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Research Article

Effect of excess zinc in soil on *Moringa oleifera* Lam. seedlings emergence

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Abstract

Zinc (Zn) is an essential micronutrient in the plant life cycle, playing catalytic, structural, and regulatory roles in various physiological processes. However, when present in excess, Zn becomes a potentially toxic element (PTE), causing adverse effects ranging from impaired seed germination to inhibited plant growth and development. Understanding plant responses to increasing Zn concentrations in the soil is fundamental for the recovery of degraded areas and for assessing phytoremediation potential. This study aimed to evaluate the effects of different Zn concentrations on the emergence of *Moringa oleifera* Lam. seedlings, comparing the responses to two Zn sources in the soil: zinc chloride ($ZnCl_2$) and zinc sulfate ($ZnSO_4$). The experiment was conducted in a greenhouse under a completely randomized factorial design with four replicates. Treatments consisted of six Zn concentrations: 0 (control), 100, 200, 300, 400, and 500 mg Zn dm^{-3} of soil, for both sources. After a 30-day stabilization period (days after element application, DAE), *M. oleifera* seeds were sown and maintained for 15 days, after which emergence and biometric parameters were evaluated. Data were subjected to analysis of variance, and when significant differences were detected, the Scott-Knott test at a 5% significance level was applied to compare treatments and sources. High Zn concentrations (400 and 500 mg Zn dm^{-3} of soil) negatively affected seedling emergence, regardless of the Zn source. $ZnSO_4$ proved to be the more phytotoxic source, significantly reducing both the emergence percentage and speed, as well as biomass accumulation in the seedlings.

Keywords: emergence speed index; Moringaceae; potentially toxic element; seed germination; soil remediation; Zn sources

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Introduction

Trace elements, also known as heavy metals or potentially toxic elements (PTEs), occur naturally in soils through the weathering of geological materials (Kabata-Pendias, 2010; dos Santos *et al.*, 2022). These elements play essential roles in ecosystem functioning, including nutrient cycling and food production, thereby contributing to a balanced and healthy environment (Cherubin and Schiebelbein, 2022). Among them, zinc (Zn) is a micronutrient required for proper plant development. It functions as an enzymatic cofactor in catalytic, structural, and regulatory pathways, and is involved in the biosynthesis of proteins, carbohydrates, lipids, and nucleic acids (Radić *et al.*, 2010; Beutler *et al.*, 2014). Additionally, Zn activates key enzymes associated with seed germination, such as phosphatases, peptidases, and lipases (Marcos-Filho, 2015; Stanton *et al.*, 2022).

However, the excessive accumulation of Zn in soils-primarily due to anthropogenic activities such as the disposal of sewage sludge (Guimarães *et al.*, 2022), animal waste (Couto *et al.*, 2016), electronic waste (Freitas *et al.*, 2021), mining residues (Souza and de Andrade-Lima, 2022), and the intensive use of agrochemicals (Beygi and Jalali, 2019; Korchagin *et al.*, 2020)-has raised serious environmental concerns. The bioavailability of Zn in soils is influenced by several factors, including pH, organic matter content, and soil texture, all of which affect its mobility and toxic potential (Bradl, 2004; Brunetto *et al.*, 2018). Although crop demands for Zn are typically below 1 kg ha^{-1}, adequate concentrations in soil range from 1 to 10 mg kg^{-1}, while toxicity is observed at levels between 40 and 100 mg kg^{-1} (Broadley *et al.*, 2007). Zinc removal from soil tends to be slow, particularly under alkaline conditions (Bradl, 2004; Casagrande *et al.*, 2008; Brunetto *et al.*, 2018).

In this context, the use of Zn-tolerant plant species-especially fast-growing trees with high biomass production-has emerged as an effective strategy for the revegetation of contaminated sites (Pereira *et al.*, 2013; da Silva *et al.*, 2016; Araújo *et al.*, 2017). Understanding how these species respond to different chemical forms and concentrations of Zn is essential for assessing their potential under stress conditions and for determining safe thresholds that prevent further environmental degradation.

Moringa oleifera Lam., a tree species from the Moringaceae family and native to India, is known for its wide adaptation to diverse soil types with pH values ranging from 5 to 9 (Duke, 1978; Souto, 2017), and for its high biomass production even under varying planting densities (Ferrari-Gualberto *et al.*, 2014). Due to these characteristics, it has been identified as a promising candidate for the recovery of degraded lands (Costa *et al.*, 2021). Nevertheless, information regarding the species' response to excess Zn during its early developmental stages remains limited, and little is known about the specific effects of different Zn compounds, such as chloride and sulfate.

Therefore, the objective of this study was to investigate the effects of increasing Zn concentrations, applied as $ZnCl_2$ and $ZnSO_4$, on the emergence and establishment of *M. oleifera* seedlings. The parameters evaluated included emergence percentage (EP), emergence speed index (ESI), total dry mass (TDM), and seed viability, assessed through the tetrazolium test.

This study was designed to address four key questions concerning the behaviour of *Moringa oleifera* under zinc (Zn) stress: (a) Can *M. oleifera* seeds successfully emerge under Zn concentrations considered toxic, up to 500 mg Zn·dm^{-3}? (b) Are there significant differences in emergence rates and biomass production depending on the Zn source applied-chloride or sulfate? (c) In cases where seedling emergence does not occur, can the tetrazolium test confirm the viability of Zn-exposed seeds? (d) Do the results on seed emergence and initial tolerance under Zn exposure support the establishment of *M. oleifera* in contaminated soils, indicating its potential as a promising species for remediation strategies in Zn-enriched environments?

Materials and Methods

Soil characterization and preparation

The soil used was predominantly sandy, classified as Typic Haplustox (Oxisol) by the USDA Soil Survey Staff classification (2022), it was collected from the surface layer (0-40 cm depth) of the experimental area at the "Fazenda de Ensino, Pesquisa e Extensão" (FEPE), plant production sector, located in Selvíria, Mato Grosso do Sul-Brazil (20° 20' 24.9" S; 51° 24' 19.7" W). The soil was sieved and homogenized, and two composite samples-each composed of ten subsamples-were collected for chemical characterization (van Raij *et al*., 2001) and textural analysis (Teixeira *et al*., 2017) (Table 1).

