualitative estimation of P and Zn solubilization by the bacterial isolates involved the inoculation of 10 µL of log-phase bacterial culture (inoculum adjusted to an optical density $(OD)_{600}$ = 0.4~2.9 × 10^8 CFU mL^{-1}) at the center of NBRIP and modified Pikovskaya media, following the same conditions as in the prescreening experiment. For qualitative estimation of P and Zn solubilization by fungal isolates, a 7 mm disk was inoculated at the center of NBRIP and modified Pikovskaya media. Uninoculated NBRIP and modified Pikovskaya media served as controls. The P-solubilizing efficiency and Zn-solubilizing efficiency were calculated by standard formula as described by Nguyen et al. [43].

Quantitative estimation of P and Zn solubilization by bacterial isolates involved the inoculation of 10 mL of NBRIP and modified Pikovskaya broth (i.e., by replacing the β-TCP with Zn sources) with 100 µL of log-phase bacterial culture [41]. These cultures were kept in an incubator–shaker at 200 rpm for seven days at 30 °C. For quantitative estimation of P and Zn solubilization by fungi isolates, the methodology described by Mittal et al. [44] was conducted. Briefly, a 7 mm disk was inoculated in 10 mL of NBRIP and modified Pikovskaya broth. After incubation, the supernatant was obtained by centrifugation at 10,000 rpm for 5 min and filtered using Whatman 1 filter paper [44]. Then, the amount of P solubilization was determined using a modified ascorbic acid method at 882 nm [40,45], and the pH was measured using a pH meter (HQ40d multi-meter, Hach, Loveland, CO, USA). The Zn solubilization was analyzed by Ward Laboratories, Inc. (Kearney, NE, USA).

2.5. Statistical Analysis

A phylogenetic tree was constructed using Mega software version 11 through the neighbor-joining method with 100 bootstrap replicates. The phylogenetic tree was visualized using the ggtree package [46]. The data were analyzed using RStudio Team 2023 version 4.2.3 (PBC, Boston, MA, USA). A one-way analysis of variance (ANOVA) was conducted, followed by a post-hoc Tukey's HSD test. Differences were considered significant at $p < 0.05$. Model assumptions, including the homogeneity of variance and normality of residuals, were evaluated using the Shapiro–Wilk test and homoscedasticity using Levene's test. Data not meeting assumptions (i.e., $p < 0.05$) were transformed using sqrt or log transformations. Additionally, correlations between pH changes and P or Zn solubilization in the broths were tested with Pearson's correlation coefficient ($p < 0.05$) for normally distributed data sets. For non-normally distributed data, Spearman's correlation coefficients were calculated.

3. Results

3.1. Initial Screening of PSMs and ZnSMs from Different Environments

A total of 25 microbes were recovered exhibiting either P- or Zn-solubilizing ability. Seven of these isolated strains were identified as bacteria and classified within the *Pseudomonas* genus (Figure 1). The remaining 18 strains were identified as fungi, representing seven genera: *Aspergillus* (2), *Circinella* (1), *Fusarium* (6), *Galactomyces* (1), *Mucor* (2), *Neucosmospora* (1), *Penicillium* (4), and *Purpureocillium* (1).

