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Assessment of Zinc Solubilizing Potentiality of *Pseudomonas* and *Bacillus* Species in Agronomic Biofortification of Cassava

Amajor, E.E¹, Nwachukwu, E. ² and Achi, O.K

¹National Root Crops Research Institute, Umudike, Abia State

Corresponding email: essyjohn2006@gmail.com

²Michael Okpara University of Agriculture Umudike, Abia State

ABSTRACT

An experiment was conducted at National Root Crops Research Institute Umudike to assess the Potentials of *Pseudomonas* and *Bacillus* spp in solubilizing zinc micronutrient for cassava biofortification. Zinc-Solubilizing rhizobacteria, produce compounds that enhance plant growth and play a vital role in the cycling of macro and micronutrients. In this study, two isolates were characterized and screened to ascertain ZSR which showed a positive result. Molecular tools were used to confirm *Bacillus* and *Pseudomonas* spp. The screen house experiment had 8 sample bags filled with sand and cassava planted in it with different treatments such as (P, B, Z, P+B, P+Z, B+Z, P+B+Z, C) replicated 3 times under CRD. The isolates were maximized in the laboratory and were inoculated in the soil planted with cassava, the inoculation was done biweekly for 6 months while the application of zinc sulphate was done bi monthly for 12 months.

The soil physical and chemical properties were evaluated on 0 and 6th month after planting. From the result, the pH values ranged from 4.70 to 5.40 on 0 day and 6 month after planting respectively, other soil parameter measured such as ECEC (Effective Cation Exchange Capacity) and others had significant changes from 0 to 6th month after planting. Zinc solubilization zones of different zinc traits (mm) was also carried out and *Pseudomonas* and *Bacillus* sp showed a significant maximum solubilization zone in zinc sulphate ($ZnSO_4$) with a diameter of 0.7mm and 0.5mm respectively when compared to zinc carbonate ($ZnCO_3$) which showed solubilization zone with diameter of 0.25mm and 0.15mm for *pseudomonas* and *bacillus* sp respectively. Zinc concentrations of different treatments in leaves, stems and roots showed that, the treatment combination of *Pseudomonas*, *Bacillus* isolates and zinc (PBZ) gave the highest content of zinc in its traits (1.08, 1.06 and 0.05mg/kg) for the root, stem and leaves respectively. From this study, there was uptake of zinc sulphate by cassava in all the treatment which increased significantly over the control. The ZSR exploitation is a potential factor for achieving the nutritional security by enhancing the yield and uptake of Zinc content of cassava.

Introduction

Cassava (*Manihot esculenta* Crantz) is a crucial staple crop for many tropical and subtropical regions, providing a significant source of calories to millions (FAO, 2013). However, cassava is often deficient in essential micronutrients like zinc (Zn), which is vital for human health. Zinc deficiency is a widespread issue, particularly in developing countries, where it leads to severe health problems (Hotz and Brown, 2004). Biofortification, which aims to increase the nutrient content of crops, has become a promising strategy to combat these deficiencies. Specifically, agronomic biofortification, which involves the use of fertilizers and beneficial microorganisms to enhance nutrient absorption in plants, is being explored as a method to increase zinc content in cassava (Cakmak, 2008).

Zinc plays a critical role in plant physiology, including enzyme activation, protein synthesis, and growth regulation (Broadley *et al.*, 2007). However, zinc in soils often exists in forms that are not easily accessible to plants. Certain soil microorganisms, especially species from the genera *Pseudomonas* and *Bacillus*, have shown the ability to solubilize zinc, converting it into forms that plants can absorb more effectively (Vaid *et al.*, 2014; Kumari *et al.*, 2016). These zinc-solubilizing bacteria (ZSB) can enhance zinc availability in soils, thereby improving zinc uptake by crops (Gontia-Mishra *et al.*, 2017).

This study focuses on evaluating the zinc-solubilizing capabilities of *Pseudomonas* and *Bacillus* species and assessing their potential for enhancing zinc biofortification in cassava. By exploring the interactions between these bacteria and cassava plants, this research aims to identify strategies to improve zinc

content in cassava tubers, contributing to better nutritional outcomes in regions where zinc deficiency is prevalent.

