Changes in Soil Zinc Chemical Fractions and Improvements in Wheat Grain Quality in Response to Zinc Solubilising Bacteria

Banafsheh Rezaei Niko
Shahid Chamran University of Ahvaz
naeimeh enayatizamir (n.enayatzamir@scu.ac.ir)
Shahid Chamran University of Ahvaz
Mojtaba Nourozi Masir
Shahid Chamran University of Ahvaz

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Abstract

This study was performed to investigate the impacts of two indigenous strains of zinc (Zn) solubilising bacteria on Zn fractionation in soil, Zn uptake and the molar ratio of phytic acid to Zn (PA/Zn) in wheat grain cv. Chamran. The experiment was implemented in a completely randomised factorial design that included the treatment of bacterial inoculation consisting of B0 (control), B1 (*Bacillus megaterium*), B2 (*Enterobacter cloacae*), B3 (a mixed inoculation of both bacteria), and $ZnSO_4$ fertiliser at three application levels: Zn0 (control), Zn1 (5.1mgkg^{-1}), and Zn2 (10.1mgkg^{-1}). *Bacillus megaterium* was isolated from the rhizosphere of *Zea mays*, which can solubilise Zn and phosphate and produce auxin. *Enterobacter cloacae*, which had been isolated from sugarcane root, was screened qualitatively and determined to be the best isolate to solubilise Zn. The results indicated the maximum values of organically bound (2.08 mg kg^{-1}) and exchangeable Zn (0.89 mg kg^{-1}) in the Zn2B3 treatment. Also, the highest amounts of carbonate bound (9.25 mg kg^{-1}), FeMn-oxides (10.70 mg kg^{-1}), and residual fractions (16.17 mg kg^{-1}) were recorded for the Zn2B0 treatment. The relative proportions of residual, FeMn-oxides, carbonate, organic, and exchangeable Zn fractions in Zn0B0 were 40.48, 29.2, 27.1, 2.18, and 0.84%, respectively. These proportions changed to 37.24, 29.51, 26.9, 4.3, and 1.75%, in the Zn0B3 treatment. Maximum values of Zn uptake and grain yield were associated with the Zn2B3 treatment, showing increases (compared to the control) of 214 and 46%, respectively. The lowest ratio of PA/Zn was obtained in the Zn2B3 and Zn2B2 treatments, which exhibited reductions of 31.38 and 30.86%, respectively, when compared to the control.

Introduction

Zinc (Zn) is an essential micronutrient that is found in almost all classes of enzymes. Its deficiency is a well-documented problem among cereal crops that causes decreased crop yields and nutritional quality. The critical level of diethylene triamine penta-acetic acid (DTPA) extractable Zn in soils is less than 1 mg kg^{-1} [1], and more than 56% of Iranian soils have DTPA extractable Zn concentrations of less than 0.75 mg kg^{-1} [2]. Zn uptake in crops is often limited due to the high soil pH, calcium carbonate content, phosphate status, high magnesium or bicarbonate concentrations, or irrigation water, which enhances Zn precipitation [3]. Zn activates the metabolism of carbohydrates and auxin in plants [4] and serves as an integral component of carbonic anhydrase, alcohol dehydrogenase, and glutamate dehydrogenase [5].

Cereals are the most important source of minerals and protein in developing countries. Among cereal crops, wheat plays an essential role in the daily diets of people in most areas of the world. It is the main food source of 35% of the world's population. Wheat is also one of the most important sources of Zn and iron for humans, especially in developing countries [6]. The reported concentrations of Zn in wheat grains are too low to meet the daily requirements of humans, especially those who consume a high proportion of cereal in their diets.

The enrichment of micronutrients, such as Zn, in crops via agronomic practices and potential microbes [7] that can increase the bioavailability of micronutrients is a sustainable method of crop bio-fortification

[8]. Bio-fortification by microbes is suggested as a green alternative within the framework of sustainable agriculture [7]. Increased Zn concentrations in wheat grains under the influence of Zn solubilising bacteria, such as *Bacillus megaterium*, *Arthrobacter chlorophenolicus*, *Enterobacter* sp. and *Pseudomonas* sp. MN12 have been reported [9, 10].The inoculation of soil with *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* has increased Zn concentrations in two bean cultivars (*Phaseolus vulgaris*) by increasing Zn dissolution from insoluble and low soluble sources and have reduced indirectly phytic acid concentration and PA/ ratio Zn [11]. The coapplication of Zn and Zn solubilizing *Enterobacter* sp. MN17 improved grain Zn concentration and reduced the phytate in grain of *kabuli* chickpea [12]. Pythic acid is the storage compound of phosphorus in grains of cereals and can chelate metal ions, especially Zn and Fe. This binding mechanism produces insoluble compounds, which reduces the availability of minerals (including Zn) [13]. Therefore, the high proportion of this substance in wheat prevents Zn absorption by the human body [14].

Khuzestan soils in the southwest of Iran are mostly calcareous and have a pH of more than 7, therefore Zn deficiency is common in these soils. Consequently, Zn deficiency has been reported in Iran [15]. Based on the above discussion, the aims of this study were: (i) to examine the effect of Zn solubilising bacteria as a supplement to $ZnSO_4$ fertiliser on Zn uptake, grain yield, and PA/Zn ratio in wheat; (ii) to investigate available Zn, pH, dissolved organic carbon (DOC), and Zn chemical fractions in soil in response to Zn solubilising bacteria, along with different Zn fertiliser application rates. To the best of our knowledge, only a few studies have investigated the role of Zn solubilising bacteria on Zn fractionation in soil. Therefore, in the present study, the impact of two indigenous isolated bacteria that can solubilise Zn from the source of zinc oxide (ZnO) were considered for different forms of Zn in soil.