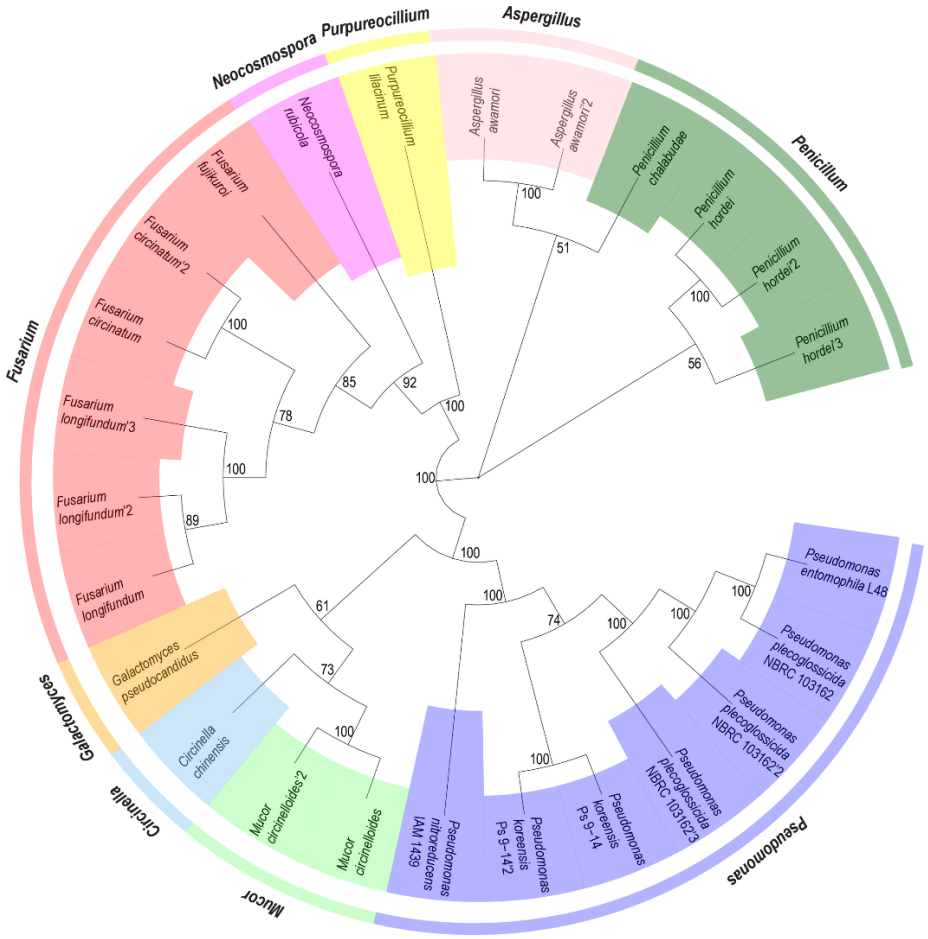

Figure 1. Neighbor-joining phylogenetic tree of 25 isolated strains with the ability to solubilize either phosphorus or zinc based on 16S rRNA or ITS gene sequences. The numbers at the nodes represent the levels of bootstrap support based on data from 100 replicates.

The phylogenetic tree grouped two *Pseudomonas korensis* Ps 9–14 strains and three *Pseudomonas plecoglossicida* NBRC 103162 strains with 100% bootstrap support (Figure 1). This grouping reduced the classification of the seven bacterial strains into four different species, providing a general overview of their relationship with their respective environments (Figure 2). Specifically, *Pseudomonas korensis* Ps 9–14 was identified in different cultivated soils, while *Pseudomonas plecoglossicida* NBRC 103162 was isolated from both cultivated soils and soil amendments. In contrast, *Pseudomonas entomophila* L48 and *Pseudomonas nitroreducens* IAM 1439 were found to be environment-specific, with the former in cultivated soils and the latter in soil amendments.

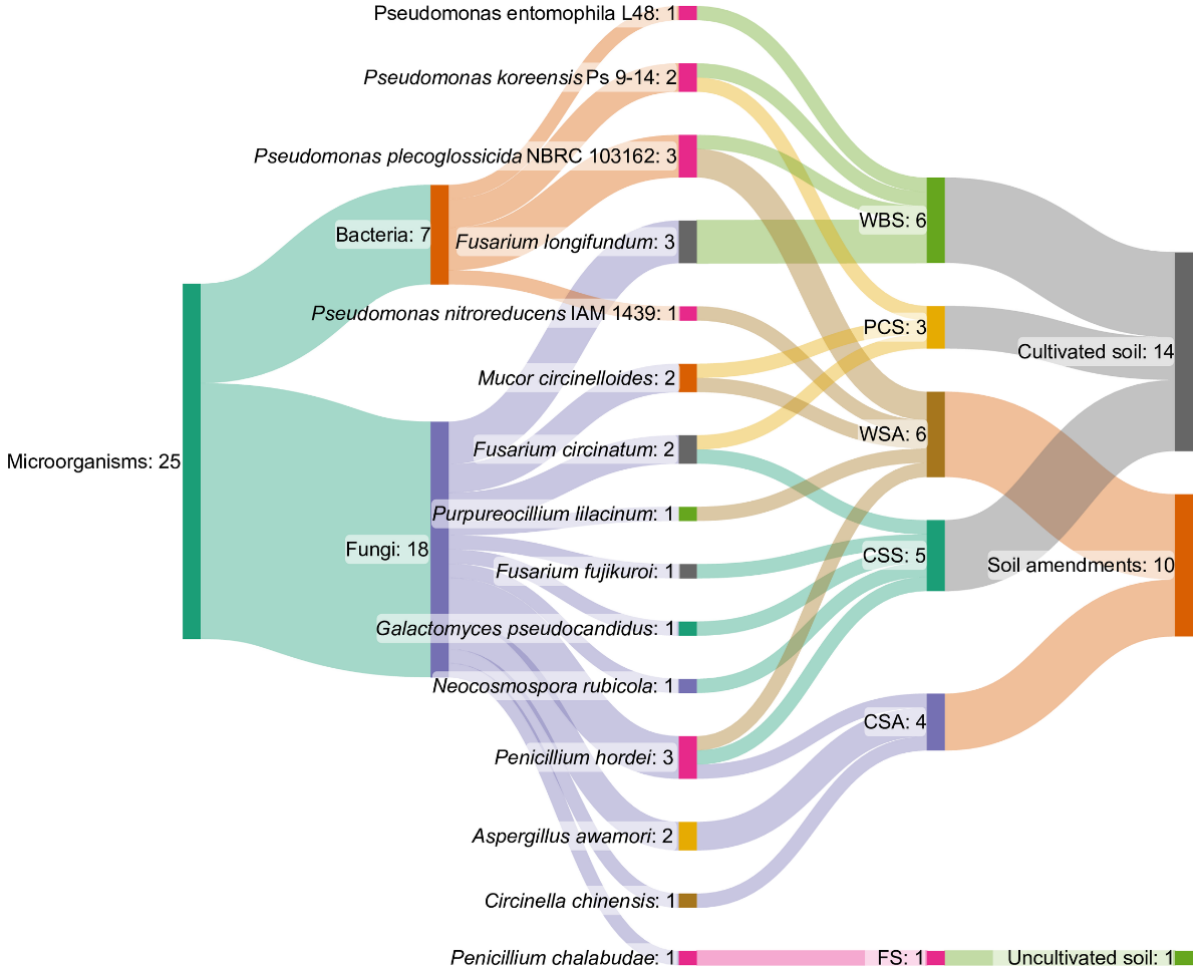

Figure 2. Sankey diagram illustrating the fungal and bacterial isolates based on their environments. The diameter of the lines is proportional to the number of unique or shared strains isolated from each of the six different environments: WBS: wheat-barley soil, PCS: pinto bean-cowpea soil, WSA: worm casting soil amendment, CSS: corn-sorghum soil, CSA: compost soil amendment, and FS: forest soil.

