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Comparative Study of Traditional and Environmentally Friendly Zinc Sources Applied in Alkaline Fluvisol Soil: Lettuce Biofortification and Soil Zinc Status

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Abstract: The use of highly effective sources of zinc (Zn) in alkaline agricultural soils is essential to achieve crop biofortification, maintain crop quality, and avoid potential environmental risks. This research examines the efficacy of environmentally friendly Zn complexes (citric acid, CIT and glycine, GLY) compared to a traditional source ($ZnSO_4$) for the lettuce cultivation in alkaline soil. The effectiveness of Zn sources was assessed based on the concentration of total and soluble Zn, plant biomass, and contents of photosynthetic pigments. The soil Zn status was also evaluated. While all Zn sources (Zn-GLY, Zn-CIT, and $ZnSO_4$) showed positive effects on lettuce growth, Zn-GLY exhibited the highest efficacy. This source exhibited increases of 230%, 502%, 296%, and 409% over the control in Zn concentration in young and mature leaves, soluble Zn, and Zn uptake, respectively. Zn-GLY also resulted in a 371% increase in soil exchangeable Zn concentration, compared to the control treatment. Our findings indicate that Zn-GLY could replace the traditional $ZnSO_4$ treatment, as it achieved high Zn biofortification of lettuce and a high concentration of Zn available in the medium-long term in the soil. The beneficial effect of the chelating agent GLY on plant chlorophyll and carotenoid contents is also remarkable.

Keywords: Zn-glycine; Zn-citric; efficiency; Zn complex; lettuce; photosynthetic pigments; Zn uptake

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1. Introduction

Zinc (Zn) is an essential micronutrient for humans; however, deficiency in the diet reaches 30% of the world population [1]. In addition, Zn is an essential micronutrient in plants, necessary for maintaining life processes and a key component of several enzyme systems, where it contributes to energy production, protein synthesis, and growth regulation. It is involved in various crucial metabolic processes, from photosynthesis to chlorophyll synthesis [2]. Plant foods differ in their Zn content. Bioenriched vegetables with Zn could, on the one hand, satisfy human needs for this nutrient, decrease the risk of contracting diseases related to this deficiency or hidden hunger [3,4] and, on the other hand, improve the added value of these products and increase plantation profits [5].

Different authors have reported the importance of green leafy vegetables, and especially lettuce, for biofortification with Zn through soil or nutrient solutions [6–8]. The importance of lettuce as a biofortified crop lies in the fact that it is the main leafy vegetable product worldwide, with an increase in production of about 62% in the last two decades [7,9]. According to Cervera-Mata et al. [10], lettuce is a good candidate for research into its possibilities in agronomic biofortification and combating hidden hunger.

Zinc deficiency in plants can be attributed to several factors, such as the inefficiency of plants to accumulate Zn and transport it to various parts of the plant, or the physical and chemical characteristics and Zn status of the soil [11–13].

Zinc availability decreases with increasing soil pH, as does the adsorption capacity of the nutrient, the creation of hydrolysed Zn forms, chemisorption on calcium carbonate, and co-precipitation on iron oxides. Alkaline, calcareous, and heavily limed soils tend to show a higher susceptibility to Zn deficiency compared to neutral or slightly acid soils [11]. The extent of soils deficient in Zn covers 50% of the cultivated soils in India and Turkey, a third of cultivated soils in China, and most soils in Western Australia are classed as Zn-deficient [11]. Large areas with predominantly alkaline soils are also found in the western United States, northern Mexico, Argentina, Chile, Bolivia, and Paraguay; North Africa, including Sudan, Ethiopia, Somalia, Namibia, and South Africa; South Asia; and the eastern Iberian Peninsula [2,11]. A total of 30% of the world's population, or 1.1 billion people, are estimated to be at risk of Zn deficiency [1].

The characteristics of these agricultural soils mentioned above require effective Zn sources. Different studies have reported foliar application of Zn sources [5,14]; however, the addition of Zn to soil allows a high rate of nutrient utilisation and accumulation of Zn for successive crops [15]. Zn fertilisers added to the soil can be transformed into unavailable forms due to factors such as the alkaline pH and the high phosphorus content. Although Zn sulphate ($ZnSO_4$) has traditionally been the most commonly used Zn source, more recently, others have proven to be very effective in soils with alkaline pH. Several Zn compounds, such as Zn salts, Zn oxides, Zn hydroxides, Zn chelates and Zn complexes, are available to treat Zn deficiency in soils and crops [5,16–19].