Table 1. Soil fertility analysis: surface layer 0-40 cm deep of typical dystrophic Red Latosol (Oxisol)

Chemical characterisation of the soil	Values
pH	5.40
P-resin (mg dm^{-3})	6.00
K^+ ($mmol_c$ dm^{-3})	1.00
Ca^{2+} ($mmol_c$ dm^{-3})	7.00
Mg^{2+} ($mmol_c$ dm^{-3})	8.00
S (mg dm^{-3})	7.00
Base Saturation V%	57.00
Sum of bases ($mmol_c$ dm^{-3})	16.00
Potential acidity ($H^+ + Al^3$) ($mmol_c$ dm^{-3})	12.00
Organic matter (g dm^{-3})	13.00
Cation exchange capacity - CTC ($mmol_c$ dm^{-3})	30.10
B (mg dm^{-3})	0.03
Cu^{2+} (mg dm^{-3})*	0.30
Fe^{2+} (mg dm^{-3})*	8.00
Mn^{2+} (mg dm^{-3})*	2.80
Zn^{2+} (mg dm^{-3})*	0.20
Physical characterisation of the soil (g kg)	**Values**
Clay (%)	14.8
Sand (%)	85.0
Silt (%)	0.2
Density (kg dm^{-3})	1.47

*Cu, Fe, Mn and Zn: extraction by DTPA pH 7.0

Artificial soil contamination was performed separately for each Zn source-zinc chloride ($ZnCl_2$) and zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$)-and for each treatment concentration. After dilution in distilled water, the solutions were applied directly to the soil of each experimental unit (3 Ldm^{-3}). The Zn doses added to the soil were calculated based on the molarity, atomic mass, and number of atoms of the contaminant source, using a proportion of 100 mL H_2O per liter of soil in 3.0 dm^3 of soil, to achieve concentrations of 100, 200, 300, 400, and 500 mg Zn dm^{-3} (Table 2). These concentrations were selected in accordance with the criteria and guideline values for agricultural soils established by CONAMA resolution 420/2009 (CETESB, 2021). The soil was manually homogenized and incubated in plastic bags for thirty days to allow element stabilization (days after element application, DAE). During the incubation period, every fifteen days, 100 mL of water per liter of soil was added to each treatment and the soil was re-homogenized to maintain moisture. No pH correction or fertilization was applied to the soil.

Table 2. Concentrations of Zn Chloride and Sulfate used to artificially contaminate the soil in each treatment

Treatments	Repetition	Soil (L-dm^{-3})	H_2O (mL)	$ZnCl_2$ (g)	$ZnSO_4{\cdot}7H_2O$ (g)
T0 - Control	4	3.0	300	0	0
T1 - 100 mg dm^{-3}	4	3.0	300	0.625	1.327
T2 - 200 mg dm^{-3}	4	3.0	300	1.250	2.655
T3 - 300 mg dm^{-3}	4	3.0	300	1.875	3.982
T4 - 400 mg dm^{-3}	4	3.0	300	2.500	5.317
T5 - 500 mg dm^{-3}	4	3.0	300	3.125	6.637
Total	**24**	**18.0**	**1.800**	**8.750**	**19.918**

Each treatment was placed in non-toxic polypropylene germination trays (52 × 26 × 2.4 cm) with a capacity of 200 cells, and one seed was sown per cell. Composite soil samples from the replicates of each treatment (germination trays) were collected both before and after seedling emergence for chemical analyses of soil pH and Zn bioavailability-that is, the fraction of Zn available for uptake by seedlings.

Total Zn content was extracted from 5 g of soil using 25 mL of a double-acid extracting solution (Mehlich 1: 0.05 M HCl + 0.0125 M H_2SO_4), following a soil-to-extractant ratio of 1:5. The mixture was shaken at 40 rpm for five minutes on a horizontal orbital shaker. Zinc concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES), using a Varian ES710A spectrometer tuned to the Zn emission line at 213.857 nm. The quantification limit for Zn in soil was estimated at 1 mg kg^{-1} (da Silva, 2009).

Available sulfur content in the soil (S-SO_4^{2-}) was determined by extraction with a 0.01 mol L^{-1} monocalcium phosphate [$Ca(H_2PO_4)_2$] solution, at a soil-to-extractant ratio of 1:2.5. Ten cm^{-3} soil samples were shaken for 30 minutes with 25 mL of extractant and 0.25 g of sulfur-free activated charcoal. After filtration, 10 mL of the extract were transferred to plastic flasks, to which 1 mL of an acidic sulfur stock solution (20 mg L^{-1} S in HCl) and 0.5 g of $BaCl_2{\cdot}2H_2O$ (60-mesh) were added. Absorbance was measured between 2 and 8 minutes after complete dissolution of the crystals, using a spectrophotometer set to 420 nm. A standard curve was constructed with 0 to 20 mg L^{-1} sulfur solutions, prepared by successive dilutions in 0.01 mol L^{-1} $Ca(H_2PO_4)_2$, and used to calculate the S-SO_4^{2-} concentration in the soil (van Raij *et al.*, 2001).

The results of Zn (mg kg^{-1}) bioavailability were used to estimate the concentration (mg dm^{-3}) of the element available in each treatment, corresponding to 3 L·dm^{-3} of soil (CET). The calculation was performed according to Equation 1, where CT_{soil} represents the Zn concentration in the soil (mg kg^{-1}), V_{soil} is the volume of soil used (dm^{-3}), and D_{soil} is the soil bulk density (1.47 kg dm^{-3}):

$$CET: (CTsoilxVsoil)xDsoil \quad (1)$$

To estimate the bioavailable sulfur content (S-SO_4^{2-}, mg dm^{-3}) in the soil, the same calculation method (CET) was employed, adjusted only for the volume of soil used.

Seed acquisition and analysis

Moringa oleifera seeds were commercially obtained from Arbocenter Comércio de Sementes LTDA, located in Birigui, SP, Brazil. The seeds belonged to batch number 00016-22 and matrix number 10, had a germination capacity of 70%, were stored at temperatures between 14 and 17 °C, originated from the 2022 harvest, and had an expiration date of December 2023.

To standardize the viability analysis, a completely randomized design was implemented using a 0.075% tetrazolium solution (França-Neto *et al.*, 2018). Two tests with different exposure times were performed, totaling 100 seeds per test distributed in four replicates of 25 seeds each. Seeds were randomly taken from the

"pure seed" portion or a representative control sample. The 2,3,5-triphenyl tetrazolium chloride salt solution was prepared at a concentration of 0.75 g L^{-1}, with a pH between 6.5 and 7.5 (Brazil, 2009).

Seeds were preconditioned between papers moistened with deionized water (2.5 times the seed mass) for 18 hours at 25 °C. Afterwards, the seed coats were removed, and a longitudinal cut was made in the distal part of the cotyledon, avoiding the embryonic axis. The seeds were then immersed in the tetrazolium solution and incubated at 30 °C. The tests varied in preconditioning times (3 and 5 hours) and immersion times (6 and 8 hours). After staining, seeds were washed, and three seeds per replicate were evaluated by photomicroscopy using a LEICA EZ4 W stereomicroscope at 8× magnification.

Experimental design and conduction

The experiment was conducted between February and March 2023 in a greenhouse equipped with automatic irrigation (suspended micro-sprinklers), under natural light conditions. The mean temperature was 26.0 ± 1.4 °C, with a maximum of 32.8 ± 2.3 °C, a minimum of 21.6 ± 0.9 °C, and an average relative humidity of 87.1 ± 5.6%. The study took place at UNESP/Campus Ilha Solteira, São Paulo, Brazil (20° 25' 58" S, 51° 20' 33" W).