Material And Methods

Screening Zn solubilising bacteria with some traits related to plant growth promotion

Soil samples were taken randomly from the *Zea mays* rhizosphere (Andimeshk 32°27' N 48°21' E, Iran) and stored in sterile envelopes in a refrigerator at 4°C. In order to isolate the bacteria, serial dilutions of soil samples were prepared, and 0.1 mL of each sample cultured on a nutrient agar medium. The bacterial growth was purified from the agar medium based on differences in the apparent properties of the colonies. The qualitative Zn dissolution ability of isolates was assayed based on the production of a clear halo around the bacterial colonies on the medium containing 0.1% ZnO as a source of insoluble Zn [16]. The ratio of total diameter (colony + halo zone) to colony diameter was considered as the zinc solubilising index (SI) [17]. A quantitative assay of Zn dissolution by isolate with maximum SI from *Zea mays* rhizosphere and some sugarcane isolated bacteria from a previous study [18] was done in Erlenmeyer flasks containing 25 mL of a liquid basal medium [16] by the inoculation of 250 µL of an overnight culture of each isolate (2×10^8 Cfu mL^{-1}). The flasks were incubated at 28°C and shaken at 120 rpm for five days. The pH of the culture medium was recorded, and then the medium was centrifuged to remove residuals and cells. The Zn concentration in the supernatant was measured using an atomic absorption spectrophotometer (GBC Scientific Equipment Ltd, Australia).

Next, a quantitative assay of phosphorus solubilisation by a selected isolate from maise rhizosphere was done using a PVK (Pikovskaya) liquid culture medium [19]. About ten days after inoculation, the pH of each culture was recorded, and the samples were centrifuged (10,000 rpm) to remove remnants and cells. Then, the phosphorus content of the culture was measured using vanadium ammonium molybdate. The intensity of the yellow colour was measured at 470 nm using a spectrophotometer (PD-303UV, Apel). The qualitative measurement of auxin production by an efficient Zn solubiliser isolate was determined through the Luria Bertani tryptophan medium [20]. The appearance of a pink colour on Sakolovsky's reagent saturated paper was considered a positive reaction of auxin production. The isolate with the better insoluble Zn dissolution ability was identified by sequencing the 16S rRNA with primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC ACG ACT T-3') [21].

Pot experiment

The effect of isolates with Zn dissolution ability was investigated on Zn fractions in soil, Zn uptake, protein concentration and PA/Zn ratio in wheat grains (*Triticum aestivum*), in Chamran cultivar under greenhouse conditions. The experiment was performed as a factorial in a completely randomised design with three replications. The three pots were provided for each treatments. Experimental factors included bacterial inoculation [without inoculation (B0), inoculation with *Bacillus megaterium* (B1), inoculation with *Enterobacter cloacae* (B2), inoculation with both *Enterobacter cloacae* and *Bacillus megaterium* (B3)] and three levels of $ZnSO_4$ application [without $ZnSO_4$ (Zn0), with 5.1 mg kg^{-1} $ZnSO_4$ (Zn1), and with 10.2 mg kg^{-1} $ZnSO_4$ (Zn2)].

Enterobacter cloacae strain sug_1 with an accession number of KX262849 was selected based on its ability to dissolve Zn in earlier research [18]. *Bacillus megaterium* with an isolate code and accession number of Z12 and MG263616, respectively, isolated from the maise rhizosphere was also selected. Soil samples were collected from a research field of Shahid Chamran University of Ahvaz (0-30 cm of soil). The collected soil was air-dried and passed through a 4-mm sieve. The chemical analysis of the soil was as follows: Electrical conductivity = 3.5 dSm^{-1}, pH = 7.4, organic matter = 0.7%, available P = 7.1 (mg kg^{-1}), available K = 290 (mg kg^{-1}), total nitrogen = 0.07%, total Zn = 32 (mg kg^{-1}), and DTPA extractable Zn = 0.55 (mg kg^{-1}) with a clay loam soil texture.

In order to supply the required nitrogen and phosphorus, fertilisation was carried out based on soil test and fertiliser recommendations for wheat plants, which was used from urea and triple superphosphate sources, respectively [22]. In 5-kg pots, ten seeds were planted at about 2 cm of soil depth, which was thinned to five plants after germination. The overnight culture of isolates was prepared in a nutrient broth at 28°C on an orbital shaker at 160 rpm. The bacterial suspension was centrifuged for 10 min at 10,000 rpm, and a pellet of bacteria was suspended in 0.85% NaCl after washing the same solution [23]. About 1 mL of the bacterial suspension (10^6 Cfu mL^{-1}) was uniformly applied on each seed and then covered uniformly with a 2-cm layer of soil. The 36 pots were kept in a greenhouse within a temperature range of 18 to 27°C and a relative humidity of 45 to 85%, with an average of 12 hours of brightness. The soil moisture content of the pots during the test period was kept at about 80% of soil field capacity.

At the end of the cultivation period (about six months), the plants were collected, and the Zn uptake by the grain was calculated after measuring grain yield and Zn concentration in grain by via the digestion of milled grain samples and reading the absorbance using atomic absorption equipment [24]. The protein content of each grain was calculated after measuring total nitrogen using Kjeldahl [25]. The phytic acid of each grain was measured based on Haug and Lantzsch's [26] method, after which the molar ratio of PA/Zn was calculated [27].

To measure five conceptual fractions of Zn, sequential extraction was done as described by Tessier et al. [28]. These Zn fractions were the exchangeable (EXCH-Zn), bound to carbonates (CAR-Zn), bound to organic matter (ORG-Zn), bound to the iron and manganese oxides (FeMnOX-Zn), and residual Zn (RES-Zn). Total Zn was extracted from soils into 5M nitric acid. Zn concentrations in the soil extracts were measured using an atomic absorption spectrometer. Zn recovery was calculated as the total fraction of Zn divided by the extracted Zn using HNO3 [29]. Soil pH was measured in a 1:1 soil: water suspension using a digital pH meter (Model 691Metrohm AG Herisau, Switzerland). DOC was determined using the method described by Bolan et al. [30].

Data analysis

Variance Analysis (ANOVA) of the data was done using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA) (SAS Institute Inc., 1988). To determine the difference in the mean values, the Duncan's Multiple Range Test was performed at $p<0.05$. The Pearson correlations between the measured properties were determined using SPSS 16.0 software.

