Universidad de Granada

Facultad de Ciencias

Departamento de Fisiología Vegetal

ESTUDIO DE DIFERENTES ASPECTOS AGRONÓMICOS Y FISIOLÓGIOS DEL ZINC EN PLANTAS HORTÍCOLAS: FITOEXTRACCIÓN Y BIOFORTIFICACIÓN

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Memoria de Tesis doctoral presentada por la licenciada en Biología

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Granada a Febrero de 2016

Director de la Tesis

Fdo. Juan Manuel Ruiz Sáez

Doctoranda

Fdo. Yurena Barrameda Medina

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Aunque este Universo poseo, nada poseo,

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si me aferro a lo conocido.

Robert Fisher (El Caballero de la Armadura Oxidada)

Indice

INDICE GENERAL

Capítulo 1

Resumen

Zn has been recognized as an essential element for growth, development and differentiation of all types of life, including microorganisms, plants and animals. This is involved in numerous physiological processes as are the activation of enzymes, protein synthesis, metabolism of carbohydrates, lipids, nucleic and auxins acids, structural and functional integrity of biomembranes, gene expression and regulation and the reproductive development (formation pollen). However, this can become toxic beyond a threshold range of soil from 100 to 400 mg kg^{-1}. Under these conditions, the Zn excess accumulates in plant tissues causing physiological alterations, growth inhibition and even reaching cause death reaching of them. Therefore, plants have developed a series of physiological and molecular to deal with different types of stress mechanisms as well as the toxic effects of metals or metalloids, maintaining metabolic homeostasis and deadening the generation of reactive oxygen species (ROS).

Furthermore, approximately 30% of the cultivated land in the world is deficient in Znso producing an alteration in various physiological processes, causing rapid growth inhibition, development and final yield of the plant. Consequently, most cultivated crops contain low concentrations of Zn which can lead to increased Zn malnutrition in humans. Recently, the Biofortification has been proposed as a way to improve the nutritional quality of food plant, and is defined as the process of increasing the bioavailable concentration of essential elements such as Zn, in the edible parts of crops, through agricultural practices or genetic selection.

Therefore, the fundamental objectives of this thesis were as follows:

1. Carrying out a study of the different strategies in plants *Lactuca sativa* and *Brassica oleracea* to the toxicity of Zn (0.5 mM), such as carboxylates metabolism and oxidative metabolism and glutathione as well as osmoprotectans compounds in order to define key physiological processes to select and / or generate resistant Zn toxicity of plants and horticultural plants using these programs decontamination (phytoextraction) in areas contaminated by this trace element.

2. Considering that the two horticultural plants used in this research are widely used in biofortification programs with trace elements, the aim of the second part of this thesis will be conducting a program of agronomic biofortification with Zn consisting of a supraoptimal application this element (10-100 uM) in order to analyze: the accumulation of Zn in the edible part of the plant and check the response of certain parameters of nutrirtional quality and NO_3^- assimilation in *L. sativa* and the synthesis and accumulation of bioactive compounds in *B. oleracea.*

- Comparative study of the toxic effect of Zn in *Lactuca sativa* and *Brassica oleracea* plants: I. Growth, distribution, and accumulation of Zn, and mebolism of carboxylates.

Zinc (Zn) is an essential micronutrient for plants, animals, and microorganisms. However, in environmental situations of heavy-metal soil pollution, Zn constitutes a major problem for worldwide agricultural production. Organic anions are compounds that have a special structure which proves indispensable to tolerate excess Zn. The aim of the present work was to determine whether carboxylate metabolism is a key physiological process to select and/or generate plants tolerance to Zn toxicity (0.5 mM). For this, we make a comparative analysis of the toxic effect of Zn between two horticultural plants of great agricultural interest, i.e. *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. The tolerance under Zn-toxicity conditions was greater in *B. oleracea* than in *L. sativa* despite to show a higher Zn concentration in shoot. Regarding organic anions metabolism, in leaves of *L. sativa* the enzyme malate dehydrogenase (MDH) notably increased its activity under Zn-toxicity conditions and both under control conditions as well as in Zn toxicity the main organic anion was malate. With respect to *B. oleracea* under Zn-toxicity conditions the MDH activity declined with respect to control increasing malate levels in leaves, also under control as well as toxicity conditions the citrate synthase (CS) activity was high and the predominant organic anion was citrate. These results suggest that both in programmes of phytoextraction as well as in biofortification with Zn that are based on the fertilization enriched with this element, *B. oleracea* is more effective than L. sativa and that the organic anion citrate could be determinant in the tolerance and greater concentration of this element in leaves.

- Role of GSH homeostasis under Zn toxicity in plants with different Zn tolerance.

Tripepthide glutathione (GSH) is a pivotal molecule in tolerance to heavy metals, including Zinc (Zn). The aim of our work is to examine the role of GSH metabolism in two different horticultural plants under Zn toxicity in order to select and/or generate plants tolerant to Zn toxicity. We show a comparative analysis of the toxic effect of 0.5 mM Zn between *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. In *L. sativa* the accumulation of Zn resulted in an increase in reactive oxygen species (ROS), while enzymes of GSH metabolism and the activities of the antioxidant enzymes were negatively affected. On the contrary, *B. oleracea* showed the existence of a detoxification mechanism of these ROS. Moreover, while in *L. sativa* increased the oxidized GSH (GSSG) and phytochelatins (PCs) concentration with the reduction of leaves biomass, in *B. oleracea* the higher concentration of reduced GSH and its use in the detoxification of ROS seems to be a major mechanism to provide tolerance to Zn toxicity without reducing leaf biomass. Our results suggested that under Zn toxicity, *B. oleracea* is more efficient and tolerant than *L. sativa* through the detoxification of lipid peroxidation products due to the reduced GSH.

- Role of some nitrogenous compounds protectors in the resistance to zinc toxicity in Lactuca satica cv. Phillipus and Brassica oleracea cv. Bronco

Zinc (Zn) pollution in the soil represents a major problem for crop production worldwide. In the present work, two horticultural plants exhibiting different tolerance to Zn, *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco, were exposed to Zn to evaluate the contribution of compatible osmolytes such as proline (Pro), glycine betaine (GB) and ɣ-aminobutyric acid (GABA) in the mechanism (s) of tolerance to Zn stress. This study confirms the higher susceptibility of *L. sativa* to Zn stress: lettuce plants experienced a strong reduction in biomass, while the levels of Pro and GB increased. These results suggest that in *L. sativa*, the increase of Pro and GB does not represent a mechanism of resistance to toxicity, but it is likely a symptom of Zn stress. Conversely, in *B. oleracea*, a slight decrease in Pro levels, mainly catalysed by degradation through proline dehydrogenase, was observed; a similar behaviour affected GB levels. On the other hand, GABA synthesis was slightly, but significantly, increased. The presence of high levels of GABA in Zn-stressed *B. oleracea* would suggest that reactive oxygen species detoxification could be essential to improve the resistance to toxicity under metal stress conditions.

The data obtained in this fourth chapter of Doctoral Thesis show as an increase in Zn induced a differential response in plants *L. sativa* and *B. oleracea*. Our results suggest that in both programs phytoextraction as biofortification programme with Zn, *L. sativa* is less effective than *B. oleracea*:

In *L. sativa* an increase of the MDH and CS activities and high levels of malate not seem to promote tolerance in this species. Likewise, the activities of enzymes and antioxidant compounds not appear to be a powerful mechanism involved in the mechanisms of tolerance. In addition, in this species the accumulation of PCs seems to be rather a survival mechanism that tolerance. Finally, the accumulation of compounds such as Pro or GB, seems to be a symptom of toxicity than an induction Zn promoted to provide tolerance to Zn in this species. Based on our results we can define *L. sativa* as species susceptible to the toxicity of Zn due to an accumulation of Zn in leaves resulted in a reduction in the biomass of this organ.

In *B. oleracea* a decrease of the MDH activity as well as high activity of the enzyme CS was accompanied by a high concentration of organic anion citrate, which could be decisive in tolerance and greater accumulation of Zn the leaves of *B. oleracea*. Furthermore, accumulation of reduced GSH and its use in the detoxification of ROS, through the induction of enzymes, such as APX, GST, and GPX, and the detoxification of toxic ompounds, such as methylglyoxal, by increasing activities Gly I and Gly II, appear to be a key mechanism to provide tolerance to Zn toxicity in this species. However, Zn stress in *B. oleracea* produced an increase in the Pro degradation and an increase in the GABA levels. These two processes resulted in improved of Zn tolerance in *B. oleracea* and suggest that the ROS detoxifying under Zn stress may be essential to provide tolerance to metals. Based on these results, we can define *B. oleracea* as a species less sensitive than *L. sativa* to Zn toxicity, since an increase of Zn did not affect negatively the biomass of the aerial part due to the chelation and transport of this trace element to the aerial part, as well as a powerful detoxification of ROS by increased production of antioxidant compounds.

In short, we can conclude that when carrying out a program of phytoextraction of Zn, *B. oleracea* be more efficient than *L. sativa*, accumulating a greater amount of this element in the aerial part without diminishing its biomass. Furthermore, these data reveal that both metabolism carboxylates, glutathione or osmoprotectants accumulation compounds such as GABA, are key processes in the tolerance in non-hyperaccumulator plants, as *B. oleracea*.

- Zinc biofortification, antioxidant properties and ionomic profile in two leafy vegetables: Lactuca sativa and Brassica oleracea

Zn deficiency is considered as a major risk factor for human health. Here, the effect of Zn biofortification a complementary solution for mineral malnutrition, were examined in two leafy vegetables, *Lactuca sativa* and *Brassica oleracea* in order to select Zn-efficient plants. Zn supply did not affected the biomass, but the concentration of malondialdehyde increased in both plants. Zn-use efficiency increased with respect to Zn concentration, total Zn accumulation (TZnA), and Zn utilization efficiency (ZnUtE). Nevertheless, Zn-use efficiency in *L. sativa* was lower than in *B. oleracea*. *L. sativa* showed lower levels of phenolic compounds, and reduced ascorbate, reduced glutathione and γ-aminobutyric acid than in *B. oleracea*. Furthermore, both plants had higher concentrations of Ca, Mg, Fe, and Mn, especially upon 20 μM Zn in *L. sativa* and 10-20 μM in *B. oleracea*. Our results indicate that the application of 20 μM Zn in *L. sativa* and 20-80 μM Zn in *B. oleracea* promoted the Zn accumulation and improved the leaf essential-nutrient quality for human health. Nevertheless, in *B. oleracea* higher Zn concentration, TZnA, phenolics, antioxidants and nutrients concentrations were observed. *B. oleracea* is emerging as a better choice and more efficient candidate in

Zn-biofortification programs. The best approach to increase human Zn levels is to encourage greater consumption of both green leafy vegetables, especially *B. oleracea*.

- Zn-biofortification enhanced nitrogen metabolism and photorespiration process in a green leafy vegetable: Lactuca sativa L.

Excessive rates of N fertilizers may result in elevated concentrations of nitrate (NO_3^-) and high NO_3^- concentrations are accumulated in the edible parts of leafy vegetables, as lettuce. The main objective of this work was to determine whether the NO_3^- accumulation and the nitrogen use efficiency (NUE) was affected by the application of different dosages of Zn, to ascertain the influence of this trace element in a biofortification programme in *Lactuca sativa* cv. Phillipus plants. For that, we analyzed the effect of Zn on NO_3^- assimilation, photorespiration, and the final products of those processes. Zn-doses in a range of 80-100 µM produced an increase on Zn concentration, provoking a decrease of NO_3^- since an increase of this element were sufficient to promote the nitrogen assimilation by an increase of the nitrate reductase (NR), glutamine synthetase (GS), aspartate aminotransferase (AAT), and the photorespiration processes. As result, we observed an increase on reduced N, total N concentration (TNC) and N utilization efficiency (NUtE). Consequently, under 80 µM-Zn the amino acid concentration increased significantly, include the concentration of amino acid essential for human health, ie; Leu, Ile and Thr. Adequate Zn fertilization is an important critical player in lettuce, making it possible to increase lettuce productivity

and quality, especially with 80 µM Zn, and could decrease the level of Zn deficiency such as the toxic level of NO_3^- in human health.

- Improved of amino acid profile and phytochemical content in Brassica oleracea cv. Bronco under a Zn-biofortification programme.

Millions of hectares of cropland are affected by Zinc (Zn) deficiency and approximately one third of the human population suffers from an inadequate intake of Zn. The main aim of the current study was to determine the potential effect of a Zn-biofortification programme on the Zn concentration, the amino acid profile and the phytochemicals content in a green leafy vegetable, such as *Brassica oleracea* cv. Bronco. Our results indicate that supplementation of 80-100 µM Zn is optimal for maintaining the normal growth of plants and to promote the Zn concentration in the edible part of *B. oleracea*. Moreover, an increase of the Zn doses can induce an accumulation for the total amino acid concentration in the same range, such as of the glucosinolates (GSLs) levels (aliphatic, indolic and total GSLs), and the phenolic concentration (sinapic acid derivatives, flavonols and total phenols). On the basic of our results, an intake of *B. oleracea* grown under 80-100 µM Zn may increase the intake of this micronutrient and other elements beneficial for the human health.

In general terms we can define the doses used in this study as optimal or supra-optimal for both plants, since a increase in the application of Zn produced no deleterious effects on the edible part of both plants maintain its quality in terms of antioxidants as well the balance of nutrients, especially with the dose of 20 µM in *L. sativa* and 20-80

µM in *B. oleracea*. However, overall we can define *B. oleracea* as the most efficient species in this type of program. These data are consistent with data obtained in the first part of this thesis, where we found that this species was able to accumulate more Zn under toxicity conditions than *L. sativa*.

In addition due to an increase in the need to increase production in recent years, it has been fertilized excess nitrogen may be toxic to the human being by its accumulation in form of NO_3^- in the edible part of plants. In this sense, and knowing that *L. sativa* is hyperaccumulator of this toxic compound, in this study it was examined whether proper fertilization with Zn could promote efficiency in the use of N. Our data reveal that *L. sativa* is able to respond efficiently in a biofortification program with Zn, increasing the concentration of this micronutrient in the aerial part promoting the nitrogen and photorespiration metabolisms, as well as increased accumulation of amino acids essential for human health, especially supplemented plants 80 µM of Zn.

In addition Zn may interact synergistically or antagonistically with other macro and micronutrients. In this sense, and knowing that *B. oleracea* is an important source of bioactive compounds, was carried out a study on the effect of Zn the amino acid profile and metabolism of glucosinolates and phenolics due to the beneficial properties that these compounds may offer human health. Based on our results, the Zn can clearly promote the synthesis of primary compounds such as amino acids, and secondary such as glucosinolates and phenolic compounds, especially when Zn dose applied in this species is in a range of 80-100 µM.

In short we can conclude that consumption of both horticultural species cultivated under a Zn biofortification program could promote increased intake of this micronutrient as well as other elements beneficial to human health. However from the point of view of quality, a consumption of *B. oleracea* could provide a greater amount of beneficial elements. While a crop of *L. sativa* under these conditions could result in a decrease in nitrogen fertilization maintaining its production and increase the synthesis of essential amino acids to humans.

Capítulo 2

Introducción general

2.1. Generalidades del Zn

El Zinc (Zn) fue descubierto por primera vez por el químico Andreas Sigismund Marggraf en 1746. Este elemento químico, es un metal de transición de número atómico 30 y es el vigésimo-tercer elemento más abundante en la tierra (Broadley et al. 2007). El Zn posee dos estados de oxidación como son el Zn^0 y Zn^{2+}, pero su forma Zn^{2+} es la forma oxidada predominante (Kaur et al. 2014). Además, el Zn^{2+} tiene un pequeño radio iónico comparable con el Mg^{2+} pero posee una acidez de Lewis más parecida a la del Cu^{2+} (Barak y Helmke 1993), y por lo tanto puede formar enlaces covalentes fuertes con los donantes S, N y O. Así, el Zn puede formar numerosas sales solubles, incluyendo haluros, sulfatos, nitratos, formiatos, acetatos, tiocianatos, percloratos, fluosilicatos, cianuros, zincatos de metales alcalinos y sales de Zn-amoníaco y compuestos poco solubles, como fosfato de Zn-amonio, hidróxido de Zn y carbonato de Zn; y una gama de complejos orgánicos solubles e insolubles (Lindsay, 1979; Barak y Helmke, 1993).

2.2. ¿Por qué el Zn es un micronutriente esencial?

El papel biológico del Zn fue identificado por primera vez por Raulin (1869). Este autor llegó a demostrar que el Zn era un nutriente necesario para el crecimiento del moho del pan ``*Aspergillus niger*´´. Posteriormente, el Zn llegó a ser identificado como un componente ubicuo tanto en los tejidos animales como vegetales, promoviendo de esto modo las sucesivas investigaciones que tendrían lugar a partir de esta observación. Así, en el año 1914, Mazé informó que el Zn era necesario para el crecimiento y desarrollo del maíz en cultivo hidropónico. Este informe promovió varios intentos para

confirmar que el Zn era requerido por las plantas, pero los resultados fueron inconsistentes, hasta que Sommer y Lipman (1926) demostraron que este micronutriente era requerido para el crecimiento y el desarrollo de plantas de cebada y girasol. Además, unos años más tarde, este elemento sería definido como indispensable para los animales (Todd et al. 1934).

Desde entonces, el Zn ha sido reconocido como un elemento esencial para el crecimiento, desarrollo y la diferenciación de todos los tipos de vida, incluyendo los microorganismos, las plantas y los animales (Vallee 1986; Dhankar et al. 2012; Sinclair y Krämer 2012).

2.2.1. Funciones fisiológicas del Zn en plantas

Alrededor del 10% de las proteínas humanas (2800) se encuentran unidas al Zn. Además, cientos de estas se encuentran implicadas en el transporte y el tráfico del Zn. En *Arabidopsis thaliana* se han encontrado 2367 proteínas relacionadas con el Zn (Broadley et al. 2007). El Zn se encuentra envuelto en numerosos procesos críticos en todos los organismos (Lim et al. 2004; Dhankar et al. 2012; Sinclair y Krämer 2012), ya que este es capaz de unirse a diferentes ligandos celulares como son; la cisteína (Cys), histidina (His), aspartato (Asp) y glutamato (Glu) (Cakmak et al. 2000; McCall et al. 2000; Broadley et al. 2007). Cabe destacar que el Zn se une a una amplia gama de otras proteínas, lípidos de membrana y moléculas de ADN/ARN (Lim et al. 2004; Broadley et al. 2007).

Además, se ha demostrado que el Zn es necesario para la actividad de más de 300 enzimas, siendo este el único metal representado en las seis clases de enzimas: hidrolasas, isomerasas, liasas, ligasas, oxidorrecductasas y transferasas (Auld et al.

1001; Broadley et al. 2007) (Tabla 1). Por lo tanto, el Zn participa como ión catalítico y co-catalítico, estructural y regulador (Auld 2001; Maret 2009) (Tabla 1 y Fig. 1). Ejemplos bien conocidos en plantas incluyen a enzimas como la anhidrasa carbónica, alcohol deshidrogenasa, ARN polimerasa, carboxipeptidasa, fosfatasa alcalina y Cu/Zn superóxido dismutasa (Guerinot y Eidet 1999; Auld et al. 2001; Sriram y Lonchyna 2009) (Tabla 1). Otras investigaciones han demostrado como el Zn puede actuar como ión estructural en dedos de Zn, giros y clusters, en factores de transcripción y en metalotioneinas (Webb 1992; Cotton et al. 1997).

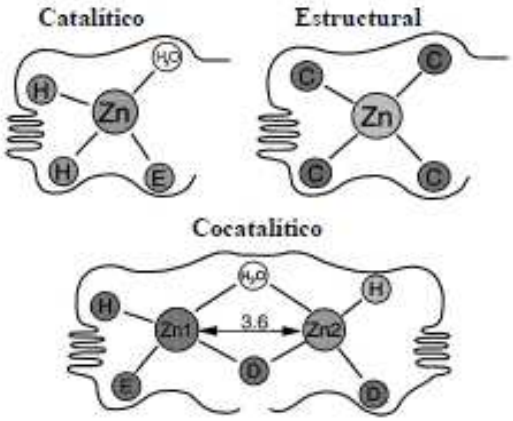


Fig. 1. Sitios de unión del Zn en enzimas: catalítico (termolisina), estructural (alcohol deshidrogenasa), cocatalítico (aminopeptidasa de *Aeromonas proteolítica*). Imagen tomada de Auld (2001). Las letras D, E y H se refieren a los aminoácidos, ácido aspártico, ácido glutámico e histidina, respectivamente.

En las plantas el Zn se requiere a concentraciones óptimas (> 15-30 µg g^{-1} pero < 700 µg g^{-1} de peso seco), tanto para el normal funcionamiento del metabolismo celular como para el crecimiento de las mismas (Broadley et al. 2007), ya que este se encuentra involucrado en numerosos procesos fisiológicos como son la activación de enzimas, síntesis de proteínas, metabolismo de hidratos de carbono, lípidos, ácidos nucleicos y auxinas, integridad estructural y funcional de las biomembranas, expresión y regulación génica y el desarrollo reproductivo (formación de polen) (Brown et al. 1993; Marschner 1995).

Tabla 1. Comparación de los ligandos y distanciadores (L1, L2, L3 y L4) y los distanciadores (X, Y y Z) entre ligandos de zinc en sitios catalíticos de zinc y estructurales (Tomado de McCall et al. 2000).

	L_1	X	L_2	Y	L_3	Z	L_4	Solvent
Catalytic zinc								
Oxidoreductases[2]								
Alcohol dehydrogenase (horse liver)[3]	C_{37}	20	H_{59}	106	C_{174}			H_2O
Alcohol dehydrogenase (*Thermoanaerobium brockii*)[4]	C_{37}	21	H_{59}	90	D_{150}		NA[5]	
Hydrolases								
Carboxypeptidase A (bovine)[6,7]	H_{69}	2	E_{72}	123	H_{196}			H_2O
Thermolysin (*Bacillus thermoproteolyticus*)[8,9]	H_{142}	3	H_{146}	19	E_{166}			H_2O
DD carboxypeptidase (*Streptomyces albus*)[10]	H_{196}	2	H_{193}	40	H_{152}			H_2O
Astacin (crayfish)[11]	H_{92}	3	H_{96}	5	H_{102}			H_2O
β-Lactamase (*Bacillus cereus*)[12]	H_{86}	1	H_{88}	121	H_{210}			H_2O
Cytidine deaminase (*Escherichia coli*)[13]	C_{132}	2	C_{129}	26	H_{102}			H_2O
Alkaline phosphatase (*E. coli*)[14]	D_{327}	3	H_{331}	80	H_{412}			H_2O
Adenosine deaminase (murine)[15]	H_{15}	1	H_{17}	196	H_{214}	80	D_{295}	H_2O
Lyases								
Carbonic anhydrase II (human)[17,16]	H_{94}	1	H_{96}	22	H_{119}			H_2O
Carbonic anhydrase (spinach)[18]	C_{213}	2	H_{210}	59	C_{150}			H_2O
Carbonic anhydrase (*Methanosarcina thermophila*)[19]	H_{81}	35	H_{117}	NA[19]	H_{122}			H_2O
Novel catalytic zinc sites								
Transferases								
Protein farnesyltransferase (rat)[20]	D_{297}	1	C_{299}	62	H_{362}			H_2O
Cobalamin-dependent methionine synthase (*E. coli*)[21]	C_{247}	62	C_{310}		C_{311}		N/O atom	
Cobalamin-independent methionine synthase (*E. coli*)[21,27]	H_{641}	1	C_{643}	82	C_{726}		N/O atom	
Nonenzymatic								
Ada repair protein (*E. coli*)[22]	C_{38}	3	C_{42}	26	C_{69}	2	C_{72}	No
Structural zinc								
Alcohol dehydrogenase (horse liver)[3]	C_{97}	2	C_{100}	2	C_{103}	7	C_{111}	No
Aspartate carbamoyltransferase (*E. coli*)[23]	C_{109}	4	C_{114}	23	C_{138}	2	C_{141}	No
Zinc finger (Zif268) (mouse)[24]	C_7	5	C_{12}	12	H_{25}	4	H_{29}	No
Glucocorticoid receptor (rat)[25]	C_{440}	2	C_{443}	8	C_{457}	2	C_{460}	No
Ferrodoxin (*Sulfolobus* sp.)[26]	H_{16}	2	H_{19}	14	H_{34}	41	D_{76}	No

2.2.2. Formas del Zn en la planta

En las plantas, el Zn no se somete a cambios de valencia, y presenta una particular afinidad en la formación de complejos tetraédricos. La mayoría del Zn en la hoja se encuentra asociado a compuestos de bajo peso molecular, metaloproteínas de almacenamiento, iones libres, y formas insolubles asociados con la pared celular. Además el Zn puede llegar a ser inactivado dentro de la célula, ya sea por la formación de ligandos o por formación de complejos con el fósforo. En este sentido, Brown et al. (1993) llegan a demostrar como dependiendo de las especies de plantas el Zn puede presentarse soluble desde un 58% al 91%.

2.2.3. Absorción, utilización, transporte y almacenamiento del Zn

El Zn es absorbido del suelo principalmente como catión divalente libre (Zn^{2+}), aunque en presencia de un pH elevado en el suelo, este puede ser absorbido como catión monovalente ($ZnOH^{+}$) (Marschner 1995). Además este elemento traza suele ser absorbido formando complejos con ligandos orgánicos (Broadley et al. 2007). Sin embargo, la absorción del Zn va a depender de su biodisponibilidad en el suelo. En este sentido, son varios los factores que afectan a la absorción del metal, como son: el pH, textura y cantidad de materia orgánica del suelo, temperatura, luz, cultivo, cultivares (Giordano y Mortvedt 1980) y la interacción con diferentes nutrientes como son el P, N, Fe, Mn, Cu, Co y Cd (Hawf y Schmid 1967; Giordano y Mortvedt 1980; Rehman et al. 2012).

En primer lugar el Zn puede ser adsorbido fuertemente a nivel radicular o bien ser absorbido. Si es absorbido, este atravesará la membrana plasmática a través de transportadores activos (Rehman et al. 2012), y será transportado desde la epidermis y córtex hacia el xilema vía simplasto a través de los plasmodesmos, siendo finalmente liberado al apoplasto (Broadley et al. 2007).

La distribución y el transporte del Zn pueden ser afectados por el suministro adecuado de este micronutriente, pero en condiciones normales el transporte del Zn no coincide con el transporte del agua. Una vez alcanzada la parte aérea, el Zn podrá acumularse y posteriormente ser removido hacia las hojas jóvenes vía floema (Lognecker y Robson 1993).