Objectives of the Study

1. To determine the effect of rhizobacteria on availability of zinc in the soil (Soil Physicochemical analysis)
2. To isolate, screen and identify zinc-solubilizing *Pseudomonas* and *Bacillus* species from cassava rhizosphere soils.
3. To evaluate the zinc-solubilizing potentials of these bacterial isolates under laboratory conditions.
4. To investigate the effect of these zinc-solubilizing bacteria on zinc uptake in cassava tissues.

Materials and Methods

1. Soil sample Collection and Location

Soil samples were collected from the rhizosphere of cassava plants in NRCRI agricultural fields.

Soil Physicochemical Analysis

Soil samples were collected from a depth of 30 cm six months after planting. These samples were air-dried, ground, sieved through a 2 mm sieve, and stored in the laboratory for analysis. Particle size distribution was determined using the Bouyoucos hydrometer method (Gee and Or, 2002; Bouyoucos, 1951), and soil texture was classified using the textural triangle (Soil Survey Staff, 2003). Soil pH was measured using a glass electrode pH meter in a 1:2.5 soil-to-water ratio (Thomas, 1996; Maclean, 1982). Organic carbon content was determined using the wet oxidation method described by Nelson and Somers (1996), and organic matter content was calculated by multiplying % organic carbon by a factor of 1.724. Total nitrogen content was analyzed using the micro-Kjeldahl apparatus (Bremner, 1996), and available phosphorus was assessed following the Olsen and Sommers method (1982).

Isolation and screening of Bacterial Strains

Isolation of zinc solubilizing bacteria Serial dilution method was used for isolating the zinc solubilizing bacteria (ZSB) from the rhizosphere soil samples. 1.0 g of cassava rhizosphere samples was suspended in 9.0 ml of saline distilled water in test tubes. From the first dilution 1 ml was transferred to test tube containing 9 ml of sterile distilled water to get 10^2 dilution. The same method was followed for preparing up to 10^8 dilution. The bacterial isolates were streaked on a solidified PVK media for 24 hours under 37°C . Isolates were purified and identified based on morphological, biochemical characteristics and confirmed using molecular tools (Schreiner *et al.*, 2012).

Molecular characterization

Molecular characterization was performed for two of effective ZSB isolates. Pure culture of the selected ZSB isolates were grown in nutrient medium until log phase (2 days) and genomic DNA was isolated according to Sambrook and Russell (2001). The amplification of 16S rRNA gene analysis was done by using universal primers 27F and 1492R as per the protocol (Pandey *et al.*, 2005). The amplified 16S rRNA gene was sequenced at Synergy Scientific Services, Chennai, India. The obtained sequence data of the 16S rRNA gene of AGM3 and AGM9 isolates was compared with sequences in the National Center for Biotechnology Information (NCBI) data bank using the BLAST program (Alschul *et al.*, 1990) aligned using the Clustal W software (Thompson *et al.*, 1997), and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei, 1987). in the MEGA version 7 program.

Screen house Inoculation and bag Experiment

Cassava cuttings were planted in bags containing different treatment. The experimental design is CRD. The treatments were; 1. *Bacillus subtilis* (BS) only

2. *Pseudomonas aerogenosa* (PA) only

3. Zn only

4. *Bacillus*+*Pseudomonas* (Consortium of BS+PA)

5. Zn+ *Pseudomonas Aerogenosa* (Zn +PA)

6. Zn+ *bacillus subtilis* (Zn +BS)

7. Zn +*Pseudomonas Aerogenosa*+ *bacillus subtilis* (Zn +PA+BS)

8. Control (No treatment).

The bags were maintained in a greenhouse under controlled environmental conditions (Li *et al.*, 2018). Soil in the bags were inoculated with maximized cultures of selected isolates before planting and biweekly for eight months, while ZnSO_4 was added monthly for 12 months.

Screening for Zinc Solubilization

The zinc-solubilizing potential of the bacterial isolates was assessed using a plate assay, with zinc oxide (ZnO) as the insoluble zinc source (Saravanan *et al.*, 2004). The solubilization index (SI) was calculated by measuring the clear halo zone surrounding the bacterial colonies on the agar plates (Ahmad and Kibret, 2014).

Assessment of Zinc Uptake and Plant growth (zinc accumulation in cassava tissues)

After 12 months of growth, the cassava crops were harvested, and zinc content in the leaves, stems and tubers was measured using AAS (Alloway, 2008).