Results

Bacterial isolates

From 18 Zn solubilising bacterial colonies (according to the appearance of a clear halo around the colonies on a plate containing ZnO), the Zn12 isolate (which had the highest SI of 2.67) was selected for further analysis. The Zn concentration in a broth medium inoculated with potent isolates of *Enterobacter cloacae* and Zn12 were 118 and 67.65 mg L^{-1}, respectively. A shift in pH with the growth medium was measured after five days of incubation with a reduction from 7.2 (control) to 5.4-5.8, with the lowest pH value being observed in flasks containing *Enterobacter cloacae*. The result of insoluble phosphorus dissolution by isolate Z12 in the liquid culture medium containing tricalcium phosphate indicated its ability to dissolve 75 mgL^{-1} of the phosphate. Subsequently, the pH of the culture medium was reduced from 7 to 4.9 due to the inoculation of the Zn12 isolate.

The qualitative consideration of auxin production by isolate Zn12 indicated a positive response to the auxin production test by changing the colour of Salkowski saturated paper to pink. The Zn solubiliser isolate Zn14 was identified using 16S rDNA primers as *Bacillus megaterium* and was deposited at the NCBI site with an access number of MG263616.

Impact of Zn solubilising bacteria on DTPA-extractable Zn, pH, and DOC in soil

Based on the critical level of Zn deficiency in soil (1 mg kg^{-1}), the tested soil suffered from DTPA-extractable Zn deficiency (so-called available). The effect of *Bacillus megaterium* and *Enterobacter cloacae* strain sugR_1 with higher Zn solubilising ability on Zn fractions in soil has been investigated. The maximum amount of available Zn was obtained in the treatment of *Enterobactercloacae* and *Bacillusmegaterium* co-inoculation, along with 10.2 mg kg^{-1} $ZnSO_4$ application (Zn2B3), which was followed by treatment of *Enterobactercloacae* in combination with 10.2 mg kg^{-1} $ZnSO_4$ (Zn2B2) (Table 1).

The significant effect of treatments on soil pH and DOC (Table 1) indicated that the minimum soil pH and maximum value of DOC were associated with the Zn2B3 treatment, which was followed by the Zn2B2 treatment. For each treatment, the pH in soil with the microbial treatment was lower than that of the soil without bacterial inoculation (Table 2). However, the application of Zn fertiliser decreased the pH; its value in the treatment of 10.2 mg kg^{-1} $ZnSO_4$ application and without bacterial inoculation (Zn2B0) was lower than that of the Zn0B0 treatment. Meanwhile, DOC and DTPA extractable-Zn concentrations were increased by Zn application and microbial inoculation.

Zn fractions in soil

The interaction effect of bacterial inoculation and $ZnSO_4$ application on EXCH-Zn, ORG-Zn, and CAR-Zn was significant ($p<0.01$). Bacterial inoculation increased the amount of EXCH-Zn and ORG-Zn when compared with non-bacterial treatments at all fertiliser levels. The highest increases in EXCH-Zn and ORG-Zn, when compared with the control, belonged to the Zn2B3 treatment (242% and 210%, respectively), followed by the Zn2B2 treatment, 213% and 180%) (Table 2). A negative correlation was found between pH and EXCH-Zn and ORG-Zn in the soil (-0.80^{**} and -0.87^{**}, respectively). Meanwhile, the correlation between DOC and EXCH-Zn and ORG-Zn was positively significant (Table 4).

An increase in the amount of CAR-Zn was observed by increasing the amount of fertiliser application. However, at all levels of fertiliser application, the amount of CAR-Zn decreased due to bacterial inoculation. The highest amount of CAR-Zn was related to the Zn2B0 treatment, with a mean of 9.25 mg kg^{-1} (Table 2). The interaction effect of bacteria and ZnSO4 application was not significant on FeMnOX-Zn, although the separate effects of bacterial inoculation and ZnSO4 application on FeMnOX-Zn were significant (Table 2).

By increasing ZnSO4 application, the FeMnOX-Zn value increased, while the inoculation of soil by bacteria caused a reduction in FeMnOX-Zn when compared with the control. This reduction was higher in the treatment containing both bacteria, followed by each the treatment containing only *Enterobacter cloacae* (B3) and the treatment containing only *Bacillus megaterium* (B2).

Mean comparison effects of treatments on RES-Zn demonstrated an increase in this form of Zn by increasing the amount of ZnSO4, while at all levels of fertilisation, bacterial inoculation reduced the

amount of RES-Zn. This reduction was maximised by soil inoculation with both bacteria, followed by *E. cloacae* inoculation (Table 2). The results of Zn chemical composition analysis showed that the recovery percentage of Zn ranged from 94 to 106.31%, with an average of 100.98%. The relative proportions of Zn in the soil for the Zn0B0 treatment (control) were 40.48, 29.2, 27.1, 2.18, and 0.84% for RES, FeMnOX, CAR, OM, and EXCH-Zn fractions, respectively.

The results demonstrated that the application of ZnSO4 in the Zn2B0 treatment caused a shift in the Zn distribution from the Fe-MnOx-Zn and CAR-Zn fractions to the exchangeable fraction (43.02, 28.47, 24.61, 2.23, and 1.68% for RES, FeMnOX, CAR, OM, and EXCH-Zn fractions, respectively). In contrast, in the Zn0B3 treatments, the co-inoculation of bacteria without ZnSO4 changed the Zn distribution from residual to exchangeable and organic matter fractions (37.24, 29.51, 26.9, 4.3, and 1.75% for RES, FeMnOX, CAR, OM, and EXCH-Zn fractions, respectively). The simultaneous application of ZnSO4 and co-inoculation of bacteria caused a shift from RES, FeMnOX and CAR fractions to OM and EXCH fractions (39.98, 27.5, 24.18, 5.83, and 2.5% for RES, FeMnOX, CAR, OM, and EXCH-Zn fractions, respectively).

Zn uptake, protein, and molar ratio of PA/Zn in wheat grains

The effects of bacterial inoculation and $ZnSO_4$ application in soil on Zn uptake, protein content, grain yield, and PA/Zn ratio in grains were significant. The maximum Zn uptake in grain was observed in the treatment of *Enterobactercloacae* and *Bacillusmegaterium* co-inoculation, along with 10.2 mg kg^{-1} $ZnSO_4$ application (Zn2B3), with an increase of 214% when compared to the control (Table 3). The next highest Zn uptake was associated with treatment of *Enterobactercloacae* inoculation, along with 10.2 mg kg^{-1} $ZnSO_4$ application (Zn2B2).