2.2.4. Líneas de investigación actuales con el Zn

La contaminación ambiental con metales pesados o metaloides así como la deficiencia de micronutrientes (especialmente el Zn) en los seres humanos son problemas globales y desafiantes que requieren esfuerzos concertados de investigadores de múltiples disciplinas, incluyendo la biología de las plantas, cultivo de plantas y la biotecnología, nutrición y ciencias ambientales.

Recientemente, una solución a estos problemas ha sido lograda gracias a la fitoextracción de metales como a la biofortificación con micronutrientes como el Zn. Tanto la fitorremediación de suelos de metal / metaloide contaminados como la biofortificación de micronutrientes minerales en los cultivos de alimentos en beneficio de la nutrición humana representan dos aplicaciones biotecnológicas potenciales que surgen de la investigación sobre la absorción de minerales, el transporte y el metabolismo de las plantas. Así, la comprensión en la variación inter e intraespecífica en cuanto a la absorción de minerales, distribución, metabolismo y tolerancia, y los mecanismos moleculares responsables de los procesos, son cruciales para ambas aplicaciones (Zhao y McGrath 2009).

2.3. Fitoextracción del Zn

2.3.1. Fitoextracción: Una tecnología eficaz y de bajo costo

Los suelos contaminados con metales pueden ser remediados mediante diversas técnicas químicas y físicas tales como: el tratamiento térmico o electro-cinético, oxidación química, o la reducción de la lixiviación ácida. Sin embargo, la mayoría de

estas tecnologías de remediación convencionales son costosas de implementar y pueden empeorar el medio ambiente ya dañado (Dhankhar et al. 2012).

Es por ello que recientemente ha habido un aumento en la investigación centrada en el uso de plantas superiores para limpiar los suelos contaminados con metales pesados (Ensley 2000; Lasat et al. 2000). Esta idea no es nueva, aunque sólo ha sido considerada recientemente debido a una serie de descubrimientos científicos combinados con un enfoque interdisciplinario en la investigación (Chaney 1993; Lasat et al. 2000; Islam et al. 2007). Además, esta tecnología ha llamado la atención en los últimos años debido a su elevada rentabilidad económica y a sus beneficios ambientales, siendo más aceptada por el público que los métodos tradicionales mencionados anteriormente (Salt et al. 1998; Dhankhar et al. 2012).

La fitorremediación puede incluir: fitoextracción, fitoestabilización, fitovolatización o rizofiltración. En este sentido, la fitoextracción es la técnica más reconocida (Dhankhar et al. 2012). Este término ha sido definido como el uso de plantas que acumulan contaminantes para eliminar los metales o compuestos orgánicos del suelo mediante la acumulación de ellos en las partes cosechables (Salt et al. 1998).

Sin embargo, el progreso hacia este objetivo se ha visto obstaculizado por la falta de la comprensión de los mecanismos bioquímicos, fisiológicos y moleculares básicos implicados en la hiperacumulación de metales pesados (Lasat et al. 2000). Por tanto, una mejor comprensión de la localización y de las vías de desintoxicación de metales en las plantas podría ayudar a reducir los riesgos y daños a de salud ambiental asociados con suelos contaminados por metales pesados. Por otra parte, ya que las plantas tienen una capacidad básica de extracción de iones inorgánicos de los suelos y la acumulación de estos en su biomasa, una mejor comprensión de la homeostasis de los metales en la planta puede ayudar en el desarrollo de tecnologías rentables basados en el

uso de las plantas para la limpieza de estos suelos (Salt et al. 1998; Krämer y Clemens 2005).

Aproximadamente hasta 400 taxones de plantas terrestres de al menos 45 familias han sido identificadas como plantas hiperacumuladoras de diferentes metales pesados en la parte aérea (Zn, Ni, Cd, Co) (Baker and Broooks 1989). Algunos de los ejemplos de estas plantas hiperacumuladoras han sido descritas en numerosos estudios: *Arabidopsis halleri*, *Brassica juncea*, *Thlaspi caerulescens*, *Thlaspi rotundifolium*, etc.. (Gisbert et al. 2006; Islam et al. 2007; Krämer 2010).

Las plantas ideales para la fitorremediación deben poseer múltiples rasgos. Deben poseer un crecimiento rápido y una gran biomasa, raíces profundas, ser fáciles de cosechar y deben tolerar y acumular una gama de metales pesados en su parte aérea y partes cosechables. Sin embargo, la mayoría de estas especies hiperacumuladoras tienen un pequeño crecimiento pequeño y lento, lo que limita gravemente su potencial para la descontaminación de suelos contaminados a gran escala (Ebbs et al. 1997). Hasta la fecha, ninguna planta ha demostrado cumplir todos estos criterios. Sin embargo, se podría ser lograr crecimiento rápido de plantas no acumuladoras de manera que se consiguiese algunas de las propiedades de las plantas hiperacumuladoras (Clemens et al. 2002). Asimismo se ha sugerido la transferencia de los genes que confieren el fenotipo de planta hiperacumuladora a plantas que producen más biomasa como una posible vía para la mejora de la fitorremediación como una tecnología comercial viable (Brown et al 1995).

2.3.2. Toxicidad de Zn en suelos

Desde el comienzo de la era industrial, las actividades humanas han dado lugar a un cambio acelerado de la composición elemental de la biosfera a través de la liberación de grandes cantidades de metales potencialmente tóxicos, tales como Zn, cadmio (Cd), y el plomo (Pb) (Nriagu y Pacyna 1988). Los seres humanos han influido mucho en las entradas Zn en los suelos. Hace dos mil años, aproximadamente eran emitidas unas 10.000 toneladas Zn por año (Nriagu 1996). Así en 1850, las emisiones aumentaron hasta 10 veces más, alcanzando hasta 3,4 megatoneladas por década en el año 1980 (Ensley 2000), y además la producción mundial de Zn sigue incrementándose día a día (Dhankhar et al. 2012).

Esta situación es originada como resultado de componentes naturales o antropogénicos derivados de numerosas actividades industriales, como son: el uso excesivo de fertilizantes y pesticidas, minería, fresado, fundiciones y refinado (Cammarota 1980; Broadley et al. 2007). Además cabe destacar que el origen de estas emisiones se suceden en una proporción 1:20 (Friedland 1990).

Entre las principales regiones productoras de Zn podemos encontrar a Asia, seguida de Europa y EE.UU (Imagen 1). Cabe destacar que China además es el mayor productor en cuanto a producción minera de Zn, con un 30% de participación en el producto total. Además, consumidores importantes de Zn son Europa, Estados Unidos, Japón y Corea (Imagen 1, U.S. Geological Survey 2011)

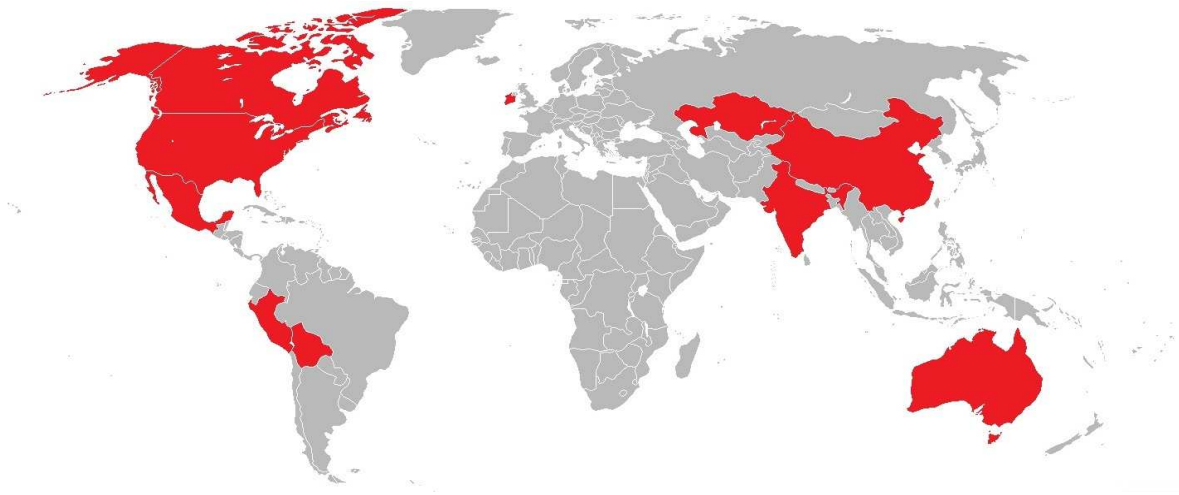


Imagen 1. Principales productores de Zn a nivel mundial. Los países sombreados de color gris se corresponden con los principales productores ordenados de mayor a menor toneladas de producción: China, Perú, Australia, India, Estados Unidos, Canadá, Mexico, Kasakhstan, Bolivia e Irlanda (Tomado de USGS: Mineral Commodity Summary 2011).

Los metales emitidos son transferidos a los compartimentos ambientales, tales como el agua, el suelo y las plantas, y, finalmente, puede entrar en el cuerpos humanos a través de las cadenas alimentarias o ingestión directa (Bi et al. 2006), provocando un gran número de enfermedades agudas y crónicas (Dhankhar et al. 2012).

2.3.3. Toxicidad de Zn en plantas

El Zn es un elemento esencial para las plantas, pero este puede convertirse en tóxico más allá de un rango de umbral del suelo de 100 a 400 mg kg^{-1} (Kabata-Pendias y Pendias 1984). Bajo estas condiciones, el Zn se acumula en exceso en los tejidos vegetales provocando alteraciones fisiológicas, inhibición del crecimiento e incluso llegando a producir la muerte de las mismas (Hoffmann 1983).

Los umbrales de toxicidad pueden ser muy variables, incluso dentro de la misma especie (Broadley et al. 2007). En general, los síntomas de toxicidad se hacen visibles a

partir de una concentración superior a 300 mg Zn kg^{-1} en el peso seco de la hoja, aunque algunos cultivos pueden mostrar síntomas de toxicidad con concentraciones menores de 100 mg Zn kg^{-1} de peso seco (Chaney 1993; Marschner 1995).

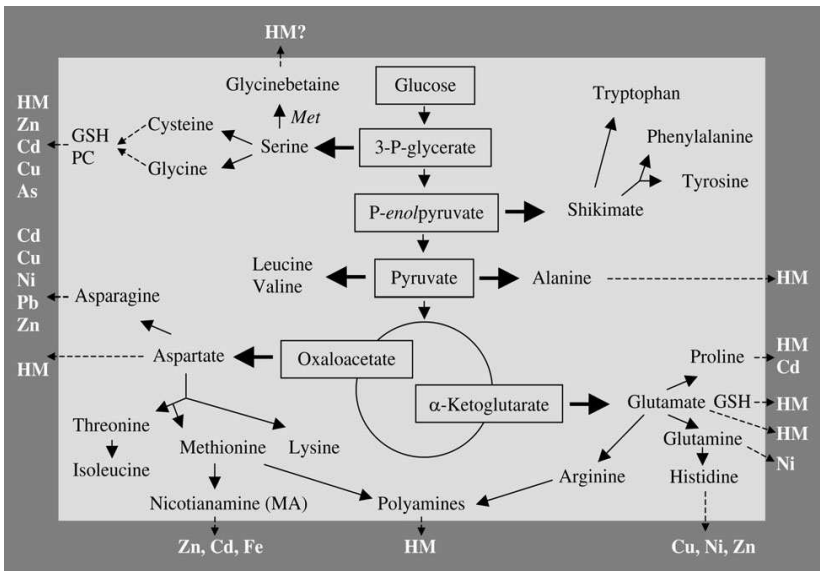
Este efecto puede ser debido a que el Zn induce, alteración del metabolismo de los carbohidratos (Foy et al. 1978), inhibición del crecimiento, disminución en el contenido de nutrientes esenciales como Fe (Connolly et al. 2002,2003; Sargodoy et al. 2009), Cu y Mn, daño oxidativo a las membranas y trastornos de la actividad fotosintética en numerosas localizaciones celulares como los pigmentos fotosintéticos, el transporte de electrones, la actividad Rubisco, etc (Clemens 2001; Broadley et al. 2007; Vassilev et al. 2007). Por lo tanto, las plantas deben regular la homeostasis de este metal para hacer frente a condiciones ambientales adversas (Krämer y Clemens 2005), lo que hace que la presencia de algún mecanismo de tolerancia sea clave para este propósito.

2.3.4. Mecanismos de tolerancia en plantas bajo condiciones de toxicidad por metales pesados

Las plantas han desarrollado una serie de mecanismos fisiológicos y moleculares para hacer frente a diferentes tipos de estrés, así como los efectos tóxicos de metales o metaloides, manteniendo la homeostasis metabólica y amortiguando la generación de especias reactivas del oxígeno (ROS) (Hossain et al. 2012).

En términos generales, la resistencia al estrés por metales pesados se puede lograr mediante la "evitación", cuando las plantas son capaces de restringir la absorción de metal, o mediante la "tolerancia" cuando las plantas son capaces de sobrevivir ante la presencia de una concentración de metal interna de alta (Hossain et al. 2012). En este sentido, la evitación implica la reducción de la concentración de metal de entrar en la

célula por precipitación extracelular, bioabsorción a las paredes celulares, reducción de la absorción, o el aumento de flujo de salida. En un segundo tipo de situación, los metales pesados pueden permanecer quelados intracelularmente a través de la síntesis de compuestos nitrogenados, ácidos orgánicos, GSH, permanecer ligados a metalotioneínas (MTs) o fitoquelatinas (PC), compartimentados dentro de las vacuolas, o mediante la regulación positiva de la defensa antioxidante y el sistema de las glyosalasas para contrarrestar los efectos perjudiciales causados por ROS y el methylgliosal (MG) (Hossain et al. 2012) (ver esquema 1).

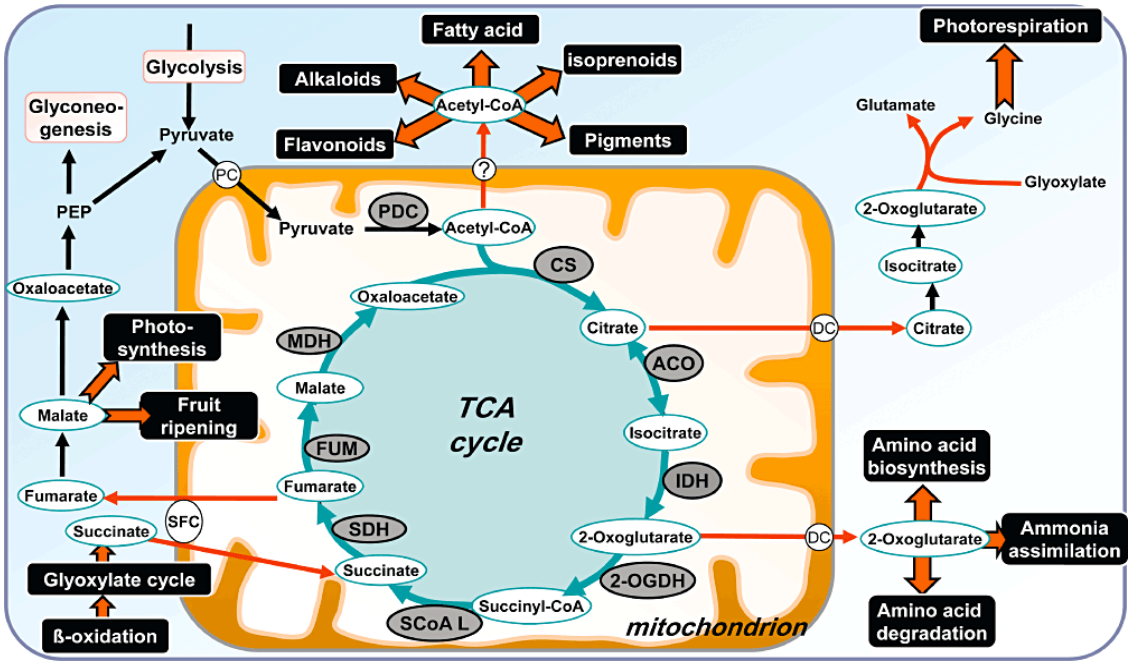


Esquema 1. Representación esquemática de las vías sintéticas que participan en la síntesis de aminoácidos y metabolitos nitrogenados relacionados con los metales pesados en las plantas. Abreviaciones: GSH, glutation; PC, fitoquelatinas; MA, ácido mugineico; HM, metales pesados (Tomado de Sharma y Dietz 2006).

A continuación describimos algunos de los posibles mecanismos de tolerancia que hemos estudiado en esta Tesis Doctoral.

2.3.4.1. Metabolismo de los ácidos tricarboxílicos

Debido a la enorme reacción de los iones metálicos con el S, N y O, los ácidos orgánicos y los aminoácidos representan ser unos potentes ligandos (Clemens 2001). La formación de estos ácidos se origina a partir del ciclo de los ácidos tricarboxílicos (TCA), comúnmente conocido como ciclo de Krebs, el cuál es un centro metabólico clave para la interacción de la respiración, asimilación de nitrógeno, y la fotorrespiración, con componentes que muestran una considerable flexibilidad en relación con las adaptaciones a las diferentes funciones de las mitocondrias en las células fotosintéticas y no fotosintéticas (Esquema 2) (Foyer et al. 2011).



Esquema 2: Metabolismo de los ácidos tricarboxílicos. Imagen tomada de Araújo et al. 2012).

En el ciclo de los ácidos orgánicos están implicadas un conjunto de ocho enzimas que unen principalmente el producto de la oxidación de piruvato y malato (generado en el citosol) a CO_2 con la generación de NADH y su posterior oxidación por la cadena

respiratoria mitocondrial (Nunes-Nesi et al. 2013). La primera reacción es experimentada por el acetil-CoA el cuál entra en el ciclo condensándose con una molécula de oxalacetato para dar citrato, reacción catalizada por la enzima citrato sintasa (CS). El citrato formado a partir del acetil-CoA se isomeriza próximo a una mezcla de cis-aconitato y la molécula asimétrica isocitrato por la enzima aconitasa. El isocitrato puede ser descarboxiladao y oxidadado a α-cetoglutarato por la isocitrato deshidrogenasa (ICDH). A partir de esta descarboxilación oxidativa se genera el succinato, el cual se convertirá en oxalacetato en tres reacciones enzimáticas consecutivas: en primer lugar el succinato se oxida a fumarato por la succinato deshidrogenasa (SDH). El fumarato es entonces hidroxilado a malato por la enzima reversible fumarasa (FUM). Finalmente, el malato es oxidado a oxalacetato por la reacción reversible de la enzima malato deshidrogenasa (MDH). Esta última reacción completa el ciclo, y nuevamente el oxalacetato podrá combinarse con otra molécula de acetil-CoA y reiniciar el ciclo formándose citrato (Kogut 1986).

Finalmente, otra enzima importante y asociada al TCA es la fosfoenolpiruvato carboxilasa (PEPC). En todos los tipos de plantas existe una PEPC C3 que se bifurca de la glucólisis para reponer intermediarios del TCA (la denominada vía anaplerótica) así como para proporcionar precursores para la síntesis de aminoácidos (Stitt, 1999).

En la actualidad son muchos los estudios que se han llevado a cabo sobre los mecanismos de tolerancia en plantas crecidas bajo condiciones de estrés por metales pesados. En este sentido, se ha demostrado que ante una elevada exposición a metales pesados, las actividades de las enzimas que intervienen en el ciclo de TCA pueden verse afectadas positiva o negativamente, en función del grado de tolerancia de la planta. En general, en plantas hyperacumuladoras o tolerantes, las actividades enzimáticas del ciclo TCA son fuertemente inducidas mientras que en las no tolerantes se produce un efecto

negativo. Además un incremento de las actividades del ciclo ha sido correlacionado con un incremento de la concentración de ácidos orgánicos así como de la concentración del metal en la parte aérea de la planta (Mathys 1980; Godbolt et al. 1984; Salt et al. 1999; López-Millán et al. 2001)

En lo que concierne al Zn, los ácidos orgánicos son compuestos que poseen una estructura especial, la cual resulta indispensable para tolerar el exceso de Zn (Jones 1998; Clemens 2001). Así, el citrato, malato y oxalacetato, se encuentran implicados en numerosos procesos, tales como, el transporte de metales a través del xilema, secuestro a nivel vacuolar, así como en la respuesta diferencial a la tolerancia de metales pesados (Clemens 2001). Asimismo, Liu et al. (2012) llegan a definir a los ácidos orgánicos como quelantes y transportadores de Zn, y por lo tanto necesarios para su absorción, translocación, acumulación y almacenamiento dentro de la planta, garantizándose de este modo una distribución eficiente del Zn en la planta y así su detoxificación en condiciones tóxicas.

2.3.4.2. Metabolismo del glutatión

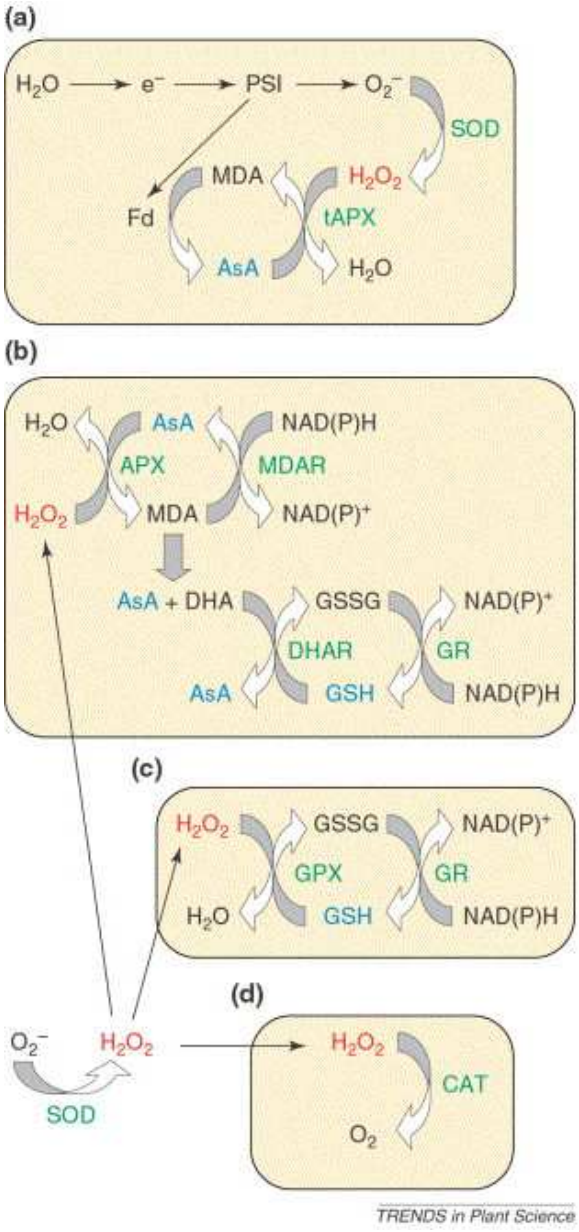
Es bien conocido que bajo condiciones ambientales normales, en las plantas se generan especies reactivas del oxígeno (ROS), tales como el anión superóxido (O_2^-), oxígeno singlete ($^1O_2$), peróxido de hidrógeno (H_2O_2) y el radical hidroxilo (OH). Sin embargo, bajo condiciones ambientales adversas el balance de la producción de ROS se desequilibra pudiendo producir efectos adversos en las células vegetales (Asada 1994). La generación elevada de estas ROS puede ser debida a numerosos tipos de estrés tales como: exceso de metales pesados, privación de nutrientes, sequía, salinidad,

refrigeración, choque térmico, radiación ultravioleta, exposición al ozono y el SO_2, tensiones mecánicas o el ataque por patógenos (Mittler 2002).

En este contexto, la eliminación de ROS y la homeostasis celular están regulados por enzimas antioxidantes tales como superóxido dismutasa (SOD), catalasa (CAT) y peroxidasas (POD) y un sistema antioxidante complejo como el ciclo ascorbato-glutatión (AsA-GSH) y varios otros antioxidantes endógenos tales como AsA, tioles, glutatión (GSH), y enzimas asociadas al metabolismo del GSH (Aravind y Prasad 2005; Anjum et al. 2012).

En este sentido, la conversión del H_2O_2 conecta directamente metabolitos tales como el AsA y el GSH con los sistemas enzimáticos. La presencia del ciclo AsA-GSH en todos los compartimentos celulares, así como la elevada afinidad de la actividad ascorbato peroxidasa (APX) por el H_2O_2 sugiere que este ciclo desempeña un papel crucial en el equilibrio de ROS (Mittler 2002; Seth et al. 2012) (Esquema 3).

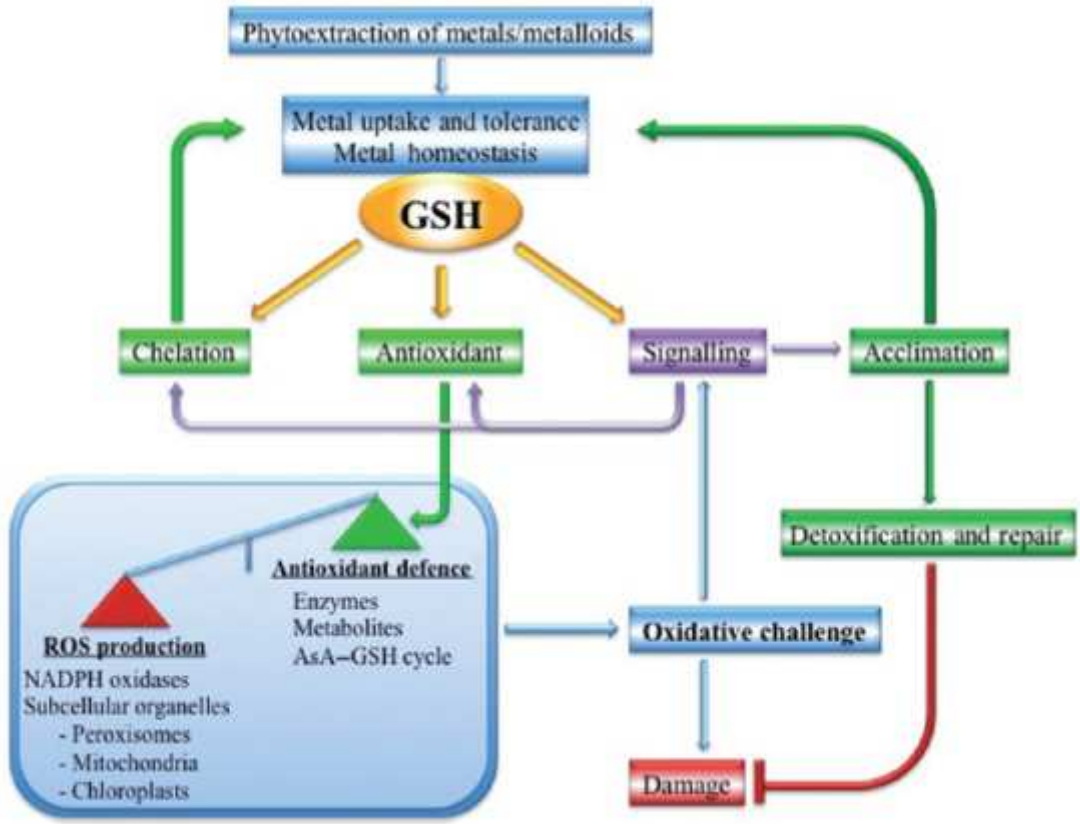
En este ciclo la enzima APX utiliza dos moléculas de AsA en el ciclo de AsA-GSH para reducir el H_2O_2 a agua con la generación de dos moléculas de monodehydroascorbate (MDHA), el cual que puede ser reducidos a AsA por la MDHA reductasa (MDHAR). Si esta molécula no se reduce de forma inmediata, el MDHA se oxida a deshidroascorbato (DHA). Posteriormente, el DHA es reducido a AsA a través de la acción de la enzima DHA reductasa (DHAR) mediante el uso de GSH, que es generado a partir del glutatión oxidado (GSSG) por la actividad GSH reductasa (GR) (Chao et al. 2010).