Data Analysis

Statistical analysis was performed using ANOVA to compare the effects of different bacterial treatments on zinc uptake and plant growth (Gomez and Gomez, 1984).

RESULT AND DISCUSSION

Soil physicochemical properties

Table 1 shows the physicochemical properties of soil before and after the application of zinc sulphate ($ZnSO_4$) and ZSR (*Pseudomonas* and *Bacillus*) at 0 days and 6 months after planting (6 MAP). The pH values ranged from 4.70 to 5.40 on 0 day and 6 month after planting respectively. The application of ZSR (Zinc solubilizing Rhizobacteria) and zinc sulfate has been shown to significantly improve soil properties. The increase in soil pH is attributed to zinc sulfate, which despite being acidic, can have complex interactions in the soil (Rengel, 2015). ZSR also play a role by producing organic acids or other compounds that can slightly alter soil pH. This increase in soil acidity helps eliminate pathogens, as ZSR can tolerate high acidic conditions (Glick, 2012). Moreover, this often makes nutrients more available to plants. Furthermore, application of zinc and ZSR reduced Exchangeable Acidity (EA) and increased the Effective Cation Exchange Capacity (ECEC) and Base Saturation (BS) by promoting plant growth and enhancing root exudation of organic compounds that can neutralize soil acidity. Some ZSR strains also produce organic acids and other metabolites that can complex with Al^{3+} , reducing its exchangeable form. (Ghosh *et al* 2003).

Biochemical characterization, screening and molecular identification of selected zsr

In this result, “+” stands for positive result “-” stands for negative result (Table2). The cultural, morphological and biochemical characteristics were done as described in the Berge’s manual of systematic Bacteriology (Krieg and Holt, 1984), attempts were made to classify them in to their respective genera. . The genomic DNA of bacterial isolates (The PCR gel electrophoresis) was given in Fig 1. The sequence was analyzed on Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information, USA (<http://www.ncbi.nlm.nih.gov>) to get the trees of the isolates (fig1 and 2). Accordingly, the isolates were found to belong to the genera of *pseudomonas* and *bacillus* species. . According to Ramette *et al.*, (2003), the biosynthesis of hydrogen cyanaide (HCN) in *Bacillus* and *Pseudomonas* sp is typically regulated by the presence of certain substrates and environmental conditions, such as the availability of glycine. These bacteria can produce hydrogen cyanaide (HCN) through the activity of the hydrogen cyanaide synthase enzyme complex. Nitrate reduction involves the conversion of nitrate (NO_3^-) to nitrite (NO_2^-) and other forms of nitrogen. This process is part of the nitrogen cycle and is essential for maintaining nitrogen balance in the soil and improves nitrogen availability for plants. Both *Bacillus* and *Pseudomonas* sp possess nitrate reductase

enzymes that enable them to reduce nitrate to nitrite. This activity is often induced under anaerobic or low-oxygen conditions, which are common in various soil microenvironments. (Philippot and Hallin, 2005).

Zinc solubilization zones of different zinc traits

Zinc solubilization of different zinc traits (Values in mm are the zone diameter on respective media and in $\mu g\ mL^{-1}$ are the respective quantities.) Bars represent standard error and values bearing different letters in the same row are significantly different from each other according to the analysis of variance ($p < 0.05$). Zinc solubilization test was done to ascertain the rate of solubilization rate of *pseudomonas* and *bacillus* sp on different zinc compounds (figure4). This result showed that, both *Pseudomonas* and *Bacillus* sp demonstrated the ability to solubilize various insoluble zinc compounds using enzyme phytase ,organic acid and siderophores and make it possible for uptake from the soil and store in cassava tissues. The findings align with research indicating that *Pseudomonas* species are generally more efficient in zinc solubilization due to their production of organic acids and other solubilizing agents Additionally, *Bacillus* species, though effective, typically produce fewer solubilizing compounds, resulting in lower solubilization rates (Singh and Prasad 2021). Inoculating cassava fields with *Pseudomonas* and *Bacillus* sp as biofertilizers can enhance zinc availability in the soil. This practice leverages the natural solubilization capabilities of these bacteria, promoting sustainable agriculture (Vassilev *et al.*, 2006).