The phylogenetic analysis also clustered two *Aspergillus awamori* strains and two *Mucor circinelloides* strains with 100% bootstrap support (Figure 1). Similar bootstrap values were observed for two strains of *Fusarium circinatum* and for three strains of *Fusarium longifundum*. Furthermore, three *Penicillum hordei* strains also formed a clade with 56% similarity, with two of them forming a terminal subclade with 100% bootstrap support. Overall, the 18 fungal strains were categorized into 11 different species, reflecting their associations with their respective environments (Figure 2). Notably, *Aspergillus awamori*, *Purpureocillium lilacinum*, and *Circinella chinensis* were exclusively isolated from soil amendments. Similarly, *Fusarium longifundum*, *Fusarium fujikuroi*, *Galactomyces pseudocandidus*, and *Neocosmospora rubicola* were solely identified in cultivated soils, whereas *Penicillum chalabudae* was only identified in uncultivated soils. In contrast, *Mucor circinelloides*, *Fusarium circinatum*, and *Penicillum hordei* were isolated from both soil amendments and cultivated soils.

This study sheds light on the prevalence of PSMs and ZnSMs in different environments, indicating their higher occurrence in cultivated soils (56%) and soil amendments (40%) compared to uncultivated soils (4%). Among cultivated soils, the highest incidence of PSMs and ZnSMs was observed in soils with cereal crop rotations, such as WBS (43%) and CSS (36%), in contrast to legume crop rotations, such as PCS (21%).

Among the 15 bacterial and fungal species, 6 demonstrated the ability to solubilize multiple insoluble P and/or Zn sources. Notably, *Pseudomonas plecoglossicida* NBRC 103162 solubilized all three insoluble P sources, while *Fusarium circinatum* and *Fusarium longifun-*

dum, among the fungal strains, solubilized two or three insoluble Zn sources (Figure 3). *Aspergillus awamori*, *Mucor circinelloides*, and *Penicillum hordei* exhibited the capacity to solubilize both insoluble P and Zn sources. Consequently, these strains underwent a second screening to assess their P and Zn solubilization abilities using all insoluble P and Zn sources.