There was a positive and significant correlation between Zn uptake in grains and EXCH-Zn (0.94^{**}) and ORG-Zn (0.95^{**}) in the soil. However, there was no significant correlation between Zn uptake in grains and FeMnOX-Zn and CAR-Zn (Table 4). The lowest ratios of PA/Zn was related to the Zn2B4, Zn2B3, Zn2B2 and Zn1B4 treatments. These treatments showed reductions of 31.38, 30.86, 28.7 and 27.4%, respectively, in the ratio of PA/Zn when compared to the control (no fertiliser and bacteria). Moreover, no significant differences were observed between these treatments (Table 3).

The maximum protein content was observed in the treatment containing a mix of both bacteria with complete fertiliser application (Zn2B3), followed by the *E. cloacae* treatment with complete fertiliser application (Zn2B2), and then the Zn1B3 treatment. The mentioned treatments increased the protein content by as much as 48, 45, and 43%, respectively, when compared to the control (Zn0B0) treatment (Table 3).

There was a positive and significant correlation between protein concentration in grains and EXCH-Zn (0.88^{**}) and ORG-Zn (0.90^{**}) in the soil. Grain yield enhanced due to treatments, with the most significant enhancement being associated with the Zn2B3 treatment (93% enhancement when compared to the

control (Zn0B0)). The increase of grain yield for the Zn0B3 treatment was almost 46% in comparison to Zn0B0.

Discussion

The production of a halo zone around the bacterial colonies in a plate containing ZnO could be due to proton extrusion, siderophore, or organic acid production by bacteria [31-32]. Quantitative Zn dissolution in flasks containing ZnO indicated the ability of isolates to release Zn, although the dissolution amount varied between the two studied isolates. The decrease in pH of the inoculated medium containing insoluble forms of Zn and phosphorus could confirm the isolates' mechanism to dissolve these mineral forms of elements.

The decrease in pH of liquid media and Zn dissolution by bacteria through different organic acid production methods have been reported previously [16]. Organic acid production by bacteria through insoluble phosphorus dissolution can be the cause of pH reduction [33]. Mumtaz et al. [34] screened 13 Zn solubilising isolates from a maise rhizosphere with maximum Zn dissolutions of 27.15 and 27.66 $\mu g\text{-}mL^{-1}$ for *Bacillus subtilis* and *Bacillus aryabhattai*, respectively. The potential of *Bacillus* sp., *Bacillusaryabhattai* (ZM31), and *Bacillus aryabhattai* (S10) to decrease the pH of a medium, as well as the dissolution of phosphorus from the source of $Ca_3(PO4)_2$, has been recorded by Mumtaz et al. [34].

Impact of Zn solubilising bacteria on Zn in soil

The results indicated a positive effect of bacteria on the increase of available Zn (DTPA-extractable Zn) in soil. Increased soil DTPA-extractable Zn could be attributed to a decrease in soil pH (Table 4). This conclusion is supported by the negative correlation between DTPA-extractable Zn values and soil pH (R^2=-0.80**). Soil microbial inoculation diminished the pH of soil [10, 35] probably by organic acids production. The results of soil pH and DOC measurements confirmed that bacteria could reduce soil pH and increase DOC by producing organic acids and proton secretion. This proton secretion could ultimately increase the amount of available Zn in the soil, as previously reported by Vaid et al. [13]. A negative and significant correlation between DOC and pH (R^2 = -0.95**) indicated the impact of DOC on reducing soil pH (Table 4). The enhancement of soluble organic carbon content due to the organic matter degradation in the soil and organic acid production by bacteria has been reported [36]. Zn solubility reduction has also been documented by sorbing Zn on functional groups of solid organic matter [37], whereas DOC has been found to increase Zn solubility [38]. Iratkar et al. [39] reported more availability of soil Zn with greater organic matter content. Our results indicate that there is a positive correlation between DTPA-extractable Zn values and DOC (Table 4). Based on the findings of this study, applied Zn-solubilizing bacteria increased EXCH-Zn and ORG-Zn and decreased CAR-Zn, FeMnOX-Zn, and RES-Zn. However, these changes varied depending on the bacteria. The increase in the concentration of EXCH-Zn in the treatment of plant growth-stimulating bacteria might be due to the transition of Zn from its less dynamic and unavailable forms to more dynamic and available forms.

In the present study, the reduction of CAR-Zn in the treatment of growth-stimulating bacteria was accompanied by an increase in EXCH-Zn and ORG-Zn when compared with the control. The increase in the solubility of carbonate-bound Zn and the conversion of this form to other forms reduced the concentration of carbonate-bound Zn in plant growth-stimulating bacteria treatment when compared with the control. It is noteworthy that the decrease in the carbonates bound form was accompanied not only by an increase in the exchangeable component but also by an increase in the organically bound form. Therefore, it seems that in addition to pH, the secretion of bacteria caused the release of Zn bounded to calcium carbonate and transformed it into exchangeable and organically bound forms, therefore Zn availability increased [40, 41].This result was confirmed by the positive correlation between DOC and ORG-Zn (R^2= 0.89**). The increased EXCH-Zn due to the influence of pH and DOC in the soil might also be related to the negative and positive relationships between EXCH-Zn values and pH (R^2= -0.80**) and DOC (R^2= 0.87**).

The role of DOC in the enhancement of Zn-exchangeable fraction in calcareous soil has been reported previously [42, 43]. Reductions in the FeMnOX and RES fractions of Zn due to the bacterial inoculation have been accompanied by increases in OM and EXCH fractions of Zn. Huang et al. [41] reported that the inoculation of soil with *Rhizobium fredii* reduced the amount of Zn bound to iron and manganese oxides. Our results agree with the findings of Bharti et al. [44], who indicated that the co-inoculation of AMF (*Glomus mosseae*), *Burkholderia cepacia*, and *Azospirillum brasilense* increased OM and EXCH fractions of Zn and reduced FeMnOX-Zn. The application of Zn fertiliser increased the RES, FeMnOX and CAR fractions of Zn, whereas bacterial inoculation reduced these fractions. These results demonstrate that Zn fertilisers, even soluble salts of Zn (Zn sulfate), dissolve slowly in soil and release Zn, which is then converted into carbonates, oxides, and residual forms [45]. Our data (Table 2) suggest that bacterial inoculation increased the EXCH and OM fractions of Zn under deficient conditions and even Zn fertiliser application. The replenishment of EXCH-Zn by OM-Zn fraction for plant uptake can be concluded by a highly positive correlation between EXCH-Zn and OM-Zn fractions (Table 4).