The experimental design was completely randomized in a factorial scheme, comprising six $ZnCl_2$ treatments (control, 100, 200, 300, 400, and 500 mg Zn dm^{-3} of soil) and six $ZnSO_4$ treatments (control, 100, 200, 300, 400, and 500 mg Zn dm^{-3} of soil), each with four replicates of 50 cells. Two factors were evaluated: Factor 1 - Zn sources; Factor 2 - Zn doses, resulting in a total of 48 experimental units (2 × 6 × 4) (Figure 1).

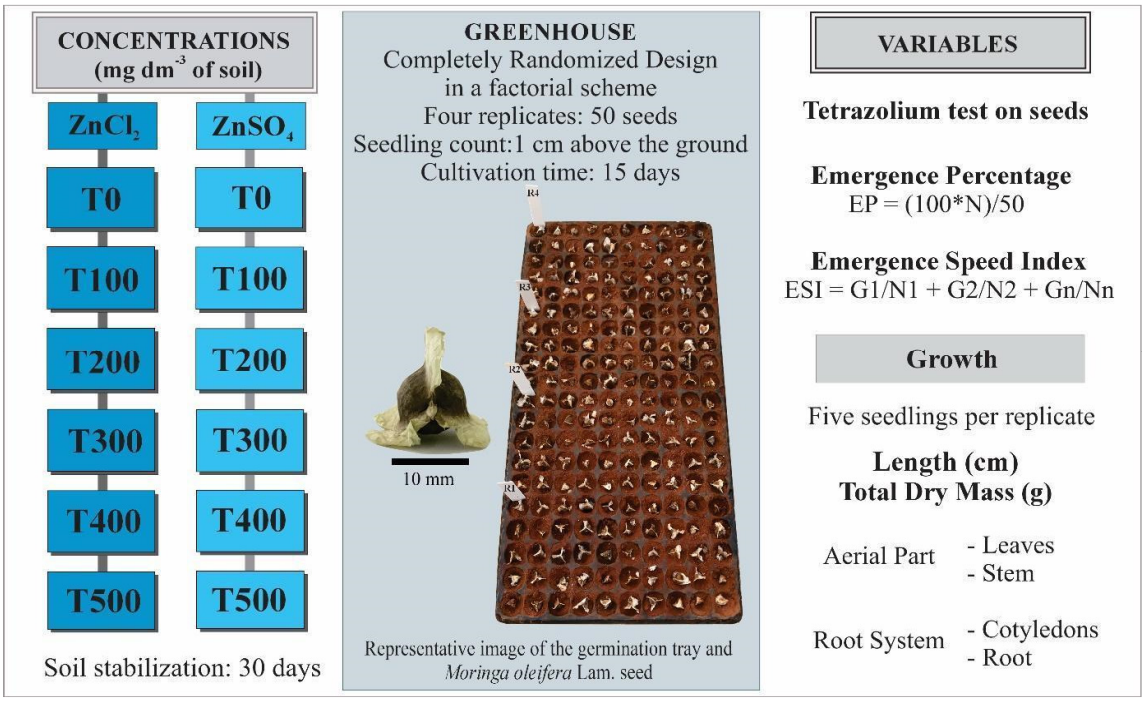

Figure 1. Schematic of the factorial experimental design and types of evaluations: emergence and growth parameters of *M. oleifera* Lam. seedlings

Emergence assessment and plant material collection

The emergence speed index (ESI) is part of a class of vigor tests based on seedling performance, including the first germination count (Brazil, 2009), germination speed, growth, and dry mass accumulation (de Lima and Marcos-Filho, 2011).

The first germination count is used to calculate the emergence percentage (EP), where *N* is the total number of emerged seedlings during the test, and 50 is the total number of seeds per replicate, according to the following equation:

$$EP = \frac{(100xN)}{50} \quad (2)$$

Normal seedlings were considered those without lesions, with the first eophylls fully expanded and the epicotyl exposed at least 1 cm above the soil surface (Cavalcante *et al*., 2018). The ESI is similar to the germination speed index (GSI), as both follow the same principle of evaluating vigorous seedlings by summing the average emergence per day. The counting frequency (daily; first and last count on the 7th and 15th days after sowing) was based on the GSI equation (Maguire, 1962), where *G* is the number of seedlings emerged per count and *N* is the number of days after sowing, as follows:

$$GSI = \frac{(G1)}{N1} + \frac{(G2)}{N2} + \frac{(Gn)}{Nn} \quad (3)$$

After the emergence period, five seedlings per replicate were randomly collected for growth measurements (shoot and root length) (Benincasa, 1988). The tissues were then dried at 60 °C for 72 hours (Malavolta, 1997), and the total dry mass (TDM) of the shoot and root system was determined.

The tolerance index (TI) was calculated for each treatment and source (Rahman *et al*., 2013; Jia *et al*., 2017), as part of assessing phytoremediation potential. It was determined by the ratio of the total biomass of each treatment to the control, as shown in the following equation:

$$TI = \frac{TDM(Treatment)}{TDM(Control)} \quad (4)$$

Statistical analysis

All comparative analyses of emergence, growth, and tolerance parameters between treatments and Zn sources were conducted using the R software version 4.4.1 (2024-06-14) - "Race for Your Life" (R Core Team, 2024), within the Integrated Development Environment (IDE) RStudio (Posit Team RStudio, 2023). The analyses utilized the packages ExpDes.pt (Ferreira *et al*., 2021) and ggplot2 (Wickham *et al*., 2025). The normality of data was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated by Bartlett's test. When the ANOVA F-test indicated significant differences among treatments ($p < 0.05$), means were compared using the Scott-Knott test at a 5% significance level.

Results

Tetrazolium test evaluation

The ideal seed coloration test demonstrated greater efficiency with an 8-hour preconditioning period followed by a 6-hour immersion in the tetrazolium solution. This protocol resulted in a low diffusion pattern of tetrazolium staining on the inner seed surface and showed no visible damage in any of the evaluated seeds (Figure 2BI).

The reddish coloration observed on the inner cotyledonary tissue is attributed to the longitudinal cut near the vascular region of the embryonic axis. Since this region remained unstained, it indicates that the seed was unaffected and therefore viable. Consequently, all seeds exhibited high germination viability (Figure 2BII).

