



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

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

Pablo Preciado-Rangel<sup>1</sup>, Adriel Campos-Ortiz<sup>1</sup>, Esteban Sánchez-Chávez<sup>2</sup>, Arturo Reyes-Gonzalez<sup>3</sup>, Francisco Higinio Ruiz-Espinoza<sup>4</sup>, Dámaris Ojeda-Barrios<sup>5</sup> and Luis Guillermo Hernandez-Montiel<sup>6\*</sup>

<sup>1</sup>Tecnológico Nacional de México/ Instituto Tecnológico de Torreón, Carr. Torreón – San Pedro, km 7.5, Ejido Ana, Torreón, Coahuila, México. C.P. 27170

<sup>2</sup>Centro de Investigación en Alimentación y Desarrollo, Av. Cuarta Sur 3820, Fracc, Vencedores del Desierto, Delicias, Chihuahua, México. C.P. 33089.

<sup>3</sup>Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental La Laguna, Blvd. Jose Santos Valdez 1200 Pte. Col. centro, Coahuila, México. C.P. 27440

<sup>4</sup>Universidad Autónoma de Baja California Sur, Carr. al Sur km 5.5. Apdo. 19-B. La Paz, Baja California Sur, México. C.P. 23000

<sup>5</sup>Facultad de Ciencias Agrotecnológicas, Universidad Autónoma de Chihuahua, Av. Pascual Orozco s/n. Campus 1, Chihuahua, México. C.P. 31310

<sup>6</sup>Centro de Investigaciones Biológicas del Noroeste, Av. Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz, Baja California Sur, México. C.P. 23096. Email. lhernandez@cibnor.mx

\*Corresponding author

#### 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 ZnSO<sub>4</sub> (0, 25, 50, 75 and 100 μM L<sup>-1</sup>) 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<sup>-1</sup> (ZnSO<sub>4</sub>). **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 ZnSO<sub>4</sub> (0, 25, 50, 75 y 100 μM L<sup>-1</sup>) en plantas de lechuga. Se cuantifico 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<sup>-1</sup> (ZnSO<sub>4</sub>). **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.

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## 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 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<sup>-1</sup> dry matter for maximum yield, but excess levels of leaf Zn in the range of 100-700 mg kg<sup>-1</sup> dry matter cause growth inhibition (White and Brown, 2010; Gupa *et al.*, 2016). The foliar application of Zn has increased the productivity of wheat (Zou *et al.*, 2019), mungbean (Haider *et al.*, 2020), triticale (Dhaliwal *et al.*, 2019), cowpea bean (López-Morales *et al.*, 2020), chickpea (Pal *et al.*, 2021), rice (Zulficar *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 (Sofa *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<sup>®</sup> 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 (ZnSO<sub>4</sub>·7H<sub>2</sub>O) was performed in five foliar doses (0, 25, 50, 75 and 100 μM L<sup>-1</sup>) 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 (%) =  $(P - p) \times 100/M$ , 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 isoamyl 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 isoamyl 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 triacid 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 ( $NH_4VO_3$ ) in an absorption range of 430 nm against a  $K_2HPO_4$  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  $3,500 \times g$  and supernatant were transferred to a Falcon tube and stored at  $-20^\circ C$  until use.

Total phenolic content was determined using a modification of the Folin-Ciocalteu method (Garcia-Nava, 2009). 150  $\mu L$  of ethanolic extract were taken, diluted in 3 mL of water (milli-Q), 250  $\mu L$  of Folin-Ciocalteu reagent (1N) was added, stirred and left in reaction for 3 min. Subsequently 750  $\mu L$  of  $Na_2CO_3$  (20%) and 950  $\mu L$  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  $\mu L$  of ethanolic extract were taken, mixed with 1.25 mL of water (milli-Q) and 75  $\mu L$  of  $NaNO_2$  (5%). After 5 min of rest, 150  $\mu L$  of  $AlCl_3$  were added. Subsequently, 500  $\mu L$  of NaOH (1M) and 275  $\mu L$  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<sup>+</sup> method (Brand-Williams *et al.*, 1995). A DPPH<sup>+</sup> solution (Aldrich, St. Louis, Missouri, USA) in ethanol was prepared, at 0.025 mg  $mL^{-1}$  concentration. 700  $\mu L$  of ethanolic extract were mixed with 1,300  $\mu L$  of DPPH<sup>+</sup> solution, after 30 min the samples were quantified in a UV-Vis spectrophotometer at 517 nm. The results were expressed in  $\mu M$  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 stimulated 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-Martin *et al.*, 2020), *Fragaria x 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).