The relative contents of Zn fractions in the soil for all treatments were in the following order: RES > FeMnOX > CAR > OM>EXCH. For all treatments, up to 37-41% of the Zn was associated with the RES fraction, and 27.5-29.5% and 24-27% resided in the FeMnOX and CAR-Zn fractions, respectively (Figure 1). The residual form of Zn is the most stable fraction in soil, and according to others, the highest and lowest proportions of Zn fractions at all treatments were the RES and EXCH fractions, respectively [44, 46, 47].

Our results also indicate that the application of Zn fertiliser and bacteria inoculation cause a shift in Zn distribution from unavailable forms to the EXCH-Zn fraction (0.84, 1.28, 1.68, 2.41, and 2.5% for Zn0B0, Zn1B0, Zn2B0, Zn1B3, and Zn2B3, respectively) (Figure 1). An increase in EXCH-Zn content was observed when Zn fertiliser treatments [46] and bacterial inoculation treatments [44] were applied.

Zn uptake, protein content, and molar ratio of PA/Zn in wheat grains

The bacterial inoculation increased Zn uptake at all $ZnSO_4$ application levels. The increase in Zn uptake might be related to the different mechanisms such as IAA production [48], root growth improvement, mineralization and transformation of Zn by isolates [49]. A close relationship was observed between EXCH and OM-Zn fractions and Zn uptake in grains, thus confirming that the exchangeable and organically bound fractions were the most significant contributors towards Zn uptake in grain (R2= 0.94** and 0.95**, respectively), followed by RES-Zn (R2= 0.33*). These results agree with the findings of Bharti et al. [44]. The EXCH and OM fractions of Zn mainly influenced the available Zn concentration and its uptake by the plant [50, 47]

Soil inoculation of *Bacillus megaterium* and *Enterobactercloacae* in combination with Zn fertilizer application was more effective than the application of each treatment lonely on Zn uptake in wheat grains. Rehman et al [10] recorded more Zn uptake by wheat using endophytic bacterial *Pseudomonas* sp. MN12 in combination with Zn application.

In our study, soil application of Zn caused to enhance grain yield, possibly due to plant growth improvement, seed establishment, nutrients uptake and Zn involvement in carbohydrate metabolism, IAA production and RNA polymerase expression [10].

Moreover, inoculation of bacteria further improved grain yield of wheat due to better root growth [10, 51, 52], photosynthesis [52, 53] and Zn uptake [51]. More improvement in grain yield, protein content and grain quality was observed by soil inoculation of *Bacillus megaterium* and *Enterobactercloacae* in combination with Zn fertilizer application. The same results have been reported by Co-application of Zn and endophyte *Enterobacter* sp. MN17 on improved nodulation, leghemoglobin, grain yield, bioavailable Zn and grain quality of chickpea [12].

The grain yield was increased by enhancing Zn uptake, which is indicated by the positive and significant correlation between Zn uptake and grain yield (R2= 0.99^{**}, Table 4B). Solubility and availability of Zn present in soil enhanced by inoculation of *Bacillus megaterium* and *Enterobactercloacae* [54].

The increases in grain protein content in treatments of bacterial inoculation and $ZnSO_4$ application are likely due to Zn's role in the expression of RNA polymerase enzymes and synthesis protein [55, 56].

Increasing Zn uptake in grains reduced the molar ratio of PA/Zn, which could be due to the negative and significant correlation between Zn uptake and PA/Zn ratio (R^2= -0.90^{**}).

According to the World Health Organization [57], foodstuffs are categorised into three groups in terms of Zn availability: low availability (molar ratio of PA/Zn > 15), moderate availability (molar ratio of PA/Zn: 5 to 15), and high availability (molar ratio of PA/Zn < 5). Hence, increasing Zn concentration in wheat is not enough to increase its nutritional value since phytate (the phosphorus storage in plants) is a combination that tends to complex iron, Zn, calcium, and magnesium and prevents their absorption in the human body [58].

In the present study, the co-inoculation of *Bacillus megaterium* and *Entrobacter cloacae*, together with $ZnSO_4$ application, decreased the molar ratio of PA/Zn in wheat grains from 17.59 (control) to 12.07 (Zn2B3) and 12.77 (Zn1B3). Based on the classification of food by the World Health Organization [57], treatments with a PA/Zn molar ratio of less than 15 are considered to have a satisfying Zn adsorption. The increase in Zn uptake and the reduction of PA/Zn ratio in wheat grains, have been associated with an increase in the amount of applied $ZnSO_4$ fertiliser [59] and inoculation of soil with Zn solubilising bacteria, as well [10, 11, 60].

Among Zn application levels and bacterial treatments, the highest soil available Zn (extracted using DTPA), Zn uptake and the lowest phytate/Zn molar ratio was recorded due to application of $10.1 mgkg^{-1}$ zinc sulfate (Zn2) and a mixed inoculation of both bacteria (B3) which confirmed the efficiency of biological methods to trigger Zn uptake and improve nutritive status of wheat grain by changing morphology of roots and solubilizing Zn in soil [61].

Conclusion

The present study investigated the effect of plant growth-stimulating bacteria on the distribution of Zn chemical forms in the soil solid phase. This study also examined these bacteria's relationship with Zn uptake and PA/Zn ratio of wheat grains, which is important from a nutritional perspective. Soils treated via bacterial inoculation had higher concentrations of available Zn and lower pH values than soils treated via $ZnSO_4$ application. There were significant differences in the concentrations of Zn chemical fractions when comparing soils with and without microbial inoculation and $ZnSO_4$ application.