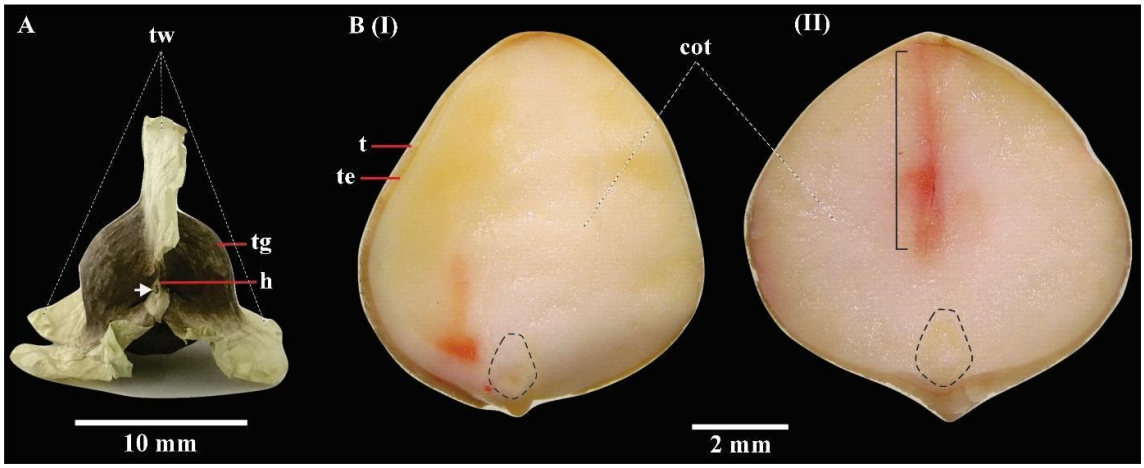

Figure 2. *M. oleifera* Lam. seed morphology
(A) Morphological characterization of the external surface of the *M. oleifera* seed. h: hilum. tg: tegument. tw: three wings. (B) I, II: Random representative image of the inner side of the seed after soaking and staining with tetrazolium solution. t: testa. te: tegmen. bracket [: longitudinal section. dotted highlight: embryo. cot: pair of cotyledons

Seedling emergence and growth parameters

The emergence of *M. oleifera* seedlings started at 7th days after sowing (DAS) in both T0 and T200 treatments of $ZnCl_2$ and $ZnSO_4$ sources (Figure 3A-B).

In the $ZnCl_2$ source, emergence occurred on the 8th day at T100 and T300. Conversely, for the $ZnSO_4$ source, emergence in T100 and T300 occurred on the 9th and 8th day, respectively (Figure 3B). However, in T400 and T500, the few emerged seeds were not evaluated as normal seedlings for counting; therefore, we considered that emergence did not occur for any of these high-concentration treatments (Figure 3A, C).

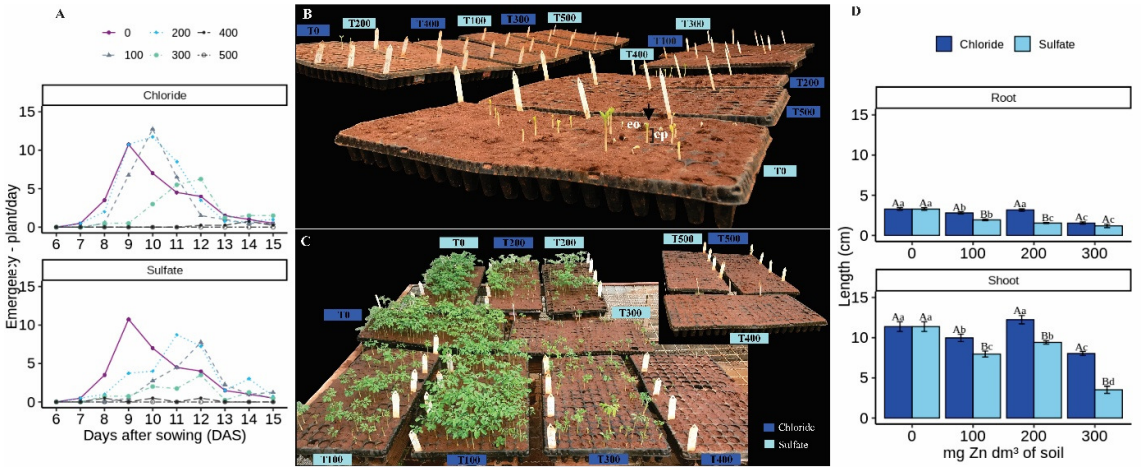

Figure 3. Emergence test of *M. oleifera* Lam. seeds in soil contaminated with Zn Chloride and Zn Sulfate
(A) Daily relation of the emergence of *M. oleifera* seedlings during the period of 15th DAS. (B) Start of seed emergence - 7th DAS. Arrow: seedling emergence considered normal for the daily count. eo: eophyll. ep: epicotyl. (C) 10th DAS. (D) Root and aerial part length (leaves and stem). Different uppercase letters indicate significant differences between sources, while different lowercase letters indicate significant differences between treatments ($p < 0.05$)

For $ZnCl_2$, the highest number of emerged seedlings (in relation to T0 and T100 to T300) was concentrated between 8th and 11th DAS. Specifically, on the 10th DAS, 51 seedlings emerged in T100 across the four replicates. This contrasts with $ZnSO_4$, where for these same treatments, the highest number of

emergences was concentrated between 9th and 12th DAS, with the maximum index in T200, showing 35 emerged seedlings on the 11th DAS (Figure 3A).

The total number of seedlings that emerged over the 15th days for each $ZnCl_2$ treatment was 120, 158, and 80 for T100, T200, and T300, respectively. For the $ZnSO_4$ source, 80, 122, and 43 seedlings emerged for the corresponding treatments. The control for both sources yielded 133 seedlings (Figure 3A).

For EP, significant differences were observed between treatments and contaminant sources of Zn. In the $ZnCl_2$ source, EP decreased only at T300. However, in the $ZnSO_4$ source, it decreased at both T100 and T300. When comparing the sources within each treatment, only the $ZnSO_4$ source showed a decrease in EP at T100 and T300 (Figure 4A).

