ZINC BIOFORTIFICATION IMPROVES YIELD, NUTRACEUTICAL QUALITY AND ANTIOXIDANT CAPACITY IN LETTUCE‡

[BIOFORTIFICACIÓN DE ZINC MEJORA EL RENDIMIENTO, CALIDAD NUTRACÉUTICA Y CAPACIDAD ANTIOXIDANTE DE LECHUGA]

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SUMMARY

Background: Zinc (Zn) is an important element in human health and is consumed through foods of animal origin. However, the biofortification of plants with Zn can be a strategy for the consumption of this micronutrient and to increase the morphology, physiology, and plant yield. Objective: Quantify the effect of Zn application on yield, nutraceutical quality and antioxidant activity of lettuce. Methodology: Foliar application of ZnSO₄ (0, 25, 50, 75 and 100 µM L⁻¹) on lettuce plants was made. Yield, nutraceutical quality and the concentration of Zn in the plant tissue was determinate. Results: The optimum Zn dose that maximized yield and nutraceutical quality, as well as the recommended consumption concentration in lettuce in this study was 75 µM L⁻¹ (ZnSO₄). Implications: Higher doses of Zn decreased bioactive compound biosynthesis. Conclusion: Zn biofortification is an alternative to increase phytochemical compound biosynthesis and yield with the possibility of improving public health. Keywords: Lactuca sativa L.; foliar fertilization; malnutrition.

RESUMEN

Antecedentes: El zinc (Zn) es un elemento importante en la salud humana y es consumido a través de alimentos de origen animal. Sin embargo, la biofortificación de las plantas con Zn puede ser una estrategia para el consumo de este micronutriente y para incrementar la morfología, fisiológica y rendimiento vegetal. Objetivo: Cuantificar el efecto de la aplicación de Zn en el rendimiento, calidad nutraceutica y actividad antioxidante de lechuga. Metodología: Se aplicó vía foliar ZnSO₄ (0, 25, 50, 75 y 100 µM L⁻¹) en plantas de lechuga. Se cuantificó el rendimiento, la calidad nutraceutica y la concentración de Zn en el tejido vegetal. Resultados: La dosis óptima de Zn que maximizó el rendimiento y la calidad nutraceutica, así como la concentración de consumo recomendada en lechuga en este estudio fue 75 µM L⁻¹ (ZnSO₄). Implicaciones: Dosis altas de Zn disminuyen la biosíntesis de compuestos bioactivos. Conclusión: La biofortificación con Zn es una alternativa para incrementar la biosíntesis y el rendimiento de compuestos fitoquímicos con la posibilidad de mejorar la salud pública. Palabras clave: Lactuca sativa L.; fertilización foliar; desnutrición.

† Submitted June 11, 2021 – Accepted July 23, 2021. This work is licensed under a CC-BY 4.0 International License.
ISSN: 1870-0462.
INTRODUCTION

Zinc (Zn) is an essential micronutrient for all organisms (Rivera-Martin et al., 2020) due to the various functions it performs within the immune, sensory, neurobehavioral development, reproductive health, growth, physical development, among others (Kumar et al., 2021). Despite its importance in human health, Zn deficiency affects 17.3% of the world population, which makes it the fifth risk factor for health in developing countries and the eleventh worldwide (World Health Organization, 2007). The National Institute of Health (NIH, 2020) points out that daily consumption of Zn in the human diet varies according to the growth stage (2-13 mg). Although this element is essential for plants (enzyme activator, involved in the transformation of carbohydrates, a source of energy in the production of chlorophyll, aid in the formation of auxins) (Reddy et al., 2019; Santis-Santis et al., 2019), its supplementation to crops is aimed at reducing the lack of this element; and since most of the foods rich in Zn derived from animal origin, there may be deficiencies of this element in the population.