Therefore, the combined influence of microbial inoculation with the application of Zn (especially $10.1 mgkg^{-1}$) improved the bio-availability of Zn in soil by changing the soil pH and increasing DOC, which promoted the distribution of Zn in its exchangeable and organically bound forms. A significant correlation between Zn uptake in grains and exchangeable and organically bound forms indicated that these fractions serve as reservoirs of Zn for plants. The combined use of Zn solubilising bacteria and $ZnSO_4$ increased Zn uptake, protein, and grain yield and reduced the molar ratio of PA/Zn in the grain to the extent that this vital element could be absorbed by the human body. However, the impact of microbe-plant interactions and the secretion of various organic compounds by bacteria on micronutrient availability in the rhizosphere is undeniable. Long-term field studies should be carried out to understand their mechanisms and confirm the supplementary role of applied bacteria in this research for chemical fertilisers. Doing so could improve the quality of grain products.

Declarations

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Conflict of Interests

The authors have declared no conflict of interests.

Author contribution statement

All authors have contributed on performing experiment and writing paper.

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Tables

Table 1 Mean comparison of some soil properties under the effect of bacterial inoculation and $ZnSO_4$ application by Duncan test.

Treatment	without inoculation	*Bacillus megaterium*	*Enterobacter cloacae*	Both bacteria	Mean
pH					
without Zn	7.39±0.010^{a}	7.11±0.015^{d}	7.08±0.01^{e}	7.05±0.005^{ef}	7.16±0.140^{A}
5.1 mg kg^{-1}	7.29±0.011^{b}	7.04±0.005^{fg}	7.02±0.005^{gh}	7.01±0.010^{hi}	7.09±0.122^{B}
10.2 mg ka^{-1}	7.25±0.010^{c}	6.98±0.005^{i}	6.95±0.005^{j}	6.92±0.005^{k}	7.02±0.135^{C}
Mean	7.31±0.062^{A}	7.04±0.055^{B}	7.02±0.053^{C}	6.99±0.058^{D}	
DOC (mgL^{-1})					
without Zn	12.38±0.062^{j}	24.69±0.060^{f}	22.49±0.639^{g}	28.91±0.105^{d}	22.19±6.35^{C}
5.1 mg kg^{-1}	15.68±0.105^{i}	25.11±0.120^{f}	27.81±0.035^{e}	32.74±0.020 ^{b}	25.33±6.48^{B}
10.2 mg ka^{-1}	21.89±0.020^{h}	31.55±0.015 ^{c}	32.95±0.050 ^{b}	34.96±0.030 ^{a}	30.34±5.24 ^{A}
Mean	16.65±4.18 ^{C}	27.11±3.33 ^{B}	27.75±4.54 ^{B}	32.20±2.65 ^{A}	
Zn-DTPA ($mgKg^{-1}$)					
without Zn	0.70±0.02^{i}	0.90±0.006^{h}	0.96±0.002^{h}	1.2±.0.000^{g}	0.94±0.17^{C}
5.1 mg kg^{-1}	0.93±0.005^{h}	1.49±0.007^{ef}	1.56±0.01^{de}	1.69±0.023^{cd}	1.42±0.30^{B}
10.2 mg ka^{-1}	1.37±0.005^{f}	1.82±0.089^{bc}	1.95±0.015^{b}	2.19±0.13^{a}	1.83±0.31^{A}
Mean	1.00±0.28^{D}	1.40±0.40^{C}	1.49±0.43^{C}	1.69±0.43^{A}	

Data are means ± standard deviation, n = 3. Numbers followed by same letters in each column and rows are not significantly different according to the Duncan's test at 5% probability level (n = 3). The lowercase and capital letters indicate interaction effects between treatments and main effects of treatments on measured data, respectively. DOC: dissolved organic carbon.

Table 2. Mean comparison of zinc different forms in soil under the effect of bacterial inoculation and $ZnSO_4$ application by Duncan test.

Treatment	without inoculation	*Bacillus megaterium*	*Enterobacter cloacae*	Both bacteria	Mean
EXCH- Zn ($mgKg^{-1}$)					
without Zn	0.26 ± 0.01^{j}	0.40 ± 0.01^{i}	0.44 ± 0.005^{h}	0.49 ± 0.01^{g}	0.39 ± 0.09^{A}
5.1 mg kg^{-1}	0.43 ± 0.005^{h}	0.67 ± 0.005^{e}	0.70 ± 0.01^{e}	0.74 ± 0.01^{d}	0.63 ± 0.125^{A}
10.2 mg ka^{-1}	0.63 ± 0.01^{f}	0.79 ± 0.011^{c}	0.83 ± 0.010^{b}	0.89 ± 0.010^{a}	0.78 ± 0.10^{C}
Mean	0.44 ± 0.160^{D}	0.62 ± 0.176^{C}	0.65 ± 0.170^{B}	0.70 ± 0.175^{A}	
CAR-Zn (mg kg^{-1})					
without Zn	8.30 ± 0.050^{e}	8.01 ± 0.036^{fg}	7.78 ± 0.028^{h}	7.59 ± 0.017^{i}	7.92 ± 0.277^{C}
5.1 mg kg^{-1}	8.35 ± 0.050^{e}	8.14 ± 0.017^{f}	8.03 ± 0.057^{fg}	7.98 ± 0.028^{g}	8.12 ± 0.151^{B}
10.2 mg ka^{-1}	9.25 ± 0.060^{a}	9.06 ± 0.057^{b}	8.89 ± 0.036^{c}	8.62 ± 0.092^{d}	8.95 ± 0.249^{A}
Mean	8.63 ± 0.468^{A}	8.40 ± 0.500^{B}	8.23 ± 0.503^{C}	8.06 ± 0.454^{D}	
ORG-Zn (mg kg^{-1})					
without Zn	0.67 ± 0.025^{h}	0.81 ± 0.028^{g}	0.89 ± 0.005^{f}	1.20 ± 0.020^{e}	0.89 ± 0.20^{C}
5.1 mg kg^{-1}	0.79 ± 0.011^{g}	1.36 ± 0.015^{d}	1.41 ± 0.017^{d}	1.60 ± 0.005^{c}	1.29 ± 0.315^{B}
10.2 mg ka^{-1}	0.84 ± 0.005^{fg}	1.62 ± 0.025^{c}	1.88 ± 0.015^{b}	2.08 ± 0.028^{a}	1.61 ± 0.490^{A}
Mean	0.77 ± 0.076^{D}	1.26 ± 0.350^{C}	1.39 ± 0.420^{C}	1.63 ± 0.380^{A}	
FeMnOX-Zn					
without Zn	9.00 ± 0.088^{ef}	8.73 ± 0.15^{fg}	8.56 ± 0.133^{gh}	8.24 ± 0.137^{h}	8.63 ± 0.307^{C}
5.1 mg kg^{-1}	9.70 ± 0.132^{cd}	9.34 ± 0.163^{de}	9.01 ± 0.173^{ef}	8.52 ± 0.195^{gh}	9.14 ± 0.474^{B}
10.2 mg ka^{-1}	10.70 ± 0.104^{a}	10.42 ± 0.068^{ab}	10.20 ± 0.094^{b}	9.80 ± 0.099^{c}	$10.28\pm.^{349A}$
Mean	9.80 ± 0.746^{A}	9.50 ± 0.748^{B}	9.26 ± 0.744^{C}	8.85 ± 0.733^{D}	