McBeath and McLaughin [19] reported similar effectiveness of synthesised Zn oxides and commercial Zn sulphate in soil with pH 8.5. The study carried out by Gangloff et al. [17] showed that Zn-lignosulfonate, $ZnSO_4$, and Zn-ethylenediaminetetraacetic (Zn-EDTA) were the most effective fertilisers in soils with pH 7.2. Other studies have reported that the use of Zn sources consisting of synthetic complexing and chelating agents such as Zn-ethylenediamine-di-(2-hydroxy-5-sulfophenylacetate), Zn-ethylenediamine tetraacetate, Zn-polyhydroxyphenylcarboxylate, Zn-N-2-hydroxyethyl-ethylenediaminetriacetate, Zn-diethylenetriaminepentaacetate, or Zn-ethylenediamine disuccinate are effective agents for providing Zn in plant-available forms in soils with alkaline pH [16]. However, the use of these synthetic chelating agents can cause environmental risks such as aquifer pollution or human risks throughout the food chain [20].

Currently, there is growing interest in the use of environmentally friendly complexing agents. For example, an amino acid such as glycine has been used as a biostimulant in plant nutrition or in the production of amino acid chelate fertilisers [21,22]. Amino acid chelate compounds improve the physiological availability of the metal nutrients and are environmentally friendly due to various physiological and metabolic cycles of plants [23]. Different studies have reported that the application of Zn bound to amino acids such as glycine is a more effective form of fertilisation compared to $ZnSO_4$ [24,25]. On the other hand, an organic compound such as citric acid is a complexing agent that rapidly biodegrades in soil [26]. Citrate is released by plant roots and by microbial metabolism, which enhances solubilisation of insoluble forms and nutrient mobilisation [27]. In addition, Zn-citrate complexes are taken up by plants. Different authors have reported that exogenous application of citric acid in problematic soils leads to an increase in Zn^{2+} uptake by crops [20,28].

Therefore, the overall objective of the present work is to study the effect of Zn complexes based on glycine or citrate compounds in an agricultural alkaline soil and their effect on lettuce biofortification. This research aims to advance the use of environmentally friendly and low-cost technologies to achieve sustainable agriculture.

2. Materials and Methods

2.1. Pot Experiment

Alkaline soil from the National Center of Irrigation Technology "CENTER", Madrid (latitude 40°24′59.2″ N, longitude 3°29′46.9″ W) was used for this experiment. Soil samples from the Ap horizon (0–28 cm) were air dried, sieved (<2 mm), and then analysed according to the Spanish official methodology [20]. This soil was classified as Fluvisol [21]. The main characteristics were as follows: pH (1:2.5), 8.3; sand, 301 g kg^{-1}; silt, 643 g kg^{-1}; clay, 56 g kg^{-1}; texture, silt loam; electrical conductivity (1:5), 160 µS cm^{-1}; extractable P ($NaHCO_3$ 0.5 M, pH 8.5), 8.2 mg kg^{-1}; oxidisable organic matter (OM) (oxidation with $K_2Cr_2O_7$ in an acid medium and subsequent titration of excess dichromate with $Fe(NH_4)_2(SO_4)_2$), 14.5 g kg^{-1}; total N (Kjeldahl method), 1.3 g kg^{-1}; C:N ratio, 6.5; exchangeable Na (washes with AcONa 1 N pH 8.2, extraction with $AcONH_4$, 1 N), 73 mg kg^{-1}; exchangeable K, 712 mg kg^{-1}; exchangeable Ca, 3503 mg kg^{-1}; exchangeable Mg, 288 mg kg^{-1}; total $CaCO_3$ (UNE 103-200-93), 80 g kg^{-1}; active $CaCO_3$ (Bouyoucos Method), 18 g kg^{-1}; pseudo total Zn concentration (acid digestion), 44.75 mg kg^{-1}; available Zn concentration [29], 0.87 mg kg^{-1}.

According to the maximum limits for heavy metals at the first 25 cm depth, in agricultural soil with a pH greater than 7, the application of Zn fertiliser would be allowed, as it does not exceed the limit of 200 mg total Zn kg^{-1} soil [30]. The value of available Zn concentration indicates a deficiency of the Zn concentration in this alkaline soil, as it does not reach the sufficient concentration proposed by Lindsay and Norwell [29] (1.0 mg kg^{-1} for alkaline soils).

A total of 3.0 kg soil pots were fertilised with the macronutrients N, P, K following the fertilisation recommendations for lettuce [31]. This fertilisation consisted of applying 100 kg N ha^{-1} as urea, 50 kg P_2O_5 ha^{-1} as K_2HPO_4 and 160 kg K_2O ha^{-1} as K_2HPO_4 and K_2SO_4. Different treatments were applied: (a) Zn-CIT, Zn complexed with citric acid (0.2% *w*/*w*): pH, 4.15; electrical conductivity (EC), 985 µS cm^{-1}, (b) Zn-GLY, Zn chelated with glycine (0.2% *w*/*w*), pH, 5.84; EC, 943 µS cm^{-1}, (c) $ZnSO_4$, $ZnSO_4 \cdot 7H_2O$: pH, 5.83; EC, 872 µS cm^{-1}, (d) CONTROL, treatment with only NPK fertilisation, (e) GLY, glycine (0.4% *w*/*w*) pH, 6.03; EC, 57 µS cm^{-1}, the same amount of glycine per pot was supplied, this time without Zn, as with treatment b.