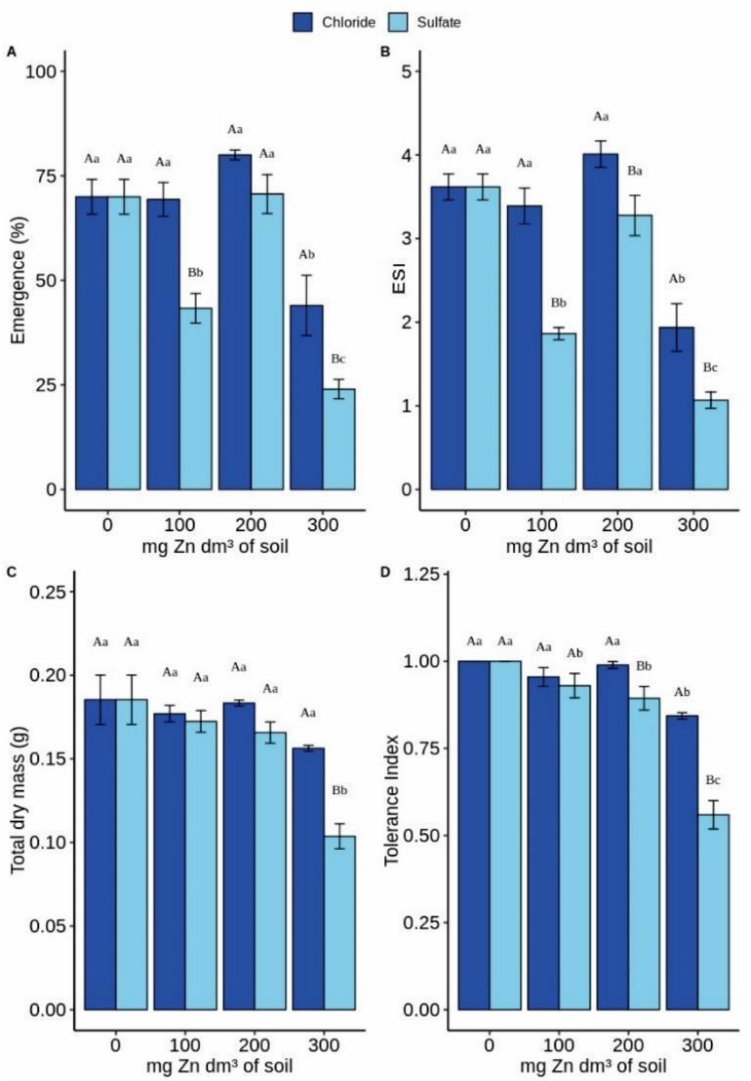

Figure 4. Emergence indices of *M. oleifera* Lam. Mean + standard error for EP (A), ESI (B), TDM (C) and TI (D) of *M. oleifera* seedlings in soil contaminated with Zn Chloride and Sulfate sources
Different uppercase letters indicate significant differences between sources, while different lowercase letters indicate significant differences between treatments ($p < 0.05$)

The same trend was observed for ESI. However, when comparing the sources within treatments, a decrease in ESI was noted from the T100 of the $ZnSO_4$ source onwards, demonstrating that this contaminant affects emergence more than chloride (Figure 4B).

Regarding TDM, the $ZnCl_2$ source did not show any significant difference between treatments. Conversely, the $ZnSO_4$ source exhibited a decrease at T300. When comparing the sources within each treatment, T300 showed a lower accumulation of biomass in the $ZnSO_4$ contamination (Figure 4C). This finding was also observed in the dry mass distribution among tissue organs. Although there were no significant differences in the $ZnCl_2$ source, the T300 treatments of both sources displayed greater mass in the cotyledons (Figure 4C).

M. oleifera exhibited a high TI in the $ZnCl_2$ source, with values close to 1 at T200. In contrast, for $ZnSO_4$, the TI decreased as the treatment concentrations increased, with the lowest index observed at T300 (Figure 4D). This demonstrates that TI is proportional to the seedling dry mass distribution (Figure 5C).

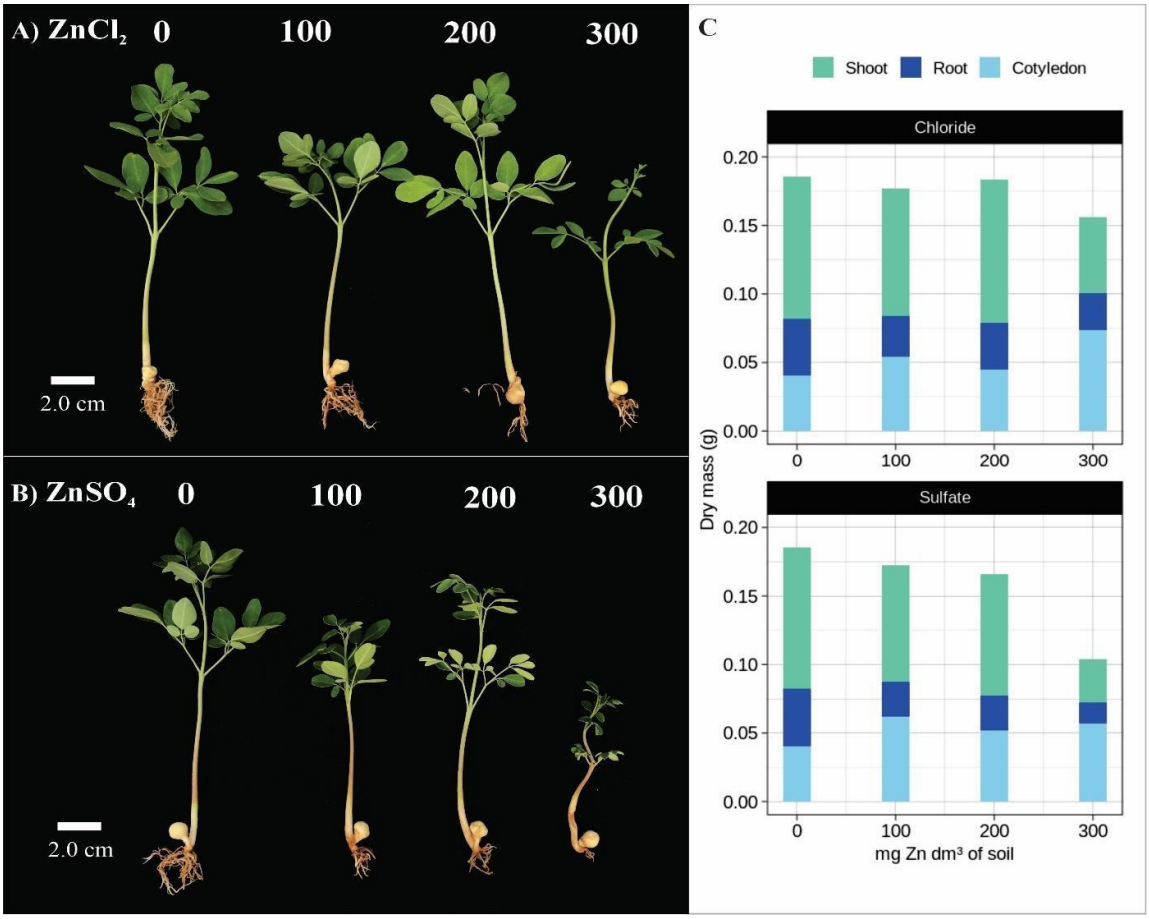

Figure 5. Collection of *M. oleifera* Lam. seedlings and biomass partitioning
Seedlings after 15th DAS in soil contaminated with Zn Chloride (A) and Zn Sulfate (B) sources. (C) Qualitative graph for the distribution of the dry mass of the aerial part (leaves and stem), root, and cotyledons

Regarding root length (Figure 3D), in the $ZnCl_2$ source, T0 and T200 they presented greater lengths when compared to T100 and T300, the latter treatment being the shortest length when compared to each other (Figure 5A), while in the $ZnSO_4$ source, from T100 onwards, all lengths were smaller when compared to T0 (Figure 5B). When comparing the source within each treatment, there was a significant difference in T100 and T200, with greater root length in the $ZnCl_2$ source, while in T0 and T300 there were no significant

differences (Figure 3D). For the shoot length (Figure 3D), the $ZnCl_2$ source showed the same behaviour as the roots, with T300 having the shortest length when compared between treatments (Figure 5A), while in the $ZnSO_4$ source, all treatments showed less growth when compared to T0, with a greater reduction in length at T300, T100, and then at T200 (Figure 5B). When comparing the source within each treatment, from T100 onwards, $ZnSO_4$ negatively affects the species shoot growth (Figure 3D).