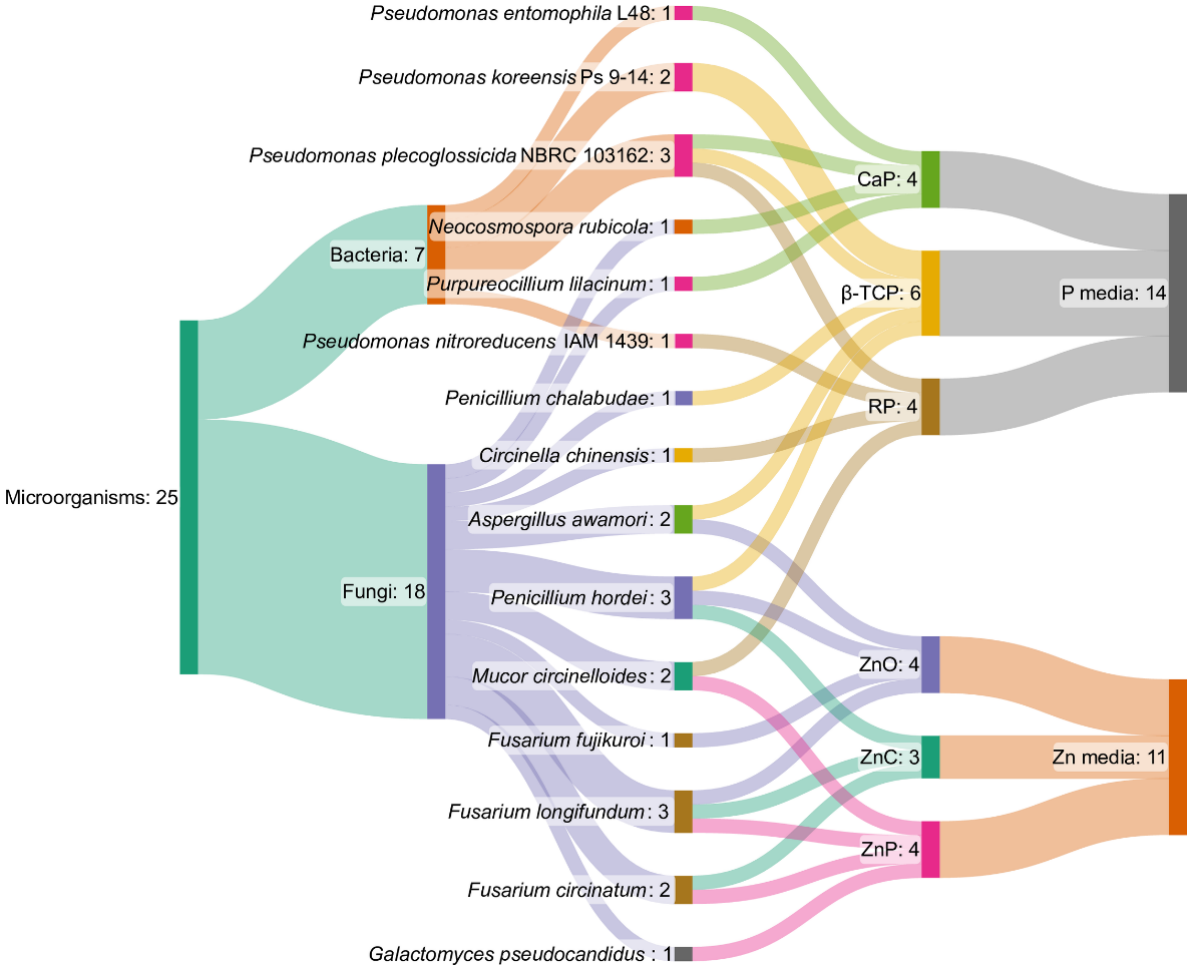

Figure 3. Sankey plot diagram illustrating the number of fungal and bacterial isolates on each node based on their P- or Zn-solubilizing ability during initial screening. The diameter of the lines is proportional to the number of unique or shared isolated strains among the six insoluble sources: CaP: calcium phytate, β-TCP: β-tricalcium phosphate, RP: rock phosphate, ZO: Zn oxide, ZnC: Zn carbonate, and ZnP: Zn phosphate.

3.2. Screening of PSMs and ZnSMs with Multiple Unavailable P and Zn Sources

The six strains exhibiting solubilization of multiple insoluble P and/or Zn sources in the initial screening (Figure 3) underwent further evaluation to assess their capability to solubilize all tested insoluble P and Zn sources in NBRIP and modified Pikovskaya media. Plate assays revealed that *Pseudomonas plecoglossicida* NBRC 103162, *Aspergillus awamori*, and *Penicillum hordei* solubilized all three tested insoluble P sources with varying efficiency (Figure 4). *Penicillum hordei* displayed high efficiency in RP solubilization, while *Pseudomonas plecoglossicida* NBRC excelled in CaP mineralization. Both strains, along with *Aspergillus awamori*, exhibited similar β-TCP solubilization. Notably, *Fusarium longifundum* might be a promising isolate for RP solubilization, even though it was unable to solubilize the other two tested insoluble P sources.

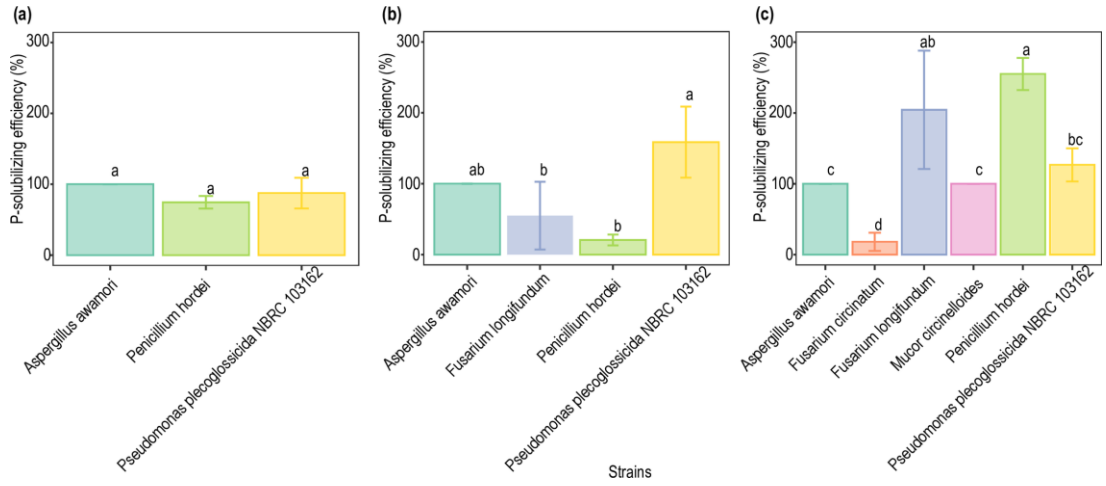

Figure 4. The P-solubilizing efficiency of 6 selected strains in solid NBRIP medium containing (**a**) β-TCP: β-tricalcium phosphate, (**b**) CaP: calcium phytate, and (**c**) RP: rock phosphate. The results represent the mean value of three replicates, with error bars representing standard errors (n = 3). The different lowercase letters above the columns denote significant difference ($p < 0.05$) according to the ANOVA test followed by Tukey's HSD test. Data for RP were normalized by square root transformation.

The results of the Zn-solubilizing assay revealed that *Aspergillus awamori*, *Penicillum hordei*, and *Mucor circinelloides* solubilized all three tested insoluble Zn sources (Figure 5). *Penicillum hordei* and *Mucor circinelloides* demonstrated high efficiency for ZnP, while *Aspergillus awamori* and *Penicillum hordei* were efficient for ZnC and ZnO, respectively. Notably, *Pseudomonas plecoglossicida* NBRC 103162 also demonstrated ZnP solubilization potential, indicating its ability to solubilize different insoluble P sources.