The Zn treatments (a, b, and c) were applied at a dose of 8 mg Zn kg^{-1} soil on the surface. This dose was selected because it is within the recommended range for Zn-deficient alkaline soils in various horticultural crops [16,32]. Three replicates were used for each treatment, with a total of 15 pots in a randomised complete block design.

Lettuce plants (*Lactuca sativa* L., Romana Verano, Ramiro Arnedo) with 25 days of growth was placed in each pot and they were placed in a controlled greenhouse environment on the Universidad Politécnica de Madrid (Madrid, Spain). The temperature in the greenhouse ranged from 4 °C at night to 38 °C during the day, with a relative humidity fluctuating between 20% and 85%. Throughout the experiment, the soil moisture was maintained at 100% of its water-holding capacity by regular irrigation with tap water, carefully controlled through weighing.

2.2. Plant Analysis

After 46 days from planting, the lettuce plants were harvested, weighed, and washed in deionised water. Young (fifth youngest leaf) and mature (second- and third-oldest leaves) lettuce leaves were separated to carry out the different analyses of the plant material. The chlorophyll and carotenoid contents in the mature leaf were determined according to AOAC Official Methods of Analysis [33] and Lichtenthaler [34] using 0.25 g of fresh leaf with 50 mL of acetone (C_3H_6O) (99.5%, Panreac, Química SLU, Barcelona, Spain). The soluble Zn concentration in fresh mature leaves was determined using 0.5 g of fresh leaf with 8 mL of 1 mM MES (2-(N-morpholino)ethanesulfonic acid) at pH 6. Details of the method can be found in Almendros et al. [35]. Subsequently, the leaves were dried in an oven at a constant temperature of 60 °C until a stable weight was reached and then stored in sealed containers for further analysis. Total Zn concentrations in dry leaves (in young and mature leaves) were determined by wet digestion in Teflon vessels using a sample preparation block system (SPB Probe, Perkin-Elmer, Waltham, MA, USA). For this procedure, 0.5 g of dry matter samples was used along with 10 mL of an acid mixture (5 mL HNO_3 (65%), 2 mL HF (48%), and 3 mL H_2O).

2.3. Soil Analysis

Following the harvest, the soil of each pot was naturally dried and homogenised. A subsample of the soil was used for laboratory analysis. Soil pH and electrical conductivity were measured using a Hamilton pH (LP238285, KCl 3 M plus glycol electrolyte) and electrical conductivity (COND50, XS Instruments, Carpi, Italy) electrodes.

Different chemical extractions were used to assess the extractability, mobility, and availability of Zn in the soil. Plant-available Zn concentration was assessed using the rhizosphere-based extraction method (Low molecular weight organic acids, LMWOAs): 2 g of soil in 20 mL of a mixture 10-mM combination of organic acid solution containing acetic, lactic, citric, malic, and formic acids in a molar ratio of 4:2:1:1:1, respectively [36]. Mobile Zn concentration was assess using double-deionised water for 24 h using a soil-to-water ratio of 1:10 [32]. Exchangeable Zn concentration was determined by weighing 1 g of dried soil mixed with 30 mL of NH_4Ac 1M, shaking for 24 h, centrifugation ($4000\times g$ for 600 s) and filtered of supernatant solution [37,38]. The pseudo total metal content was determined by acid digestion: 0.5 g of soil was digested (220 °C, 340 bar) with 10 mL HNO_3 (65%) and 5 mL HF (48%) in a microwave oven (Milestone Ethos, Sorisole, BG, Italy). The Zn concentration in the extracts was quantified using atomic absorption spectrometry (AAnalyst 900, Perkin Elmer, Waltham, MA, USA).

The extraction efficiency (EF) was estimated according to Hall and Chang-Yen [39]. It involved calculating the percentage of the total content extracted by each extractant, as specified below:

$$EF = 100\ Ce/Ct$$

where Ce and Ct are, respectively, the metal extracted and (pseudo) total metal content. Different EF values could indicate the ability of each extractant to target the potential soil phases responsible of metal availability for the crop in this soil [33].

2.4. Statistical Analysis

Statistical analyses were performed using Statgraphics Centurion 19 software (Manugistic, Rockville, MD, USA). Multifactor analyses of variance were conducted using the optimized Box–Cox General Linear Model. The main effects were differentiated using Fisher's LSD test at a probability level of $p \leq 5\%$.