Esquema 3. Sistemas antioxidantes de las plantas. (a) Ciclo ascorbato-glutation. (b) Ciclo glutatión peroxidasa. (c) Catalasa (CAT). ROS en rojo, antioxidantes en azul y enzimas detoxificadoras en verde. Abreviaciones: ascorbato peroxidasa (APX), dehidroascorbato (DHA), DHA reductasa (DHAR), glutaion reductasa (GR), glutatión oxidado (GSSG), monodehidroascorbato (MDA), MDA reductasa (MDAR), superóxido dismutasa (SOD) (Tomado de Mittler 2002).

Por otro lado, el aumento de la síntesis de compuestos de defensa azufrados tales como el GSH y las PCs bajo estas condiciones de estrés por metales pesados, han sido considerados como un mecanismo primordial de tolerancia y supervivencia en las plantas (Cobbett y Goldsbrough 2002; Grill et al. 2006; Mishra et al. 2009). En las plantas, el tripéptido GSH (γ-L-glutamina-L-cisteina-glicina) es el compuesto tiólico más abundante, y por lo tanto este se encuentra involucrado en numerosas funciones tales como: la regulación del desarrollo, división celular y floración, transporte y

almacenamiento de azufre reducido, traducción de señales y defensa contra patógenos (Flocco et al. 2004; Yadav 2010). Además este compuesto se encuentra implicado tanto en la regeneración de AsA así como en la síntesis de PCs (Srivastava et al. 2006; Wang et al. 2009), en la defensa contra ROS, quelación de metales pesados y en la detoxificación de xenobióticos (metilglioxal) y herbicidas (Yadav et al. 2008; Szalai et al. 2009) (Esquema 4). Por lo tanto, tanto su síntesis como su catabolismo juegan un papel clave en la planta bajo condiciones estresantes por la presencia de elevadas concentraciones de metales pesados.



Esquema 4. Modelo generalizado del papel central del glutation (GSH) en la fitoextracción de metal, para la cual la absorción y la tolerancia son requisitos previos esenciales ya que ambos en la homeostasis. (Tomado de Seth et al. 2012).

En este respecto, la enzima serina acetiltransferasa (SAT) es clave en la asimilación del S promoviendo la síntesis de cisteína (Cys), uno de los sustratos necesarios para la síntesis de GSH (Droux et al. 2004). Posteriormente, el GSH es sintetizado a través de dos reacciones dependientes de ATP, donde la enzima γ-glutamilcisteína sintetasa (γ-ECS) cataliza la formación de un enlace peptídico entre el grupo carboxilo del glutamato y el grupo amino de la cisteína formada dando lugar a γ-glutamilcisteína. En segundo lugar la enzima glutation sintetasa (GS) dará lugar a la formación de GSH mediante la adicción de glicina (Gly) al carbono terminal de la γ-glutamilcisteína (Yadav 2010). Posteriormente, tanto la enzima GPX como GST pueden utilizar el pool de GSH como sustrato para detoxificar el H_2O_2 así como xenobióticos mediante la conjugación de estos con el GSH (Ramakhrisna y Rao 2013).

Además, se ha demostrado como el GSH se encuentra involucrado en la detoxificación de otros metabolitos que se sobre-producen tras la exposición a metales pesados o metaloides (Yadav et al. 2008). Uno de estos compuestos es el MG, este compuesto al igual que las ROS es altamente tóxico para la célula vegetal, y en ausencia de cualquier mecanismo de protección pueden reaccionar con proteínas, lípidos, ADN e inactivar los sistemas antioxidantes de defensa. En las plantas, el MG es principalmente detoxificado mediante la homeostasis a través del sistema de las glioxalasas dependientes de GSH (Yadav et al. 2005). La glioxalasa I (Gly I) utiliza una molécula de GSH reducido para convertir el metilglioxal (MG) a S-D-lactoilglutation (SLG). Posteriormente, la enzima glioxalasa II (Gly II) convierte el SLG a D-lactato y una molécula de GSH reducido es reciclado de nuevo en el sistema (Yadav 2010).

Dado que el Zn no es un metal redox y no es capaz de generar ROS directamente a través de reacciones Haber-Weiss, la sobreproducción de ROS y la aparición del estrés oxidativo en plantas podrían ser la consecuencia indirecta de la toxicidad de Zn.

Además, las ROS poseen una fuerte capacidad oxidante y puede atacar a los ácidos nucleicos, pigmentos y proteínas causando peroxidación lipídica y alterando las actividades y contenidos enzimáticos y finalmente originar la muerte celular (Gomes et al. 2013; Li et al. 2013). Por lo tanto la rápida activación de los mecanismos de detoxificación es de gran importancia para aliviar los efectos tóxicos ocasionados por el estrés por Zn. (Wang et al. 2009). En este sentido, la eficiencia en la fitorremediación se encuentra fuertemente influenciada por la capacidad de la planta para escapar de concentraciones perjudiciales del metal y de ROS que podrían generarse en el tejido (Bittsánszky et al. 2005).

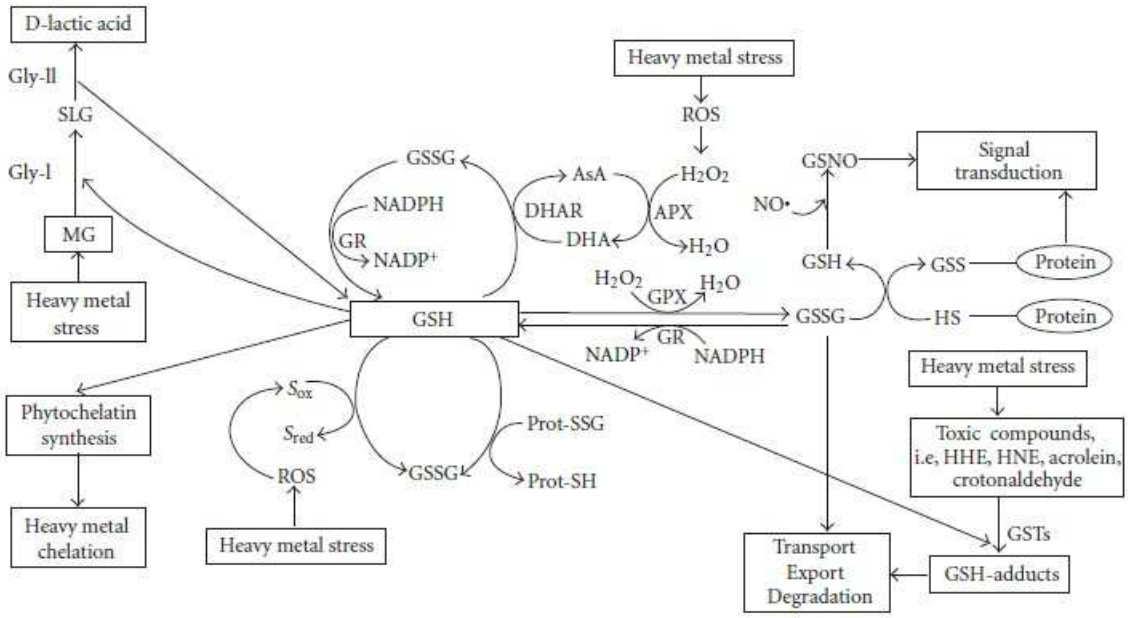
Con respecto al Zn, varios autores han correlacionado una modulación de los radicales libres y sus procesos relacionados mediaada por la participación de las vías metabólicas del AsA-GSH (Aravind y Prasad 2005). Así, Cuypers et al. (2001) observaron cómo ante toxicidad de Zn, tuvo lugar un incremento de las 4 enzimas implicadas en el ciclo AsA-GSH en hojas de *Phaseolus vulgaris*.

Como se ha mencionado anteriormente, un aumento de producción de GSH puede contribuir a la protección antioxidante de las células de la planta contra el estrés oxidativo ocasionado por diversos factores ambientales (Noctor y Foyer 1998). Es por ello que la síntesis de GSH y las actividades de las enzimas que participan en las funciones de defensa, tales como la glutatión peroxidasa (GPX) y la glutatión-S-transferasas (GST), muestran una fuerte respuesta a ROS (Levine et al. 1994).

En concreto, bajo condiciones de estrés por Zn, se ha demostrado como un incremento de este elemento en la planta puede promover tanto la síntesis como la utilización de GSH incrementando las actividades de las enzimas SAT, γ-ECS, GPX and GST incrementándose de este modo la tolerancia en diversas plantas (Aravind y Prasad 2005; Yadav 2010; Ramakhrisna and Rao 2013).

Además varios autores han demostrado como ante una exposición alta a metales pesados, incluido el Zn, una elevada actividad de las enzimas Gly I y Gly II es capaz de mantener niveles elevados de GSH y PCs en la planta ofreciendo mostrando de este modo una mayor tolerancia frente a la exposición a estos metales pesados, incluido el Zn (Singla-Pareek et al. 2006).

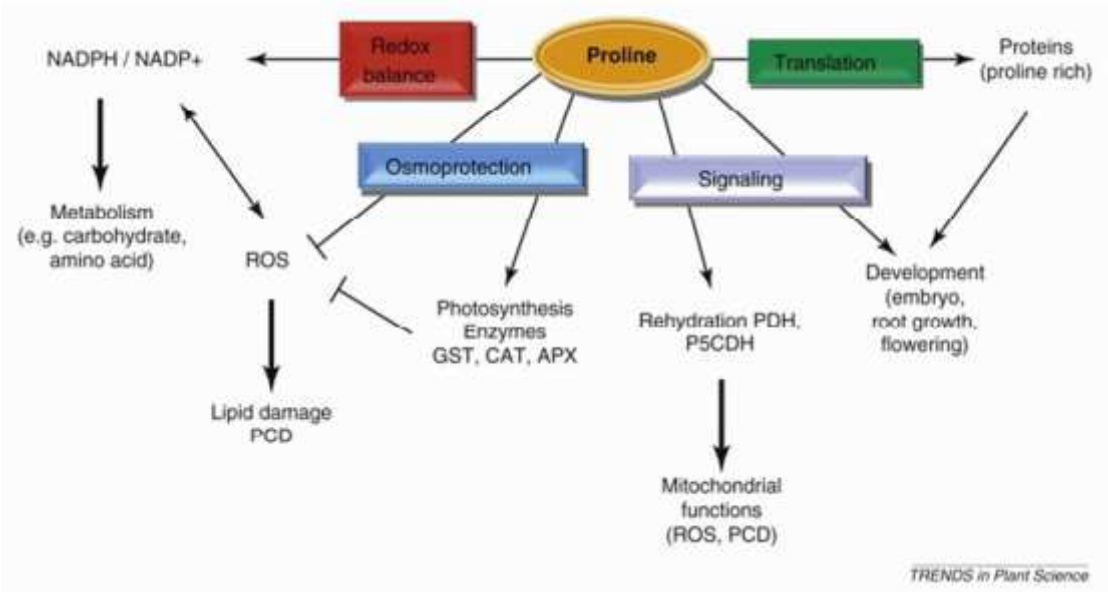
Finalmente, se ha demostrado que las inducciones o regulaciones coordinadas tanto de la vía antioxidante y enzimas de la vía glioxalasa son necesarias para obtener una tolerancia sustancial contra el estrés oxidativo generado bajo la toxidad por metales pesados (Hossain et al. 2012; Hasanuzzaman y Fujita 2013) (Esquema 5). Así, Di Baccio et al. (2005) llegan a concluir que la síntesis de GSH, su consumo y el estado redox juegan un papel central en la respuesta a altas concentraciones de Zn en plantas de álamo.



Esquema 5: Múltiples funciones y regulación del glutatión (GSH) y de las enzimas relacionadas que metabolizan la producción excesiva de especies reactivas del oxígeno (ROS) y del metilglioxal (MG) producidos bajo la toxicidad por metales pesados en las plantas (Tomado de Hossain et al. 2012).

2.3.4.3. Síntesis de compuestos nitrogenados

La acumulación de osmolitos puede ser parte de una adaptación general de las plantas frente a condiciones adversas, incluido la exposición a metales pesados como el Zn. (Schwache et al. 1999; Dhir et al. 2012). Los principales osmolitos presentes en las plantas superiores son: aminoácidos (prolina, ácido γ-aminoburítico, glutamato), amonios cuaternarios (glicina betaína, poliaminas), azúcares reductores (sacarosa, trehalosa, fructosa, rafinosa y glucosa) y polioles (glicerol, sorbitol y manitol) (Schwache et al. 1999; Dhir et al. 2012). Estos osmolitos son pequeñas moléculas orgánicas, muy solubles en agua y se pueden acumular a concentraciones elevadas sin resultar tóxicas para las células (Chen y Murata 2011). La principal contribución de estos osmolitos radica en la protección de las plantas a través de diversos mecanismos como son: el ajuste osmótico celular, detoxificación de ROS, mantenimiento de la integridad de membranas y estabilización de proteínas y enzimas. Además, estos compuestos son conocidos ampliamente por promover la protección de los componentes celulares ante una lesión o deshidratación, así como ser una fuente importante de nitrógeno y actuar como reservorios de energía y reducir la acidez celular (Schwache et al. 1999; Kavi-Kishor et al. 2005; Dhir et al. 2012). Schwache et al. (1999) y Dhir et al. (2012) demuestran como un aumento de estos compuestos ante situaciones de estrés puede ser regulado ya sea alterando el metabolismo (incremento de la síntesis y/o disminución de la degradación) o mediante su transporte (incremento de la absorción y/o disminución de la exportación).



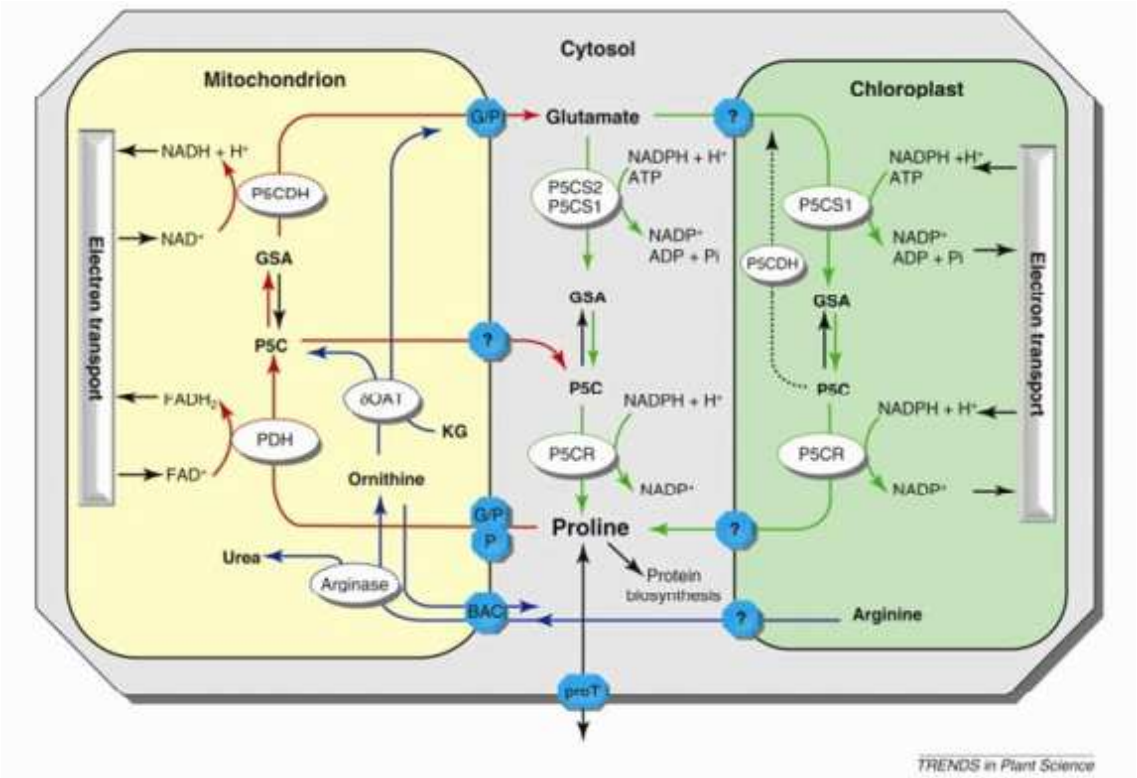
Esquema 6. Varias funciones de la prolina en plantas. Abreviaciones: APX, ascorbato peroxidasa; CAT, catalasa; PCD, muerte celular programada (Tomado de Szabados y Savouré 2010).

Entre los osmolitos mencionados antiormente, la prolina (Pro) es un imino ácido protenoigénico con una rigidez conformacional excepcional y este es esencial en el metabolismo primario (Szabados y Savouré 2010). Este compuesto es acumulado predominantemente en los plastos y citosol, y en menor medida en la vacuola. Este hecho ha revelado su papel como osmoprotector en plantas expuestas a una amplia variedad de tensiones ambientales (Schwache et al. 1999). Además la Pro es transportada a través del floema entre las distintas partes de la planta (Schwache et al. 1999; Verslues y Sharma 2010). Por ello se ha visto que además de su papel osmoprotector, la Pro puede actuar como molécula de señalización, modulando funciones mitocondriales, influyendo en la proliferación celular o desencadenando la muerte de las células así, como en la regulación de la expresión de genes específicos que pueden ser esenciales en la recuperación de las plantas sometidas a un estrés (Szabados y Savouré 2010) (Esquema 6). Además, se ha demostrado que este osmolito se encuentra implicado en el desarrollo de las plantas, acumulándose en órganos y

estructuras reproductivas tales como flores, anteras y polen (Biancucci et al. 2015). Sin embargo, pese a tener una función protectora importante en la planta ante diversos tipos de estrés, una correlación entre su acumulación y tolerancia sólo ha sido comprobada en ciertos tipos de estrés tales como la salinidad o la sequía. Sin embargo, en la actualidad su papel en situaciones de estrés por metales pesados no ha sido elucidada (Szabados y Savouré 2010; Verslues y Sharma 2010; Biancucci et al. 2015).

Como se mecionó anteriormente, los niveles intracelulares de este soluto compatible están determinados por su biosíntesis, catabolismo y el transporte entre las células y diferentes compartimentos celulares. En las plantas, la Pro se sintetiza principalmente a partir de glutamato en el citosol, que es reducido a glutamato semialdehído (GSA) por la enzima pirrolina-5-carboxilato sintetasa (P5CS), y este es espontáneamente convertido a pirrolina-5-carboxilato (P5C). Finalmente la enzima P5C reductasa (P5CR) reduce la P5C hasta formar Pro (Szabados y Savouré 2010). Posteriormente, la Pro formada puede ser transportada a la mitocondria a través de transportadores para su catabolismo por la enzima prolina deshidrogensa (PDH), la cual cataliza la conversión de la prolina a P5C, que es convertida a continuación en glutamato por la pirrolina-5-carboxilato deshidrogenasa (P5CDH) (Qamar et al. 2015) (Esquema 7).

Además, existe otra posible ruta de síntesis de la Pro, como es la llamada ``Vía de la ornitina´´. En esta vía actúan las enzimas, ornitina δ-aminotransferase (δ-OAT) y, posiblemente, la ornitina α-aminotransferase (α-OAT), (Biancucci et al. 2015). El catabolismo de arginina a ornitina por la arginasa y la transaminación posterior de la ornitina por la OAT produce P5C y glutamato en las mitocondrias. Finalmente, la P5C mitocondrial podrá ser reciclada a Pro en el citosol por la P5CR (Szabados y Savouré 2010; Qamar et al. 2015) (Esquema 7).



Esquema 7. Modelo propuesto para el metabolismo de la prolina en las plantas superiores. La ruta de la biosíntesis está marcada con líneas verdes, la vía catabólica con líneas rojas y la vía ornitina con líneas azules. Las enzimas se representan como elipses y proteínas transportadoras como octógonos azules (Tomado de Szabados y Savouré 2010)

Con respecto a las enzimas relacionadas con el metabolismo de la Pro, se ha comprobado como bajo condiciones de estrés la enzima P5CS puede acumularse en los cloroplastos favoreciendo de este modo la síntesis de Pro y mejorando la respuesta de la planta ante situaciones de estrés (Szabados y Savouré 2010). Además, la Pro puede ser catabolizada en respuesta al estrés osmótico, pero una vez se supera el estrés esta es oxidada por la PDH en las mitocondrias, regenerando el balance NADP/NADPH o el balance redox (Kavi-Kishor et al. 2005). Además se ha comprobado que la vía de la ornitina se incrementa enormemente ante condiciones ambientales desfavorables en plantas de *Arabidopsis thaliana*, *Brassica napus y Medicago truncatula* (Szabados y Savouré 2010, revisión). Sin embargo, algunos autores han demostratado recientemente

que la Pro no ejerce un papel protector y no se correlaciona con la toxicidad de Zn en plantas de *Brassica* y *Cajanus* (Alia et al. 1995)

Por otro lado, el ácido γ-aminobutírico. (GABA) es un aminoácido no proteico que comparte muchas características como osmolito compatible con la Pro (Shelp et al. 1999; Verslues y Sharma 2010; Anjum et al. 2014). Este compuesto se encuentra implicado en el metabolismo de la planta, incluyendo el balance C/N, balance energético, señalización celular, regulación del pH, defensa contra patógenos y en el crecimiento y el desarrollo (Anjum et al. 2014; Biancucci et al. 2015). Además, al igual que la Pro, el GABA puede acumularse rápidamente ante diferentes condiciones adversas, incluida la toxicidad por metales pesados (Obata y Fernie 2012).

En plantas y animales, el GABA es principalmente metabolizado por una vía compuesta por 3 enzimas, llamada GABA-shunt ya que pasa por dos etapas del ciclo TCA (Bouché y Fromm 2004; Michaeli y Fromm 2015). Por otra parte, la síntesis de este compuesto puede verse promovida por elevados niveles de glutamato (Shelp et al. 1999). Sin embargo, es conocido que pueden darse fenómenos antagónicos con la Pro, ya que las enzimas de síntesis de ambos compuestos utilizan el glutamato como sustrato.

El GABA es sintetizado en el citosol por la enzima glutamato descarboxilasa (GAD) y posteriormente se cataboliza en la mitocondria por la acción del la GABA transaminasa (GABA-T) para producir succínico semi-aldehído (SSA) con la posible participación de varios aceptadores amino tales como α-cetoglutarato (α-KG), piruvato o glioxilato. Posteriormente, la SSA es convertido a succinato por otra enzima mitocondrial, la SSA deshidrogenasa (SSADH). Finalmente, el succinato generado podrá actuar tanto como un donante de electrones a la cadena de transporte de electrones mitocondrial (ETC) y como una componente del TCA (Michaeli y Fromm 2015).

Existen otras dos rutas biosínteticas del GABA. Una de ellas es por la degradación de poliaminas por las enzimas diamino oxidasa (DAO) y poliamina oxidasa (PAO) dando lugar a la formación de aminoácidos tales como la β-alanina y el GABA (Shelp et al. 2012) y, la otra es por una conversión no enzimática de la Pro a GABA bajo condiciones de estrés oxidativo (Signorelli et al. 2015).

En cuanto a los metales pesados, el papel protector del GABA ha sido ampliamente discutido. Con respecto al Zn, una elevada exposición a este metal en plantas de *Nicotiana tabaccum* produjo una disminución del GABA, así como de las actividades del GABA-shunt sin promover la homeostasis celular (Daş et al. 2016).

Finalmente, la glicina betaína (GB) es uno de los solutos compatibles más estudiados. La GB es un amonio cuaternario que se encuentra presente en una gran variedad de plantas (Chen y Murata 2011; Krasensky y Jonak 2012). Este es un osmoprotector efectivo que es sintetizado naturalmente y puede acumularse en respuesta a varios tipos de estrés, tales como la salinidad, sequía y temperatura (Ashraf y Foolad 2007; Anjum et al. 2014) y metales pesados (Sharma y Dietz 2006).

En general, la GB se encuentra involucrada en la protección de la maquinaria fotosintética manteniendo la integridad de las membranas, respuestas ante el estrés ambiental y osmótico (actuando como agente antioxidante) y previniendo la acumulación excesiva de ROS (Chen y Murata 2011; Dhir et al. 2012; Anjum et al. 2014).

En plantas superiores este osmolito se sintetiza en los cloroplastos a partir de la serina, etanolamina, colina, betaína aldehído. La colina es oxidada por la colina monooxigenasa (CMO) dando lugar a la betaína aldehído la cual también será oxidada por la enzima betaína aldehído deshidrogenasa (BADH) (Ashraf y Foolad 2007). Esta vía de síntesis

es bien conocida, sin embargo, la vía de degradación de la GB no ha sido demostrada en plantas superiores (Barra et al. 2006).

Con respecto a las respuestas de las plantas ante toxicidad de Zn, Sharma y Dietz (2006) demostraron como ante condiciones de suelos contaminados por Zn las plantas acumulaban una mayor cantidad de GB. Sin embargo, en la actualidad el papel de la GB en condiciones de toxicidad por metales pesados ha sido profundamente cuestionado (Ali et al. 2015).