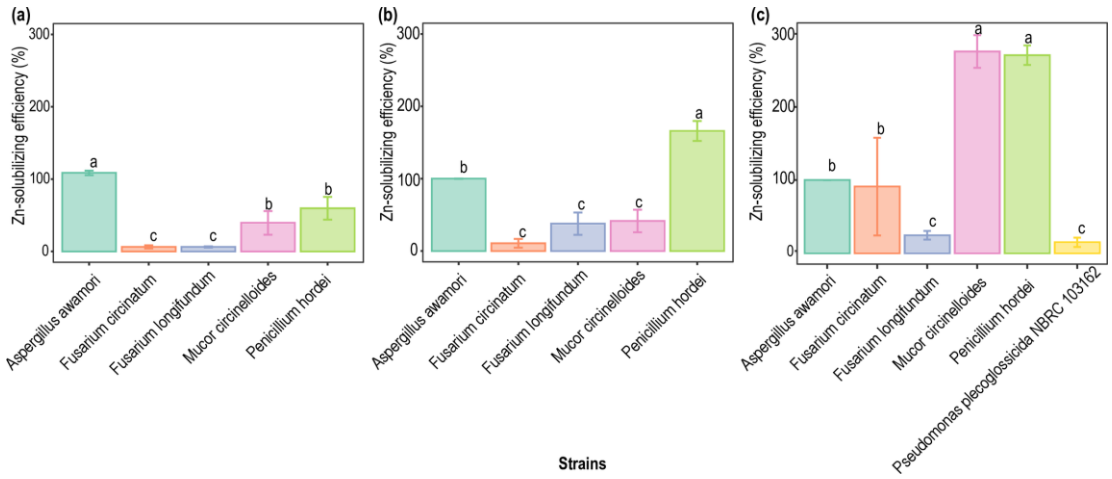

Figure 5. The Zn-solubilizing efficiency of 6 selected strains in solid modified Pikovskaya. Medium containing (**a**) ZnC: Zn carbonate, (**b**) ZnO: Zn oxide, and (**c**) ZnP: Zn phosphate. The results represent the mean value of three replicates, with error bars representing standard errors (n = 3). The different lowercase letters above the columns denote significant difference ($p < 0.05$), according to the ANOVA test followed by Tukey's HSD test. Data for ZnC and ZnP were normalized by square root and log transformations, respectively, before ANOVA analysis.

3.3. Screening of PSMs and ZnSMs in Liquid Broth and pH Changes

The six selected isolates underwent a quantitative test of all insoluble P sources, revealing varying efficiencies of P solubilization depending on both the insoluble P sources and the inoculated strain. *Aspergillus awamori*, *Fusarium circinatum*, *Fusarium longifundum*, and

Mucor circinelloides demonstrated significant solubilization and mineralization capabilities in NBRIP broths containing β-TCP, CaP, and RP compared to the control (Figure 6). Notably, *Mucor circinelloides* displayed the highest solubilized P concentration for NBRIP broths containing β-TCP, CaP, and RP compared to the control, with $\log_2$-fold changes of 3.7, 1.8, and 8.9, respectively. In contrast, *Penicllum hordei* and *Pseudomonas plecoglossicida* NBRC 103162 could only solubilize one insoluble P source each (i.e., CaP and β-TCP, respectively) compared to the control, confirming their limited ability to solubilize different insoluble P sources.

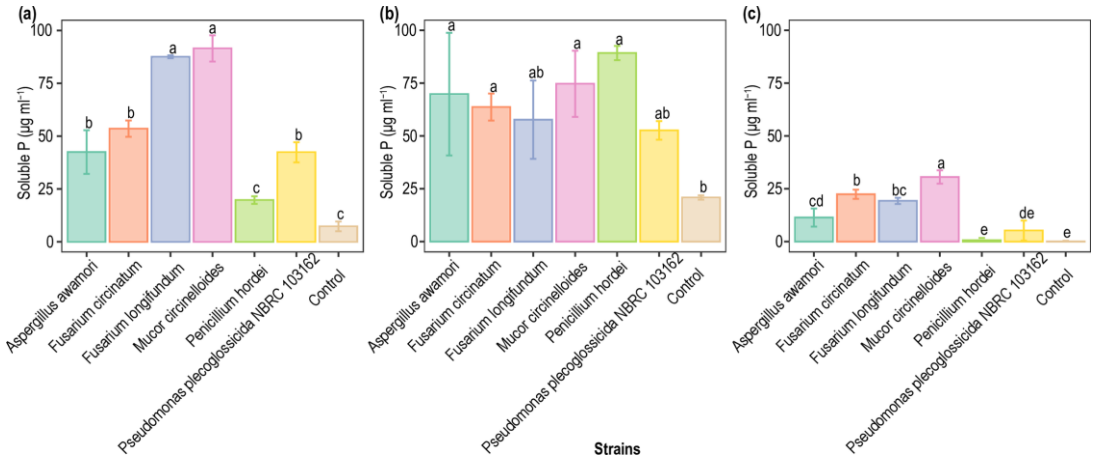

Figure 6. Solubilized P concentration for six selected strains and the control in NBRIP broth containing (**a**) β-TCP: β-tricalcium phosphate, (**b**) CaP: calcium phytate, and (**c**) RP: rock phosphate after seven days of incubation. The results represent the mean value of three replicates, with error bars representing standard errors (n = 3). The different lowercase letters above the columns indicate a significant difference ($p < 0.05$) according to the ANOVA test followed by Tukey's HSD test.