3. Results

Figure 1 shows the effects of the different treatments on Zn biofortification of lettuce (total Zn concentration in mature and young leaves, and Zn uptake by the plant) and leaf soluble Zn concentration. Significant differences between treatments were obtained for total Zn concentration in mature and young leaves and Zn uptake ($p < 0.001$, $p < 0.0001$, and $p < 0.0001$, respectively) and soluble Zn concentration in the leaf ($p < 0.001$). The Zn concentrations (both total and soluble) in the leaves and Zn uptake by plant were higher in lettuce treated with Zn treatments compared to the nil-Zn treatments (Figure 1). The highest Zn uptake and total Zn concentration in mature leaves was obtained with the Zn-GLY treatment, followed by $ZnSO_4$, while Zn-CIT reached the lowest concentration among the Zn treatments. The Zn concentration in the young leaves was statistically similar for all Zn treatments. Total Zn concentrations in mature and young leaves were similar when nil-Zn treatments were applied. However, in all treatments with added Zn, the Zn concentrations in the mature leaves were higher than the accumulated Zn concentrations in the young leaves, with increments of 1.13, 1.52, and 1.27-fold more Zn for Zn-CIT, Zn-GLY, and $ZnSO_4$, respectively. The highest concentration of soluble Zn in mature leaves was obtained with $ZnSO_4$ and Zn-GLY sources (Figure 1C). The soluble Zn concentration in mature leaves in the treatments in which Zn was applied reached values of up to 56.38% ($ZnSO_4$) of the total Zn concentration in the leaf. This percentage reached 49.49% and 55.33% for Zn-GLY and Zn-CIT, respectively.

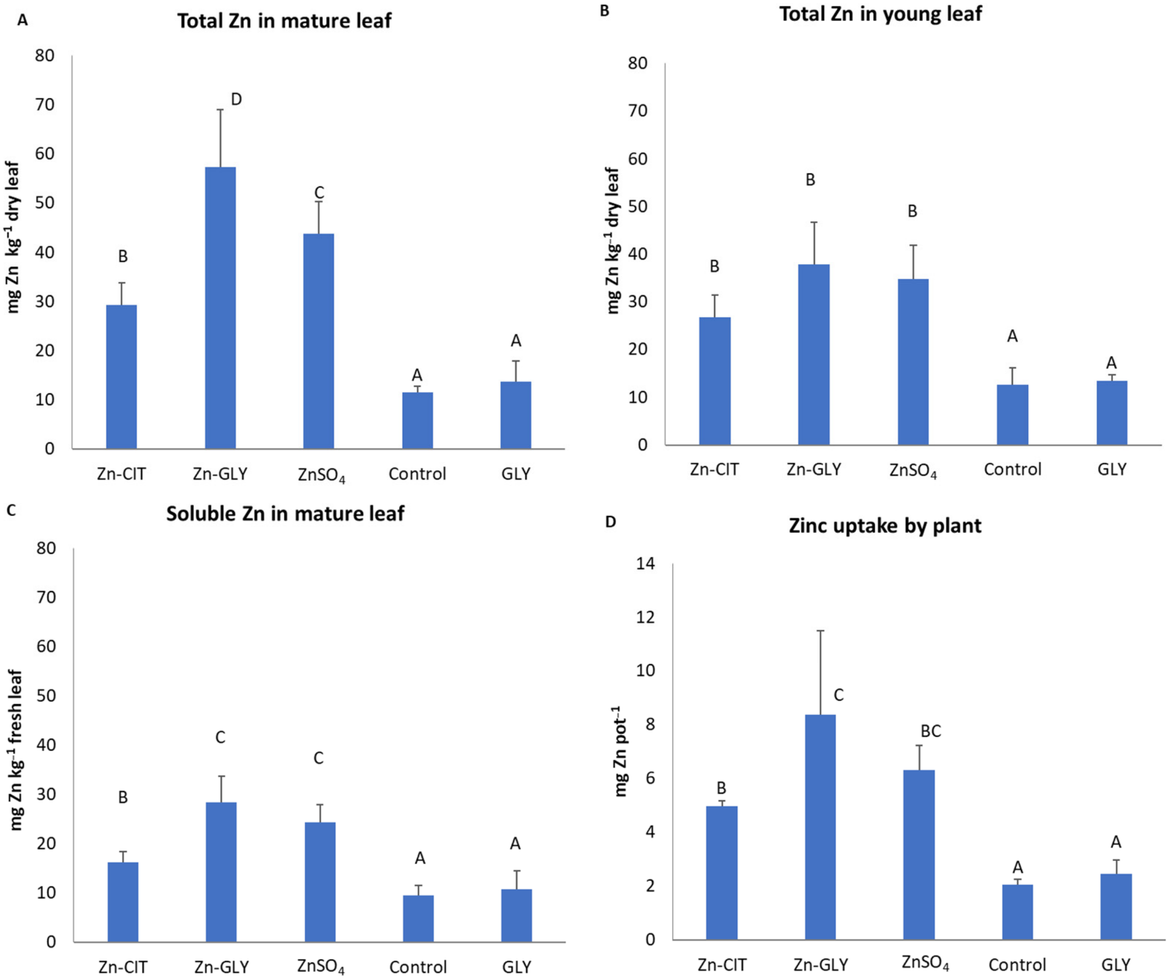

Figure 1. Total Zn concentration in mature (**A**) and young (**B**) leaves, soluble Zn concentration in mature leaf (**C**), and Zn uptake (**D**) for each treatment: Zn-CIT, Zn complexed with citric acid; Zn-GLY, Zn chelated with glycine; $ZnSO_4$, Zn sulphate heptahydrate; Control; and GLY, glycine without Zn fertilisation. Statistical differences at $p \leq 5\%$ (LSD test) are indicated by different letters. The vertical line in each of the data represents the standard deviation from the mean.