2.4. Biofortificación con Zn en plantas

2.4.1. Deficiencia de Zn en suelos y cultivos

Aproximadamente el 30% de la tierra cultivada del mundo es deficiente en Zn (Liu et al. 2012). De hecho, la deficiencia de Zn en el suelo se encuentra muy extendida en el mundo llegando a afectar a grandes áreas de suelos cultivados en India, Turquía, China, Brasil, Bangladesh y Australia (Humayan-Kabir et al. 2014). En una muestra mundial de 190 suelos de 25 países se llegó a encontrar que el 49% de ellos eran bajos en Zn. Ello es debido a que a diferencia de otros micronutrientes la deficiencia de Zn puede presentarse de forma ubicua, es decir, esta ser producida tanto en climas fríos como cálidos, en suelos drenados e inundados y en suelos ácidos y alcalinos (Guerinot y Eidet 1999).

Bajo esta situación puede ocasionarse una deficiencia aguda de Zn en las plantas superiores (Alloway 2004), llegando a producir una alteración de varios procesos fisiológicos y provocando una rápida inhibición del crecimiento, desarrollo y rendimiento final de la planta (Cakmak 2000). De hecho la deficiencia de este

micronutriente es probablemente la más extendida limitando la producción y calidad en cultivos tales como el arroz (Guerinot y Eidet 1999).

Los síntomas que se encuentran en las plantas de cultivos que sufren deficiencia de Zn pueden producrise en diferentes grados de severidad y en diversas combinaciones en diferentes especies de plantas. En general, los síntomas en las plantas se pueden clasificar como: síntomas visuales, histológicos y bioquímicos (Humayan-Kabir et al. 2014). En este sentido cabe destacar que la deficiencia de Zn puede conducir a un aumento de la producción de ROS como resultado de una disminución de las actividades Cu-Zn superóxido dismutasa (Cu-Zn/SOD), anhidrasa carbónica (AC) y alcohol deshidrogenasa (ADH), inhibición proteíca y un aumento de la acumulación de Fe, provocando daños en las membranas, proteínas, clorofilas y otras enzimas (Sinclair y Krämer 2012).

Como se ha mencionado anteriormente, en todo el mundo, existen muchos suelos que son deficientes en Zn o con baja biodisponibilidad de este elemento. En consecuencia, la mayoría de las cosechas cultivadas contienen bajas concentraciones de Zn lo cual puede conducir a un incremento en la malnutrición de Zn en humanos (Barabasz et al. 2010) (Imagen 2).

2.4.2. Deficiencia de Zn en humanos

Hace unos 50 años el Zn fue reconocido como un micronutriente esencial para la salud humana por el Dr. Ananda Prasad, químico en la Universidad Estatal de Wayne en Detroit, Michigan. Actualmente, la deficiencia de Zn se reconoce como un problema nutricional en todo el mundo tanto en los países desarrollados como en los países en vías de desarrollo (Nriagu 2007).

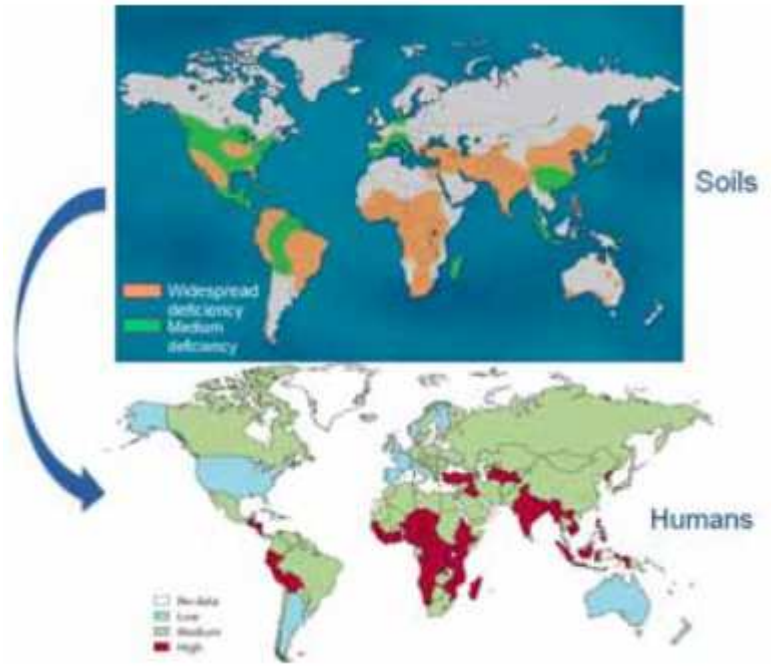


Imagen 2. La deficiencia de zinc en el mundo en los suelos y los seres humanos (Tomado de Alloway 2008)

Las deficiencias de Zn y Fe son las deficiencias de micronutrientes más comunes en las poblaciones humanas llegando a afectar a la salud de más de tres mil millones de personas en todo el mundo (Welch y Graham 2004; Cakmak et al. 2010). De acuerdo con un informe publicado en el año 2002 por la Organización Mundial de la Salud, la deficiencia de Zn se encuentra en el quinto puesto en términos de una de las principales causas de enfermedad en el desarrollo de alta mortalidad en los países. Además, cabe destacar que alrededor de 800.000 personas mueren cada año debido a la deficiencia de este micronutriente, de los cuales 450.000 son niños menores de cinco años (OMS, 2002).

El espectro de efectos clínicos ante una deficiencia de Zn depende de la dosis, la edad, estadio de desarrollo, la deficiencia del metal y de otros micronutrientes relacionados, así como de la susceptibilidad individual. Respecto a los síntomas, estos pueden incluir:

disfunción del sistema inmunitario primario (que conduce a neoplasias, infecciones víricas y fúngicas); infecciones oportunistas frecuentes (por virus, toxinas y venenos); alergias respiratorias y de la piel; asma; diarrea crónica; cambios neurosensoriales anormales; falta de apetito (especialmente en los jóvenes y ancianos); letargo mental; problemas de fertilidad (hipogonadismo, insuficiencia de la madurez sexual, prostatitis, cólicos menstruales e hinchazón); defectos de nacimiento; enanismo y retraso del crecimiento; envejecimiento prematuro; problemas de visión; pérdida del gusto; dolor en articulaciones; hipertensión; angina de pecho; isquemia esfuerzo; retraso de la cicatrización; esclerodermia; pérdida de color del pelo; anemia; estrías; ceguera nocturna; acné; tejido conectivo defectuoso y degeneración macular; apatía e irritabilidad. Además la deficiencia de Zn también ha sido vinculada con el pecho excavado, el VIH y el SIDA, enfermedad de las células falciformes (ECF), ciertas enfermedades relacionadas con el envejecimiento del cerebro y enfermedades neurodegenerativas como el Parkinson y el Alzehimer, así como una serie de síndromes como: Marfan, Ehlers-Danlos (EDS), Wilson y el síndrome del prolapso de la válvula mitral (Nriagu 2007; Kaur et al. 2014).

2.4.3. Biofortificación agronómica con Zn: Una herramienta eficaz para paliar la malnutrición por Zn

Se ha demostrado como la suplementación con Zn puede llegar a mitigar las enfermedades anteriormente mencionadas (Nriagu 2007). En EE.UU. la cantidad diaria recomendada (RDA, o la ingesta adecuada) de Zn ha sido definida como 8,0 a 13,0 mg. Desafortunadamente, las dietas de muchas personas en todo el mundo carecen del Zn suficiente para una nutrición adecuada. Este hecho ha sido atribuido a la compra de

productos con baja fitodisponibilidad de minerales, al consumo de cultivos con concentraciones del mineral inherentemente bajas en los tejidos o al uso de alimentos procesados (White y Broadley 2011).

En este sentido, mejorar el estado nutricional de Zn en las plantas puede proporcionar beneficios adicionales tanto para la producción de cultivos como para la nutrición humana. Por ello, la contribución a la salud humana a través de la fertilización de los cultivos de alimentos básicos parecer ser una herramienta excelente para aliviar los problemas de la deficiencia de Zn en los humanos de todo el mundo (Gibson y Ferguson 1998; Cakmak 2008). En este contexto, las estrategias tradicionales de intervención nutricional para mitigar las deficiencias de minerales y vitaminas han basado principalmente su interés en la suplementación, fortificación y diversificación / modificación de la dieta (DMD) (Gibson y Ferguson 1998; Welch y Graham 2004).

Recientemente, la biofortificación ha sido propuesta como una forma de mejorar la calidad nutricional de los alimentos de la planta, y se define como el proceso de incrementar la concentración biodisponible de los elementos esenciales, como el Zn, en las partes comestibles de los cultivos, a través de prácticas agrícolas o de la selección genética (White y Broadley 2005; HarvestPlus, 2012). En general, esta herramienta va dirigida a una eficiencia en la absorción, principalmente en suelos pobres, así como en una translocación eficiente hacia las partes comestibles de las plantas de cultivo (Barabasz et al. 2010). Además, se ha demostrado que un programa de biofortificación puede ser un método sostenible y rentable para mejorar el estado de Zn a nivel nacional en los países donde la deficiencia de este micronutriente es endémica. Alternativamente, la biofortificación puede ser dirigida en regiones específicas y / o para ciertos grupos de alto riesgo (por ejemplo, alimentos de destete para lactantes) dentro de un país. Esta técnica no requiere ningún cambio en las creencias y prácticas de los consumidores de

alimentos existentes y, a diferencia de la suplementación, no impone una carga para el sector de la salud. Por otra parte, debido a que el coste de la biofortificación es asumido por la industria y el consumidor, los costes para los gobiernos son generalmente bajos (Gibson y Ferguson 1998; Cakmak 2008).

En segundo lugar, la biodisponibilidad de los alimentos de origen vegetal es generalmente más baja debido a la fibra dietética y el ácido fítico, que inhiben la absorción de Zn. En este sentido, son muchos los estudios que han focalizado sus esfuerzos en incrementar la concentración de Zn fundamentalmente en cereales, tales como el arroz. Sin embargo, los resultados obtenidos en estos programas no han tenido demasiado éxito, ya que el grano no llega a acumular cantidades elevadas de Zn. Además, debido a que el grano presenta un alto contenido de fitatos esto puede promuever una menor biodisponibilidad de este micronutriente en el ser humano. En tales contextos, la DMD podría ser la mejor manera para incrementar el consumo tanto de alimentos ricos en Zn como para la absorción de promotores de éste en el cuerpo humano (Gibson 2012; Das y Green 2013).

Una variedad amplia de estrategias pueden ser incorporadas en un programa de DMD. Estos incluyen: (a) aumento de la producción y consumo de alimentos con un alto contenido de micronutrientes, como el Zn (b) aumento de la producción y el consumo de alimentos conocidos por mejorar la biodisponibilidad de del elemento; (c) reducción del contenido de fitatos; (d) aumento la densidad energética en las dietas (Gibson 2012). En tales contextos, la biofortificación y la DMD pueden ser las opciones preferidas, ya que son más accesibles a las personas que consumen alimentos básicos desde un nivel local o mediante autoproducción (Gibson 2012).

2.4.4.1. Importancia del Zn en la producción y calidad de los cultivos

La deficiencia de micronutrientes no sólo va a afectar a la producción de los cultivos destinados al consumo humano sino también a su calidad (Grotz y Guerinot 2006). Es por ello que muchos plantas cultivadas en zonas con déficit de micronutrientes pueden llegar a presentar concentraciones de metabolitos secundarios bajas y por tanto la ingesta de estos compuestos llegan a ser inferiores al óptimo necesario para promover la mejora de la salud humana (Messias et al. 2015). Por lo tanto, en un programa de biofortificación con Zn, no sólo bastará con promover la concentración del elemento en la parte comestible sino que las plantas objeto de estudio deberán mantener su calidad.

Es por ello que en la actualidad, otro aspecto de gran interés es la relación entre el consumo de frutas y verduras y la relación con los beneficios en la salud reduciendo el riesgo de cáncer y enfermedades cardiovasculares (Liu 2004). Estas propiedades han sido atribuidas a los alimentos que son fuentes ricas de numerosos compuestos bioactivos tales como fitoquímicos con propiedades antioxidantes conocidos por conferir beneficios que promueven la salud (Cartea et al. 2011; Pérez-López et al. 2013).

En los últimos años, el papel de los antioxidantes dietéticos naturales en la prevención de las enfermedades en la salud humana ha llevado a la investigación en el campo de la horticultura y en la ciencia de los alimentos a evaluar los fitoquímicos antioxidantes en frutas y vegetales (Singh et al. 2006; Volden et al. 2009; Messias et al. 2015). Estos antioxidantes incluyen vitaminas solubles, tales como AsA, carotenoides y tocoferoles, así como compuestos fenólicos (Kurilich et al. 2002). Estos antioxidantes inhiben las reacciones de las ROS y por lo tanto pueden proteger a las células contra el daño oxidativo (Kurilich et al. 2002).

Cabe destacar que es de gran interés seleccionar la planta idónea así como llevar a cabo un estudio de los metabolitos secundarios de la planta en un programa de biofortificación, con el fin de asegurar las propiedades nutricionales de las plantas.

En este contexto, la lechuga es una hortaliza de la familia de las Asteráceas cuyo cultivo se ha visto incrementado enormemente tanto en los países orientales como occidentales. Por ejemplo, en China tras la revolución verde o en EEUU desde la década de 1970 (Mulabagal et al. 2010). Entre los principales países productores que se reflejan en la Tabla 1, cabe destacar a Asia, EEUU, India y España (FAO, 2012). En Europa, la lechuga es el principal cultivo alimenticio y es el segundo vegetal consumido fresco tras la patata en USA (Baslam et al. 2013; Bouain et al. 2014).

Tabla 1. Principales países productores (en toneladas) de *Lactuca* y *Brassica* según datos de la FAO (2012).

País productor	Cultivo de *Lactuca*	País productor	Cultivo de *Brassica*
China	14,000,00	China	32,800,000
Estados Unidos	3,875,543	India	8,500,000
India	1,075,000	Rusia	3,309,315
España	877,000	Japón	2,300,000
Italia	755,697	Sur de Corea	2,118,930
Irán	570,000	Ucrania	1,922,400
Japón	566,100	Indonesia	1,487,531
Alemania	420,595	Polonia	1,198,726
Turquía	419,066	Rumania	990,154
Méjico	335,337	Estados Unidos	964,830

De acuerdo con la Comisión Europea Eurostat (2011), la cantidad total de la producción de lechuga en la Unión Europea alcanzó casi tres millones de toneladas en 2010, con España (809.000 toneladas) como el país productor importante en la Unión Europea y el principal exportador del mundo (Pérez -López et al. 2013). Esta hortaliza es una verdura de hoja importante en la dieta que se consume principalmente fresca o mezcladas en ensalada (Liu et al. 2007). Además, junto con la escarola, es una de las hortalizas más populares en las ensaladas que se consumen en cantidades cada vez mayores debido a su percepción de estar entre los alimentos más saludables y tener un alto valor nutritivo (DuPont et al., 2000;. Pérez-López et al, 2013). El efecto de la lechuga en la salud se debe principalmente a sus propiedades reductoras del colesterol (Huang 1980; Anderson y Hanna 1999) y enfermedades cardiovasculares (Serafini et al. 2002; Nicolle et al. 2004). Además, en la medicina popular las semillas de lechuga se utilizan en el tratamiento del asma, la tos y como analgésico (Mulabagal et al., 2010). Estas propiedades saludables se atribuyen a una gran cantidad de compuestos antioxidantes (por ejemplo, la vitamina E, polifenoles, antocianinas, carotenoides, AsA) y el contenido de fibra (Serafini et al., 2002; Nicolle et al. 2004; Llorach et al., 2008; Li et al, 2010). Además, la lechuga es una importante fuente de minerales y posee un bajo aporte calórico en la dieta (Bouain et al. 2014) (Tabla 2).

A pesar de su importancia nutricional de la lechuga, un aspecto a considerar en el consumo de lechuga es que esta planta es definida como una planta hiperacumuladora de nitratos (NO_3^-). Esta hortaliza puede llegar a acumular valores superiores a los límites permitidos por la Comunidad Europea (3500-4500 mg kg^{-1} PF) (EU, 2011). Los NO_3^- no son relativamente tóxicos per se, pero aproximadamente un 5% de los NO_3^- ingeridos son convertidos en la saliva y el tracto gastrointestinal en una forma más

tóxica como son los nitritos (NO_2^-) llegando a causar patologías severas en humanos como la metahemoglobinemia o ``síndrome del bebé azul´´ (Santamaria 2006).

Tabla 2. Valor nutricional en 100 gramos (g) de *Lactuca sativa y Brassica oleracea* según el Departamento de Agricultura de los Estados Unidos (USDA).

	Lactuca sativa	*Brassica oleracea*
Aporte energético	13 Kcal	25 kcal
Carbohidratos:	2.23 g	5.8 g
Azúcares	0.94 g	3.2 g
Fibra	1.1 g	2.5 g
Grasas	0.22 g	0.1 g
Proteínas	1.35 g	1.28 g
Vitaminas	7,7823 mg	37,39 mg
Minerales	325,619 mg	266,81 mg
Agua	95.63 g	-
Fluoruro	-	1 µg

Por lo tanto la aplicación de técnicas agrícolas y/o la selección de genotipos eficientes en el uso del nitrógeno, podrían promover una menor fertilización nitrogenada así como una mayor producción y un mayor aporte de compuestos esenciales para la salud humana.

Otra especie de plantas ampliamente cultivadas son aquellas dentro del género *Brassica* (Tabla 1). Esta especie se ha cultivado en Europa y el norte de Asia durante al menos 600 años y desde la primera parte del siglo XX se ha incrementado su cultivo en América del Norte (Horn 1985). El género *Brassica* es el más importante dentro de la familia Brassiceae, que incluye algunos cultivos y especies de gran importancia económica a nivel mundial, tales como *Brassica oleracea*. Los Brassicaceae representan

una parte importante de la dieta humana en todo el mundo y son considerados cultivos alimentarios muy importantes en China, Japón, India y los países europeos (Cartea et al. 2011). Vegetales de *Brassica* se consumen todo el año como ingredientes de diferentes ensaladas o después de la cocción de verduras crudas y congeladas (Podsedek 2007). Además, la popularidad y el consumo de especies de *Brassica* se encuentran en aumento debido su contribución a la nutrición humana y otros beneficios para la salud (Salunkhe y Kadam 1998). Este efecto sobre la salud *de Brassica* son las propiedades de prevención de enfermedades cardiovasculares así como el cáncer (Cartea et al., 2011). Por otra parte, la col se ha valorado con fines medicinales en el tratamiento de dolores de cabeza, gota, diarrea y úlceras pépticas (Cheney 1950).

En *Brassica*, estas propiedades promotoras de la salud son debidas a que esta especie contiene una gran cantidad de fitoquímicos: antioxidantes, azúcares libres, lípidos, minerales y aminoácidos libres o formando proteínas (Podsedek 200; Park et al. 2014) (Tabla 4). Particularmente, *Brassica* contiene muchos antioxidantes, incluyendo carotenoides, compuestos fenólicos, antocianina, AsA y GABA (Wang et al. 1996; Galati & O ' Brien 2004).

Asimismo, ciertos aminoácidos aromáticos, tales como la fenilalanina (Phe), tirosina (Tyr) y triptófano (Trp) son moléculas centrales en el metabolismo de la planta. Además de ser precursores de las proteínas, estos tres aminoácidos son precursores de una amplia gama de metabolitos secundarios (glucosinolatos y fenoles) con múltiples funciones biológicas y valor en la promoción de la salud, medicina e industria alimentaria. Además, están implicados en la síntesis de hormonas tales como: auxina y salicilato (Tzin y Galili 2010). Especialmente, el género *Brassica* presenta un alto contenido en glucosinolatos (GSL) y fenoles (Bravo 1998; Vrchovská et al. 2006; Kinsella et al. 2014).

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Capítulo 3

Objetivos

El Zn es un micronutriente esencial necesario para las plantas, los animales y los microorganismos. Sin embargo, debido al incremento de las actividades industriales las deposiciones de este elemento traza se ha incrementado. Bajo estas circunstancias, el Zn puede llegar a acumularse en exceso en los tejidos vegetales provocando alteraciones fisiológicas e inhibición del crecimiento. Por otra parte, la deficiencia de Zn en los suelos representa hasta un 30% de las tierras cultivadas. Bajo esta situación puede ocasionar una deficiencia aguda en plantas superiores lo cual puede conducir a un incremento de la malnutrición de Zn en humanos llegando a afectar a la salud de más de tres mil millones de personas en todo el mundo. La fitorremediación de suelos de metal / metaloide contaminados como la biofortificación de micronutrientes minerales en los cultivos de alimentos en beneficio de la nutrición humana representan dos aplicaciones biotecnológicas potenciales. Así, la comprensión en la variación inter e intraespecífica en cuanto a la absorción de minerales, distribución, metabolismo y tolerancia, y los mecanismos moleculares responsables de los procesos, son cruciales para ambas aplicaciones. Por tanto los objetivos fundamentales de esta Tesis Doctoral han sido los siguientes:

1. Realización de un estudio de las diferentes estrategias en plantas de *Lactuca sativa* y *Brassica oleracea* ante la toxicidad de Zn (0.5 mM), tales como el metabolismo de los carboxilatos y el metabolismo oxidativo y del glutatión así como de compuestos osmoprotectores con el fin de definir procesos fisiológicos claves para seleccionar y/o generar plantas resistentes a la toxicidad de Zn, así como utilizar estas plantas hortícolas en programas de descontaminación (fitoextracción) en aquellas zonas contaminadas por este elemento traza.

2. Considerando que las dos plantas hortícolas utilizadas en este trabajo de investigación son muy utilizadas en programas de biofortificación con elementos traza, el objetivo de la segunda parte de esta Tesis Doctoral será la realización de un programa de biofortificación agronómica con Zn consistente en una aplicación supraóptima de este elemento (10-100 μM) con el fin de analizar: la acumulación de Zn en la parte comestible de la planta y comprobar la respuesta de determinados parámetros de calidad nutricional así como la asimilación del NO_3^- en *L. sativa* y la síntesis y acumulación de compuestos bioactivos en *B. oleracea*.

Capítulo 4

Estudio comparativo del efecto tóxico de Zn en plantas hortícolas

4.1. Comparative study of the toxic effect of Zn in Lactuca sativa and Brassica oleracea plants: I. Growth, distribution, and accumulation of Zn, and metabolism of carboxylates

Abstract

Zinc (Zn) is an essential micronutrient for plants, animals, and microorganisms. However, in environmental situations of heavy-metal soil pollution, Zn constitutes a major problem for worldwide agricultural production. Organic anions are compounds that have a special structure which proves indispensable to tolerate excess Zn. The aim of the present work was to determine whether carboxylate metabolism is a key physiological process to select and/or generate plants tolerance to Zn toxicity (0.5 mM). For this, we make a comparative analysis of the toxic effect of Zn between two horticultural plants of great agricultural interest, i.e. *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. The tolerance under Zn-toxicity conditions was greater in *B. oleracea* than in *L. sativa* despite to show a higher Zn concentration in shoot. Regarding organic anions metabolism, in leaves of *L. sativa* the enzyme malate dehydrogenase (MDH) notably increased its activity under Zn-toxicity conditions and both under control conditions as well as in Zn toxicity the main organic anion was malate. With respect to *B. oleracea* under Zn-toxicity conditions the MDH activity declined with respect to control increasing malate levels in leaves, also under control as well as toxicity conditions the citrate synthase (CS) activity was high and the predominant organic anion was citrate. These results suggest that both in programmes of phytoextraction as well as in biofortification with Zn that are based on the fertilization enriched with this element, *B. oleracea* is more effective than L. sativa and

that the organic anion citrate could be determinant in the tolerance and greater concentration of this element in leaves.

Keywords: Zn toxicity, carboxylate metabolism, malate, citrate.

Abbreviations: CS, citrate synthase; DC, distribution coefficient; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PVP, polyvinylpyrolidone; TCA, tricarboxylic acid cycle.

4.1.1. Introduction

Zinc (Zn) is an essential micronutrient for plants, animals, and microorganisms (Dhankhar et al., 2012). In plants, Zn is required at optimal concentrations, both for the normal functioning of cell metabolism as well as for plant growth (Broadley et al., 2007). This is involved in many physiological processes such as the metabolism of carbohydrates, lipids, and nucleic acids; gene expression and regulation; enzyme activation; protein synthesis; and reproductive development such as pollen formation (Brown et al., 1993; Cakmak, 2000).

However, in environmental situations of heavy-metal soil pollution, Zn constitutes a major problem for worldwide agricultural production (Miretzky et al., 2004). Under these conditions, Zn accumulates in excess in the plant tissues, reaching toxic concentrations (> 300 $\mu g\ g^{-1}$) for plants, provoking physiological alterations and growth inhibition by altering carbohydrate metabolism (Foy et al., 1978), lowering the content in essential nutrients such as Fe, Cu, and Mn (Connolly et al., 2002, 2003; Sagardoy et al., 2009), causing oxidative damage to membranes, altering photosynthetic activity,

and inhibiting the synthesis of photosynthetic pigments, electron transport, and Rubisco activity, among other processes (Clemens, 2001; Broadley et al., 2007; Vassilev et al., 2007). This situation of Zn toxicity arises as a result of natural or anthropogenic components derived from numerous industrial activities, including: excessive fertilizer and pesticide use, mining, smelting, and refining (Cammarota, 1980; Broadley et al., 2007).

Under deficiency and toxicity conditions of Zn, plants maintain the homeostasis of this element by regulating its uptake processes, utilization, and storage (Liu et al., 2012). Many works suggest that in stress situations involving trace elements, the plant produces low-molecular-weight compounds involved in the long-distance transport in the xylem and it subsequent detoxification in the cytoplasm and/or vacuole (Küpper et al., 1999, 2000; Assunção et al., 2003). Notable among these compounds are organic anions, phytochelatins, histidine, nictotinamine, and phytosiderophores (Sinclair and Krämer, 2012). With respect to organic acids, these are compounds that have a special structure which proves indispensable to tolerate excess Zn, as these contain predominantly carbon, hydrogen, and oxygen, and have one or more carboxyl groups (Jones, 1998). Therefore, organic anions may function as chelants that sequester Zn ions in the cytosol and subcellular compartments (Haydon and Cobbett, 2007). In this sense, Sun et al. (2006) have indicated that the accumulation of organic anions in the vacuole against an excess of metals could explain the role of these compounds in the subcellular compartmentalization of these elements under toxicity conditions. Recently, Liu et al. (2012) defined organic anions as chelants and transporters of Zn and therefore necessary for the uptake, translocation, accumulation, and storage within the plant, thereby guaranteeing efficient Zn distribution within the plant and thus detoxification under toxic conditions.