Plant nutritional analysis (zinc accumulation in cassava tissues)

The results (Figure 5) showed that, the treatment combination of *Pseudomonas*, *Bacillus* isolates and zinc (PBZ) gave the highest content of zinc in its traits (1.08, 1.06 and 0.05mg/kg) for the root, stem and leaves respectively. This was followed by treatment of *Bacillus* inoculant and zinc (BZ) (0.58, 0.51 and 0.5mg/kg) for the root, stem and leaves respectively which was not significantly higher than the treatments of *Pseudomonas* and zinc (PZ). The treatment of zinc only (Z) gave a higher content of zinc (0.07mg/kg) in cassava leaves than other traits while there was a significant lower content of zinc in cassava traits observed in the control (C) treatment. However, iron (Fe) was also evaluated and the content of (Fe) iron observed in the cassava traits were significantly lower than that of zinc. The application of zinc in conjunction with these bioinoculants enhances the bioavailability of zinc, facilitating its transport and storage within the plant tissues (Lugtenberg and Kamilova, 2009). The lower iron accumulation compared to zinc might be due to low level and competitive uptake mechanisms or

specific transport pathways favoring zinc over iron in the presence of these microbes (Ghasemi *et al.*, 2014). Overall, the integration of *Bacillus* and *pseudomonas* with zinc fertilization represents a promising strategy to biofortify cassava with essential micronutrients, thereby improving its nutritional quality and potential health benefits. However, this work aligns with the studies done by Graham *et al.*, (2014) in Australia where they investigated the Agronomic Biofortification of Cassava with Zinc and Other Micronutrients to improve human Health.

Conclusion

The *Bacillus* sp and *Pseudomonas* sp are best zinc solubilizers and an ideal candidate for cassava production and biofortification. The enhancement of cassava traits through agronomic fortification with

zinc, *pseudomonas*, and *bacillus* represents a promising strategy to improve crop productivity and nutritional value, ultimately benefiting both farmers and consumers, especially in regions vulnerable to nutrient deficiencies.

This approach is environmentally sustainable compared to traditional zinc fertilization methods. It also offers a cost-effective alternative for farmers, reducing dependency on chemical fertilizers. This biofortification method can play a crucial role in combating micronutrient malnutrition, particularly in developing countries where zinc deficiency is prevalent and also promotes sustainable agricultural practices, improving soil health and reducing the environmental impact of chemical fertilizers.

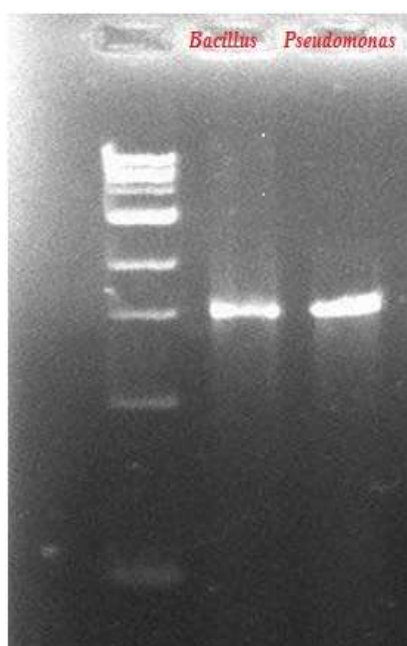
Table 1: Soil physicochemical properties

Samples parameters	0DAY	6MAP
PH	7.70	5.40
Sand%	73.00	73.50
Silt%	14.00	14.00
Clay%	11.50	11.50
Textural Class	SL	SL
Zinc content(ppm)	0.62	2.98
P(%)	3.7	9.86
OC (%)	1.00	2.16
OM (%)	1.04	3.42
N(%)	0.110	2.256
Mg (cmol/kg)	0.21	1.46
Ca (cmol/kg)	2.02	3.54
Na (cmol/kg)	0.11	1.49
K (cmol/kg)	0.112	1.155
EA (cmol/kg)	0.88	0.86
ECEC (cmol/kg)	2.436	5.815
BS(%)	58.64	90.54

Table 2: Determination of morphology, gram, screening and biochemical characteristics of isolates.