Fresh matter (FM) yields and photosynthetic pigments (chlorophylls and carotenoids) are shown in Figure 2. The results showed that the treatments applied in this study did not influence lettuce yield, with no significant differences observed between the various fertiliser treatments ($p > 0.05$). The average yield value reached 172.5 g of lettuce per pot (on a pot area of 219 cm^2). The trend of the total chlorophyll content shows a lower value for the control treatment. Although no significant differences were obtained between the treatments applied, it is noteworthy that the orthogonal analysis between different pairs showed significant statistical differences: Zn-GLY vs. control, $ZnSO_4$ vs. control, and GLY vs. control (mean difference: 1.41, 1.57, and 1.37, respectively; cut-off 1.29). These treatments showed a percentage increase in chlorophyll of 31.3, 29.8, and 32.9%, (GLY, Zn-GLY, and $ZnSO_4$, respectively).

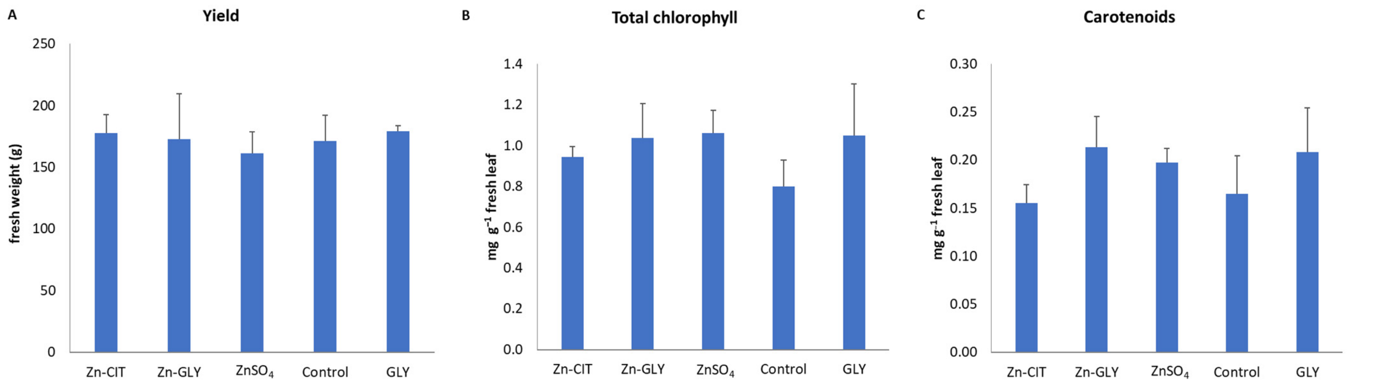

Figure 2. Yield (fresh matter) (**A**) and total photosynthetic pigments (chlorophylls (**B**) and carotenoids (**C**)) for each treatment: Zn-CIT, Zn complexed with citric acid; Zn-GLY, Zn chelated with glycine; $ZnSO_4$, Zn sulphate heptahydrate; Control; and GLY, glycine without Zn fertilization. The vertical line at each of the data represents the standard deviation from the mean.

Plant bioavailable (LMOWAs), mobile (water soluble), and exchangeable (extracted with NH_4Ac 1M) Zn forms are shown in Figure 3. These Zn concentrations showed significant differences ($p < 0.001$) between treatments. As expected, the concentrations of bioavailable Zn in the soil were higher in treatments in which a Zn source was applied. There were no significant differences in the bioavailable and mobile concentrations between the Zn sources used. However, the exchangeable Zn concentration showed statistical differences between the Zn sources, with a higher concentration when the Zn-GLY source was applied. The LMWOAs method showed the lowest EF values, with less than 0.80% of total Zn released (Table 1). The proportions of total Zn released through water-soluble extraction were greater compared to those of LMWOAs. This amounted to an average of 2% of the total Zn released. The highest EF values were obtained with NH_4Ac extraction, reaching a mean value of 7%.