Among the organic anions found in plants, coming from the tricarboxylic acid cycle (TCA), the following acids can be distinguished: citrate, aconitate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, and oxalate. All these anions are produced in catalytic amounts in plant tissues, although only citrate, malate, and oxalate are accumulated (Sousa et al., 2009). Different studies indicate that detoxification by organic anions in leaf did not correlate with the different Zn treatments applied, while in root, a positive relation was found in citrate and malate (Zhao et al., 2000) as well as with oxalate (Liu et al., 2012). Confirming this hypothesis, it has recently been confirmed in *Erica andevalensis* plants that tolerance against high Zn concentrations appears to be promoted by the immobilization of the metal in the root system together with a low interference with nutrient uptake and increased production of organic ligands such as citrate and aminoacids (Rossini-Oliva et al., 2012). On the contrary, other authors indicate a positive relation of citrate (Salt et al., 1999) or malate (Mathys, 1977; Godbold et al., 1984) in leaves and the resistance to Zn toxicity, although this correlation depends on the plant species and the Zn concentration available in the culture medium. In this sense, by a comparative analysis between *Thlaspi caerulescens* and *Thaspi ochroleucum*, Shen et al. (1997) demonstrated that in the former species (a Zn-accumulating plant and therefore resistant to this trace element) there was a greater Zn concentration in leaf after the application of 500 μM Zn in comparison to control (10 μM Zn), which was associated with a rise in the concentration of malate in leaf, whereas in *T. ochroleucum* (a Zn-sensitive plant) the Zn concentration increased more in root with respect to control in *comparison with T. caerulescens,* with higher citrate levels in leaf in *T. ochroleucum* than in *T. caerulescens*. However, Wójcik et al. (2006), working with *T. caerulescens* detected an increase both in malate as well as in citrate in leaf, the accumulation level depending on the metal concentration. On the other hand, in leaves

of beetroot (*Beta vulgaris*) with a greater capacity to accumulate heavy metals, Sagardoy et al. (2011) demonstrated that the citrate and malate concentrations changed at the level of the leaf according to the Zn concentration used in the experiment. Finally, more recent work (Schneider et al., 2013) with *T. caerulescens* confirms the results of Shen et al. (1997), noting that the application of 500 μM of Zn increased the malate concentration in leaf, permitting the sequestering of Zn in the vacuole and conferring avoidance to the plant without toxicity symptoms in this organ.

In addition to all the works cited above, where the Zn concentration is related to the presence of different organic anions (mainly malate and citrate), differences have also been documented in sensitivity of TCA enzymes regarding exposure to several metals. With respect to Zn, the activity of malate dehydrogenase (MDH), which catalyses the reversible reaction of oxidation of malate to oxalate, is extremely compatible with this element, and it has been demonstrated that tolerant ecotypes possess a higher number of isoenzymes than non-tolerant ones in seeds and leaves (Mathys,1980). In addition, both MDH as well as citrate synthase (CS), responsible for this latter enzyme from the condensation of acetyl-CoA with a molecule of oxalate to give rise to citrate, appear to be highly sensitive to ions and also trigger competitiveness phenomena between the two enzymes (D'Souza and Srere, 1983). Another important enzyme associated with TCA is phosphoenolpyruvate carboxylase (PEPC). In plants, PEPC it is an enzyme involved in multiple physiological processes (Andreo et al., 1987). In all types of plants, there is a PEPC C3 that splits from glucolysis to replenish TCA intermediaries (the so-called anaplerotic pathway), thereby also providing precursors for amino acid synthesis (Stitt, 1999). Greater activity of this enzyme has been positively correlated with a higher Zn content in plants, because an increase in the metal decreases Fe, and the malate formed from this enzyme could improve CO_2 fixation (López-Millán et al., 2001). Furthermore,

an increase in PEPC and in isocitrate dehydrogenase (ICDH), which catalyses the formation of α-ketoglutarate through the decarboxylation and oxidation of isocitrate, has also been correlated with a greater accumulation of organic anions in the root in the presence of high Zn concentrations (Sagardoy et al., 2011).

In view of the above findings, the aim of the present work was to determine whether carboxylate metabolism is a key physiological process to select and/or generate plants tolerant to Zn toxicity, with the aim of using them in decontamination (phytoextraction) programmes in areas contaminated with this trace element. For this, we make a comparative analysis of the toxic effect of Zn between two horticultural plants of great agricultural interest, i.e. *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. Furthermore, taking into account that the two plants used in this research are widely used in biofortification programmes with trace elements, a comparison of the two in terms of the toxic effect of Zn will enable us to select the species that is more suited to agricultural biofortification programmes with Zn, consisting of the application of a supraoptimal amount of this element. The specific implementation of this type of programme is fundamental, given that some 30% of the world population suffers symptoms of Zn deficiency, a percent-age that could be reduced by the intake of agricultural products enriched in Zn.

4.1.2. Material and methods

4.1.2.1. Plant-growth conditions

Seeds of *L. sativa* cv. Phillipus and *B. oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant S.L.). After 35 days, the seedlings were

transferred to a growth chamber under the following controlled environmental conditions, with relative humidity of 50%, day/night temperature of 25/15◦C, a photoperiod of 16/8 h at a photosynthetic photon flux density (PPFD) of 350 µmol $m^{-2}s^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, Nebraska, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) in a volume of 3 L. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2 \cdot 4H2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2H_2O$, 2 µM $MnCl2 \cdot 4H_2O$, 10 µM $ZnSO_4 \cdot 7H_2O$, 0.25 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2H_2O$, 5 ppm Fe-chelate (Sequestrene; 138 FeG100) and 10 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was changed every three days.

4.1.2.2. Experimental design

Treatments were initiated 35 days after germination and were maintained for 21 days. Received control treatment nutrient solution, while treatment of toxicity was supplemented with the dose of 0.5 mM of Zn as $ZnSO_4$. The shape of the experimental design consisted of randomized complete block with four treatments (*L. sativa*-control, *B. oleracea*-control, *L. sativa*-0.5 mM, *B.oleracea*-0.5 mM), eight plants per treatment and three replicates per treatment.

4.1.2.3. Plant sampling

Plants of each treatment were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the roots and leaves from each treatment were frozen at −30◦C for later performance of

biochemical assays and the other half of the plant material sampled dried in a forced air oven at 70◦C for 24 h to obtain the dry weight (DW) and the subsequent analysis of the concentration of Zn.

4.1.2.4. Zn concentration and distribution coefficient

For the determination of Zn concentration, a sample of 30 mg dry material was subjected to a process of mineralization by the method of Wolf (1982). Zn concentration was determined by ICP-MS and expressed as mg kg^{-1}DW.Distribution coefficient (DC) was calculated as the quotient between Zn concentration in leaves and Zn concentration in roots (Zhu et al., 2003).

4.1.2.5. Concentrations of organic anions by HPLC

Approximately 0.1 g of samples frozen roots and leaves were homogenized with 1 ml milliQ water. The resulting mixture was centrifuged at 2.000 rpm for 25 min and diluted 1:5 with 10 mM KH_2PO_4 pH 2.6, then filtered through a 0.45 µm membrane filter and the samples were analyzed by HPLC–DAD. The organic acids analysis were carried out using an Agilent HPLC 1100 series equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). Samples were injected into a Luna C18 column (150 mm × 1.0 mm, 3 µm particle size; Phenomenex, Macclesfield, UK) operating at 25◦C. Mobilephase (10 mM KH_2PO_4, pH 2.6) was pumped with a 0.6 ml min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, citrate, and malate, were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. Quantification was made with known amounts of each organic anions using peak areas (Scherer et al., 2012).

4.1.2.6. Carboxylate metabolism

Extracts for measuring enzyme activities were made following the method of Li et al. (2000), modified by grinding 0.1 g of plant material in liquid N with 1 ml of extraction buffer containing 1 mM EDTA-Na, 10% glycerol, 1% Triton X-100, 5 mM DTT and 1% polyvinylpyrrolidone (PVP) in 100 mM Tris–HCl pH 8.0. The slurry was centrifuged for 5 min at 14,700 rpm and 4◦C, and the supernatant was collected and analyzed immediately. The activities of all enzymes were analyzed in 0.2 ml (final volume) of the media indicated below. The activity of MDH (EC1.1.1.37) was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (Dannel et al., 1995). The reaction was carried out with 0.1 mM NADH, 0.4 mM oxalate and 46.5 mM Tris–HCl, pH 9.5. CS (EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1969) by monitoring the reduction of acetyl coenzyme A (CoA) to CoA with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm. The reaction was carried out in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalate and 100 mM Tris–HCl, pH 8.1. ICDH (EC 1.1.1.42) activity was determined by monitoring the reduction of NADP at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM NADP, 0.55 mM isocitrate and 88 mM imidazole buffer pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) was assayed following the increase in optical density at 240 nm due to the formation of fumarate in 50 mM malate and 100 mM phosphate buffer, pH 7.4 (Bergmeyer et al., 1974). Finally PEPC (EC 4.1.1.31) activity was measured in a coupled enzyme assay with the MDH in 2 mM phosphoenolpyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH and 100 mM of N,N-bis[2-hydroxyethyl]glycine(Bicine)-HCl, pH 8.5 (López-Millán et al., 2001).

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

4.1.2.7. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics 5.1 program. Means were compared by Fisher's least-significant differences (LSD). The significance levels for both analyses were expressed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ or NS (not significant).

4.1.3. Results

4.1.3.1. Biomass, Zn concentration and DC

L. sativa as well as *B. oleracea* plants accumulated fundamentally Zn in the root when they were applied 0.5 mM de Zn (Table 1), producing the same level of toxicity in this organ with similar reductions in root biomass. However, the behaviour of leaves differed between plants. That is, while in L. sativa the application of 0.5 mM of Zn raised the Zn concentration with respect to control (+ 270%) and sharply lowered biomass, in *B. oleracea* this greater Zn concentration, although exceeding that of *L. sativa* (+ 622%), did not lower aerial biomass (Table 1) despite accumulation to a level considered toxic (Marschner, 1995).Moreover, in both plants the DC fell drastically with the application of 0.5 mM de Zn (Table 1), since, as we confirmed, most of the Zn under these conditions accumulated in the root (Table 1). However, given the Zn toxicity, the DC in *B. oleracea* was again greater than that of *L. sativa*, despite to show a higher Zn concentration in leaves. Furthermore, less decrease in this coefficient with respect to its control (*L. sativa* −96% and *B. oleracea* −87%; Table 1).

Table 1. Zn concentration root and leaf biomass and distribution coefficient of Zn (DC) in plants of *L. sativa* and *B. oleracea*.

	Root Zn concentration (µg g^{-1} DW)	Root biomass (g DW/plant)	Leaf Zn concentration (µg g^{-1} DW)	Foliar biomass (g DW/plant)	DC
***Lactuca sativa* cv. Phillipus**					
Control	585.7 ± 68.7	0.135 ± 0.003	80.78 ± 4.39	2.11 ± 0.19	0.138 ± 0.007
0.5 mM	34,949.1 ± 376.1	0.042 ± 0.010	218.4 ± 35.7	1.43 ± 0.04	0.006 ± 0.001
p-value	***	***	**	*	***
$LSD_{0.05}$	10,440.9	0.03	83.02	0.53	0.02
***Brassica oleracea* cv. Bronco**					
Control	895.1 ± 104.3	0.083 ± 0.003	61.43 ± 0.66	0.70 ± 0.06	0.069 ± 0.001
0.5 mM	42,540.7 ± 234.2	0.033 ± 0.006	382.4 ± 60.6	0.61 ± 0.08	0.009 ± 0.001
p-value	***	**	***	NS	***
$LSD_{0.05}$	862.77	0.02	139.86	0.29	0.004

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2. Enzymes of the tricarboxylic acid cycle in leaves of *L. sativa* and *B. oleracea.*

	PEPC	Fumarasa	MDH	CS	ICDH
	(ΔAbs mg^{-1} prot min^{-1})				
***Lactuca sativa* cv. Phillipus**					
Control	0.20 ± 0.03	0.81 ± 0.06	3.37 ± 0.97	0.26 ± 0.02	0.81 ± 0.07
0.5 mM Zn	0.24 ± 0.02	0.44 ± 0.11	6.43 ± 1.94	0.13 ± 0.02	0.70 ± 0.12
p-value	NS	*	*	*	NS
$LSD_{0.05}$	0.11	0.34	2.03	0.08	0.39
***Brassica oleracea* cv. Bronco**					
Control	0.12 ± 0,02	0.29 ± 0.08	13.07 ± 1.77	1.95 ± 0.02	0.013 ± 0.00
0.5 mM Zn	0.17 ± 0,04	0.32 ± 0.02	6.25 ± 0.65	1.94 ± 0.10	0.015 ± 0.00
p-value	NS	NS	*	NS	NS
$LSD_{0.05}$	0.11	0.22	5.24	0.29	0.006

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$.

4.1.3.2. Carboxylate metabolism and concentrations of organic anions

For *L. sativa* under a concentration of 0.5 mM Zn, Table 2 indicates a decline in fumarase and CS activities with respect to control, while the enzyme MDH notably increased its activity. With respect to the activities of PEPC and ICDH, the Zn toxicity in *L. sativa* signified no changes with respect to control (Table 2).

Table 3. Organic anions concentration in leaves of *L. sativa* and *B. oleracea*.

	Malate (µmol/ g FW)	Oxalate (µmol/ g FW)	Citrate (µmol/ g FW)
***Lactuca sativa* cv. Phillipus**			
Control	36.59 ± 0.52	0.82 ± 0.06	5.90 ± 1.30
0.5 mM Zn	30.56 ± 3.48	1.11 ± 0,01	10.51 ± 1.74
p-value	NS	**	**
$LSD_{0.05}$	9.78	0.15	3.02
***Brassica oleracea* cv. Bronco**			
Control	2.16 ± 0.07	1.56 ± 0.19	35.67 ± 4.49
0.5 mM Zn	11.15 ± 0.26	1.71 ± 0.16	36.65 ± 2.05
p-value	***	NS	NS
$LSD_{0.05}$	0.73	0.69	13.70

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, ** $p < 0.01$, *** $p < 0.001$.

With respect to *B. oleracea* under Zn-toxicity conditions, it should be indicated that MDH activity declined with respect to control, although despite the decrease this activity continued to be high compared that in L. sativa (Table 2). With respect to the enzymatic activities analyzed (PEPC, fumarase, CS, and ICDH), the Zn toxicity in *B. oleracea* prompted no significant changes with respect to the activities found in control

plants (Table 2). It bears mentioning that in *B. oleracea*, both under control as well as Zn-toxic conditions, the enzymes MDH as well as CS presented activities which were in general far higher than those of *L. sativa* (Table 2).

Table 3 and Fig. 1 reflect the concentration and distribution of the different organic anions in L. sativa. As can be seen, both under control conditions as well as in Zn toxicity, the main organic acid was malate, representing under both growth conditions more than 70% with respect to the total. Finally, with respect to the *L. sativa* plants, it is worth emphasizing that Zn toxicity results in increased citrate (Table 3 and Fig. 1), although this increase is not due to greater CS activity in the leaves of these plants, which diminishes under Zn-toxicity conditions (Table 2). The study of the behavior of TCA in the roots of L. sativa shows that the only significant results were greater CS activity in the root ($p < 0.05$, Fig. 2A) and a lower citrate concentration ($p < 0.001$, Fig. 2B).

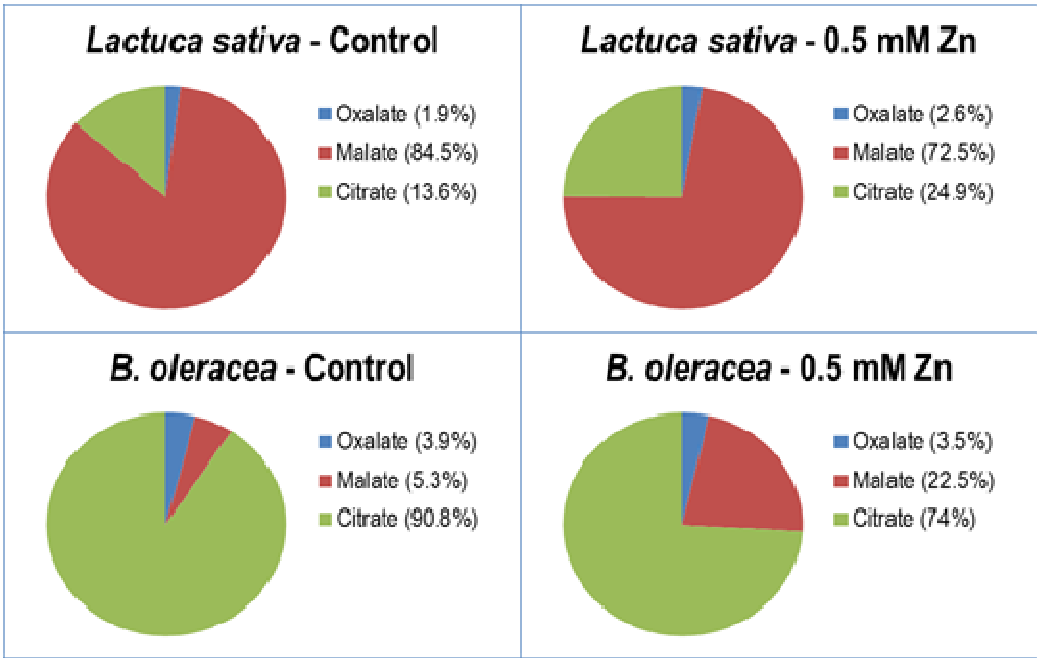

Fig. 1. Distribution of the major organic anions in *L. sativa* and *B. oleracea* leaves.

With regard to *B. oleracea*, the predominant organic acid under control as well as toxicity conditions was citrate (Table 3 and Fig. 1). However, it should be noted that under Zn-toxicity conditions in *B. oleracea*, MDH activity declined (Table 2) and levels of malate rose in the leaves (Table 3 and Fig. 1).

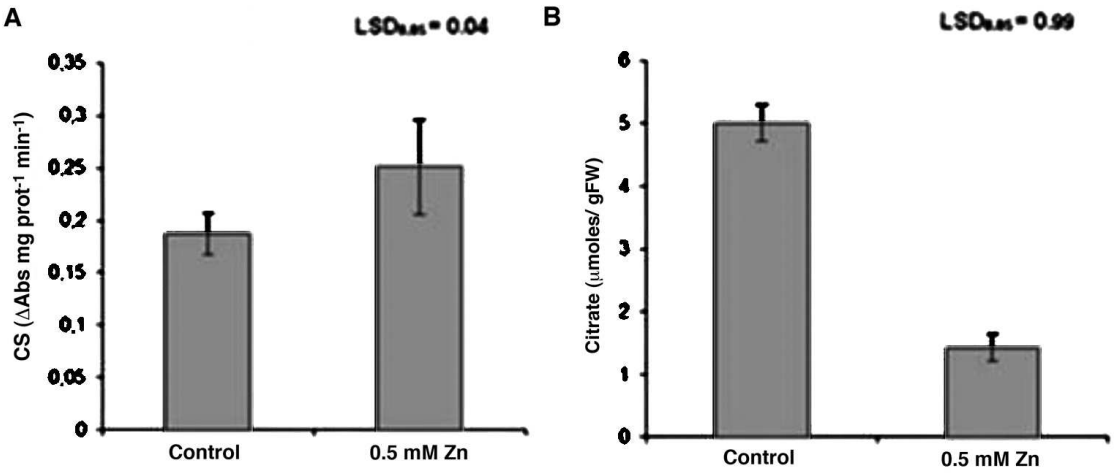

Fig. 2. Toxicity effect of Zn on the citrate synthase (CS) activity (A) and citrate concentration (B) in roots of *L. sativa*. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

4.1.4. Discussion

Numerous works demonstrate that the increased application of Zn in the medium augmented the accumulation of this element both in shoot as well as in the root (Zeng et al., 2011), values reaching some 80% (Ozdener and Aydin, 2010), due to possible mechanisms of compartmentalization (Ma et al., 2005; Wójcik et al., 2005; Gisbert et al., 2006; Ozdener and Aydin, 2010) or to the precipitation of the element in the apoplast (Küpper et al., 2000). In our work, both plants registered a higher Zn concentration in the 0.5 mM treatment, especially in the root (Table 1), this accumulation being greater in *B. oleracea* (Table 1). The effect caused in the biomass in

the presence of toxic Zn levels has been widely studied and known to be especially harmful to root biomass (Islam et al., 2007), as confirmed in our work (Table 1). On the other hand, the results for shoot biomass show a broad variation in tolerance or non-tolerance of the plant under study. In species of the family Brassicaceae, the biomass of this part of the plant does not appear to be negatively affected (Baker and Brooks, 1989; Gisbert et al., 2006), while in the family Crassulaceae, such as *Sedum alfreddi*, the biomass increased at 0.5 mM Zn (Yang et al., 2006) without any toxic effect in the aboveground part. On the contrary, in the species *Apium graveolens*, subjected to increments of Zn in the growth solution, the aerial biomass declined with respect to control (Islam et al., 2007). Our results clearly indicate that under these conditions of Zn toxicity, *B. oleracea* is much more tolerance than *L. sativa*, since in this latter species the root as well as foliar biomass was negatively affected (Table 1). On the contrary, *B. oleracea*, despite accumulating a greater Zn concentration in the roots and leaves, showed a toxic effect only in the root biomass, without variation in the foliar biomass (Table 1). These data suggest that *B. oleracea* under Zn-toxicity conditions is more effective in Zn distribution this being reflected in the greater increase in the concentration of this element in the leaves of *B. oleracea* with respect to that found in *L. sativa* (Table 1).

In short, considering these results we can affirm that the tolerance under Zn-toxicity conditions was greater in *B. oleracea* than in *L. sativa*, despite that the former species presented higher Zn concentration in leaves. In this sense, different studies have revealed findings contrary to those of our experiment, given that the plants tolerant to heavy metals showed restricted metal uptake in the root in tolerant plants (Zeng et al., 2011). On the contrary, other works have demonstrated a rise in the levels of the metal in leaves as a tolerance mechanism (Baker et al., 1994). In these plants, values

correspond to greater synthesis of organic anions such as citrate or malate, which increase the long-distance transport of Zn as well as its foliar storage (Salt et al., 1999; Monsant et al., 2011).

Regarding to carboxylate metabolism in *L. sativa* plants the MDH activity appears to be key in organic anions synthesis under Zn-toxicity conditions. This enzyme shows reversible activity and thus can generate oxalate or, from this, can produce malate. These results can be explained by the high MDH activity in these plants (Table 2), which form malate from oxalate, and this possibly does not form part of the TCA, thus also explaining the low CS activities in these plants (Table 2). However, in *L. sativa* plants Zn toxicity increased the concentration of citrate in leaves (Table 3 and Fig. 1), although this increase is not due to greater CS activity in the leaves of these plants, which diminishes under Zn-toxicity conditions (Table 2). The study of the behaviour of TCA in the roots of *L. sativa* shows that the only significant results were greater CS activity in the root and a lower citrate concentration (Fig. 2A and Fig. 2B). These results imply that with Zn toxicity the *L. sativa* root induces citrate synthesis that in turn could be transported to leaves with the aim of improving the response of tolerance to high concentrations of this element.

With regard to *B. oleracea*, the predominant organic anion under control as well as toxicity conditions was citrate (Table 3 and Fig. 1), due to a high CS and low ICDH and fumarase activities that reduce the recycling of citrate in the TCA. It has been confirmed that under heavy-metal toxicity, malate increases to facilitate the transport of metal towards the shoot (Yang et al., 2006; Wójcik et al., 2006; Monsant et al., 2011), and afterwards the metal would be sequestered in the vacuole by its bonding to citrate (Salt et al., 1999; Monsant et al., 2011) or complexed with citrate in the cytosol to protect the cell from high Zn concentrations. These mechanisms could explain in our work why the

B. oleracea plants faced with Zn toxicity showed a greater Zn concentration in the leaves together with high levels of citrate and an increase in malate. In short, according to these results, it appears that the maintenance of high citrate levels in *B. oleracea* may be essential to maintain tolerance to toxicity of this micronutrient, which as a chelant at the level of the leaves facilitates the accumulation of a greater quantity of Zn in this organ, without which this would a negatively affect biomass production. Furthermore, the greater synthesis of malate could be involved in the increased translocation of this metal to the leaf. On the contrary, in L. sativa plants, the main organic acid both under control conditions as well as under an application of 0.5 Mm of Zn was malate, which did not appear to be involved in an effective way in tolerance to high Zn amounts. Therefore, *L. sativa* plants applied with 0.5 mM of Zn may have increased the quantity of citrate, this appearing to be inadequate to provide tolerance to this element.

4.1.5. Conclusion

These results suggest that both in programmes of phytoextraction as well as in biofortification with Zn that are based on the fertilization enriched with this element, *B. oleracea* is more effective than *L. sativa* (defining effectiveness as greater Zn accumulation in leaves with less reduction of biomass production). In *L. sativa* an increase of MDH and CS activities and high levels of malate seems not promote tolerance in this species since the accumulation of Zn in leaves resulted in a reduction of the biomass of this organ. However, in *B. oleracea* a decrease of MDH activity and high CS activity concomitant with the high concentration of organic anion citrate could be determinant in the tolerance and greater accumulation of this element in leaves without which this would a negatively affect biomass production. Therefore, one step in the improvement of these cultivars could be based on the selection or use of techniques of

genetic manipulation with the aim of developing plants with a greater capacity to synthesize citrate, given that this is one of the most powerful chelating agents in this group of organic anions.

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4.2. Role of GSH homeostasis under Zn toxicity in plants with different Zn tolerance

Abstract

Tripepthide glutathione (GSH) is a pivotal molecule in tolerance to heavy metals, including Zinc (Zn). The aim of our work is to examine the role of GSH metabolism in two different horticultural plants under Zn toxicity in order to select and/or generate plants tolerant to Zn toxicity. We show a comparative analysis of the toxic effect of 0.5 mM Zn between *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. In *L. sativa* the accumulation of Zn resulted in an increase in reactive oxygen species (ROS), while enzymes of GSH metabolism and the activities of the antioxidant enzymes were negatively affected. On the contrary, *B. oleracea* showed the existence of a detoxification mechanism of these ROS. Moreover, while in *L. sativa* increased the oxidized GSH (GSSG) and phytochelatins (PCs) concentration with the reduction of leaves biomass, in *B. oleracea* the higher concentration of reduced GSH and its use in the detoxification of ROS seems to be a major mechanism to provide tolerance to Zn toxicity without reducing leaf biomass. Our results suggested that under Zn toxicity, *B. oleracea* is more efficient and tolerant than *L. sativa* through the detoxification of lipid peroxidation products due to the reduced GSH.