Chemical characterization of soil after treatment application

The bioavailability of Zn, S ($S\text{-}SO_4^{2-}$), and soil pH before (initial) sowing and after (final) the emergence of *Moringa oleifera* seedlings in treatments with both $ZnCl_2$ and $ZnSO_4$ sources are presented in Table 3. For treatments where emergence occurred (from T0 to T300) in the $ZnCl_2$ source, the Zn concentrations after cultivation were 4.70, 67.40, 267.00, and 452.00 mg Zn kg^{-1}, respectively. The calculated estimated total (CET) of bioavailable Zn (mg) in the soil for these treatments was 14.81, 212.31, 841.05, and 1423.80 mg Zn dm^{-3}. The difference between initial and final CET bioavailability for T100 to T300 was 140.49, 418.95, and 1786.00 mg Zn dm^{-3}, corresponding to percentage reductions of 39.82%, 33.25%, and 55.64%, respectively.

Table 3. Results of the soil chemical analysis, obtained from composite samples of each treatment, before and after the cultivation period of the emergence test

Treatments	Chemical Characterization			Treatments	Chemical Characterization				
		Zn^{2+}				Zn^{2+}		S	
$ZnCl_2$ (mg dm^{-3})	pH	Mehlich (mg kg^{-1})	CET (mg dm^{-3} in 3 L·dm^{-3})	$ZnSO_4$ (mg dm^{-3})	pH	Mehlich (mg kg^{-1})	CET (mg dm^{-3} in 3 L·dm^{-3})	Mehlich (mg dm^{-3})	CET (3 L·dm^{-3})
Contaminated soil before cultivation									
T0 Control	5.9	3.40	10.71	**T0 Control**	5.9	3.40	10.71	7.00	21.00
T100	5.6	112.00	352.80	**T100**	5.7	96.10	302.72	55.00	165.00
T200	5.7	400.00	1260.00	**T200**	5.7	369.00	1162.35	228.00	684.00
T300	5.6	1019.00	3209.85	**T300**	5.6	924.29	2911.53	484.00	1452.00
T400	5.5	1524.00	4800.60	**T400**	5.5	1535.00	4835.25	949.00	2847.00
T500	5.5	1636.00	5153.40	**T500**	5.4	1602.00	5046.30	1406.00	4218.00
Contaminated soil after cultivation									
T100	6.4	67.40	212.31	**T100**	6.5	79.80	251.37	15.00	45.00
T200	6.4	267.00	841.05	**T200**	6.3	243.00	765.45	16.00	48.00
T300	6.4	452.00	1423.80	**T300**	6.2	494.00	1556.10	24.00	72.00

$Zn^{2+}_{(Mehlich)}$: bioavailable Zn content in the soil for the environment (seedling) in mg Kg^{-1}; $Zn^{2+}_{(CET)}$: bioavailable Zn content (mg) in the soil for the seedling in 3 L·dm^{-3}; $S_{(Mehlich)}$: bioavailable S content in the soil for the environment (seedling) in mg dm^{-3}; $S_{(CET)}$: bioavailable S content (mg) in the soil for the seedling in 3 L·dm^{-3}

In the $ZnSO_4$ source, bioavailable Zn concentrations in the soil after cultivation at T0 to T300 were 4.70, 79.80, 243.00, and 494.00 mg Zn kg^{-1}, respectively. The CET of bioavailable Zn (mg) in the soil for these treatments was 14.81, 251.37, 765.45, and 1556.10 mg Zn dm^{-3}. The difference between initial and final CET bioavailability for T100 to T300 was 51.35, 396.90, and 1354.50 mg Zn dm^{-3}, representing percentage reductions of 16.96%, 34.15%, and 46.55%, respectively.

For bioavailable S in the soil after cultivation, concentrations at T100 to T300 were 15.00, 16.00, and 24.00 mg dm^{-3}, respectively. The CET of bioavailable S (mg) in the soil for these treatments was 45.00, 48.00, and 72.00 mg dm^{-3}. The difference between initial and final bioavailable CET for T100 to T300 was 120.00, 636.00, and 1380.00 mg S dm^{-3}, with percentage reductions of 72.73%, 92.98%, and 95.04%, respectively.

Soil solution pH for both $ZnCl_2$ and $ZnSO_4$ sources showed moderate changes relative to the T0 soil across treatments before and after cultivation, with an average pH of 5.9 ± 0.4. Notably, sulfur did not alter soil pH.

These results clearly indicate that Zn bioavailability decreased after seedling emergence for both contaminant sources. Furthermore, the percentage reduction in CET of bioavailable Zn was lower in the

$ZnSO_4$ treatments compared to $ZnCl_2$ at T100 and T300. A similar trend was observed for CET of bioavailable sulfur, with significant percentage reductions in the same treatments following seedling emergence.

Discussion

Germination represents a critical phase in the plant life cycle, particularly under environmental stress conditions such as heavy metal contamination (Stanton *et al.*, 2022). The effects of zinc (Zn), in different chemical forms and soil concentrations, on the emergence and establishment of *Moringa oleifera* Lam. seedlings revealed both the element's potential toxicity and the species' capacity to acclimate to abiotic stress. This highlights the germination rate as a valuable indicator to assess seedling performance probability (Maguire, 1962) in various contexts, including environmental pollution.

M. oleifera seeds exhibited emergence up to a concentration of 300 mg Zn dm^{-3} of soil for both Zn sources ($ZnCl_2$ and $ZnSO_4$). Above this concentration, emergence was completely inhibited, possibly due to reduced mobilization of nutritional reserves during germination, caused by decreased activity of hydrolytic enzymes regulated by Zn (Marcos-Filho, 2015; Stanton *et al.*, 2022). Additionally, excess trace elements can increase the osmotic potential of the solution surrounding the seed, impeding water uptake and consequently compromising germination processes (Kranner and Couvillion, 2011).