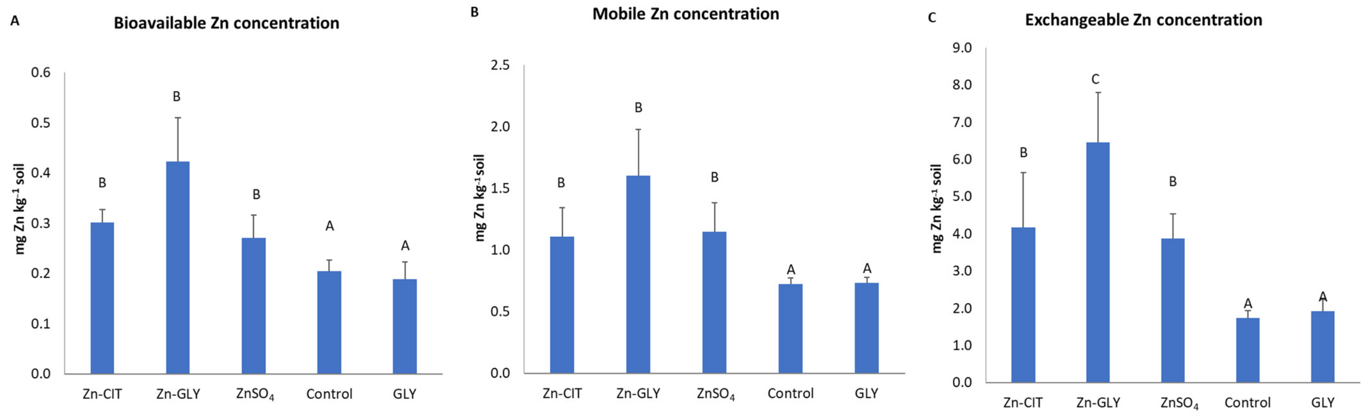

Figure 3. Bioavailable (**A**), mobile (**B**), and exchangeable (**C**) Zn concentration in soil for each treatment: Zn-CIT, Zn complexed with citric acid; Zn-GLY, Zn chelated with glycine; $ZnSO_4$, Zn sulphate heptahydrate; Control; and GLY, glycine without Zn fertilization. Statistical differences at $p \leq 5\%$ (LSD test) are indicated by different letters. The vertical line at each of the data represents the standard deviation from the mean.

There were not significant differences between the different fertiliser treatments ($p \geq 0.05$) in soil pH and electrical conductivity. The mean values were 8.33 for pH and 163.01 µS cm^{-1} for electrical conductivity.

Table 1. Extraction efficiencies (EF) for each of the extraction methods (rhizosphere-based extraction method, LMWOAs; water-soluble, double-deionised water for 24 h using a soil-to-water ratio of 1:10; and NH4Ac 1M, shaking for 24 h) and treatment (Zn-CIT, Zn complexed with citric acid; Zn-GLY, Zn chelated with glycine; $ZnSO_4$, Zn sulphate heptahydrate; Control; and GLY, glycine without Zn fertilization).

Treatment	LMWOAs	Water-Soluble	NH_4Ac 1 M
Zn-CIT	0.56 ± 0.05	2.05 ± 0.44	7.72 ± 2.75
Zn-GLY	0.79 ± 0.16	2.97 ± 0.70	11.95 ± 2.50
$ZnSO_4$	0.50 ± 0.08	2.13 ± 0.43	7.17 ± 1.24
Control	0.49 ± 0.04	1.74 ± 0.19	4.18 ± 0.66
GLY	0.44 ± 0.08	1.62 ± 0.22	4.03 ± 0.20

Simple linear regression analysis (Table 2) showed high correlations between soil and plant Zn concentrations. The highest linear correlation coefficient between mobile Zn concentration (water soluble) and total Zn concentration in mature leaves. Significant negative correlations were observed between pH and Zn concentrations in both soil and plant, except for soluble Zn concentration in plant. A significant negative correlation between pH and electrical conductivity was also observed.

Table 2. Linear correlation coefficient (r) for relationships between bioavailable, mobile, and exchangeable Zn concentration, soil pH, electrical conductivity, and Zn concentrations in plant.

Plant and Soil Factors	Bioavailable Zn Concentration (LMWOAs)	Mobile Zn Concentration (Water Soluble)	Exchangeable Zn Concentration (NH_4Ac 1 M)	Soil pH	Electrical Conductivity
Total Zn concentration in mature leaves	0.839 **	0.890 ***	0.827 **	−0.591 *	NS
Total Zn concentration in young leaves	0.741 *	0.857 ***	0.769 **	−0.487 *	NS
Soluble Zn concentration in mature leaves	NS	0.616 *	0.727 *	NS	NS
Soil pH	−0.573 *	−0.512 *	−0.523 *	--	−0.827 **

***, ** and * significant at 0.01., 0.1, and 5% levels, NS: not significant.

4. Discussion

Lettuce (*Lactuca sativa* L.) is considered a crop with a medium relative sensitivity to Zn deficiency [11,12]. Different authors have reported that this crop presents a notable response to the addition of Zn [6,7]. Our results showed that the application of Zn to this alkaline soil produced an increase in Zn concentration in lettuce leaves, indicating a biofortification effect on the edible part of the crop. However, the extent of this increase depended on the specific Zn fertilisers used and the maturity level of the lettuce leaf analysed. The Zn-GLY treatment reached the highest total Zn concentration in mature leaves, followed by $ZnSO_4$ and Zn-CIT. In contrast, the Zn concentration in young leaves showed statistically similar concentrations for all Zn treatments (Zn-CIT, Zn-GLY and $ZnSO_4$). It is remarkable that the Zn concentrations in the nil-Zn treatments (control and GLY) did not reach the Zn ranges considered suitable for the plant according to Broadley et al. [2] (Table 3). On the contrary, the Zn concentration in mature and young leaves reached optimal leaf Zn concentrations [40] in all treatments where Zn was applied, which could make a significant contribution as a biofortified crop. According to the concentration of Zn contained in the biofortified lettuce, it would be necessary to consume 149 to 480 g of lettuce (for children over 7 years of age consuming mature lettuce treated with Zn-GLY and men consuming young lettuce treated with Zn-CIT, respectively) to reach the daily dietary Zn requirement of the European Commission (Table 3).