RES-Zn (mg kg^{-1})					
without Zn	12.40±0.035^{g}	11.71±0.070^{h}	11.13±0.121^{i}	10.40±0.121^{j}	11.41±0.770^{C}
20 kg ha^{-1}	14.13±0.056^{d}	13.50±0.160^{e}	12.74±0.098^{f}	11.77±0.171^{h}	13.03±0.92^{B}
40 kg ha^{-1}	16.17±0.030^{a}	15.67±0.071^{b}	15.06±0.078^{c}	14.25±0.10^{d}	15.29±$^{0.750A}$
Mean	14.23±1.63^{A}	13.63±1.71^{B}	12.98±1.71^{C}	12.14±1.69^{D}	

Data are means ± standard deviation, n = 3. Numbers followed by same letters in each column and rows are not significantly different according to the Duncan's test at 5% probability level (n = 3). The lowercase and capital letters indicate interaction effects between treatments and main effects of treatments on measured data, respectively.

EXCH- Zn: exchangeable Zn, CAR-Zn: Carbonate bound Zn, ORG-Zn: organic bound Zn, RES-Zn: residual Zn, FeMnOX-Zn: iron and manganese oxide bound Zn.

Table 3 Mean comparison of some properties of grain under the effect of bacterial inoculation and $ZnSO_4$ application by Duncan test.

Treatment	without inoculation	*Bacillus megaterium*	*Enterobacter cloacae*	Both bacteria	Mean
Zinc uptake in grain (mg pot^{-1})					
without Zn	0.28±0.01^{i}	0.48±0.003^{g}	0.50±0.003^{g}	0.57±0.001^{e}	0.46±0.11^{C}
5.1 mg kg^{-1}	0.46±0.01^{h}	0.58±0.005^{e}	0.63±0.00^{d}	0.71±0.003^{bc}	0.59±0.09^{B}
10.2 mg ka^{-1}	0.53±0.004^{f}	0.72±0.003^{c}	0.78±0.01^{b}	0.88±0.017^{A}	0.72±0.13 ^{A}
Mean	0.42±0.11^{D}	0.59±0.10^{C}	0.63±0.12^{B}	0.72±0.13 ^{A}	
Molar ratio of PA/Zn					
without Zn	17.59±1.224 ^{a}	14.00±0.079 ^{bc}	14.11±0.071 ^{bc}	13.25 ±0.041^{cde}	14.73±01.88 ^{A}
5.1 mg kg^{-1}	15.04±0.456 ^{b}	13.62±0.120 ^{cd}	13.60±0.059 ^{cd}	12.77±0.113 ^{def}	13.75±0.876 ^{B}
10.2 mg ka^{-1}	14.04±0.071 ^{bc}	12.54±0.106 ^{def}	12.16±0.128 ^{ef}	12.07±0.172 ^{f}	12.70±0.835^{C}
Mean	15.56±1.714 ^{A}	13.39±0.659 ^{B}	13.29±0.880 ^{B}	12.70±0.525 ^{C}	
Protein (%)					
without Zn	8.92±0.068 ^{l}	11.19±0.063 ^{h}	10.14±0.057^{j}	11.90±0.060^{e}	10.54±1.175^{C}
5.1 mg kg^{-1}	9.17±0.015 ^{k}	11.38±0.0152 ^{g}	11.69±0.040 ^{f}	12.82±0.020 ^{c}	11.26±1.38^{B}
10.2 mg ka^{-1}	10.95±0.050 ^{i}	12.66±0.015 ^{d}	12.97±0.025 ^{b}	13.22±0.020 ^{a}	12.45±0.929 ^{A}
Mean	9.68±0.959 ^{D}	11.74±0.691 ^{C}	11.60±1.226 ^{B}	12.65±0.588 ^{A}	
		Grain yield (g pot-1)			
without Zn	4.49±0.00^{j}	5.84±0.01^{i}	6.21±0.04^{h}	6.58±0.00^{g}	5.78±0.82^{C}
5.1 mg kg^{-1}	6.00±0.10^{i}	6.93±0.04^{f}	7.26±0.00^{e}	7.65±0.02^{d}	6.96±0.63^{B}

10.2 mg ka^{-1}	6.59 ± 0.05^{g}	7.89 ± 0.01^{c}	8.23 ± 0.12^{b}	8.68 ± 0.03^{a}	7.85 ± 0.81^{A}
Mean	5.69 ± 0.94^{D}	6.89 ± 0.88^{C}	7.23 ± 0.87^{B}	7.64 ± 0.90^{A}	

Data are means ± standard deviation, n = 3. Numbers followed by same letters in each column and rows are not significantly different according to the Duncan's test at 5% probability level (n = 3). Numbers followed by same letters in each column and rows are not significantly different (P<0.05). The lowercase and capital letters indicate interaction effects between treatments and main effects of treatments on measured data, respectively.