## 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  $\mu\text{M L}^{-1}$  of  $\text{ZnSO}_4$  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).

**Table 1. Effect of foliar application of  $\text{ZnSO}_4$  on lettuce weight and proximal composition.**

ZnSO <sub>4</sub> ( $\mu\text{M L}^{-1}$ )	Weight g	Ash ----- % -----	Protein %	Crude fiber
0	294.80±34 <sup>b*</sup>	23.91±0.02 <sup>a</sup>	14.55±0.02 <sup>d</sup>	16.63±0.02 <sup>b</sup>
25	412.67±17 <sup>ab</sup>	21.93±0.01 <sup>b</sup>	16.16±0.03 <sup>b</sup>	16.27±0.02 <sup>c</sup>
50	453.60±53 <sup>ab</sup>	19.92±0.02 <sup>d</sup>	16.26±0.02 <sup>b</sup>	17.04±0.06 <sup>a</sup>
75	488.37±79 <sup>ab</sup>	18.90±0.05 <sup>e</sup>	16.44±0.03 <sup>a</sup>	14.75±0.03 <sup>e</sup>
100	551.87±12 <sup>a</sup>	20.67±0.02 <sup>c</sup>	16.13±0.02 <sup>b</sup>	15.19±0.03 <sup>d</sup>

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

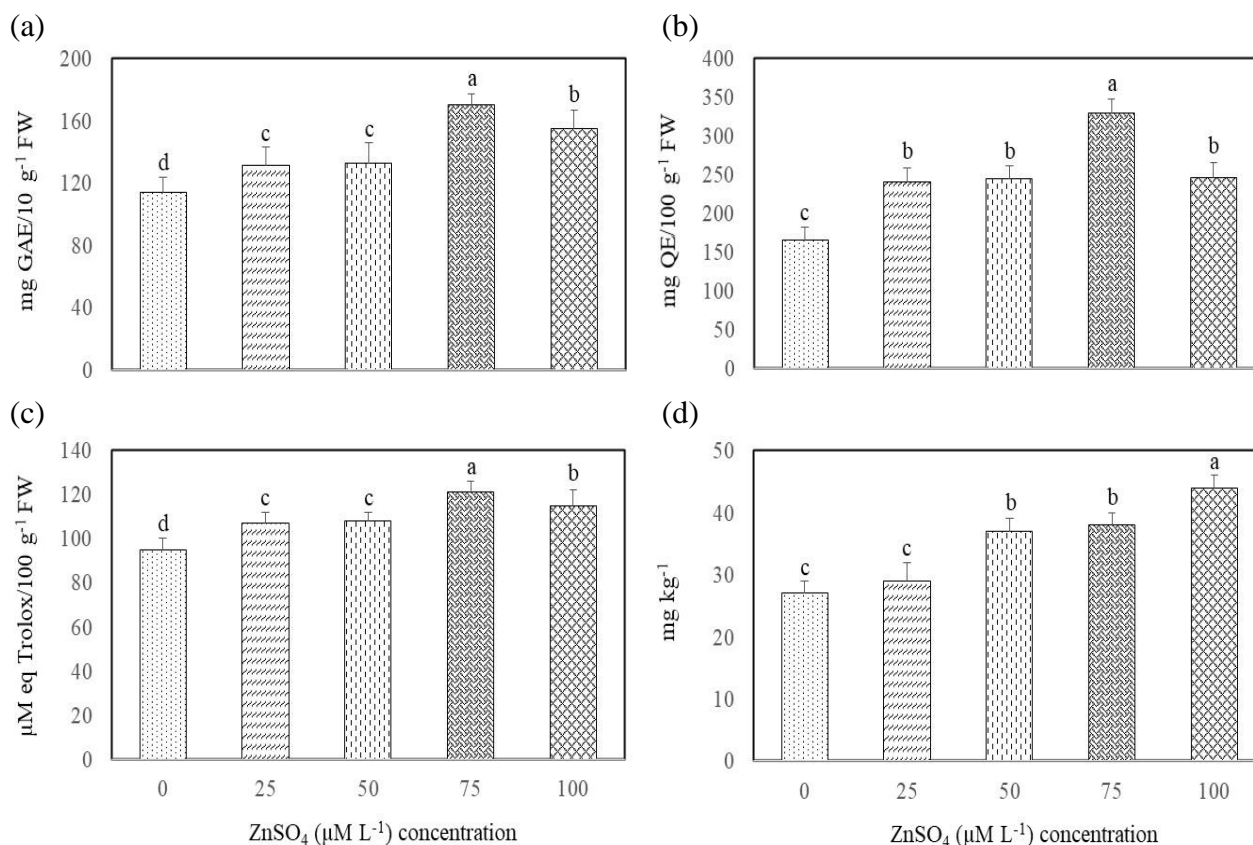
**Table 2. Effect of the foliar application of  $\text{ZnSO}_4$  on the mineral content of the lettuce crop.**