Keywords: Glutathione, zinc, horticultural plants, tolerance

Abrevviations: AsA, ascorbate; APX, ascorbate peroxidase; CDNB, 1-chloro-2,4-dinitrobenzene; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DTNB, 5,5´-dithiobis-2-nitrobenzoic acid; GSH, glutathione; GSSG, oxidized glutathione; Gly I, glyoxalase I; Gly II, glyoxalase II; GPX, glutathione peroxidase; GR, glutathione

reductase; GS, glutathione synthetase; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; LOX, lipoxygenase; MDA, malondialdehyde; MDHAR, monodehydoascorbate reductase; NBT, nitroblue tetrazolium; $^1O_2$, singlet oxygen; $O_2^{\bullet-}$, superoxide anion; OH, hidroxyl radical; PCs, phytochelatins; PPFD, photosynthetic photon flux density; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SAT, serine acetyltransferase; SLG, S-d-lactoylglutathione; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TF, transfer factor; TNP-SH, non-protein thiols; ɣ-ECS, ɣ-glutamylcysteine synthetase.

4.2.1. Introduction

Zinc (Zn) is an essential micronutrient for plants, animals, and microorganisms [1]. In plants, Zn is required at optimal concentrations, both for the normal functioning of cell metabolism as well as for plant growth [2]. As cofactor of several enzymes, Zn is involved in many physiological processes such as the metabolism of carbohydrates, lipids, and nucleic acids, gene expression and regulation, enzyme activation, protein synthesis, and reproductive development such as pollen formation [3]. However, in environmental situations of heavy-metal soil pollution, Zn constitutes a serious problem for worldwide agricultural production [4]. Under these conditions, Zn accumulates in excess in the plant tissues, reaching toxic concentrations (> 300 µg g^{-1}) for plants, causing physiological alterations and growth inhibition [5]. Particularly, the deleterious effects of Zn on photosynthesis has been associated with Zn-induced oxidative damage to membranes [2]. In this situation, the primary response of plants is the generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hidroxylradical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), causing the lipid peroxidation [6,7]. The non-redox-active metal ions, Zn and Cd, have been reported to increase lipid

peroxidation via ROS generation in plants. Increased lipid peroxidation in plants exposed to toxic Zn levels has been attributed to stronger activity of membrane-bound lipoxygenase (LOX), which is known to oxidize polyunsaturated fatty acids and produce free radicals with concomitant increased production of malondialdehyde (MDA) [8].

The main enzymes and antioxidant compound determining the tolerance to the oxidative stresses might dependent on the plant species and metal toxicity [9]. However, several studies have suggested that the tripeptide glutathione (GSH) is a pivotal molecule in tolerance to heavy metals, include Zn [6,10,11]. The amount of GSH in a given organism is the result of the combined action of biosynthesis, consumption, and degradation [12]. Data suggest that excess of Zn in plants induces the generation of GSH, which in turn accelerates the enzyme activities of GSH synthesis [10,13]. The enzyme serine acetyltransferase (SAT), is the key enzyme in cysteine synthesis, thereby providing one of the substrates of GSH synthesis [14]. GSH is synthesized in two ATP-dependent steps catalyzed by ɣ-glutamylcysteine synthetase (ɣ-ECS), a rate-limiting enzyme,and glutathione synthetase (GS) [12,15]. First, ɣ-ECS catalyzes the formation of a peptide bond between the ɣ-carboxyl group of glutamate and the α-amino group of cysteine. Following, GS catalyzes the formation of a peptide bond between the cysteinyl carboxyl group of ɣ-glutamylcysteine and the α-amino group of glycine [16]. Several works have demonstrated that the overexpression of SAT or ɣ-ECS in various plants has triggered higher tolerance and accumulation of heavy metals such as Zn [6].

On the other hand, GSH is consumed in a number of redox reactions to combat oxidative stress, resulting in its oxidation to GSSG [17]. In this sense, in the ascorbate–glutathione cycle, the enzyme ascorbate peroxidase (APX) reduces H_2O_2 into water using ascorbate (AsA) as the electron donor and the resulting dehydroascorbate (DHA)

is cycled back to ascorbate using reduced GSH as the electron donor, while the GSSG formed is converted back to reduced GSH by NAD(P)H-dependent glutathione reductase (GR) [11]. Some authors have demonstrated that the ascorbate–glutathione cycle plays a major role in tolerance to excess of heavy metals [9]. Recently, some studies have shown an increase in these enzymes in plants tolerant of Zn-toxicity conditions [18, 19]. Regarding to Zn, Cuypers et al. [20] have suggested that an improvement in the activity of these enzymes could alleviate the toxic effects of 50 μM of $ZnSO_4$ in primary leaves of *Phaseolus vulgaris*.

Another highly reactive compound that is detoxified by the use of glutathione is the methylglyoxal. This compound is formed spontaneously in plants by non-enzymatic mechanisms under physiological conditions from glycolysis and from photosynthesis intermediates. However, under stress conditions, the rate of glycolysis increases, leading to an imbalance in the pathway [21]. Several reports indicate the overproduction of methylglyoxal under heavy-metals toxicity, including Zn [22,23]. Methylglyoxal is detoxified mainly by the maintenance of GSH homeostasis via glyoxalase system, which consists of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I uses one molecule of reduced GSH to convert methylglyoxal to S-d-lactoylglutathione (SLG). Then Gly II converts SLG to d-lactate and one molecule of reduced glutathione is recycled back into the system [23]. Singla-Pareek et al. [22] demonstrated as both enzymes promoted stress tolerance in tobacco plants (*Nicotiana tabacum*) subjected to Zn stress (5 mM $ZnCl_2$).In addition, the enzymes glutathione-S-transferase (GST) and glutathione peroxidase (GPX) use the pool of GSH as substrate to detoxify H_2O_2 and sequestration of heavy metals by acting as a precursor for phytochelatins (PCs) synthesis [8,24]. In this sense, overexpression of GST and GPX in transgenic tobacco enhanced seedling growth under a variety of stressful conditions [25]. With respect Zn,

in *Hydrilla verticillata* exposed to a higher Zn dosage, GST activity increased with respect the control with the enhanced Zn-stress tolerance [26].

Finally, many works suggest that in stress situations involving trace elements, the plants produce low-molecular-weight compounds involved in the long-distance transport in the xylem and their subsequent detoxification in the cytoplasm and/or vacuole [27–29]. Notable among these compounds are phytochelatins (PCs), organic anions, histidine, nictotinamine, and phytosiderophores [30]. With respect to PCs, due to their ability to bind metals, generally these have been considered to be important cellular chelating agents, which function as heavy-metal detoxification and/or homeostasis agents [31]. GSH is a key compound for the synthesis of PCs. Gasic and Korban [32] observed that PCs function as important chelators of Zn excess due to slightly elevated Zn tolerance in *Brassica juncea* plants transformed with *AtPCS1*. However, recently, many studies have questioned the role of this compound as a tolerance mechanism in *Sedum alfredii* submitted Cd stress [33–35] or *Arabis paniculata* under different Zn stress [36].

Many studies have shown that there are great differences between the capacity to accumulate and tolerance mechanisms in different plants species under metal-toxicity conditions [1,2,5,7,21]. Therefore, in this work we performed a comparative study of two species of horticultural plants of great agricultural interest, i.e. *Lactuca sativa* and *Brassica oleracea*, determining the effect Zn toxicity on: (i) Zn concentration in root and leaves, Zn accumulation and transfer factor; (ii) oxidative stress in terms of lipid peroxidation; (iii) the response of GSH and its metabolism as a possible key role in tolerance to Zn toxicity in both species. These information will be useful to understand Zn-tolerance and to establish physiological markers of plants suited for phytoremediation of Zn-contaminated soils. Moreover, and considering that both

horticultural plants used in this research are widely used in biofortification programs trace elements, comparing the toxic effect of Zn between them allow us to select which plant is more suited to agricultural biofortification programmes with Zn, consisting of the application of a supraoptimal amount of this element.

4.2.2. Materials and methods

4.2.2.1. Plant materials and experimental design

Seeds of *L. sativa* cv. Phillipus and *B. oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant S.L.). After 35 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions, with relative humidity of 50%,day/night temperature of 25/15◦C, a photoperiod of 16/8 h at a photosynthetic photon flux density (PPFD) of 350 µmol $m^{-2}s^{-1}$(measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) in a volume of 3 l. At 35 days after germination and throughout the experiment, control plants received a growth solution, which was composed of 4 mM KNO3, 3 mM Ca $(NO3)_2 \cdot 4\ H_2O$, 2 mM $MgSO_4 \cdot 7\ H_2O$, 6 mM KH_2PO^4, 1 mM $NaH_2PO_4 \cdot 2\ H_2O$, 2 µM $MnCl_2 \cdot 4$ H2O, 10 µM $ZnSO_4 \cdot 7\ H_2O$, 0.25 µM $CuSO_4 \cdot 5\ H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2\ H_2O$, 5 ppm Fe-chelate (Sequestrene; 138 FeG100) and10 µM H_3BO_3. At the same time, treatments were applied with the same growth solution amended with 0.5 mM of ZnSO4. This solution, with a pH of 5.5–6.0, was changed every three days.

The experimental design was a randomized complete block with 4 treatments (*L. sativa*-control, *B. oleracea*-control, *L. sativa*-0.5 mM, *B. oleracea*-0.5 mM) arranged in individual trays with eight plants per treatment and three replications each, so that the total number of plants was 96.

4.2.2.2. Plant sampling

L. sativa and *B. oleracea* plants were sampled after 21 days further growth under these conditions. Plants of each treatment were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the roots and leaves from each treatment were frozen at −30◦C for later performance of biochemical assays and the other half of the plant material sampled dried in a forced air oven at 70◦C for 24 h to obtain the dry weight (DW) and the subsequent analysis of the concentration of Zn.

4.2.2.3. Zn concentration and transfer factor

For the determination of Zn concentration, a sample of 30 mg dry material was subjected to a process of mineralization by the method of Wolf [37]. Mineral analysis was conducted using ICP-MS. Briefly, samples were further diluted 1-in-10 with milli-Q water and analyzed using an ICP-MS (X-Series II; Termo FisherScientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml^{-1}) and Ir (5 ng ml^{-1}) in 2% TAG HNO_3. External multielement calibration standards (Claritas-PPT grade CLMS-2, SPEXCerti-Prep Ltd, Stanmore, Middlesex, UK) included Al, As, Ba, Bi,Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, U, V and Zn, in the range 0–100 µg L^{-1}. Zn concentration was expressed as µg g^{-1} DW.

Transfer factor (TF) was calculated using both the Zn concentrations (ZC) of the fresh leaves and the ZC of the solution [38]. Thus, the TF was calculated in the following way:

$$\text{TF solution} - \text{leaf} = \frac{[\text{ZC}]\text{fresh leaf}}{[\text{ZC}]\text{solution}}$$

4.2.2.4. SOD activity, $O_2^{\bullet -}$, and H_2O_2, and lipid peroxidation

SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the methods of Giannopolitis and Ries [39] and Beyerand Fridovitch [40], with some modifications [41]. Leaves material were homogenized in liquid N_2 with buffer Heppes-HCl 50 mM pH7.6 containing 5 mM EDTA and 1% polyvinylpyrrolidone (PVP) and centrifuged at 4◦C for 10 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at A_{560} nm. Determination of $O_2^{\bullet -}$ in leaf extracts was based on the ability to reduce NBT [42]. Optical density was measured at a wavelength of A_{580} nm and the $O_2^{\bullet -}$ concentration was expressed as A_{580} g^{-1} FW. The H_2O_2 concentration in leaf extracts was colorimetrically measured as described by Mukherjee and Choudhuri [43]. Leaf samples were extracted with cold acetone to determine the H_2O_2 levels. The intensity of yellowcolour of the supernatant was measured at A415nm. The result of H_2O_2 concentration was expressed as nmol g^{-1} FW.

LOX (EC 1.13.11.12) activity in leaf extracts was measured according to Minguez-Mosquera et al. [44], using 50 mM K-phosphate buffer (pH 6.0) containing 5 mM EDTA and 1% PVP for extraction. The LOX activity was calculated following the rise in the extinction at A_{234} using an extinction coefficient of 25,000 $M^{-1}cm^{-1}$ [45]. For the MDA assay, leaves were homogenized with 3 ml of 50 mM solution containing 0.07%

$NaH_2PO_4 \cdot 2H_2O$ and 1.6% $Na_2HPO_4 \cdot 12\ H_2O$ and centrifuged at 20,000 × g for 25 min in a refrigerated centrifuge. For measurement of MDA concentration 3 ml of 20% trichloroacetic acid (TCA) containing 0.5%thiobarbituric acid (TBA) was added to a 1-mL aliquot of the supernatant. The mixture was heated at 95◦C for 30 min, quickly cooled in an ice bath and then centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was read at A_{532} and A_{600} nm. The concentration of MDA was calculated using the MDA extinction coefficient of 155 $mM^{-1}cm^{-1}$ [46]. The result of MDA was expressed as μmol g^{-1} FW.

4.2.2.5. GSH synthesis and concentration

The SAT (EC 2.3.1.3.0) activity was performed following Tripati et al. [12]. The reaction mixture contained 63 mM Tris–HCl (pH7.6), 1.25 mM EDTA, 1.25 mM DTNB (5,5′-dithiobis-2-nitrobenzoic acid), 0.1 mM acetyl-CoA, 1 mM and L-Serine. The rate of reaction was followed at A_{412} nm. For the assay of ɣ-ECS, plants were homogenized in 100 mM Tris–HCl (pH 8.0) containing 5 mM EDTA and 1% PVP. The reaction mixture contained 0.1 M Tris–HCl (pH 8.0), 150 mM KCl, 2 mM EDTA, 20 mM $MgCl_2$, 5 mM Na_2ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 0.2 mM NADH, 7 unit ml^{-1}pyruvate kinase (ICN, USA) and 10 unit ml^{-1} L-lactic dehydrogenase (Sigma, USA) [47]. The reaction was initiated by the addition of enzyme extract. Activity of ɣ-ECS was determined from the rate of formation of ADP (assumed to be equal to the rate of NADH oxidation; ε = 6.2 $mM^{-1}cm^{-1}$, monitored at 340 nm).

Reduced GSH, GSSG and total GSH (reduced GSH + GSSG) were assayed according to the GSSG-recycling method [48]. Leaves material were homogenized in liquid N_2 with metaphosphoric acid at 5% (w/v) and centrifuged at 13,500 × g and 4◦C for 15

min. GSSG and total GSH were assayed in the same extract. A standard curve was analyzing in the same manner as for the extracts. Reduced GSH levels were estimated as the difference between total GSH and GSSG.

4.2.2.6. Ascorbate–glutathione cycle

The activities of the enzymes APX (EC 1.11.1.11) and GR (EC1.6.4.1) were assayed in leaf extracts following Rao et al. [49]. APX activity was determined by registering the absorbance change at A_{290} nm and GR activity was measured after monitoring the oxidation of NADPH at A_{340} nm for 3 min. MDHAR (EC 1.6.5.4) was assayed by registering the change in absorbance of the samples at a wavelength of A_{340} nm [50]. In addition, DHAR activity (EC 1.8.5.1) in leaf extracts was measured at A_{265} nm for 3 min following the change in absorbance resulting from the formation of AsA [51].

The determination of total AsA concentration (reduced AsA and DHA forms) in leaf extracts was following the method of Law et al.[52], modified from Okamura [53]. This method is based on the reduction of $Fe^{3+}$to Fe^{2+} by AsA in acid solution. Leaves material were homogenized in liquid N_2 with metaphosphoric acid at 5% (w/v) and centrifuged at 4◦C for 15 min. Absorbance was measured at A_{525} nm against a standard AsA curve that followed the same procedure as above. The results were used to quantify the total AsA concentration, while the reduced AsA was quantified in the same way as the previous procedure, replacing 0.1 ml of DTT with 0.1 ml of distilled H_2O. Finally, the DHA concentration was deduced from the difference between total AsA and reduced AsA. The result of AsA forms were expressed as $\mu g\ g^{-1}$ FW.

4.2.2.7. GSH metabolism enzymes

Glyoxalase I (EC 4.4.1.5) assay was carried out according to Hasanuzzaman et al. [54]. The increase in absorbance was recorded at A_{240} nm for 1 min. Glyoxalase II (EC 3.1.2.6) activity was determined according to the method of Principato et al. [55] by monitoring the formation of GSH at A_{412} nm for 1 min. GST (EC2.5.1.18) activity was determined spectrophotometrically by the method of Hossain et al. [56] with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). The increase in absorbance was measured at A_{340} nm for 1 min. GPX (EC 1.11.1.9) activity was measured as described by Elia et al. [57] with slight modification using H_2O_2 as a substrate. The oxidation of NADPH was recorded at A_{340} nm for 1 min. The protein concentration of the extracts was determined according to the method of Bradford [58], using bovine-serum albumin as the standard.

4.2.2.8. Non-protein thiols and PCs determination

Non-protein thiols (TNP-SH) were determined as described by Garg and Kaur [8]. One hundred microlitres of the supernatant was taken in a microfuge tube, to which 0.5 ml reaction buffer (0.1 M K-phosphate buffer (pH 7.0), 0.5 mM EDTA) and 0.5 ml of DTNB (1 mM) were added. The reaction mixture was incubated for 10 min, and absorbance was read at A_{412} nm. Values were corrected for the absorbance by preparing a blank without extract. A standard curve was prepared from varying concentrations of cysteine to calculate non-protein thiols content in samples. PC levels were estimated from the difference between TNP-SH and reduced GSH [59].

4.2.2.9. Statistical analysis

The data were analyzed by a simple variance analysis (ANOVA) and differences between the means were compared by Fisher's least-significant difference test (LSD). In addition, to ascertain whether the different plants and Zn-doses used in the experiment significantly influenced the results, a two-way ANOVA was used and the means were compared by Fisher's LSD test.

4.2.3. Results

4.2.3.1. Zn concentration, biomass and transfer factor

After 21 days of treatment, *L. sativa* as well as *B. oleracea* plants accumulated fundamentally Zn in the root when treated with 0.5 mM of Zn, resulting in the same toxicity level in this organ with similar reductions in root biomass (69% and 60% respectively) (Table 1). However, the behaviour of leaves differed between plants. While in *L. sativa* the application of 0.5 mM of Zn raised the Zn concentration with respect to control and sharply lowered biomass (32%), in *B. oleracea* this greater Zn concentration, although exceeding that of *L. sativa*, did not lower shoot biomass (13%) (Table 1).

For both plants studied in the present work, the application of the treatment 0.5 mM Zn resulted in a sharp reduction in the TF (Table 1).

Table 1. Zn concentration and root and leaf biomass in plants of *L. sativa* and *B. oleracea*. Transfer factor (TF).

	Root Zn concentration (μg g^{-1} DW)	Root Biomass (g DW/plant)	Leaf Zn concentration (μg g^{-1} DW)	Leaf Biomass (g DW/plant)	TF
***Lactuca sativa* cv. Phillipus**					
Control	585 ± 68	0.13 ± 0.00	80.78 ± 4.39	2.11 ± 0.19	6.22 ± 0.34
0.5 mM Zn	34949 ± 376	0.04 ± 0.01	218.4 ± 35.7	1.43 ± 0.04	0.40 ± 0.07
p-value	***	***	**	*	***
$LSD_{0.05}$	10440	0.03	83.02	0.53	0.79
***Brassica oleracea* cv. Bronco**					
Control	895 ± 104	0.08 ± 0.00	61.43 ± 0.66	0.70 ± 0.06	6.85 ± 0.07
0.5 mM Zn	42540 ± 234	0.03 ± 0.00	382.4 ± 60.6	0.61 ± 0.08	1.20 ± 0.19
p-value	***	**	***	NS	***
$LSD_{0.05}$	862.77	0.02	139.36	0.29	0.47
Analysis of variance					
Plants (P)	NS	**	NS	***	**
Doses (D)	***	***	***	***	***
P x D	NS	**	*	***	NS

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.3.2. SOD activity, $O_2^{\bullet-}$ and H_2O_2, and lipid peroxidation

ROS levels constitute a good indicator of stress in plants. In our study, the levels of $O_2^{\bullet-}$ and H_2O_2 increased in *L. sativa* under stress conditions compared with the control (Table 2). However, *B. oleracea* showed no significant differences at 0.5 mM with respect to control in either ROS (Table 2). SOD activity in *L. sativa* was lower than in control whereas, in *B. oleracea*, there was a marked increase in activity of this enzyme under Zn toxicity (Table 2). Fig. 1 shows a different LOX-activity pattern for the two species; that is, in *L. sativa* its activity significantly increased with the application of 0.5 mM of Zn (Fig. 1A, $p < 0.001$) but decreased in *B. oleracea* when compared to control (Fig. 1A´, $p < 0.01$). In addition, the MDA content, wich is the final product of lipid

peroxidation also increased in *L. sativa* (Fig. 1B), while in *B. oleracea*, MDA concentration decreased under Zn toxicity with respect to control plants (Fig. 1B´).

Table 2. Effect of Zn toxicity on SOD activity and ROS concentration in leaves of *L. sativa* and *B. oleracea*. Superoxide dismutase (SOD), anion superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2).

	SOD (U mg^{-1} prot min^{-1})	$O_2^{\cdot-}$ (A_{580} g^{-1} FW)	H_2O_2 (nmol g^{-1} FW)
***Lactuca sativa* cv. Phillipus**			
Control	9.13 ± 0.52	0.51 ± 0.01	1712.79 ± 34.84
0.5 mM Zn	6.67 ± 0.35	0.54 ± 0.01	1898.27 ± 29.41
p-value	***	*	**
$LSD_{0.05}$	1.34	0.03	101.61
***Brassica oleracea* cv. Bronco**			
Control	2.81 ± 0.04	0.51 ± 0.01	3542.39 ± 264.21
0.5 mM Zn	3.14 ± 3.06	0.53 ± 0.01	4231.54 ± 430.19
p-value	**	NS	NS
$LSD_{0.05}$	0.20	0.03	0.20
Analysis of variance			
Plants (P)	***	NS	***
Doses (D)	**	**	NS
P x D	***	NS	NS

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.3.3. GSH synthesis and total GSH concentration

The effects of Zn on GSH synthesis differed in the two species. In *L. sativa*, SAT and ɣ-ECS activities decreased drastically in plants grown under 0.5 mM Zn, and the total GSH levels in these plants fell with respect to control (Table 3). However, in *B. oleracea* these results were the opposite. In both cases, the enzymatic activities registered no significant differences while the levels of total GSH rose markedly with 0.5 mM Zn (Table 3).

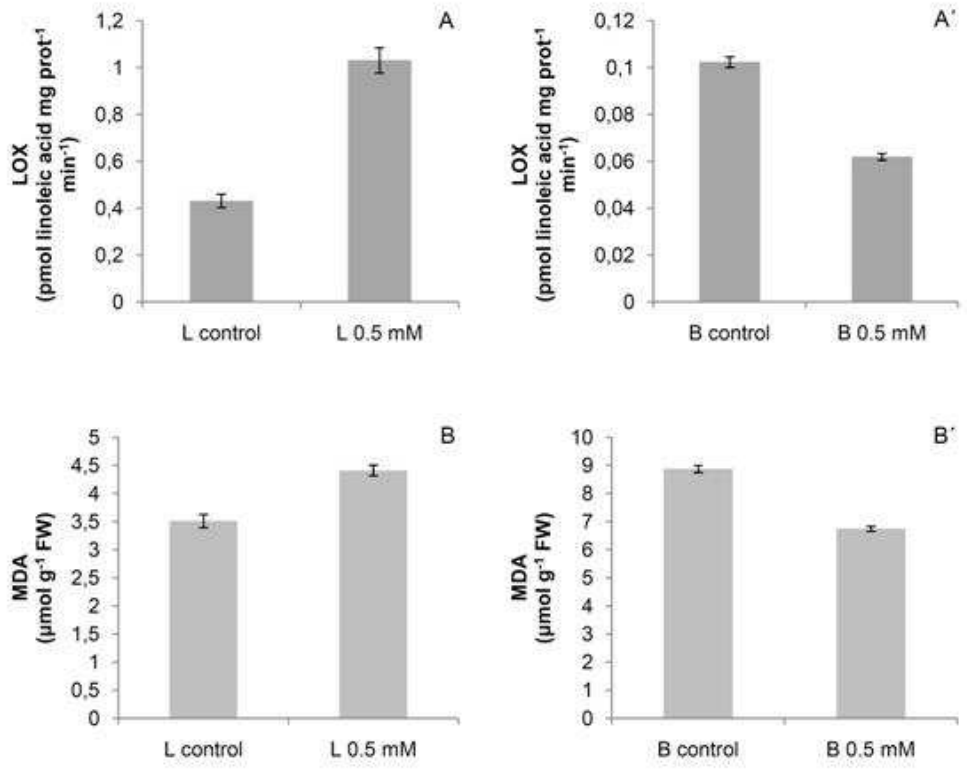

Fig. 1. Determination of lipid peroxidation in leaves of *L. sativa* and *B. oleracea* under Zn-toxicity. Lipoxygenase activity (LOX) (A and A´) and malondialdehyde concentration (B and B´). Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

4.2.3.4. Ascorbate–glutathione cycle

The Zn treatment did not significantly influence in APX and DHAR activities in *L. sativa* (Table 4). However, MDHAR and GR activities markedly diminished in *L. sativa* treated with 0.5 mM Zn (Table 4). By contrast, in *B. oleracea* plants under Zn toxicity, only APX activity notably intensified under these conditions, with no significant differences in MDHAR, DHAR, or GR under Zn toxicity (Table 4).

Table 3. Influence of Zn on glutathione synthesis in leaves of *L. sativa* and *B. oleracea*. Serine acetyltransferase (SAT), and γ-glutamylcysteine synthetase (γ-ECS) and concentration of total glutathione (GSH).

	SAT (nmol mg^{-1} prot min^{-1})	γ-ECS (nmol mg^{-1} prot min^{-1})	Total GSH (μg g^{-1} FW)
***Lactuca sativa* cv. Phillipus**			
Control	1.19 ± 0.12	0.70 ± 0.04	56.96 ± 3.95
0.5 mM Zn	0.69 ± 0.05	0.50 ± 0.02	44.66 ± 2.25
p-value	***	***	*
$LSD_{0.05}$	0.27	0.10	12.63
***Brassica oleracea* cv. Bronco**			
Control	0.31 ± 0.01	0.49 ± 0.03	126.32 ± 7.64
0.5 mM Zn	0.29 ± 0.01	0.54 ± 0.04	172.57 ± 9.50
p-value	NS	NS	*
$LSD_{0.05}$	0.04	0.10	33.85
Analysis of variance			
Plants (P)	***	**	***
Doses (D)	***	*	*
P x D	***	**	**

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The cellular redox status of AsA and GSH pools strongly responded to Zn phytotoxicity. The total AsA concentration increased in both species with respect to control, although this increase was significant only in *L. sativa* under 0.5 mM Zn (Fig. 2A, $p < 0.001$). Also, the reduced AsA concentration increased in *L. sativa* and *B. oleracea* under Zn toxicity with respect to the controls (Fig. 2B, $p < 0.001$; Fig. 2B´, $p < 0.001$). However, while in *L. sativa* DHA concentration significantly increased (Fig. 2C, $p < 0.01$), in *B. oleracea* DHA concentration strongly decreased (Fig. 2C´, $p < 0.001$) in plants treated with 0.5 mM Zn with respect to the control.