The observed P solubilization coincided with a pH shift towards the acidic range after seven days of incubation (Table 2). *Mucor circinelloides* induced the most significant pH reduction for β-TCP, CaP, and RP compared to the control (−0.5, −0.3, and −1.0 $\log_2$-fold changes, respectively). This strain exhibited the lowest pH value along with high concentrations of soluble P, which might suggest organic acid production and subsequent media acidification. Correlation analysis further supported this relationship between the pH decrease in the broths and the increase in P solubilization. Negative correlations were observed for NBRIP broths containing β-TCP (r = −0.71; $p < 0.001$) and RP (r = −0.94; $p < 0.0001$) according to the non-parametric Spearman's correlation test. However, a non-significant correlation (r = −0.38; $p > 0.05$) was observed for CaP according to Pearson's correlation test.

Table 2. Mean pH of NBRIP broth after seven days of incubation with each of the six selected strains. Data represent mean ± error standard (n = 3). The different letters in the same columns denote significant difference ($p < 0.05$) according to the ANOVA test followed by Tukey's HSD test.

Strains	β-TCP	CaP	RP
	pH	pH	pH
Aspergillus awamori	5.30 ± 0.07 ^{d}	6.01 ± 0.03 ^{c}	5.11 ± 0.05 ^{c}
Fusarium circinatum	5.50 ± 0.02 ^{c}	5.84 ± 0.03 ^{d}	4.65 ± 0.12 ^{d}
Fusarium longifundum	5.25 ± 0.02 ^{d}	5.78 ± 0.04 ^{d}	4.52 ± 0.16 ^{d}
Mucor circinelloides	4.87 ± 0.03 ^{e}	5.51 ± 0.08 ^{e}	3.69 ± 0.13 ^{e}
Penicillum hordei	5.88 ± 0.08 ^{b}	6.37 ± 0.01 ^{b}	6.27 ± 0.09 ^{b}
Pseudomonas plecoglossicida NBRC 103162	5.14 ± 0.02 ^{d}	5.83 ± 0.02 ^{d}	5.09 ± 0.01 ^{c}
Control	6.77 ± 0.14 ^{a}	6.75 ± 0.07 ^{a}	7.53 ± 0.16 ^{a}

Among the six strains assessed for their ability to solubilize all tested insoluble Zn sources, *Aspergillus awamori*, *Fusarium circinatum*, and *Fusarium longifundum* demonstrated proficiency in solubilizing all of them (Figure 7). Among these strains, *Aspergillus awamori* displayed the highest ZnC and ZnO solubilization compared to the control, exhibiting a 2.1 and 3.0 $\log_2$-fold change, respectively. Meanwhile, *Mucor circinelloides* presented the highest ability to solubilize Zn in broths containing ZnO and ZnP compared to the control, with $\log_2$-fold changes of 7.8 and 2.4, respectively. Notably, *Penicllum hordei* and *Pseudomonas plecoglossicida* NBRC 103162 were unable to solubilize more Zn than the control.

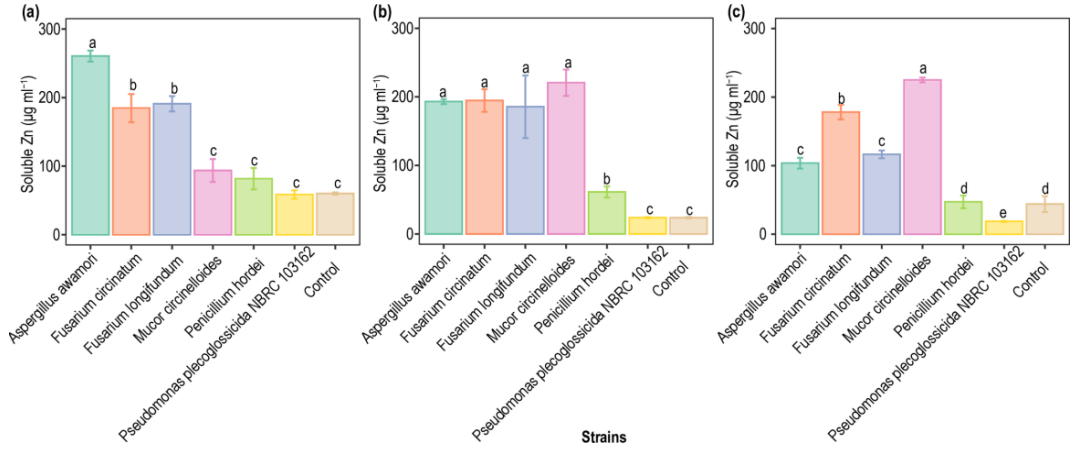

Figure 7. Solubilized Zn concentration for the six selected strains and the control in modified Pikovskaya broth containing (**a**) ZnC: Zn carbonate, (**b**) ZnO: Zn oxide, and (**c**) ZnP: Zn phosphate. The results represent the mean value of three replicates, with error bars representing standard errors (n = 3). The different lowercase letters above the columns denote significant difference ($p < 0.05$) according to the ANOVA test followed by Tukey's HSD test. Data for ZnO were normalized by log transformations before ANOVA analysis.