Agronomic biofortification with numerous nutrients is considered a profitable and sustainable strategy to minimize micronutrient deficiencies in the population (Zou et al., 2019; De Groote et al., 2021); since it aims to improve the agronomic characteristics and increase the content of essential elements in the edible parts of plants, through application of these elements in edaphic and/or foliar pathways (Jha et al., 2020). Foliar fertilization with Zn is an effective method to obtain desirable concentrations of the element in crops intended for human consumption (Ram et al., 2016; Rehman et al., 2021), furthermore, Zn deficiency is one of the severe micronutrient deficiencies in most of the soils cultivated soils around the world (Noulas et al., 2018; Xie et al., 2019), its application in the plants increase the photosynthesis, antioxidant function, growth, yield and fruit quality through the improvement of plant metabolism (Gomez-Coronado et al., 2016). Zn is an important element in various enzymes that include transferases (Alam et al., 2020), lyases (Esra et al., 2018), isomerases (Chao et al., 2021) and ligases (Kud et al., 2019) and acts as cofactor of more than 300 proteins (Chasapis et al., 2020). The plants require Zn concentrations of their leaves greater than 15-30 mg kg⁻¹ dry matter for maximum yield, but excess levels of leaf Zn in the range of 100-700 mg kg⁻¹ dry matter cause growth inhibition (White and Brown, 2010; Gapa et al., 2016). The foliar application of Zn has increased the productivity of wheat (Zou et al., 2019), mungbean (Haider et al., 2020), tircitale (Dhalwal et al., 2019), cowpea bean (López-Morales et al., 2020), chikpea (Pal et al., 2021), rice (Zulfiqar et al., 2021), broccoli (Rivera-Martin et al., 2021), maize (Augustine and Kalyanasundaram, 2021), among others.

Lettuce (Lactuca sativa L.) is one of the most consumed leafy vegetables in the world, mainly in fresh, and is a base ingredient of many salads (SIAP, 2018; Medina-Lozano et al., 2021). Lettuce is rich in fiber, fatty acids, amino acids, vitamins A, C, E, B1, B2, and B3, proteins, and minerals (Cu, Al, Na, Mg, K and Ca) (Kim et al., 2016). In addition, it has phytochemical compounds (Sofo et al., 2016; Lee et al., 2021), which provide antioxidant properties (Zapata-Vahos et al., 2020). The micronutrients application through the biofortification of crops is a very useful tool not only to increase the amount of minerals, but also, the production of bioactive compounds is considerably improved. Therefore, the aim of this study was to determine the effect of biofortification with Zinc on yield, nutraceutical quality and antioxidant capacity in lettuce.

MATERIALS AND METHODS

Plant Material and Treatments

Lettuce seed (Lactuca sativa L.) "Parris Island cos" of Heirloom Seeds® were germinated in agricultural foamy plate, 40 days after sowing the seedlings were taken to a hydroponic system NFT and the crop nutrition was carried out using the Steiner nutrient solution (Steiner, 1961). The application of zinc sulfate (ZnSO₄.7H₂O) was performed in five foliar doses (0, 25, 50, 75 and 100 µM L⁻¹) proposed by Meneghelli et al. (2021). Applications were made with manual sprinklers during early hours in the morning, three sprayings were performed every 15 days after transplantation.

Sampling

Lettuces were harvested at 60 DAT, measured and weighted to determinate total growth and total crop yield. At the same time, samples were collected to quantify the biochemical variables and Zn content, obtaining five samples by each treatment.

Yield

In order to determine total crop yield, the lettuce heads were harvested and weighed on an analytical scale (Ohaus Corporation, Pine Brook, NJ, USA) to determine the fresh weight.

Proximate composition analysis

Ash quantification was conducted under the Official Mexican Standard (NOM, 1978), in a melting pot at a constant weight, for which 1 g of each sample was weighed and placed in a muffle furnace at 600°C for 24 h, until calcination. Ash results were expressed as a percentage and were estimated as follows: Ash (%) \(= (P − p) × 100/M,\) where \(P\) is the weight (g) of the melting pot with the ashes, \(p\) is the weight (g) of the empty melting pot, and \(M\) is the weight of the sample (g). The protein content was determined with the
Dumas method (Calvo et al., 2008) by placing 3 g of each sample in nickel capsules, to which 9 g of vanadium pentoxide ($V_2O_5$) was added and then introduced into the Flash 2000 kit (Thermo Scientific, Waltham, MA, USA). The protein concentration was expressed as a percentage (%).