Tests	Isolate1	Isolate2
Shape	Rod	Rod
Gram's reaction	—	+
Oxidase	+	+
Motility	+	+
Catalase	+	+
Urease	-	-
Citrate Utilization	-	+
Methyl red	-	+
Spore Formation	-	+
Glucose Fermentation	+	+
Lactose Fermentation	-	-
Sucrose Fermentation	-	-
Maltose Fermentation	-	+
Starch Fermentation	—	+
Mannitol Fermentation	-	+
Gelatin Hydrolysis	+	+
Nitrate Reduction	+	+
Indole acetic acid (IAA)	+	+
Ammonia(NH ₃) production	+	+
Hydrogen cyanide (HCN) production	+	+

Figure 1: A picture of agrose gel electrophoresis (Total genomic DNA of *pseudomonas* and *bacillus* species)



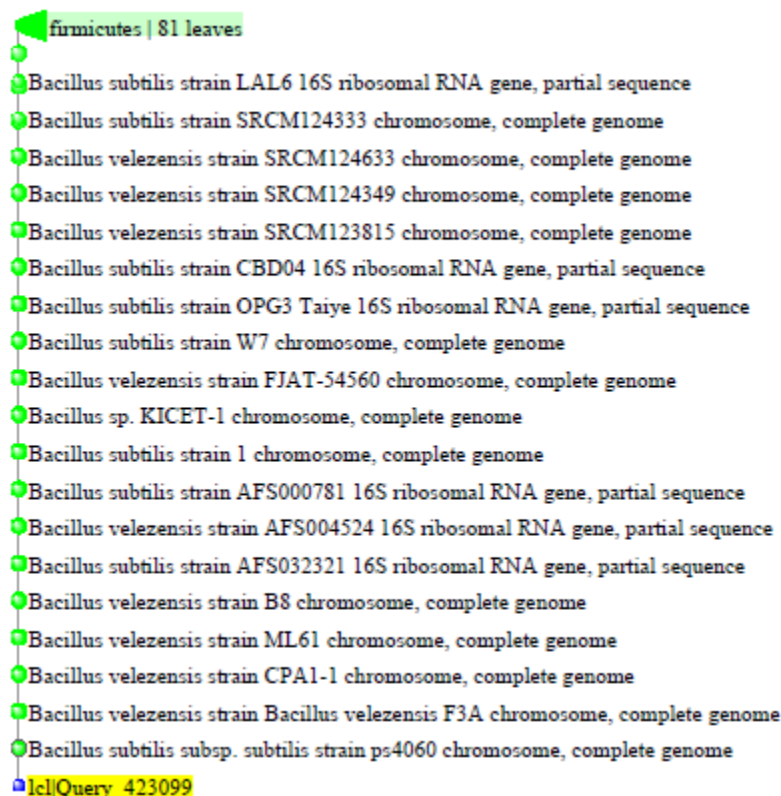


Figure 2: Tree for *Bacillus subtilis*



Figure 3: Tree for *Pseudomonas* species