Zn tolerance in tree species during early growth stages varies widely. For example, *Khaya senegalensis* (Desv.) A.Juss. tolerates up to 20 mg Zn dm^{-3} of soil from germination (Araújo *et al.*, 2017); *Corymbia citriodora* (Hook.) K.D.Hill & L.A.S. Johnson resists concentrations up to 1,500 mg Zn kg^{-1} of soil (da Silva *et al.*, 2016); and *Enterolobium contortisiliquum* (Vell.) Morong., a native Brazilian species, tolerates up to 600 mg Zn kg^{-1} (da Silva *et al.*, 2018). These differences highlight the importance of investigating species-specific tolerance, such as that of *M. oleifera*.

The reduction in *M. oleifera* seedling emergence speed compromised biomass accumulation, particularly at 300 mg Zn dm^{-3} from the $ZnSO_4$ source, indicating limitations in root and shoot growth that resulted in lower total dry mass. Considering that adequate Zn levels in seeds range between 25 and 100 mg Zn kg^{-1} of dry mass (Stanton *et al.*, 2022), these results reinforce the species' sensitivity to high Zn doses. Besides the toxic effects attributed to excess Zn, accumulation of sulfur originating from $ZnSO_4$ ($S\text{-}SO_4^{2-}$) may have intensified physiological stress in seedlings, contributing to additional secondary phytotoxic effects. High doses of sulfur, especially in the form of sulfate, negatively affect germination, growth, and grain yield (Beutler *et al.*, 2014). This hypothesis is further supported by the observed reduction in soil sulfur content after cultivation between T100 and T300, suggesting active element uptake and consequent limitations to seedling development.

Toxicity caused by sulfur accumulation can compromise essential physiological processes during the early developmental stages. This mainly occurs due to increased ionic competition in the soil, which hinders the absorption of other essential nutrients (Viégas *et al.*, 2004). Such nutritional imbalances can specifically induce iron deficiency, leading to symptoms such as leaf chlorosis (Missio *et al.*, 2004). In soil, the transport of $S\text{-}SO_4^{2-}$ to the roots occurs through mass flow and diffusion, mechanisms dependent on the water potential gradient generated during water uptake by plants (Barber, 1984; Silva *et al*., 2002).

In this context, the superior performance observed in *Oryza sativa* L. seeds treated with $ZnCl_2$-manifested by faster germination and greater biomass accumulation compared to $ZnSO_4$ treatments-supports the hypothesis that the absence of sulfur in the $ZnCl_2$ formulation avoids the deleterious effects of excess sulfate (Beutler *et al.*, 2014).

The differential biomass allocation among shoots, roots, and cotyledons in *M. oleifera*, under varying chemical forms and concentrations of soil Zn, suggests the activation of adaptive mechanisms against metal stress (Fig. 5C). Under high heavy metal concentrations, plants commonly allocate more resources to root

systems as a strategy to limit contaminant translocation to shoots (Bomfim *et al.*, 2021), thereby optimizing water and nutrient uptake (Kranner and Couvillion, 2011). This pattern is particularly evident in phytostabilizing species (Hunt *et al.*, 2014; Bomfim *et al.*, 2021, 2023) capable of accumulating trace elements at concentrations exceeding natural soil levels without exhibiting visible phytotoxic symptoms (Broadley *et al.*, 2007).

Classified as species with phytoremediation potential, these plants possess intrinsic tolerance and detoxification mechanisms that allow them to sustain growth and physiological functioning even under adverse environmental conditions (Sharma and Pandey, 2014; Nedjimi, 2021).

Phytoremediation is an environmentally sustainable technique that employs plants to remove, immobilize, or degrade contaminants from soil and water (Sharma and Pandey, 2014). Within this approach, different strategies are applied based on plant-contaminant interaction mechanisms. Two primary physiological strategies include phytoextraction, where plants absorb and translocate contaminants to aerial parts for subsequent removal-typically performed by hyperaccumulating species, many of which exhibit slow growth and low biomass production (Robinson *et al.*, 2009; Hunt *et al.*, 2014)-and phytostabilization, in which plants immobilize contaminants within the root zone, reducing their mobility and bioavailability, usually associated with species exhibiting high biomass production (Pilon-Smits, 2005; do Nascimento *et al.*, 2021). These strategies may vary according to the contaminant, as its dynamics in the soil and the plant's absorption and transport capacities differ. Therefore, selecting an appropriate plant species is fundamental for successful phytoremediation, requiring knowledge of tolerance mechanisms and biomass allocation in contaminated environments.

Although Zn-phytostabilizing species are infrequently reported in the literature, some forest species exhibit notable metal tolerance, often demonstrated by deeper root systems and higher root biomass relative to shoots. Examples include *Enterolobium contortisiliquum* Vell., which accumulates 187 mg Zn kg^{-1} in shoot dry mass and 200 mg Zn kg^{-1} in roots (da Silva *et al.*, 2018), and *Cordia africana* Lam., which shows Zn accumulation in roots (57.1 mg Zn kg^{-1}), stem (17.1 mg Zn kg^{-1}), and leaves (49.1 mg Zn kg^{-1}) (Pereira *et al.*, 2013). These cases indicate that, although not classical phytostabilizers, these species can exhibit effective tolerance mechanisms, underscoring the importance of biomass allocation when evaluating phytoremediation potential.

Regardless of how *M. oleifera* may be classified, data such as those presented herein-focusing on early developmental stages essential for species establishment in contaminated soils-are both highly relevant and scarce in the literature. The qualitative image (Figure 5C) illustrates that, under 300 mg Zn dm^{-3} concentrations from both $ZnCl_2$ and $ZnSO_4$ sources, total seedling dry mass is reduced; however, cotyledon biomass, located in the root system, remains relatively high compared to the shoot. It was also observed that at this concentration, root dry mass was reduced while cotyledons retained a larger biomass proportion, indicating restricted biomass partitioning. This developmental inhibition-evidenced by suppressed root biomass and reduced mobilization of reserves from cotyledons-is a common plant strategy under heavy metal stress (Muccifora and Bellani, 2013), reflecting a metabolic containment mechanism and energy redirection aimed at cellular protection. Alternatively, it may represent phenotypic plasticity, involving adjustments in form, structure, or function in response to environmental changes (Pollard *et al.*, 2002). Such strategic biomass allocation adjustments reinforce the species' resilience in contaminated environments.

In this context, the Tolerance Index (TI) emerges as a valuable parameter for evaluating seedling performance under stress conditions. Based on accumulated biomass data, *M. oleifera*'s TI to Zn was calculated. According to Jia *et al.* (2017), TI values between 0 and 0.5 indicate low tolerance, while values between 0.5 and 1.0 indicate tolerance. In *M. oleifera*, a high TI was observed in seedlings treated with $ZnCl_2$, especially at T200, where values approached 1.0, suggesting total biomass was not significantly affected. Conversely, under $ZnSO_4$, TI decreased with increasing concentrations, reaching the lowest value at T300. Nonetheless, all treatments remained within the tolerant range, indicating that *M. oleifera* can withstand the tested Zn concentrations.