The crude fiber content was determined according to the Official Mexican Norm (NOM, 1978) using a degreased sample, which was transferred to fiberglass papers. Then, 200 mL of 1.25% sulfuric acid ($H_2SO_4$) and 1 mL of isooamyl alcohol as antifoam were added, allowing the mixture to boil for 30 min. Next, the samples were washed to remove the $H_2SO_4$ and isooamyl alcohol residues, as well as to neutralize the mixture. Subsequently, 200 mL of 1.25% sodium hydroxide (NaOH) was added, and the sample was boiled for 30 min. At the end of that time, the sample was washed in fiberglass until becoming neutral. Then, the fiberglass with the sample was placed in a capsule, put in an oven, and allowed to dry for 12 h. Subsequently, the capsule with the fiberglass and the sample were weighed, and the percentage of fiber was determined by the weight difference.

### Nutrient Content Analysis

The micronutrient content was determined by triacid digestion. One gram of each sample was weighed on an analytical balance (HR-120), with an accuracy of 0.0001 g. The sample was then placed in a 250 mL beaker with boiling beads, and 25 mL of triazide mixture (1 L of HNO$_3$, 100 mL of HCl, 25 mL of $H_2SO_4$) was added. Following this, the sample was placed in a digester grill in a fume hood for one hour. At the end, the resulting samples were filtered into 50 mL volumetric flasks (stock solution), gauged, and stirred with triple distilled water. Finally, samples were poured into 50 mL tubes to centrifuge them. The concentrations of Zn, Mn, Cu, Fe and Ni were determined by atomic absorption spectrophotometry (AAS, iCE 3000 Series, Thermo Scientific, Waltham, MA, USA), and the results were expressed in ppm. Macronutrients (K, Ca and Mg) were determined by atomic absorption spectrophotometry (AAS, iCE 3000 Series, Thermo Scientific, Waltham, MA, USA), by reporting the concentration in percentage. Phosphorus (P) was determined by the colorimetric method of ammonium metavanadate (NH$_4$VO$_3$) in an absorption range of 430 nm against a K$_2$HPO$_4$ calibration curve. In total, 3.5 mL of distilled water, 500 L of the stock solution, and 1 mL of phosphorus reagent (P) were added to test tubes. Each tube was shaken in a vortex and allowed to stand for one hour. At the end, the reading was performed on a visible light spectrophotometer (Spectrophotometer, Genesis 10s UV/Vis, Thermo Scientific, Waltham, MA, USA). The P concentration was expressed as a percentage.

### Bioactive Compounds

For ethanolic extracts, 100 g de fresh lettuce pulp per treatment were ground and used to assess nutraceutical quality of the lettuce. 1 g of sample was placed in a 15 mL Falcon tube and 10 mL of ethanol reagent grade was added. After 1 min of stirring in Vortex, they were allowed to rest for 24 h. The ethanolic extracts were subsequently decanted at 3,500 × g and supernatant were transferred to a Falcon tube and stored at -20°C until use.