Table 4. Pearson correlation coefficients (r) between different fractions of Zn and pH and DOC (A), and between wheat grain quality parameters and different forms of Zn (B). (n=36)

(A)

	EXCH-Zn	CAR-Zn	ORG-Zn	RES-Zn	FeMnOX-Zn	DTPA-Zn	pH	DOC
EXCH- Zn	1							
CAR-Zn	0.47**	1						
ORG-Zn	0.92**	0.25ns	1					
RES-Zn	0.53**	0.96**	0.29ns	1				
FeMnOX-Zn	0.48**	0.95**	0.26ns	0.98**	1			
DTPA-Zn	0.98**	0.45**	0.95**	0.49**	0.45**	1		
pH	-0.80**	0.02ns	-0.87**	-0.01ns	-0.00ns	-0.80**	1	
DOC	0.87**	0.11ns	0.89**	0.11ns	0.09ns	0.86**	-0.95**	1

EXCH- Zn: Exchangeable Zn, CAR-Zn: Carbonate bound Zn, ORG-Zn: Organic bound Zn, RES-Zn: Residual Zn, FeMnOX-Zn: Iron and manganese oxide bound Zn, DOC: Dissolved organic carbon

**, * are significant at 1 % probability level, and ^{ns}, not significant.

(B)

	Zn uptake	Grain yield	Protein	PA/Zn
Zn uptake	1			
Grain yield	0.99**	1		
Protein	0.93**	0.91**	1	
PA/Zn	-0.90**	-0.88**	-0.89**	1
EXCH- Zn	0.94**	0.97**	0.88**	-0.81**
CAR-Zn	0.29ns	0.34*	0.21ns	-017ns
ORG-Zn	0.95**	0.94**	0.90**	0.78**
RES-Zn	0.33*	0.40*	0.20ns	-0.20ns
FeMnOX-Zn	0.31ns	0.37*	0.18ns	-0.20ns
DTPA-Zn	0.96**	0.98**	0.89**	-0.78**

PA/Zn: Molar ratio of phytic acid to zinc, EXCH- Zn: Exchangeable Zn, CAR-Zn: Carbonate bound Zn, ORG-Zn: Organic bound Zn, RES-Zn: Residual Zn, FeMnOX-Zn: Iron and manganese oxide bound Zn,

**, * are significant at 1 % probability level, and ns, not significant.

Figures

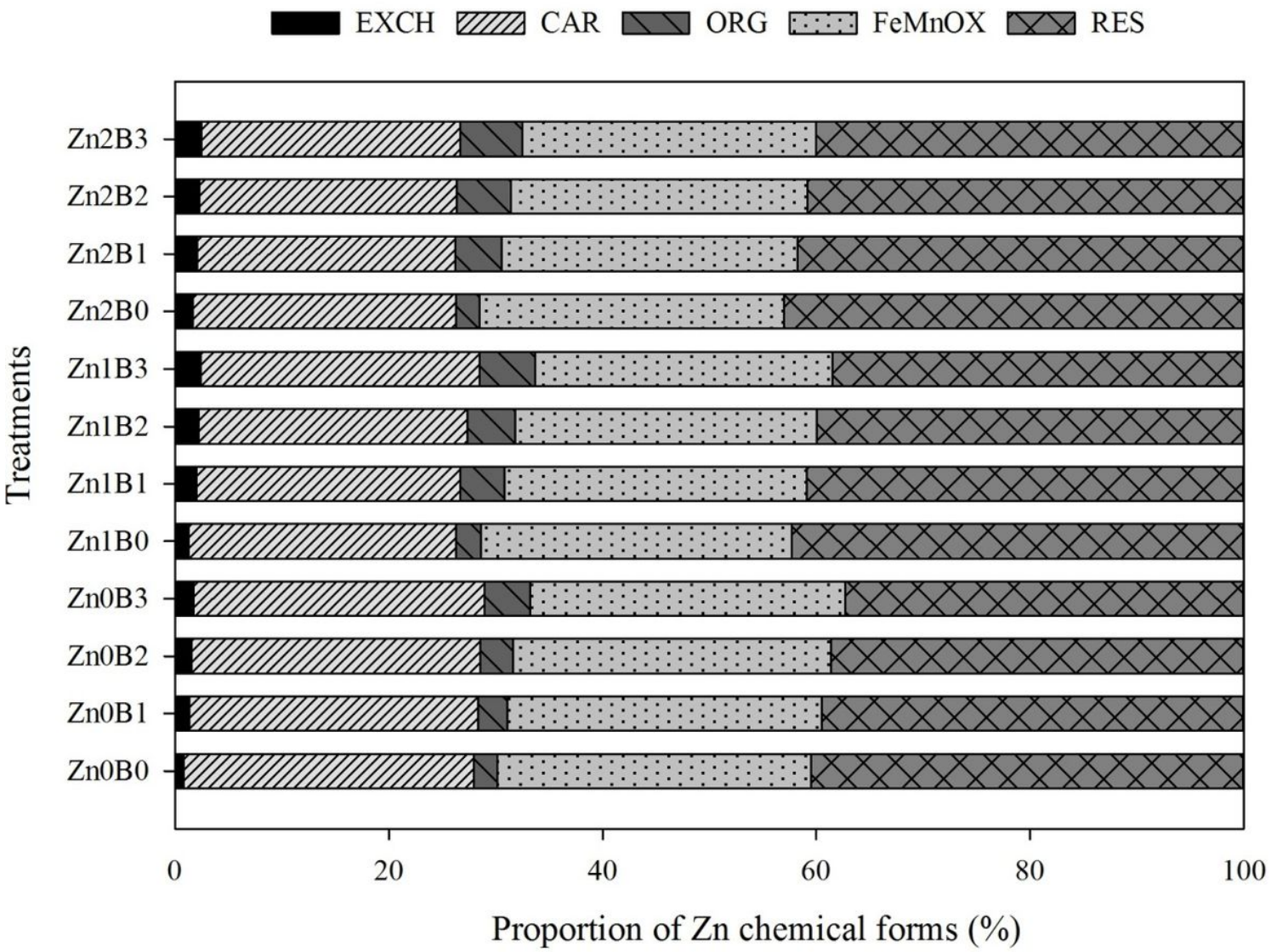

Figure 1

Proportion of Zn chemical fractions in different treatments (EXCH: exchangeable, CAR: bound to carbonates, FeMnOX: bound to iron–manganese oxides, ORG: bound to organic matter, RES: residual). B0 (without inoculation), B1 (Bacillus megaterium), B2 (Enterobacter cloacae), B3 (a mixed inoculation of both bacteria), Zn0 (without ZnSO4 fertiliser application), Zn1 (application of 5.1mgkg-1 ZnSO4), and Zn2 (application of 10.1mgkg-1 ZnSO4).