Table 3. Adequate and optimal levels of plant Zn concentration and daily dietary Zn requirements reported in the literature.

Level	Reference Value	Reference
Zn concentration in plant:		
Appropriate for the plant	15–20 mg Zn kg^{-1} of leaves	Broadley et al. [2]
Optimal for the plant	20–60 mg Zn kg^{-1} of leaves	Maynard and Hochmuth [40]
Daily dietary Zn requirement:		
Women	10.1 mg	
Men	12.85 mg	European Commission [41]
Children over 7 years old	8.55 mg	

The Zn concentrations in lettuce plants were in line with the Zn concentrations found in this alkaline soil. As observed in Figure 3, the estimated Zn concentrations in soil follow the same trend as the Zn concentration in the leaves, with a significant linear correlation between the soil and plant concentrations (Table 2). The highest Pearson correlation coefficient was shown between mobile Zn concentration (water soluble) and the Zn concentrations of mature and young leaf. This correlation coefficient provides information on the appropriate analysis method to assess Zn levels in the plant. Almendros et al. [32] reported that weak extractants such as water or dilute mild acid solutions (LMWOAs) have a greater predictive ability for Zn concentrations in different parts of the plants than stronger reagents, such as buffered salt solutions. In our study, the correlation coefficient and the low EF values with LMWOAs indicate that the Zn concentration estimated as bioavailable by this method extracts concentrations lower than what the plant actually takes up. EF values also indicate that the Zn concentration estimated by NH_4Ac 1M as exchangeable extracts has higher Zn concentrations than are available to the plant in the short term. The NH_4Ac solution extracts the metal ions that bind to the soil colloids by ion exchange. It is a widely used procedure to extract the water-soluble and easily exchangeable fractions by displacing them from the exchange sites with NH_4^+ [38].

The different sources of Zn did not show significant differences in potentially bioavailable (extracted by the LMOWAs method) or mobile (water soluble) Zn concentrations. However, significant differences were obtained with respect to the exchangeable Zn concentration (extracted with NH4Ac 1M), with the Zn-GLY source showing the highest exchangeable Zn concentration in the soil. This behaviour indicates a higher long-term Zn availability with the Zn-GLY treatment. Zinc bound to the soil exchange complex with Zn-GLY reaches almost 12% of its total content in the soil (Table 1). This concentration of Zn bound to the soil exchange complex (or adsorbent complex) is of the utmost importance due to its direct influence on soil fertility in the medium to long term [42]. This behaviour could be related to the differences in the stability constant (K) of the sources. The high stability of the Zn-GLY chelate (log K = 9.81, at 25 °C and 0.1 M ionic strength) [43] may explain the higher exchangeable concentration of Zn compared to the Zn-CIT source (logK = 5.9, at 25 °C and 0.1 M ionic strength), where Zn is more retained in the soil due to the lower stability of the source. On the other hand, the high solubility of the $ZnSO_4$ source justifies the Zn^{2+} retention in this alkaline soil due to the sorption processes associated with this nutrient, as this source lacks a protective structure for the metal (chelating agent). Metal chelating agents play an important role in solubilising Zn and transporting it from solid phases in soils to the surface of plant roots [44]. Furthermore, Zn in soil solution is not only taken up by the plant as Zn^{2+}, but is also complexed with organic ligands [2].

The characteristics of this alkaline soil lead to a retention of Zn^{2+} by the soil components. The solubility of Zn^{2+} in soil is highly dependent on pH. Broadley et al. [2] reported that soluble Zn and the proportion of complexed Zn^{2+} increased at low soil pH. As shown in Table 1, there was a significant negative correlation between soil pH and bioavailable, mobile and exchangeable concentrations of Zn in the soil, suggesting that a decrease in the pH of this alkaline soil increases the availability of Zn in the soil. This decrease in soil pH also has a positive effect on the biofortification of the crop with Zn, as it increases the concentration of this nutrient in the plant, as shown by the significant and negative correlations between both parameters (pH and Zn concentration in young and mature leaves) (Table 1).