Total phenolic content was determined using a modification of the Folin-Ciocalteau method (Garcia-Nava, 2009). 150 µL of ethanolic extract were taken, diluted in 3 mL of water (milli-Q), 250 µL of Folin-Ciocalteau reagent (1N) was added, stirred and left in reaction for 3 min. Subsequently 750 µL of Na$_2$CO$_3$ (20%) and 950 µL of water (milli-Q) were added. The solution was allowed to stand for 2 h and the samples were quantified in a UV-Vis spectrophotometer at 760 nm. The standard was prepared with gallic acid. The results were expressed in mg GAE/100 g$^{-1}$ fresh weight. Total flavonoids were determined by colorimetry (Garcia-Nava, 2009). 200 µL of ethanolic extract were taken, mixed with 1.25 mL of water (milli-Q) and 75 µL of NaNO$_2$ (5%). After 5 min of rest, 150 µL of AlCl$_3$ were added. Subsequently, 500 µL of NaOH (1M) and 275 µL of water (milli-Q) were added. It was vigorously stirred and the samples were quantified in a UV-Vis spectrophotometer at 510 nm. The standard was prepared with quercetin dissolved in absolute ethanol ($y = 0.0122x - 0.0067$; $r^2 = 0.965$). The results were expressed in mg QE/100 g$^{-1}$ fresh weight. Total antioxidant capacity was measured by the in-vitro DPPH$^+$ method (Brand-Williams et al., 1995). A DPPH$^+$ solution (Aldrich, St. Louis, Missouri, USA) in ethanol was prepared, at 0.025 mg mL$^{-1}$ concentration. 700 µL of ethanolic extract were mixed with 1,300 µL of DPPH$^+$ solution, after 30 min the samples were quantified in a UV-Vis spectrophotometer at 517 nm. The results were expressed in µM equivalent in Trolox/100 g$^{-1}$ fresh weight.

### Statistical Analysis

The experimental design was completely randomized with six replicates per treatment on each variable, with one plant considered as an experimental unit. All variables measured as explained above were evaluated by one-way ANOVA by the GLM method of SAS statistical package version 9.1 (SAS Institute, 2009). Tukey simultaneous test was used for comparing statistical means ($P < 0.05$).
RESULTS AND DISCUSSION

Yield

In lettuce yield (weight), an increase of 46% was observed with the highest dose of Zn compared to the treatment without Zn (Table 1). These results are due to the Zn stimulate a greater absorption of nitrogen and therefore production of greater biomass. Hulagur and Dangarwala (1983), showed that the N application influences the Zn absorption, improving yield. Other reports indicate increases in yield with the foliar application of Zn in various crops such as Lactuca sativa L. (Yuri et al., 2006), Ocimum basilicum L. (Abbasifar et al., 2020), Brassica oleracea var. italica L. (Rivera-Martín et al., 2020), Fragaria × ananassa Duch (López-Herrera et al., 2018). This effect is attributed to the Zn is a nutrient strongly related to plant growth, since it is necessary for the synthesis of nucleic acids, proteins and carbohydrates (Marschner and Rengel, 2012). Likewise, it is part of different processes such as photosynthesis and respiration, which ensures higher yield (Ramzan et al., 2020).

Table 1. Effect of foliar application of ZnSO₄ on lettuce weight and proximal composition.

<table>
<thead>
<tr>
<th>ZnSO₄ (µM L⁻¹)</th>
<th>Weight g</th>
<th>Ash %</th>
<th>Protein %</th>
<th>Crude fiber %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>294.80±34ᵃᵇ</td>
<td>23.91±0.02ᵃ</td>
<td>14.55±0.02ᵈ</td>
<td>16.63±0.02ᵇ</td>
</tr>
<tr>
<td>25</td>
<td>412.67±17ᵃᵇ</td>
<td>21.93±0.01ᵇ</td>
<td>16.16±0.03ᵇ</td>
<td>16.27±0.02ᶜ</td>
</tr>
<tr>
<td>50</td>
<td>453.60±53ᵃᵇ</td>
<td>19.92±0.02ᵈ</td>
<td>16.26±0.02ᵇ</td>
<td>17.04±0.06ᵃ</td>
</tr>
<tr>
<td>75</td>
<td>488.37±79ᵃᵇ</td>
<td>18.90±0.05ᵃ</td>
<td>16.44±0.03ᵃ</td>
<td>14.75±0.03ᵃ</td>
</tr>
<tr>
<td>100</td>
<td>551.87±12ᵃ</td>
<td>20.67±0.02ᶜ</td>
<td>16.13±0.02ᵇ</td>
<td>15.19±0.03ᵈ</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviation (SD). Different letters indicate significant difference (P < 0.05) according to Tukey’s test.

Table 2. Effect of the foliar application of ZnSO₄ on the mineral content of the lettuce crop.