This pattern is directly related to biomass redistribution among seedling tissues, with a larger proportion retained in cotyledons under greater stress (Figure 5C). The combined analysis of TI and biomass allocation provides a comprehensive view of the species' tolerance and acclimatization to different Zn chemical forms.

Given these findings, *M. oleifera*'s initial tolerance potential deserves emphasis, particularly due to the maintenance of emergence rates in soils with high Zn concentrations (up to 300 mg Zn dm^{-3}). This suggests the species' potential application in phytoremediation. Despite these promising initial data, knowledge gaps remain regarding the species' specific phytoremediation strategy. Further studies are required to investigate growth and biomass accumulation during vegetative development, as well as the organs responsible for Zn accumulation post-absorption, to determine whether *M. oleifera* acts as a phytostabilizer or phytoextractor. Although not classified as a hyperaccumulator (Nedjimi, 2021), its ability to maintain germination and early development under high Zn concentrations, combined with its growth potential and biomass production, positions *M. oleifera* as a promising candidate for sustainable remediation technologies in Zn-contaminated areas. Complementary studies are needed to explore Zn uptake and translocation within the plant.

Dormancy period and Tetrazolium test

Regarding the dormancy period of *Moringa oleifera*, few studies provide detailed information. Costa *et al.* (2021) concluded that the species exhibits tegumentary and photoblastic dormancy, showing greater germinative vigor under light conditions at 30 °C, a temperature similar to that used in the emergence tests of the present study. Identifying dormancy in tree seeds is particularly challenging, especially for slow-germinating species such as many forest trees (Fogaça *et al.*, 2006). However, the tetrazolium test provides a rapid alternative for evaluating seed viability and vigor in seed lots, offering crucial data on physiological quality to support decision-making, as demonstrated for *Ocotea porosa* (Kalil-Filho *et al.*, 2008) and *Peltophorum dubium* (Oliveira *et al.*, 2005), both species used in native seedling propagation for the recovery of degraded areas (Nogueira *et al.*, 2014).

The application of the tetrazolium test for qualitative evaluation of *M. oleifera* seed viability revealed the need for optimized protocols to ensure accurate and feasible results, avoiding costly and time-consuming errors. The light pink coloration observed in the cotyledonary tissues after immersion in tetrazolium solution, without staining the embryonic axis (vascular cylinder), aligns with the findings of Nogueira *et al.* (2014) for *Enterolobium contortisiliquum*, indicating viable and high-vigor seeds with normal respiratory activity (Brazil, 2009). These results complement the germination tests under varying Zn concentrations and sources, where the absence of emergence-especially at higher Zn levels (400 and 500 mg Zn dm^{-3})-is linked to the element's toxicity.

The practical relevance of these findings is significant when considered alongside the reference quality values (VRQ) for Zn in soil (60 mg kg^{-1} dry soil) and prevention values (VP) (86 mg Zn kg^{-1} soil) established by the environmental legislation of São Paulo state (CETESB, 2021). Zn concentrations equal to or exceeding 400 mg Zn dm^{-3} inhibited *M. oleifera* germination, indicating a critical threshold for the species' emergence in contaminated environments, where element absorption and accumulation in plant tissues may occur (Kranner and Couvillion, 2011). Nonetheless, *M. oleifera*'s capacity to tolerate substantial Zn concentrations and adjust biomass allocation underscores its potential for use in the restoration of degraded areas and phytoremediation strategies (Nedjimi, 2021), especially in Zn-contaminated sites.

Limitations and perspectives for future research

Conducting germination tests in a greenhouse presents certain challenges, particularly in controlling the optimal duration of automatic irrigation to avoid water stress during seedling emergence. As *Moringa oleifera*

is a tropical species tolerant to dry soils, constant monitoring of soil moisture in the experimental units is essential to prevent waterlogging, which often necessitates manual interruption of irrigation systems. For consistent assessment of seed vigor, it is crucial to standardize the length of emerged seedlings considered in daily counts and to perform evaluations consistently at the same time.

M. oleifera is capable of emergence in soils containing up to 300 mg Zn dm^{-3}. This finding highlights the species' potential for further studies involving medium-term monitoring (six months to one year) of growth and development under these soil conditions. Such research would provide insights into its tolerance and phytoremediation capacity for Zn and other potentially toxic elements, in addition to its suitability for reforestation initiatives.

To ensure accuracy in qualitative analyses of seed lots, it is recommended that, following an 8-hour preconditioning period at 25 °C, the seed tegument be removed and a lateral cut be made opposite the embryonic axis, followed by immersion in tetrazolium solution for 6 hours at 30 °C. Notably, the coloration pattern revealed in plant tissues by this method presents an innovative and effective approach to identifying seed dormancy in *M. oleifera*, serving as a valuable complement for assessing seed viability.

Conclusions

The species *Moringa oleifera* Lam. exhibited seedling emergence in both $ZnCl_2$ and $ZnSO_4$ sources at soil concentrations up to 300 mg Zn dm^{-3}. No emergence was observed at higher concentrations. Among the two sources, $ZnSO_4$ demonstrated greater toxicity, reducing both the percentage of emergence and the emergence speed index starting at 100 mg Zn dm^{-3}, and negatively impacting the total dry mass of seedlings at 300 mg Zn dm^{-3}. This heightened toxicity of $ZnSO_4$ is likely attributable not only to zinc toxicity but also to potential nutritional imbalances caused by excess sulfur ($S\text{-}SO_4^{2-}$) in the soil, which may have influenced the availability of zinc and other nutrients.

The tetrazolium test provided a valuable complement to the germination test for assessing seed viability. At the highest tested concentrations (400 and 500 mg Zn dm^{-3}), where emergence was completely inhibited, the tetrazolium test confirmed seed viability, indicating that the lack of germination was a consequence of zinc toxicity rather than an intrinsic deficiency in seed vigor.

These findings have important practical implications for the restoration and phytoremediation of zinc-contaminated soils. The tolerance of *M. oleifera* to elevated zinc concentrations during seedling emergence, combined with its capacity for biomass allocation, highlights its potential as a promising candidate for further research on the recovery of degraded areas and phytoremediation strategies, particularly in environments impacted by zinc contamination.

Authors' Contributions

Conceptualization: MLGO and TCF; Methodology: MLGO, TCF, GIS, GSR, MAA, FAAM, ARC; Formal analysis: MLGO, TCF, BSS, GIS, GSR, FAAM, ARC; Investigation: MLGO, TCF, BSS, NCPB; Data curation: GSR, TCF, MLGO; Writing-original draft preparation: MLGO, TCF, BSS, GIS, NCPB; Writing-review and editing: MLGO, TCF, FAAM, NCPB, ARC, LSC; Supervision: LSC; Project administration: LSC; Funding acquisition: LSC.

All the authors have read and approved the final manuscript.

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Conflict of Interests

The authors declare no competing interests.

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