The Zn solubilization was accompanied by a notable shift in pH towards the acidic range after seven days of incubation (Table 3). *Mucor circinelloides* induced the most significant pH decrease for ZnO and ZnP compared to the control (−0.1 and −0.7 $\log_2$-fold changes, respectively). Similarly, *Aspergillus awamori* showed the greatest reduction in pH compared to the control treatment (−0.1 $\log_2$-fold change). These strains presented the highest concentration of soluble Zn along with the lowest pH value, which might suggest organic acid production and subsequent media acidification. Correlation analysis further supported the inverse relationship between the pH decrease in the broths and the amount of soluble Zn, showing negative correlations for the modified Pikovskaya broths containing ZnC ($r = -0.65$; $p = 0.01$), ZnO ($r = -0.60$; $p < 0.01$), and ZnP ($r = -0.93$; $p < 0.0001$) according to the non-parametric Spearman's correlation test.

Table 3. Mean pH of modified Pikovskaya broth after seven days of incubation with each of the six selected strains. Data represent mean ± error standard (n = 3). The different letters in the same columns denote significant difference ($p < 0.05$) according to the ANOVA test followed by Tukey's HSD test.

Strains	ZnC	ZnO	ZnP
	pH	pH	pH
Aspergillus awamori	6.77 ± 0.02 ^{b}	6.73 ± 0.07 ^{cd}	5.11 ± 0.06 ^{c}
Fusarium circinatum	7.16 ± 0.14 ^{a}	7.06 ± 0.03 ^{b}	4.71 ± 0.11 ^{d}
Fusarium longifundum	7.18 ± 0.04 ^{a}	7.01 ± 0.09 ^{b}	5.00 ± 0.06 ^{c}
Mucor circinelloides	7.18 ± 0.05 ^{a}	6.68 ± 0.06 ^{d}	4.40 ± 0.01 ^{e}
Penicillum hordei	7.26 ± 0.04 ^{a}	6.89 ± 0.09 ^{bc}	5.96 ± 0.09 ^{b}
Pseudomonas plecoglossicida NBRC 103162	7.22 ± 0.06 ^{a}	6.99 ± 0.08 ^{b}	6.74 ± 0.06 ^{a}
Control	7.29 ± 0.05 ^{a}	7.26 ± 0.05 ^{a}	6.94 ± 0.09 ^{a}

4. Discussion

Given the diverse chemical compositions of soils, the use of β-TCP as a universal insoluble P source for isolating PSMs is not reliable [24]. Therefore, different insoluble P sources (β-TCP, CaP, and RP) or Zn sources (ZnC, ZnO, and ZnP) were utilized to isolate 25 bacterial and fungal strains with either P- or Zn- solubilizing abilities during the initial screening in media. Among the 25 isolated microbial strains, 6 strains from the *Aspergillus*, *Fusarium, Mucor*, *Penicillum*, and *Pseudomonas* genera were found to possess the ability to solubilize multiple insoluble P and/or Zn sources in media. These findings are consistent with previous studies that found microbes belonging to the *Aspergillus*, *Penicillum*, and *Pseudomonas* genera as dominant PSMs and ZnSMs in agricultural soils [32–34,44,47–52].

In order to establish an effective process for selecting PSMs and ZnSMs, the six species from the initial screening were tested on all insoluble P and Zn sources under different conditions. Differences were found between media and broths. For instance, while *Mucor circinelloides*, *Fusarium circinatum*, and *Fusarium longifundum* exhibited no halos for all the insoluble P sources in media, they were able to solubilize all the insoluble P sources in broths. *Pseudomonas plecoglossicida* showed halos for all the insoluble P sources in media but only solubilized β-TCP in broths. This aligns with previous reports that the *Pseudomonas* genus has poor solubilization and mineralization of P [53].

Similar trends were observed for Zn media. *Pseudomonas plecoglossicida* NBRC 103162 exhibited a halo in media but no Zn solubilization in broths. In contrast, *Aspergillus awamori* showcased the highest percentage of Zn-solubilizing efficiency in media containing ZnC, similar to its ability to solubilize the greatest amount of Zn in broth with the same insoluble source. Similar results were found for *Mucor circinelloides* in media and broths containing ZnP. However, this trend was not observed for media or broths containing ZnO. Consistent with these findings, other researchers have reported a poor correlation between media and broths for PSMs due to variations in the diffusion rates of different organic acids secreted on media [24,47].