<table>
<thead>
<tr>
<th>ZnSO₄ (µM L⁻¹)</th>
<th>P %</th>
<th>K %</th>
<th>Ca %</th>
<th>Mg %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.295±0.02ᵃᵇ</td>
<td>11.17±3.27ᵇ</td>
<td>0.746±0.05ᵃ</td>
<td>0.146±0.02ᵃ</td>
</tr>
<tr>
<td>25</td>
<td>0.291±0.05ᵇ</td>
<td>9.82±0.73ᶜ</td>
<td>0.710±0.02ᵇ</td>
<td>0.148±0.03ᵇ</td>
</tr>
<tr>
<td>50</td>
<td>0.269±0.03ᵇ</td>
<td>11.67±0.93ᵇ</td>
<td>0.473±0.13ᵇ</td>
<td>0.114±0.03ᵇ</td>
</tr>
<tr>
<td>75</td>
<td>0.279±0.04ᵃᵇ</td>
<td>13.01±0.08ᵃᵇ</td>
<td>0.561±0.06ᵇ</td>
<td>0.106±0.04ᵇ</td>
</tr>
<tr>
<td>100</td>
<td>0.282±0.03ᵇ</td>
<td>8.20±1.45ᵇ</td>
<td>0.577±0.09ᵇ</td>
<td>0.079±0.05ᵇ</td>
</tr>
</tbody>
</table>

Table 3. Effect of the foliar application of ZnSO₄ on the proximal composition of lettuce crops.

<table>
<thead>
<tr>
<th>ZnSO₄ (µM L⁻¹)</th>
<th>Fe mg kg⁻¹</th>
<th>Mn mg kg⁻¹</th>
<th>Ni mg kg⁻¹</th>
<th>Cu mg kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>304.38±26ᵃ</td>
<td>142.74±13ᵃ</td>
<td>4.9±0.11ᵃᵇ</td>
<td>5.78±0.72ᵇ</td>
</tr>
<tr>
<td>25</td>
<td>138.70±80ᵃ</td>
<td>142.46±40ᵃ</td>
<td>4.2±0.21ᵇ</td>
<td>6.64±0.6ᵃ</td>
</tr>
<tr>
<td>50</td>
<td>133.83±14ᵃ</td>
<td>110.42±14ᵃᵇ</td>
<td>5.1±0.22ᵇ</td>
<td>5.64±0.5ᵃ</td>
</tr>
<tr>
<td>75</td>
<td>135.34±17ᵃ</td>
<td>107.35±28ᵃᵇ</td>
<td>4.8±0.32ᵃᵇ</td>
<td>5.64±0.6ᵃ</td>
</tr>
<tr>
<td>100</td>
<td>210.35±20ᵇ</td>
<td>123.92±12ᵃ</td>
<td>5.3±0.24ᵃ</td>
<td>5.82±0.71ᵇ</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviation (SD). Different letters indicate significant difference (P < 0.05) according to Tukey’s test.

Proximal composition

Ash percentage, which represents the mineral content of the food, a decrease was quantified in the treatments where Zn was applied, in relation to the treatment without Zn (Table 1). Regarding proteins and fiber, an increase in protein was observed in plants treated with 75 and 50 µM L⁻¹ of ZnSO₄ for crude fiber content with respect to the treatment without Zn. Similar results are reported by Yuan et al. (2016), who showed that biofortification with Zn in pea shoots improves the nutritional quality determined by the content of crude fiber, protein and content of soluble sugars. Estrada-Dominguez et al. (2018), also report an improvement in the nutritional quality of beans (Vigna unguiculata L) after the foliar application of Zn. In lettuce, adequate Zn fertilization increases the concentration of Zn in the tissue, amino acids and proteins, all with beneficial properties for human health (Barrameda-Medina et al., 2016). However, higher doses of Zn can cause oxidative stress to the plant, which increases the production of active oxygen species, which damages cellular components and protein production (Imran et al., 2016; Hosseini et al., 2021).
Figure 1. Effect of different doses of ZnSO$_4$ on the content of total phenols (a), total flavonoids (b), antioxidant capacity (c), and Zn concentration (d) in lettuce. Columns with different letters were significantly different according to Tukey’s test ($P < 0.05$).