ZnSO <sub>4</sub> ( $\mu\text{M L}^{-1}$ )	P ----- % -----	K ----- % -----	Ca ----- % -----	Mg ----- % -----
0	0.295±0.02 <sup>a*</sup>	11.17±3.27 <sup>b</sup>	0.746±0.05 <sup>a</sup>	0.146±0.02 <sup>a</sup>
25	0.291±0.05 <sup>a</sup>	9.82±0.73 <sup>c</sup>	0.710±0.02 <sup>ab</sup>	0.148±0.03 <sup>a</sup>
50	0.269±0.03 <sup>d</sup>	11.67±0.93 <sup>b</sup>	0.473±0.13 <sup>b</sup>	0.114±0.03 <sup>ab</sup>
75	0.279±0.04 <sup>c</sup>	13.01±0.08 <sup>a</sup>	0.561±0.06 <sup>ab</sup>	0.106±0.04 <sup>ab</sup>
100	0.282±0.03 <sup>b</sup>	8.20±1.45 <sup>d</sup>	0.577±0.09 <sup>ab</sup>	0.079±0.05 <sup>b</sup>

ZnSO <sub>4</sub> ( $\mu\text{M L}^{-1}$ )	Fe ----- mg kg <sup>-1</sup> -----	Mn ----- mg kg <sup>-1</sup> -----	Ni ----- mg kg <sup>-1</sup> -----	Cu ----- mg kg <sup>-1</sup> -----
0	304.38±26 <sup>a</sup>	142.74±13 <sup>a</sup>	4.9±0.11 <sup>ab</sup>	5.78±0.72 <sup>b</sup>
25	138.70±80 <sup>c</sup>	142.46±40 <sup>a</sup>	4.2±0.21 <sup>b</sup>	6.64±0.6 <sup>a</sup>
50	133.83±14 <sup>c</sup>	110.42±14 <sup>ab</sup>	5.1±0.22 <sup>ab</sup>	5.64±0.51 <sup>c</sup>
75	135.34±17 <sup>c</sup>	107.35±28 <sup>b</sup>	4.8±0.32 <sup>ab</sup>	5.64±0.65 <sup>c</sup>
100	210.35±20 <sup>b</sup>	123.92±12 <sup>ab</sup>	5.3±0.24 <sup>a</sup>	5.82±0.71 <sup>b</sup>

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



**Figure 1.** Effect of different doses of ZnSO<sub>4</sub> 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 (Amezcu-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<sup>-1</sup>) 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<sup>-1</sup> (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.*,

2017). 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.*, 2020).

### 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  $\mu\text{M L}^{-1}$  of Zn ( $\text{ZnSO}_4$ ) since higher doses decrease bioactive compound biosynthesis in lettuce. Foliar zinc biofortification in lettuce is an effective tool for increasing the zinc content and thus helping to combat micronutrient malnutrition.

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**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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