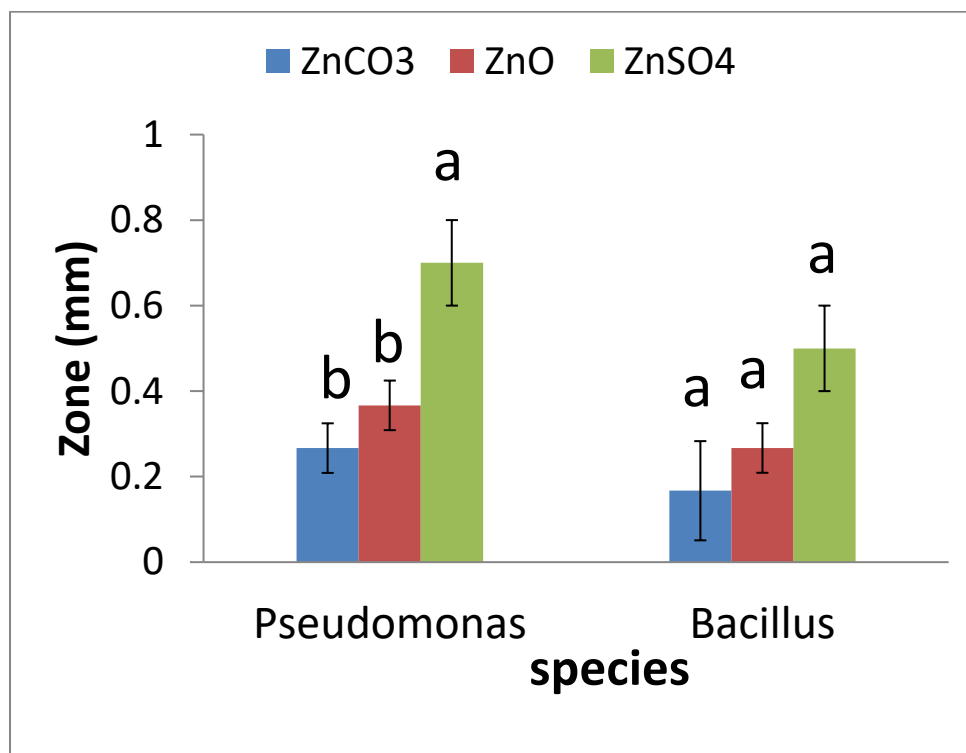


Figure 4: Zinc solubilization of different zinc traits (Values in mm are the zone diameter on respective media and in $\mu\text{g mL}^{-1}$ are the respective quantities.) Bars represent standard error and values bearing different letters in the same row are significantly different from each other according to the analysis of variance ($p < 0.05$).

Assessment of Zinc Uptake and Plant Growth

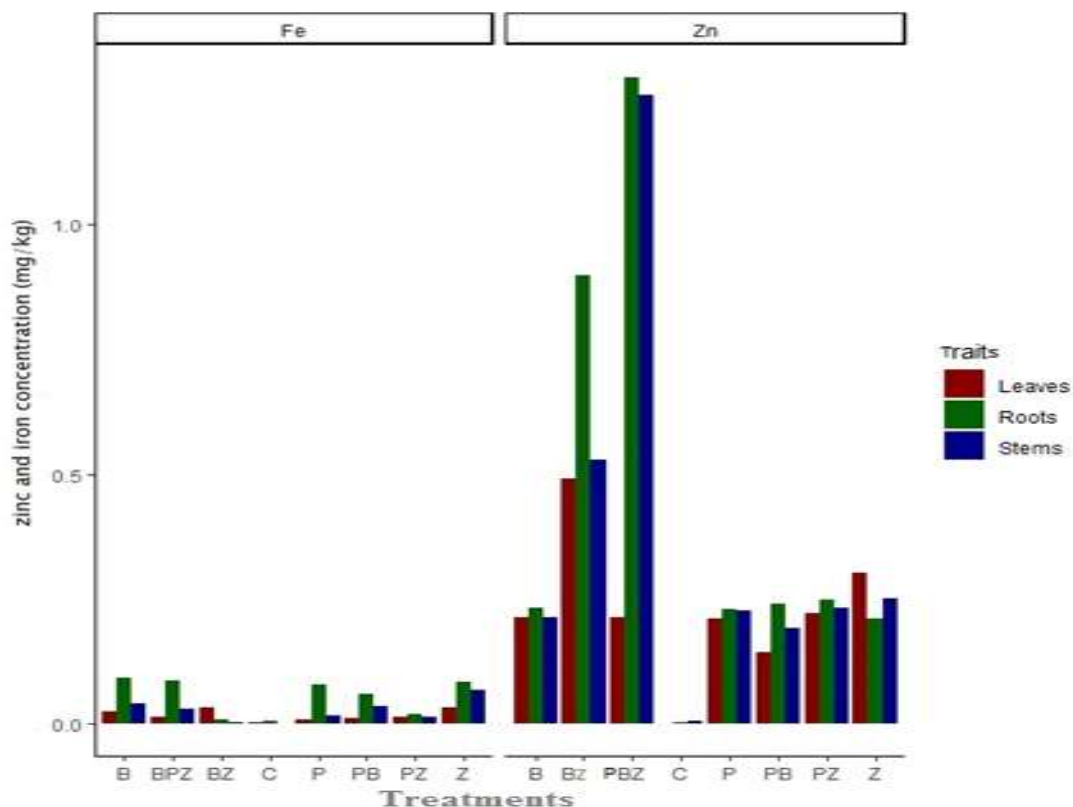


Figure 5: Zinc concentrations of different treatments in leaves, stems and roots (mg/kg) Values are mean of three replicates

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