**Nutrient Content**

Excess of Zn application can inhibit the absorption of other nutrients such as Fe, Mg, and Mn (Amezcua-Romero and Lara-Flores, 2017). The effect before mentioned was reflected in the mineral content of the lettuces evaluated (Table 2). The total concentration of Fe, Mn, Mg and Ca, showed a significant decrease in comparison to the control treatment. Likewise, an increase in the absorption of N was observed, because Zn is related to the metabolism of N in the plant, since it is correlated with the activity of the enzyme nitrate reductase (Marschner and Rengel, 2012; Sun et al., 2020). Zn deficiency or toxicity has been shown to inhibit the enzyme nitrate reductase, leading a decrease in N content (Luna et al., 2000) and a decrease in the incorporation of N in amino acids and proteins (Sutter et al., 2002). Hulagur and Dangarwala (1983) showed that N influences in the absorption of Zn by plants and vice versa, in fact both nutrients have a synergistic effect and higher yields are obtained.

The zinc content of the lettuce leaves was influenced by the dose used. Higher doses of zinc application promote increases the zinc content of the leaf (Figure 1d). According to the NIH (2020), the daily intake of Zn for adults is an average of 15 mg, based on this value, the foliar application of Zn increases its content in lettuce, in addition, the applied doses do not cause an accumulation of Zn resulting toxic for consumption because its content is within the limit (21-70 mg kg$^{-1}$) that can be consumed by people without harming health (World Health Organization, 2006). Zinc is found in many foods, especially those in the animal kingdom and often these sources of Zn are not accessible to low-income people; therefore, the biofortification lettuce is a good alternative to meet nutritional needs by humans daily.

**Bioactive Compounds**

Foliar application of Zn, promoted an increase in the biosynthesis of bioactive compounds, obtaining the highest values of these metabolites with the dose of 75 µM L$^{-1}$ (Figure 1a-1d).

Foods Production rich in phytochemicals is desirable since these compounds delay oxidation and degradation of lipids which increase the nutritional quality of food (Schiavon et al., 2020) and its consumption is helpful for human health (Gupta and Gupta, 2017); due to its potential to protect people against reactive oxygen species (Wilson et al., 2006).
Higher doses cause a decrease of these compounds, because depending on the form and dose, Zn can act as an antioxidant or as a toxic agent (Estrada-Domínguez et al., 2020). Yuan et al. (2016) indicate that Zn improves the biosynthesis of compounds because they increased the zinc content, chlorophyll, phenolic compounds and amino acids, so that biofortification with zinc could increase the nutritional quality and antioxidant activity of crops. In the same way Zhu et al. (2013) and Sida et al. (2017) have shown that biofortification with zinc improves the biosynthesis of bioactive compounds, but the improvement depends on the abundance of this ion. Other studies have also reported that the foliar application of Zn increases the content of bioactive compounds (Abbasifar et al., 2020; Młynarczyk et al., 2021).

CONCLUSION

Agronomic biofortification with zinc applied via foliar improved yield, nutraceutical quality, antioxidant capacity and Zn concentration in lettuce. The optimum dose that maximized yield and nutraceutical quality, as well as the recommended Zn consumption concentration in lettuce in this study was 75 µM L⁻¹ of Zn (ZnSO₄) since higher doses decrease bioactive compound biosynthesis in lettuce. Foliar zinc biofortification in lettuce is an effective tool for increasing the zin content and thus helping to combat micronutrient malnutrition.

Acknowledgements

Adriel Campos Ortiz thank the National Council for Science and Technology of Mexico (CONACYT) for supporting his graduate studies.

Funding. This research was funded by the Tecnológico Nacional de México/ Instituto Tecnológico de Torreón, Coahuila, México.

Conflict of Interests. The authors declare that there are no conflicts of interest related to this article.

Compliance with ethical standards. Does not apply.

Data availability. Data are available with <Pablo Preciado Rangel, ppreciado@gmail.com> upon reasonable request.

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