With respect GSH concentration, the two species differed. Under Zn toxicity, in *L. sativa* the concentration of reduced GSH significantly decreased (Fig. 3A, $p < 0.01$)

with a concomitant increase of GSSG concentration compared to control (Fig. 3B, $p < 0.01$), whereas in *B. oleracea* the concentration of reduced GSH increased notably with respect to the control (Fig. 3A´, $p < 0.01$) while the GSSG concentration decreased significantly under 0.5 mM Zn (Fig. 3B´, $p < 0.05$).

Table 4. Effect of Zn on ascorbate-glutathione cycle in leaves of *L. sativa* and *B. oleracea*. Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR).

	APX	MDHAR	DHAR	GR
	(nmol mg^{-1} prot min^{-1})			
***Lactuca sativa* cv. Phillipus**				
Control	0.56 ± 0.09	3.31 ± 0.52	0.96 ± 0.20	0.10 ± 0.01
0.5 mM Zn	0.52 ± 0.07	0.48 ± 0.06	1.00 ± 0.16	0.05 ± 0.01
p-value	NS	***	NS	**
$LSD_{0.05}$	0.26	1.16	0.58	0.03
***Brassica oleracea* cv. Bronco**				
Control	0.40 ± 0.06	0.25 ± 0.08	5.96 ± 0.23	0.27 ± 0.02
0.5 mM Zn	0.72 ± 0.07	0.24 ± 0.09	5.09 ± 0.33	0.40 ± 0.04
p-value	**	NS	NS	NS
$LSD_{0.05}$	0.21	0.27	0.93	0.12
Analysis of variance				
Plants (P)	NS	***	**	***
Doses (D)	NS	***	NS	*
P x D	*	***	*	***

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

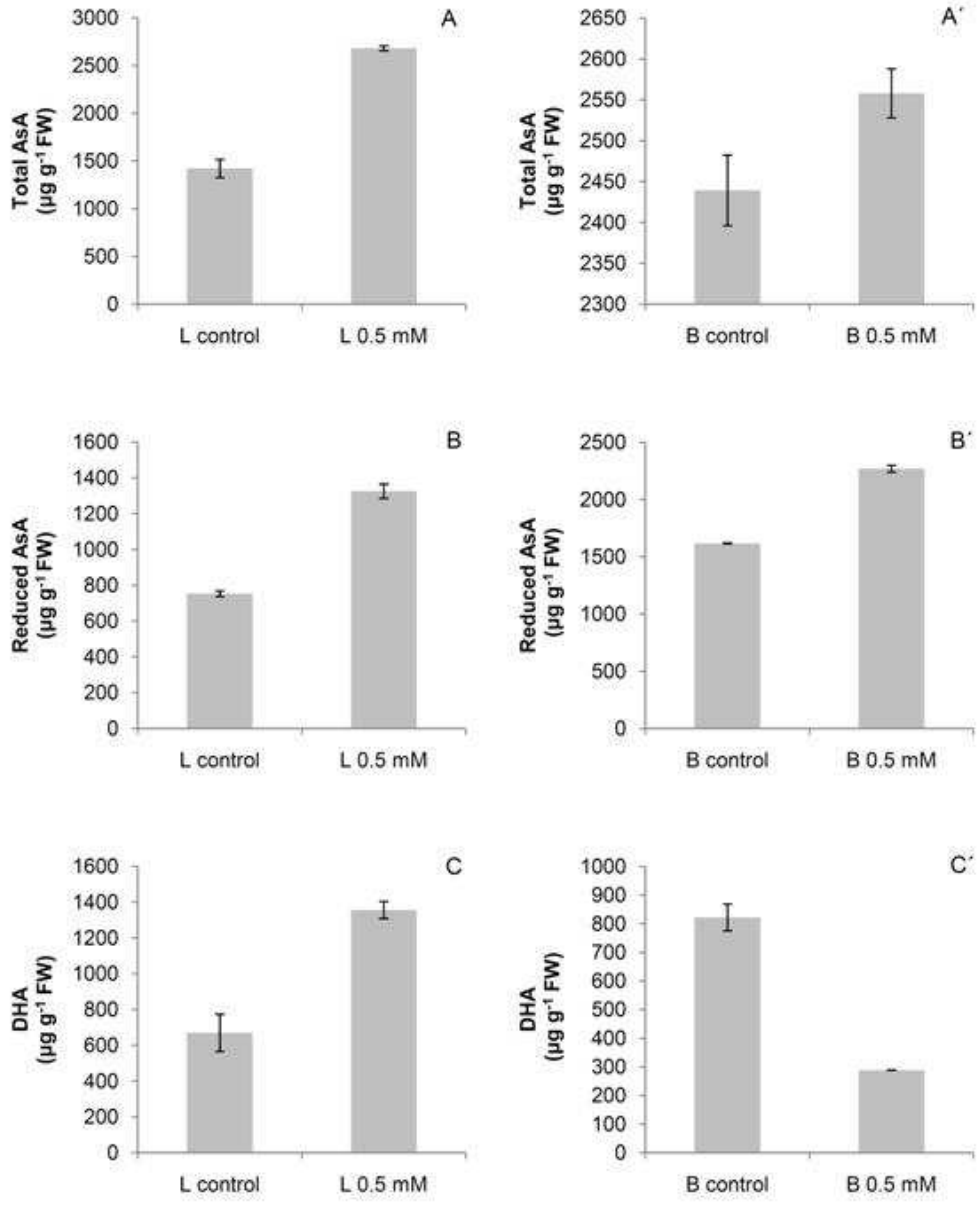

Fig. 2. Effect of Zn on concentration of ascorbate in leaves of *L. sativa* and *B. oleracea*. Total ascorbate (total AsA) (A and A´), reduced ascorbate (reduced AsA) (B and B´) and oxidized ascorbate (DHA) (C and C´). Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

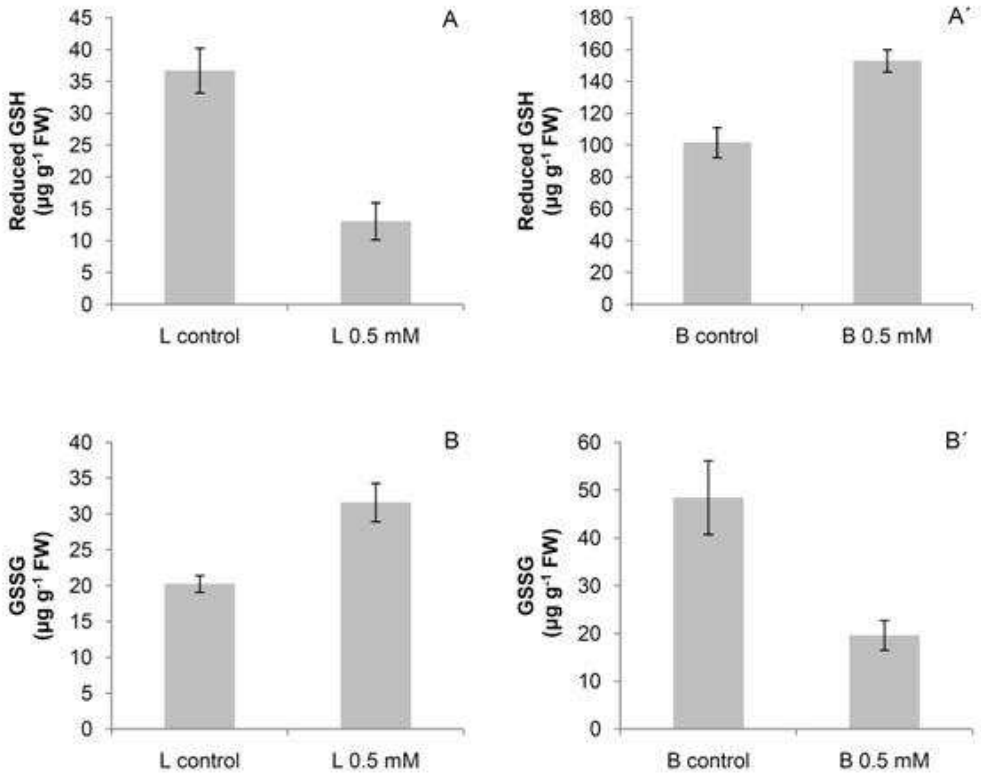

Fig. 3. Effect of Zn on concentration of glutathione in leaves of *L. sativa* and *B. oleracea*. Reduced glutathione (reduced GSH) (A and A´) and oxidized glutathione (GSSG) (B and B´). Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

4.2.3.5. GSH metabolism enzymes

In terms of detoxifying enzymes of methylglyoxal (Gly I and Gly II) under Zn toxicity, the response in two plants proved opposite. That is, in *L. sativa*, Gly I and Gly II decreased significantly when plants were exposed at 0.5 mM Zn with respect to control, while in *B. oleracea* both enzymatic activities increased under Zn toxicity (Table 5). GST activity showed no changes in L. sativa whereas GPX activity was negatively affected compared to control when these plants were exposed to Zn treatment (Table 5). However, in *B. oleracea*, GST and GPX activities intensified significantly under 0.5 mM Zn with respect to control (Table 5).

Table 5. Effect of Zn on some enzymes implicated in GSH metabolism in leaves of *L. sativa* and *B. oleracea*. Glyoxalase I (Gly I), glyoxalase II (Gly II), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) in leaves of *L. sativa* and *B. oleracea*..

	Gly I	Gly II	GPX	GST
	(nmol mg^{-1} prot min^{-1})			
***Lactuca sativa* cv. Phillipus**				
Control	2.50 ± 0.04	1.26 ± 0.08	0.43 ± 0.03	0.04 ± 0.00
0.5 mM Zn	2.26 ± 0.14	0.99 ± 0.08	0.30 ± 0.05	0.03 ± 0.01
p-value	*	*	*	NS
$LSD_{0.05}$	0.23	0.02	0.13	0.02
***Brassica oleracea* cv. Bronco**				
Control	3.17 ± 0.07	0.55 ± 0.02	0.64 ± 0.03	0.04 ± 0.00
0.5 mM Zn	6.04 ± 0.10	0.64 ± 0.03	0.82 ± 0.05	0.05 ± 0.00
p-value	***	*	*	**
$LSD_{0.05}$	0.27	0.08	0.13	0.01
Analysis of variance				
Plants (P)	***	***	***	NS
Doses (D)	***	NS	NS	NS
P x D	***	**	***	NS

Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, p = 0.05). Significance levels are represented by $p > 0.05$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.3.6. NPT-SH and PC content

Fig. 4 shows the levels of TNP-SH and PCs in both plants. *L. sativa* presented a significant rise in both compounds ($p < 0.01$), while *B. oleracea* showed no significant differences for plants exposed to Zn toxicity.

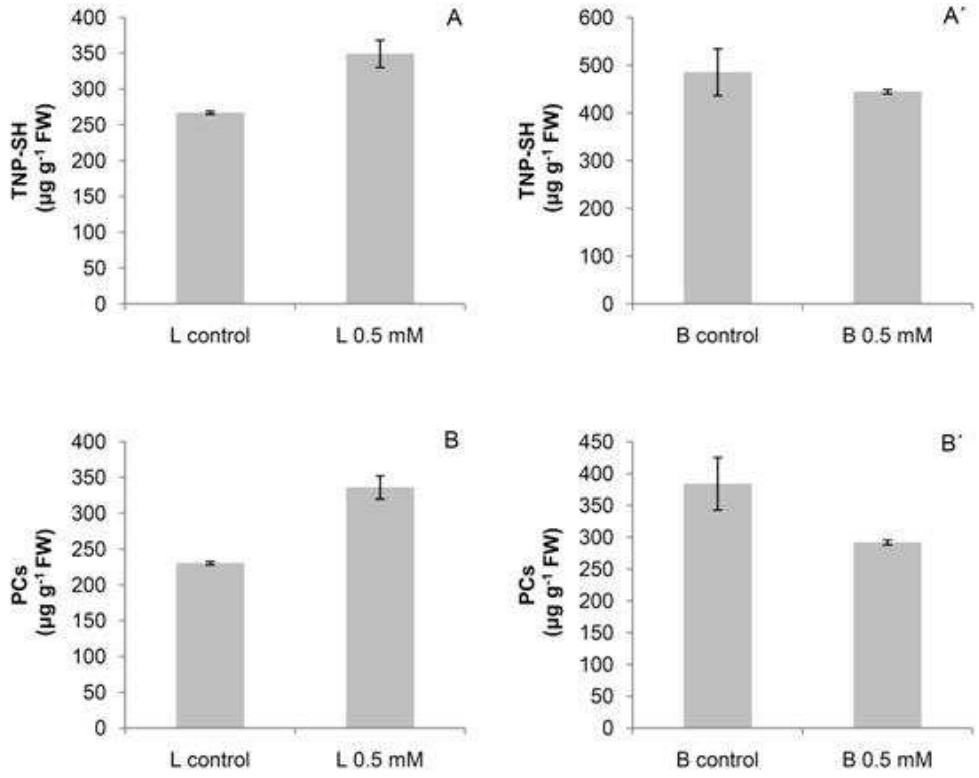

Fig. 4. Effect of Zn on phytochelatins synthesis in leaves of *L. sativa* and *B. oleracea*. Non-protein thiols (TNP-SH) (A and A´) and phytochelatins (PCs) (B and B´). Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, p = 0.05).

4.2.4. Discussion

Several works demonstrate that increasing applications of Zn in the growth media increase the accumulation of this element both in shoot as well as in the root [36]. Values can reach about 80% [60] due to possible compartmentalization mechanisms [60–62] or to the precipitation of the element in the apoplast [28]. In our work, both plants studied, *L. sativa* and *B. oleracea*, registered a higher Zn concentration in the 0.5 mM treatment, especially in the root (Table 1), this accumulation being greater in *B. oleracea* (Table 1). The effect caused on the biomass by toxic Zn levels has been widely studied and is known to be especially harmful to root biomass [63], as confirmed in our

work (Table 1). On the other hand, the results for shoot biomass show a broad variation in tolerance or non-tolerance of the plant species under study. In species of the family *Brassicaceae*, the shoot biomass does not appear to be negatively affected [61], while in the family *Crassulaceae*, such as *S. alfredii*, the biomass increased at 0.5 mM Zn [64] without any toxic effect in the aboveground part. On the contrary, in the species *Apium graveolens*, subjected to increments of Zn in the growth solution, the aerial biomass declined with respect to control [63]. Our results clearly indicate that under these conditions of Zn toxicity, *B. oleracea* is much more tolerant than *L. sativa*, since in this latter species the root as well as foliar biomass was negatively affected (Table 1). On the contrary, *B. oleracea*, despite accumulating a greater Zn concentration in the roots and leaves, showed a toxic effect only in the root biomass production, without variation in the foliar biomass (Table 1). These data suggest that *B. oleracea* under Zn-toxicity conditions is more efficient in Zn distribution, this being reflected in the stronger boost in the concentration of this element in the leaves of *B. oleracea* (622%) with respect to that found in *L. sativa* (270%) (Table 1).

The TF offers an idea both of the bioavailability as well as the mobility of Zn from the outside solution towards the aerial part of the plant. High TF values indicate greater efficiency in the uptake and accumulation of the element in question in the aerial part of the plant [65]. In our work, the different degree of toxicity provoked by the application of 0.5 mM of Zn both in *L. sativa* as well as in *B. oleracea* was clearly visible with the TF. Thus, *B. oleracea*, being more tolerant to Zn toxicity, was able to take up and accumulate a greater quantity of Zn in the aerial part, as it presented less reduction of TF with respect to control (82%) than that presented by *L. sativa* with respect to its control (94%). These data suggest that *B. oleracea* under Zn-toxicity conditions is more efficient in translocating Zn to the aerial part, this being reflected in the greater increase

in the concentration of this element in the leaves of *B. oleracea* with respect to that found in *L. sativa* (Table 1).

The mechanism to detoxify, compartmentalize or exclude heavy metals plays a key role in plant survival under heavy-metal exposure. Several studies have shown that the greater accumulation of Zn induces oxidative stress by stimulating ROS production [7], leading to lipid peroxidation. Under these conditions, the activation of detoxification mechanisms is of great importance to alleviate the toxic Zn effects [26]. SOD has been proposed to be important in plant-stress tolerance, providing the first line of defense against the toxic effects of elevated levels of ROS, because this enzyme can catalyze the dismutation reaction of $O_2^{\bullet-}$ into molecular oxygen and H_2O_2 [66]. In our study in L. *sativa*, when plants were subjected to Zn stress, there was an accumulation of $O_2^{\bullet-}$ due to lower SOD activity with respect to control (27%). However, despite the decrease in SOD activity, in these plants under Zn toxicity, H_2O_2 registered an accumulation of 111% (Table 2) compared to control plants, suggesting the non-detoxification of this ROS in these plants. However, in *B. oleracea* the $O_2^{\bullet-}$ levels did not rise significantly because SOD activity intensified with Zn toxicity compared to control (112%). This greater SOD activity in *B. oleracea* at 0.5 mM Zn did not prompt a rise in the foliar concentration of H_2O_2, implying the existence of a detoxification mechanism of this ROS in this species (Table 2). The accentuation of SOD activity at high Zn dosages was also found in various stressed plants, i.e. tolerant and hyperaccumulator plants, such as *Myracrodruon urundeuva* [11], *H. verticillata* [26], *Thlaspi caerulescens* [62] or *B. juncea* [67]. On the contrary, Weckx and Clijters [68] reported that Zn did not affect the SOD activity in *P. vulgaris*, causing oxidative damage in this plant.

On the other hand, excess of Zn promoted MDA production in plants due to increased lipid peroxidation through excessive generation of ROS [67]. In this sense, some studies

suggested that this greater lipid peroxidation in plants exposed Zn is due to accentuated LOX activity [68]. Our results agree with the above works; that is, in *L. sativa* LOX activity augmented (239%) with a concomitant rise in MDA concentration (126%) in the presence of 0.5 mM Zn with respect to control (Fig. 1). In fact, these situation could be related with the significantly decreased of leaf biomass (Table 1). However, in *B. oleracea* the MDA concentration decrease significantly since the LOX activity decreased when the plants were exposed Zn toxicity, and the leaf biomass of this species was not affected (Table 1). Therefore, ROS accumulation positively correlated with increasing Zn in *L. sativa*, indicating an insufficient defense system in this sensitive species, since the formation of ROS and thus the lipid peroxidation was induced in these plants. By contrast, *B. oleracea* showed an efficient defense under Zn-stress conditions. Similar to our results, an increase in $O_2^{\bullet-}$, H_2O_2, and MDA concentration under Zn toxicity has been reported in sugarcane (*Saccharum* spp.), provoking a marked reduction of biomass [69].

GSH occurs abundantly in reduced form in plant tissues and is localized in all cell compartments, such as the cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as in apoplast, and plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics and the expression of stress-responsive genes [66]. Some studies have suggested that the higher production of GSH contributes to the antioxidative protection against oxidative stress caused under adverse conditions in plants [15]. Tsuji et al. [13] demonstrated that in *Duniella tertiolecta* under Zn stress, GSH was increased by the induction of the enzymatic synthesis of this compound. On the basis of our results, SAT and ɣ-ECS activities, limiting enzymes in glutathione synthesis process, markedly declined in *L. sativa* with a consequent

decrease in total GSH (Table 3). On the contrary, in *B. oleracea* the activity of both enzymes remained constant under Zn-toxicity conditions, promoting the accumulation of total GSH in these plants (Table 3). The induction of the synthesis of GSH has been observed in plants in response to heavy metals [6]. Recent research has demonstrated an increase in both enzyme activities in a tolerant ecotype of rice while these activities diminished in a sensitive ecotype under stress conditions [12]. Therefore, since GSH can reduce oxidative stress by binding to heavy metals [70], these results suggest that GSH could help ROS detoxification and metal quelation in *B. oleracea* leaves, thereby preventing the oxidative damage in this organ.

The ascorbate–glutathione cycle or "Foyer-Halliwell-Asada"pathway is key player in H_2O_2 metabolism [71] and appears to be a mechanism of great importance to maintain the cell redox status in plants, especially after the application of heavy metals such as Zn or Cd [20,72]. Cuypers et al. [20] and Smeets et al. [72] concluded that the ascorbate–glutathione cycle plays a prominent role in the defense against Zn-imposed oxidative stress, since they observed an increase of these enzymes under toxicity conditions in the primary leaves of P. vulgaris. In this cycle, H_2O_2 scavenging involves a series of reactions with four enzymes, APX, MDHAR, DHAR, and GR [51]. In this work, in *L. sativa* we found that the excess of Zn altered the enzymes of this cycle, depressing MDHAR and GR activities (Table 4). In addition, APX and DHAR enzymes were not affected under toxicity conditions (Table 4). These results are consistent with data from the H_2O_2 concentration in this species (Table 2), and this increased ROS was not accompanied by more intense activities of this cycle, so that the consequent accumulation of this compound provoked oxidative damage and concomitant reduction of leaves biomass in *L. sativa* at 0.5 mM Zn (Table 1). By contrast, *B. oleracea* showed an intensification of the APX and GR activities (176%and 146%, respectively) while

MDHAR and DHAR were not affected under toxicity conditions (Table 4). The high activity of APX could explain why there was no increase in H_2O_2 in *B. oleracea* under toxicity conditions (Table 2). Several works have demonstrated the rise in the activities of various enzymes in *P. vulgaris* and *B. juncea* grown under Zn stress; that is, these results showed a coordinated increase in enzymatic antioxidants (APX, MDHAR, DHAR, GR) which play an essential role in the tolerance in these species [20,67]. However, the enzyme APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants because this enzyme has high affinity for H_2O_2 [66]. Our results agree with the pivotal role of this enzyme in tolerance to excess metal, as tolerant species in the induction of this enzyme promote high detoxification of H_2O_2, a situation that did not occur in the sensitive species.

Removal of ROS and cell homeostasis are regulated by ascorbate–glutathione cycle end endogenous antioxidant such as AsA and GSH [73]. The cellular redox status of the AsA and GSH pools responds strongly to Zn phytotoxicity [20]. In our studied plants, as previously described, the response of both antioxidants significantly differed. In *L. sativa*, there was a major increase in the forms of oxidized AsA and GSH (Figs. 2C and 3B) since in this species the MDHAR activity (Table 4) accelerated while that of GR abated (Table 4), favouring the accumulation of these oxidized forms at 0.5 mM Zn. On the contrary, in *B. olerecea* the reduced forms of both compounds increased (Figs. 2B´ and 3A´) showing a significant decrease in their oxidized forms (Figs. 2C´ and 3B´). In this species, the high activity of APX is consistent with our results in the determination of non-enzymatic antioxidant. That is, *in B. oleracea* it promotes higher values reduced AsA (140%) recycling through constant MDHAR and DHAR activities (Table 4) this fact in turn promoting the recycling of reduced GSH (151%) by the higher GR activity (Table 4).

A role for increased AsA content in amelioration of oxidative stress has been reported by Wang et al. [26] and Michaeland Krishnaswamy [74] to withstand Zn stress in aquatic and bean plants. Also, reduced GSH is a crucial compound for the effective scavenging of ROS and for the maintenance of other antioxidants such as AsA and tocopherol [75]. In this sense, the hyperaccumulating *S. alfredii* showed more reduced GSH and less ROS production than did the non-hyperaccumulating under excess Cd [76] preventing the loss of aerial biomass in this species. On the basis of our results, we conclude that the antioxidant enzymes APX and GR as well as the reduced forms of AsA and GSH have a major role as a detoxification mechanism of ROS, thereby diminishing the accumulation of H_2O_2 in leaves, thereby avoiding biomass production decreases in this organ by acting as an efficient tolerance mechanism in *B. oleracea* plants under Zn toxicity.

Based on several studies, it is clear that the oxidative metabolism is altered greatly under Zn stress. Recent studies have revealed that stress conditions also significantly induce methylglyoxal metabolism [23]. The function of the glyoxalase pathway has been extensively studied. Gly I and Gly II act coordinately to convert methylglyoxal and other 2-oxoaldehydes to their 2-hydroxyacids using GSH as a cofactor in a two-step reaction. The spontaneous reaction between GSH and methylglyoxal forms hemithioacetal, which is then converted to SLG by Gly I. The second reaction is the hydrolysis of SLG to D-lactate catalyzed by Gly II and GSH is recycled back into the system. Methylglyoxal detoxification is therefore strongly depends on the availability of cellular GSH, and the deficiency of GSH limits the production of hemithioacetal, leading to the accumulation of methylglyoxal [21]. In several plant species, a high activity of Gly I and Gly II bolstered the tolerance against abiotic stresses [77,78]. In this sense, Singla-Pareek et al. [22] demonstrated the role glyoxalase system

maintenance of GSH homeostasis in transgenic plants during exposure to excess Zn (5 mM) as the possible mechanism behind this tolerance. As a result, we observed that in *L. sativa* both activities Gly I and Gly II declined significantly in plants grown under 0.5 mM Zn (Table 5). By contrast, *B. oleracea* showed more vigorous activities of both enzymes under stress conditions (Table 5), thereby removing methylglyoxal formed. It has been demonstrated that in transgenic plants, the overexpression of Gly I and Gly II results in a major detoxify of methylglyoxal and low lipid peroxidation under Zn toxicity [22], and more recently in rice leaves higher activity of both enzymes has been detected in tolerant species than sensitive species under salt stress [79]. In light of the results found in the present study and supporting evidence from literature, we conclude that *B. olereacea* are more powerful components that allow detoxify the toxic compound methylglyoxal and, hence, may contribute to its better tolerance against Zn stress.