The broths containing PSMs and ZnSMs revealed an inverse relationship between soluble P or Zn and pH. This trend was particularly evident in *Aspergillus awamori* and *Mucor circinelloides*, emphasizing the potential role of organic acid production in the solubilization of P and Zn, as previously reported by other authors [24,47,49,54]. Even though fungi produce more organic acids than bacteria [49,53], the type and quantity of organic acids produced may vary depending on the incubation time and temperature, as well as on the insoluble source [47,55]. This could potentially explain why fungi exhibited a greater ability to solubilize P and Zn compared to bacteria in this study, as well as the differences in solubilization capabilities among isolates. This outcome is consistent with other studies where fungi demonstrated greater effectiveness in solubilizing insoluble sources of P, such as β-TCP and RP [56] and Zn sources, including ZnO, ZnP, and Zn sulphate [29], compared to bacteria. For *Aspergillus awamori*, *Fusarium circinatum*, *Fusarium longifundum*, and *Mucor circinelloides*, which also exhibited the ability to mineralize insoluble organic P sources, this capability might be attributed to enzymes such as phosphatases and phytases [24,47,53]. While some studies have also reported the ability of *Mucor* and *Fusarium* genera to solubilize P [47,55,57–60], there is no information regarding their ability to solubilize Zn. This research, however, has demonstrated for the first time that strains of the *Mucor* and *Fusarium* genera can solubilize Zn.

This study unveiled that fungi showed greater potential efficiency as PSMs and ZnSMs than bacteria in agricultural soil under crop rotation systems and soil amendments. *Fusarium circinatum* and *Fusarium longifundum* were isolated from cultivated soils under crop rotation systems. *Mucor circinelloides* was also identified in both cultivated soils and soil amendments, while *Aspergillus awamori* was found in soil amendments. However, the diverse soil conditions in uncultivated soils and soil amendments posed challenges in determining how these conditions affect the presence and efficiency of various microbial species. Similar results have been demonstrated in studies showing that efficient PSMs and ZnSMs are more abundant in crop rotation systems than in uncultivated environments due

to crop plant roots providing specific nutrients that promote differential microbial growth, which in turn impacts the composition and density of the soil microbial community [35]. Another study compared the population density and biogeographic distribution of PSMs from 40 different sites across China and reported that PSMs are more abundant in agricultural soils than in desert, forest, grassland, and mined soils [34]. Similarly, a previous study also demonstrated that land uses (e.g., uncultivated soil and cultivated soils) can have a pronounced effect on the ability of solubilization among strains, even those of the same genus [20,61,62]. In that study, the authors reported that uncultivated soils showed more efficient PSMs compared to cultivated soils due to the low ability of PSMs in cultivated soils resulting from intensive agricultural practices. To promote efficient PSMs, the study recommended the adoption of sustainable agricultural practices and organic fertilizers. Thus, this study demonstrates that soil organic amendments and good agricultural practices, such as crop rotation, may promote efficient PSMs and ZnSMs. Nevertheless, to evaluate the relationship between their prevalence and parameters such as available P and Zn levels, as well as organic matter percentage, proved challenging due to variations in these parameters, as soil amendments exhibited higher values compared to cultivated soils.

The present study also highlighted that while it is important to screen PSMs and ZnSMs by using different insoluble P and Zn sources in media, qualitative screening should be complemented by a quantitative screening in broths to identify efficient PSMs and ZnSMs. This study also underscores the potential of using microbial consortia to solubilize/mineralize diverse insoluble soil sources, which could significantly enhance crop yields and quality. The inclusion of specific fungi, such as *Fusarium circinatum* and *Fusarium longifundum*, along with *Mucor circinelloides* and *Aspergillus awamori*, enriches this potential. These findings highlight the importance of diverse microbial species in improving soil biofortification and, consequently, agricultural productivity.

5. Conclusions

In conclusion, adopting a more efficient approach to the identification of efficient PSMs and ZnSMs involves isolating strains from different soils or soil management practices, such as crop rotation systems or soil amendments. Our results revealed that some fungal strains did not produce halo zones on agar plates but exhibited solubilization in liquid media, whereas bacterial strains produced halo zones but showed lower solubilization in liquid media compared to the fungal strains. This highlights that the solubilization of P and Zn varies depending on the insoluble sources and the inoculated strains. Thus, while tests in media with various insoluble P and Zn sources in tandem serve as a valuable prescreening method to narrow down strains, additional tests in broths are advisable to provide an acceptable indication of P and Zn solubilizing ability, as well as to identify efficient PSMs and ZnSMs. Out of the 25 isolates, *Aspergillus awamori, Fusarium circinatum, Fusarium longifundum,* and *Mucor circinelloides* were selected as the most efficient strains to solubilize P and Zn. This study also showed that strains of the Mucor and Fusarium genera can solubilize Zn. Despite their capability to release P from both organic and inorganic sources, as well as Zn from different sources, given the complexity of soil conditions compared to in vitro settings, further studies on their interactions should be carried out before considering applications. Further studies should also explore the combined effects of these PSMs and ZnSMs from different environments, aiming to develop a composite biofertilizer with higher-quality and multifunctional properties. This might enhance P and Zn bioavailability and improve their effectiveness under field conditions, despite challenging factors such as soil properties, environmental conditions, and competition with native soil microorganisms.

Author Contributions: Conceptualization, S.I.-V., A.A. and J.M.V.; methodology, S.I.-V. and A.A.; formal analysis, S.I.-V.; writing—original draft preparation, S.I.-V.; writing—review and editing, S.I.-V., A.A., B.R. and J.M.V.; visualization, S.I.-V.; supervision, J.M.V.; funding acquisition, J.M.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by USDA-ARS- Pulse Crop Health Initiative grant number 58-30602032.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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