The total Zn concentrations in mature and young leaves were similar to those of nil-Zn treatments (control and GLY). However, Zn concentrations in mature leaves exceeded those in young leaves in all treatments with added Zn (Zn-CIT, Zn-GLY and $ZnSO_4$). According to Longnecker and Robson [13] with a high Zn supply, Zn tends to accumulate in the older leaves of the plants, resulting in significantly higher Zn concentrations in these older leaves compared to the young leaves. In our study, this effect was especially observed with the Zn-GLY treatment (resulting in a 1.52-fold increment in the Zn concentration in mature leaf with respect to Zn concentration in young leaf), indicating a high effectiveness of the treatment with respect to Zn biofortification of this crop in this alkaline and Zn-deficient soil.

The concentration of soluble Zn is related to the nutritional quality of the plant products, as it is this soluble Zn that is absorbed into the gastrointestinal tract of humans [4]. This soluble Zn is often considered the physiologically active fraction and is considered a better indicator of Zn status than the total Zn content in the plant [45]. The highest soluble Zn concentrations were obtained with $ZnSO_4$ and Zn-GLY sources. The $ZnSO_4$ is a highly soluble source which solubilizes in soil water, giving rise to Zn^{2+} ions. In the Zn-GLY source, the Zn forms a chelate with the amino acid glycine: Zn-glycinate. On the other hand, the Zn-CIT source formed by the complexing agent citrate also significantly increased the concentration of soluble Zn in the leaf with respect to the nil-Zn sources. Free Zn ions constitute only a small proportion of the total soluble content, which may explain why high leaf-soluble Zn concentrations are achieved with the complexed sources, since the fraction of soluble Zn in lettuce contains sulphur, reducing sugars and amino acids [46]. These authors reported that in plant leaves, soluble Zn exists largely as an anionic compound possibly associated with amino acids. Among these soluble forms of Zn, low molecular weight complexes are often the most abundant and probably the most significant form of active Zn in the plant. These authors indicated that the isolated low molecular weight fractions accounted for 73% of the total soluble Zn, which was the to 58% of the total Zn in lettuce leaf. In our study, we obtained a soluble Zn concentration ranging from 49.5% to 55.4% of the total Zn in the leaf.

Our results suggest that a higher proportion of the accumulated Zn in lettuce is readily bioavailable. Leafy vegetables typically have low levels of phytic acid, a compound that has a strong ability to chelate multivalent metal ions, especially Zn, Ca, and Fe. Binding can result in very insoluble salts with poor bioavailability of minerals [47]. Therefore, a daily ration of 50 g of biofortified lettuce can provide more Zn than larger rations of grains and cereals, as these foods contain higher amounts of phytic acid [48].

Cakmak and Marschner [45] reported that leaf soluble Zn concentration was closely correlated with chlorophyll levels. In our study, the chlorophyll concentrations obtained with the treatments were low, but not deficient [49]. Carotenoids reached average values for soil production [50]. It should be noted that the statistical analysis performed with all treatments except GLY showed statistical correlations between both factors ($p \leq 0.05$, r = 0.62). The chlorophyll and carotenoid contents in the GLY treatment exhibited a slight tendency to be higher than those in the control treatment. This indicates that although the application of this treatment did not respond to an increase in the availability of Zn present in the soil nor in the concentration of Zn in the plant, it may have had a beneficial effect on the chlorophyll and carotenoid contents of the plant.

5. Conclusions

The findings of this study indicate that, although the application of Zn-citrate and Zn-glycinate sources in this alkaline Zn-deficient Mediterranean soil has no effect on yield, it has a beneficial effect on Zn biofortification of the edible part of the lettuce. This effect was remarkable on Zn concentration in mature leaves and Znuptake for Zn-GLY and in young leaves for all Zn treatments (Zn-CIT, Zn-GLY and $ZnSO_4$). Although Zn concentrations in the crop without Zn treatments did not reach adequate levels for the crop, lettuce grown under Zn treatments reached optimal Zn concentrations. This implies that a reasonable consumption of lettuce fertilised with these Zn treatments provides a large percentage of the Zn intake needed by humans. Furthermore, the concentration of soluble Zn in the Zn-treated plants suggests that a higher proportion of the Zn accumulated in lettuce is readily bioavailable or absorbed in the human gastrointestinal tract. Different parameters such as the degree of maturity of the tested leaves, the stability of the Zn sources and the soil pH influenced the effect of the Zn fertilizers used. Differences in Zn concentrations in mature and young leaves indicated a low mobility of this element in the plant. The stability of the Zn sources played an important role in the concentration of exchangeable Zn in the soil. The stability of the complexes (Zn-citrate and Zn-glycinate) and the high solubility of the traditional source used ($ZnSO_4$) explained the Zn status in the soil and the availability of Zn to the plant. Additionally, low decreases in soil pH increased the availability of this micronutrient, which also positively impacted Zn concentration in plants. Although the application of GLY did not respond to increasing the availability of Zn present in the soil or in the Zn biofortification of lettuce, it had a beneficial effect on the chlorophyll and carotenoid content of the plant. Further research will be necessary to study the possible influence of glycine application on the agricultural soil-plant systems and on different crop parameters.

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