Finally, enzymes involved in the GSH metabolism may have vital roles in plant tolerance to environmental stress. Besides the essential role of the redox state of GSH, several GSH-related enzymes GST and GPX were found to be indispensable in ROS detoxification ROS [21,24]. GST catalyses the conjugation of GSH with toxic compounds and may relieve the oxidative stress by scavenging various ROS [80]. In this regard, Aravind and Prasad [73] demonstrated that Zn supplementation activates GST activity. Zn, by triggering GSTs, detoxifies endogenously produced electrophiles such as 4-hydroxyalkenals and base propenals. GSTs conjugate themselves into these products formed by oxidative degradation of lipids and nucleic acids and eliminate them from propagating further damage. GST activity in *L. sativa* fell (Table 5), probably promoting an accumulation of these electrophile products in these plants, while in *B. oleacea* GST increased (142%), encouraging the use of reduced GSH and the elimination of lipid-peroxidation products (Table 5). Many authors suggest the role of

this enzyme in detoxification under Zn stress, but the use of GSH might contribute to the depletion of GSH pool in this condition [26]. In our study, we found an imbalance in the redox state, so that there was a decline in reduced GSH as well as an accumulation of GSSG in *L. sativa* (Fig. 3A and B). However, in this study *B. oleracea* showed an increase in GSH, with reduced GSH being the predominant form (Fig. 3A´). We explain these results due to the high GR activity in these plants (Table 4), which would be responsible for regenerating GSH and maintaining the redox status in *B. oleracea*.

GPX is another GSH-dependent enzyme that serves as defense and catalyzes H_2O_2 detoxification [66]. In *L. sativa*, GPX activity does not appear to show a detoxifying role since treated plants showed a decline in this enzymatic activity (Table 5), which would be consistent with the accumulation of H_2O_2, despite there was a decrease of SOD activity, without using the reduced GSH for subsequent incorporation into proteins and result in signal transduction. However, in *B. oleracea* the rise GPX activity under Zn toxicity was pronounced (127%) (Table 5), stimulating the detoxification of H_2O_2 and using reduced GSH. In agreement with our results, Gill and Tuteja [66] indicated an improvement in the activity of both enzymes under various abiotic stresses. Similar to what occurred in *B. oleracea*, in transgenic tobacco the overexpression of GST/GPX increased GSH-dependent peroxide scavenging and alterations in GSH and AsA metabolism that lead to less oxidative damage [25]. Also, tobacco plants with enhanced Gly I and Gly II activities showed higher tolerance and additional increase of GST, GPX, and GR activities, indicating the interaction between the antioxidant system and the glyoxalase system in stress tolerance [21].

Similar to some hyperaccumulators, where metal chelation and sequestration form a basis for metal tolerance, translocation, and storage of metals within vacuoles constitute an important phenomenon found in non-accumulators, where it is an efficient defense

strategy against metal stress [81]. Accumulated metal ions can be detoxified by vacuolar compartmentalization or complexing with organic ligands, such as metal-binding peptides: PCs and MTs, organic ligands or amino acids, which could greatly alleviate metal toxicity in plants [82,83]. Formation of complexes between metal ions and thiol compounds is considered to be a specific defense strategy against heavy metal toxicity [84]. A rise in the levels of TNP-SH in *L. sativa* thus explains the increase in the content of PCs, which may be acting as a survival mechanism in these plants. However, this would not be a mechanism for providing powerful tolerance in this species, given that we saw earlier that this biomass was negatively affected (Fig. 4A and B). In *B. oleracea*, the TNP-SH and PCs concentrations did not increase (Fig. 4A´and B´) and as we have seen in previous work, another mechanism for tolerance in *B. oleracea* was Zn chelation by organic acid citrate (data not shown). Recently, it has been suggested that PCs are important chelators of excess Zn and that PC formation is crucial for Zn tolerance [85]. However, many studies do not agree with the implication of PCs in tolerance mechanisms. Sun et al. [33] demonstrated that in *S. alfredii* under Zn stress PC synthesis was not stimulated. In a more recent work, the sensitive plant of *S. alfredii* showed an increase of PCs without low of biomass, while the tolerant plant had a more pronounced increase in GSH [34]. These authors concluded that GSH might serve as an antioxidant or a metal chelator. Moreover, Zn hyperaccumulation and tolerance in *S. alfredii* and *T. caerulescens* additional detoxification mechanisms have been detected [33,86]. In this sense, it has been suggested that increased Zn tolerance in the shoot are associated with malate or citrate [35]. On the basis of our work, the present results support the idea that PCs are not involved in metal tolerance in *B. oleracea* plants.

4.2.5. Conclusions

Our results show that the increase in Zn induced a differential response in *L. sativa* and *B. oleracea*. In *L. sativa* under Zn stress the role of enzymatic and non-enzymatic antioxidants does not seem to be powerful enough as a tolerance mechanism. Also the accumulation of PCs in this species seems to be a survival mechanism rather than a tolerance mechanism, since Zn accumulation of in leaves resulted in a biomass reduction in this organ. On the contrary, in *B. oleracea* the accumulation of reduced GSH and its use in the detoxification of ROS, through induction of enzymes such as APX, GST, and GPX, and the detoxification of toxic compounds, such as methylglyoxal, by an increase in Gly I and Gly II activities, seems a pivotal mechanism to provide tolerance to Zn toxicity in these species, since reduced GSH is not used for PCs synthesis. Our results suggest that under Zn toxicity *B. oleracea* will be more efficient and tolerant than *L. sativa* through the detoxification of lipid peroxidation products due to the reduced GSH. These results indicate that B. *oleracea* is a better choice than *L. sativa* in phytoremediation programmes.

Acknowledgements

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4.3. Role of some nitrogenous compounds protectors in the resistance to zinc toxicity in Lactuca satica cv. Phillipus and Brassica oleracea cv. Bronco

Abstract

Zinc (Zn) pollution in the soil represents a major problem for crop production worldwide. In the present work, two horticultural plants exhibiting different tolerance to Zn, *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco, were exposed to Zn to evaluate the contribution of compatible osmolytes such as proline (Pro), glycine betaine (GB) and ɣ-aminobutyric acid (GABA) in the mechanism (s) of tolerance to Zn stress. This study confirms the higher susceptibility of *L. sativa* to Zn stress: lettuce plants experienced a strong reduction in biomass, while the levels of Pro and GB increased. These results suggest that in *L. sativa*, the increase of Pro and GB does not represent a mechanism of resistance to toxicity, but it is likely a symptom of Zn stress. Conversely, in *B. oleracea*, a slight decrease in Pro levels, mainly catalysed by degradation through proline dehydrogenase, was observed; a similar behaviour affected GB levels. On the other hand, GABA synthesis was slightly, but significantly, increased. The presence of high levels of GABA in Zn-stressed *B. oleracea* would suggest that reactive oxygen species detoxification could be essential to improve the resistance to toxicity under metal stress conditions.

Keywords: Proline, glycine betaine, ɣ-aminobutyric acid, Zn toxicity, stress tolerance

Abrevviations: Pro, proline; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; OAT, ornithine-δ-aminotransferase; PDH, proline dehydrogenase; GB, glycine betaine; BADH, betaine-aldehyde dehydrogenase; ROS, reactive oxygen species; GABA, ɣ-aminobutyric acid; GAD, glutamate decarboxylase; DAO, diamine oxidase; PAO, polyamine oxidase.

4.3.1. Introduction

Metal toxicity is one of the major environmental threats to ecosystems, because of its continuous increase by both natural sources and human activities; among the many metals, Zinc (Zn) represents one of the most widespread pollutants in the environment.

It is noteworthy that Zn is an essential micronutrient required as a cofactor in many enzymatic reactions and is involved in various physiological and metabolic processes. Zn also has a stabilizing and protective effect on biomembranes during oxidative damage by the preservation of plasma membrane integrity and permeability (Bettger and O'Dell 1981). Moreover, Zn alleviates other metal-induced oxidative stress (Upadhyay and Panda 2010). It is generally available in the soil for plant uptake as zinc sulphate, chelated and/or linked zinc (Thounaojam et al. 2012).

Zinc emissions from industrial activities are the main source of pollution in the crops worldwide. Zn accumulates in plant tissues, reaching toxic levels (> 300 µg/g), and thus provoking physiological alterations and growth inhibition. These effects are caused, among other processes, by changes in carbohydrate metabolism (Foy et al. 1978), decrease in the content of essential nutrients such as Fe, Cu and Mn (Sargadoy et al. 2011), oxidative damage and the alteration of photosynthetic activity (Vassilev et al. 2007).

There is a broad variability in the susceptibility and tolerance to exposure to Zn, as well as in the tolerance mechanisms in plants (Zorrig et al. 2010). It is well known that, under metal stress, plants are able to synthesise lowmolecular- weight compounds involved in long-distance transport in the xylem and in heavy metal detoxification.

Commonly, these compounds are compatible organic solutes, usually harmless at high cellular concentrations (Sharma et al. 1998; Chen et al. 2007). These molecules can protect plants from stress in different ways, including cellular osmotic adjustment, detoxification of reactive oxygen species (ROS), protection of membrane integrity and stabilization of proteins (Chen et al. 2007). Furthermore, some of them preserve cellular components from dehydration, and thus commonly recognized as osmoprotectants. These solutes include proline (Pro) and quaternary ammonium compounds, such as glycine betaine (GB) and ɣ-aminobutyric acid (GABA) (Rhodes and Hanson 1993).

Levels of Pro are able to control the expression of genes coding of enzymes involved in stress response and supply of reductants. It has been demonstrated that an increase in Pro levels are linked to glucose-6-phosphate dehydrogenase activities in salt-stressed barley (Cardi et al. 2015); similarly, Zn-induced inhibition of glucose-6-phosphate dehydrogenase (G6PDH) is reduced by the increase of Pro levels (Sharma et al. 1998).

GB plays different protective roles: (1) the stabilization of the structures of enzymes and proteins; (2) the reduction of ROS levels under stress; (3) the preservation of the stability of membranes under non-physiological conditions (Chen and Murata 2002). In addition to these direct protective roles, GB levels play a part in signal transduction during environmental stress: both the exogenous supply of GB and the genetically engineered biosynthesis of GB increase the tolerance of plants to abiotic stress (Chen and Murata 2002). It has been demonstrated that, in transgenic plants under water stress,

GB reduces ROS accumulation, increasing the resistance to drought (Kathuria et al. 2009).

GABA has pivotal important roles in nitrogen metabolism (Fait et al. 2007) and in the response to different stressors (Bor et al. 2009). Intriguingly, the time-scale accumulation of GABA in response to environmental stress follows a similar pattern in different species, which raises questions on its possible role as a unique signal for stress in plants (Kinnersley and Turano 2000).

It has been hypothesized that GABA could be involved in the resistance to heavy metals; its degradation could limit the accumulation of ROS under oxidative stress conditions, by inhibiting enzymes of the TCA cycle and lowering the levels of ROS, by transfer of reactive intermediates from the cytosol to mitochondria (Fait et al. 2007).

Many studies have previously highlighted several differences between the capability to store osmo-protectants and the tolerance mechanism(s) in different plants under metal stress.

In this work, a comparative study has been made on two species of great agricultural interest, *Lactuca sativa* (lettuce) and *Brassica oleracea* cv. Bronco (white cabbage), where the effects of Zn toxicity on the levels of som nitrogenous compounds and their metabolism have been investigated. The possible key role of these pathways in establishing tolerance to Zn toxicity in plants will be discussed. These data will be useful to identify possible osmolytes and/or enzymatic activities able to confer tolerance to Zn effects in plants, to select varieties suitable for phytoremediation of Zn-contaminated soils.

4.3.2. Materials and methods

4.3.2.1. Growth of plants and experimental design

Seeds of *L. sativa* cv. Phillipus and *B. oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 x 3 x 10 cm filled with a perlite mixture substrate. The flats were placed on benches in an experimental greenhouse located in Spain (Granada, Motril, Saliplant S.L.). After 35 days, seedlings were transferred to a growth chamber under controlled environmental conditions, with relative humidity of 50 %, day/night temperature of 25/15 ºC, and a photoperiod of 16/8 h under a photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm-diameter top, bottom diameter 60 cm and height 7 cm) with a volume of 3 l. Throughout the experiment, the plants were treated with a growth solution composed as follows: 4 mM KNO_3, 3mM $Ca(NO_3)_2 \cdot 4H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2H_2O$, 2 µM $MnCl_2 \cdot 4H2O$, 10 µM $ZnSO4 \cdot 7H_2O$, 0.25 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2H_2O$, 5 ppm Fe-EDTA (Sequestrene; 138 FeG100, Syngenta) and 10 µM H_3BO_3. The nutrient solution was checked for a pH of 5.5–6.0 and changed every 3 days. Treatments were initiated 35 days after germination and maintained for 21 days. Control plants were grown in the basal nutrient solution, while stressed plants were supplemented with 0.5 mM of Zn as $ZnSO_4$.

The experimental design consisted of randomized complete block with four treatments (*L. sativa*—control, *B. oleracea*—control, *L. sativa*—0.5 mM Zn, *B. oleracea*—0.5 mM Zn) and eight plants per treatment in three independent biological replicates (fresh weights in Supplemental Table 1).

4.3.2.2 Zn concentration

For the determination of Zn concentration, a sample of 30 mg dry material was subjected to a process of mineralization by the method of Wolf (1982). The detailed procedure has been described elsewhere (Barrameda-Medina et al. 2014).

4.3.2.3 Measurements of proline, glycine betaine, and ɣ-aminobutyric acid

For the determination of Pro, leaves were homogenized in 5 ml of 96 % ethanol. The insoluble fraction was washed with 70 % ethanol. The extract was centrifuged at 3500 g for 10 min and the supernatant stored at 4ºC (Irigoyen et al. 1992). 1 ml aliquot of the supernatant was taken and, after adding reactive ninhydrin acid reagent (ninhydrin, 6 M phosphoric acid, 60 % glacial acetic acid) and 2.5 ml of 99 % glacial acetic acid, was placed in a water bath at 100ºC. After 45 min, the tubes were cooled on ice and 5 ml of benzene was added; after 5–10 min the absorbance of the organic phase was measured at 515 nm.

GB was determined as described by Grive and Gratton (1983): GB was extracted from 200 mg of dry plant material in 10 ml of distilled water, gently shaking for 24 h. The extract was filtered and 2 ml of 2 N H_2SO_4 was added. Then the solution was incubated for 16 h at 4ºC and centrifuged at 9000 g for 15 min at 0ºC. The pellet was suspended in 1,2 dichloroethane; after 2 h, the GB content was measured by reading the absorbance at 365 nm and quantified using a standard curve of GB.

ɣ-Aminobutyric acid (GABA) was measured by the method of Zhang and Bown (1997): frozen leaves were ground in liquid nitrogen, and 100 mg of the powdered tissue was extracted in 0.1 ml methanol for 10 min at room temperature. The slurry was vacuum dried with a Vacufuge concentrator 5301 (Eppendorf, Hamburg, Germany). The dried powder was dissolved in 1 ml 70 mM lanthanum chloride, shaken for 15 min,

and then centrifuged at 13,000g for 5 min. The clear supernatant was transferred to a new tube, and 0.8 ml of the supernatant was mixed with 0.16 ml 1 M potassium hydroxide, shaken for 5 min, and centrifuged at 13,000g for 5 min. The supernatant was used for the determination of GABA.

GABA content was measured using GABase (Sigma, St. Louis, MO, USA): 1 ml assay mixture contained 0.6 Mm $NADP^+$, 0.1 unit GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate, and 550 µl sample. The reduction of $NADP^+$ to NADPH was monitored at 340 nm in a spectrophotometer after 10 min incubation at room temperature.

4.3.2.4 Extraction and determination of enzymatic activities

Δ^1-Pyrroline-5-carboxylate synthetase (P5CS) extraction was carried out according to Sumithra et al. (2006). Leaves (100 mg) were homogenized in the extraction buffer [100 mM Tris–HCl (pH 7.5), 10 mM β-mercapto-ethanol, 10 mM $MgCl_2$ and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF)], and then centrifuged at 10,000 g for 15 min; the clear supernatant was used for enzyme assays. P5CS activity was measured as described by Charest and Phan (1990): the reaction mixture contained 100 mM Tris–HCl (pH 7.2), 25 mM $MgCl_2$, 0.4 mM NADPH, 5 mM ATP, and extract. The reaction was initiated by the addition of 75 mM sodium glutamate and the activity measured as the rate of consumption of NADPH monitored as absorbance decrease at 340 nm.

Ornithine-δ-aminotransferase (OAT) and proline dehydrogenase (PDH) were extracted from leaves (100 mg) homogenized in 100 mM potassium phosphate buffer (Ph 7.8). The homogenate was filtered and centrifuged at 12,000 g for 20 min (4ºC). OAT was assayed according to Charest and Phan (1990) in 0.2 M Tris–KOH buffer (pH

8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate, and 0.25 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with the addition of the extract. PDH activity was assayed by the reduction of NAD^+ at 340 nm. The reaction mixture contained 0.15 M Na_2CO_3–HCl buffer (pH 10.3) containing 2.67 mM L-proline and 10 mM NAD^+.

Protein extraction and NAD^+-dependent betaine aldehyde dehydrogenase (BADH) activity measurements were made as in Tang (1999). One gram of fresh leaf tissue was ground in a mortar on ice with 2 ml of cold extraction buffer (50 mM Hepes-KOH, pH 8.0, 5 mM dithiothreitol). Leaf debris were removed by centrifugation at 14,500 g for 10 min at 4ºC. The BADH activity was determined measuring at 340 nm at 37ºC the rate of formation of NADPH in a 3 ml mixture consisting of 50 mM HEPES (4-(2 hydroxyethyl)-1-piperazine ethanesulfonic acid)–KOH buffer (pH 8.0), 5 mM DTT, 1 mM EDTA, 1 mM betaine aldehyde, 1 mM NAD^+, and extract. Each assay was made in duplicate.

Cytosolic glutamate decarboxylase (GAD) was extracted from plant material, as described previously, in a buffer containing 0.1 M Tris–HCl (pH 9.1), 10 % (v/v) glycerol, 1 mM dithiothreitol (DTT), 5 mM ethylene-diamine-tetraacetic acid (EDTA), 0.5 mM pyridoxal phosphate (PLP), and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF). Fresh tissue was added to the pre-cooled extraction buffer at a ratio of 1:3 (w/v) and homogenized at 4ºC. The homogenate was filtered through one Miracloth layer and then centrifuged at 24,500 g at 4ºC for 30 min. The supernatant was used for GAD activity determination using the method of Bartyzel et al. (2003): the enzyme rate was assayed by incubating the extract at 30ºC for 60 min in 400 µl assay mixture containing 0.1 M potassium phosphate buffer (pH 5.8), 40 µM PLP, and 3 mM L-glutamate. The reaction was stopped by adding 0.1 ml of 0.5 mM HCl; the amount of

GABA in the samples was determined as previously described.

Diamine oxidase (DAO) and polyamine oxidase (PAO) activities were determined by measuring H_2O_2 production, as described by Su et al. (2005), with few modifications. The plant material (700 mg) was homogenized in 100 mM potassium phosphate buffer (pH 6.5); the homogenate was centrifuged at 10,000 g for 20 min at 4ºC and the supernatant used for the assays. The reaction mixture contained 2.5 ml of potassium phosphate buffer (100 mM, pH 6.5), 0.2 ml of 4-aminoantipyrine/*N*,*N*-dimethyl-aniline reaction solution, 0.1 ml of horseradish peroxidase (250 U/ml), and 0.2 ml of the enzyme extract. The reaction was initiated by the addition of 0.15 ml of 20 mM putrescine for DAO determination and 20 mM spermidine plus spermine for PAO determination. One unit of enzyme activity corresponded to a change in 0.001 absorbance unit at 555 nm per min under the condition described.

4.3.2.5 Statistical analysis

Data were analysed by ANOVA test; differences between means were compared by Fisher's least significant difference (LSD).

4.3.3. Results and discussion

Zinc (Zn) is a micronutrient playing an essential role in many physiological and biochemical processes; when exposed to an excess of Zn, plants accumulate this element in their tissues, where it can reach toxic concentrations, thus provoking metabolic alterations and inhibition of growth (Gisbert et al. 2006).

In natural environments, Zn occurs in water, air and soil, but its concentrations are increasing, largely due to anthropic activities. Plants are not able to manage the excess of Zn by their physiological uptake systems, thus resulting in a serious threat to

agricultural production (Vassilev et al. 2007). *L. sativa* and *B. oleracea* accumulated Zn in the roots when supplied with 0.5 mM Zn, up to 35–45 mg g^{-1} DW (Table 1); a corresponding decrease in root biomass (about 35 %) was observed in both species.

In the leaves, differences were observed between the two species: in *L. sativa* the application of 0.5 mM Zn caused a 2.7-fold accumulation of Zn, with a related reduction in biomass (- 33 %); in *B. oleracea*, a sixfold Zn accumulation was detected, without a significant change in biomass (Table 1), although Zn levels reached values considered as toxic (Marschner 1995).

Table 1. Levels of Zn in roots and leaves and changes in biomass in *L. sativa* and *B. oleracea* in control plants and plants exposed to 0.5 mM Zn.

Cultivar/Treatment	`[Zn] root concentration (mg g^{-1} DW)	Root biomass (g DW/plant)	[Zn] shoot (µg g^{-1} DW)	Leaf biomass (g DW/plant)
***Lactuca sativa* cv. Phillipus**				
Control	0.586 ± 0.069	0.135 ± 0.003	80.78 ± 4.39	2.11 ± 0.19
0.5 mM	34.95 ± 0.376	0.042 ± 0.010	218.4 ± 35.7	1.43 ± 0.04
p-value	***	***	**	*
$LSD_{0.05}$	10.44	0.03	83.02	0.53
***Brassica oleracea* cv. Bronco**				
Control	0.895 ± 0.104	0.083 ± 0.003	61.43 ± 0.66	0.70 ± 0.06
0.5 mM	42.54 ± 0.234	0.033 ± 0.006	382.4 ± 60.6	0.61 ± 0.08
p-value	***	**	***	NS
$LSD_{0.05}$	0.863	0.02	139.86	0.29

DW; dry weight. Values are means (n = 9) ± standard error. $LSD_{0.05}$ Fisher's least significant difference (LSD) ($p > 0.05$), NS not significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

It has been suggested that exposition to Zn caused its accumulation both in leaves and roots (Ozdener and Aydin 2010), due to compartmentation (Gisbert et al. 2006) or to the adsorption in the apoplast (Küpper et al. 2000).

Our results suggest that the effects of toxic Zn levels on the biomass are particularly harmful in the roots. On the other hand, changes in the leaf biomass are mutable, depending on the susceptibility to stress of the different species: Brassicaceae seem

unresponsive to Zn in the leaves (Gisbert et al. 2006), while Crassulaceae, such as *Sedum alfredii*, showed an increase in biomass at 0.5 mM Zn, without any toxic effects in the leaves (Yang et al. 2006). Our results clearly suggest that under exposure to toxic conditions of Zn, *B. oleracea* accumulates Zn in both roots and leaves, but toxic effects are evident only in the roots, while *L. sativa* showed a marked decrease in both roots and leaves biomass.

Pro is possibly the most known osmolyte produced by plant cells in response to abiotic stress. In plants, Pro is synthesized from either glutamate or ornithine, by Δ^1-pyrroline-5-carboxylate synthetase (P5CS), or ornithine- δ-aminotransferase (OAT), respectively. The accumulation of Pro in the response to different types of stress is directly related to enzyme activities and/or transcriptional activation of the genes encoding these enzymes, thus inducing an improved capability to survive under stress conditions (Strizhov et al. 1997).

Table 2. Proline levels, and activities of enzymes of proline metabolism in *L. sativa* and *B. oleracea* control plants, and plants exposed to 0.5 mM Zn.

Cultivar/ Treatment	`Pro (µmol g^{-1} FW)	P5CS (ΔAbs mg^{-1} prot min^{-1})	OAT (ΔAbs mg^{-1} prot min^{-1})	PDH (ΔAbs mg^{-1} prot min^{-1})
***Lactuca sativa* cv. Phillipus**				
Control	0.27 ± 0.03	0.084 ± 0.007	0.10 ± 0.008	0.10 ± 0.008
0.5 mM	0.39 ± 0.01	0.081 ± 0.005	0.14 ± 0.009	0.08 ± 0.006
p-value	**	NS	**	NS
$LSD_{0.05}$	0.07	0.02	0.02	0.05
***Brassica oleracea* cv. Bronco**				
Control	0.42 ± 0.02	0.083 ± 0.003	0.05 ± 0.003	0.08 ± 0.005
0.5 mM	0.37 ± 0.01	0.033 ± 0.006	0.05 ± 0.002	0.15 ± 0.009
p-value	*	**	NS	**
$LSD_{0.05}$	0.03	0.02	0.02	0.03

FW; fresh weight, Pro; proline, P5CS; Δ^1-pyrroline-5-carboxylate synthetase, OAT; ornithine-δ-aminotransferase, PDH; proline dehydrogenase. Values are means (n = 9) ± standard error. $LSD_{0.05}$ Fisher's least significant difference (LSD) ($p > 0.05$), NS not significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Pro levels notably increased upon treatment with 0.5 mM Zn in *L. sativa* (+ 44 %) and are correlated to an increase of OAT (+ 40 %). In contrast, Zn induced a slight reduction of Pro levels in *B. oleracea* with respect to control plants (Table 2); correspondingly, Zn-treated *B. oleracea* did not exhibit major changes in P5CS and OAT activities.

Lactuca sativa is more sensitive than *B. oleracea* to Zn stress; thus, our results do not support the hypothesis that proline accumulation could be associated with an increased tolerance against heavy metal stress, as previously proposed (Tripathi and Gaur 2004). In *L. sativa*, Pro increase would represent a symptom of Zn stress, rather than a mechanism of resistance against Zn toxicity; this is in agreement with the hypothesis of Schat and Vooijs (1997), who suggested that metal-induced Pro accumulation is a consequence, and not a cause, of metal tolerance in plants.

Furthermore, the increase in Pro levels upon metal stress can be possibly caused by a decrease in the Pro degradation process (Kavi Kishor et al. 2005), due to mitochondrial proline dehydrogenase reaction (PDH) (Lin and Kao 2007). In *L. sativa*, PDH activity remained substantially unaltered in Zn-treated plants, thus confirming that the synthesis (OAT), and not the degradation (PDH), is the cause of the increased Pro levels under Zn stress; similar results were obtained in Zn-exposed wheat seedlings, where Zn induced Pro accumulation was associated with a prompt rise of OAT activity and an inhibition of PDH (Li et al. 2013).

In contrast, the improved Zn tolerance in *B. oleracea* is supported by a twofold increase in PDH activity, thus decreasing Pro levels.

GB accumulates in response to heavy metal stress in plants; GB is synthesized from choline by a two-step oxidation reaction catalysed by choline monooxygenase (CMO) and NAD^+-dependent betaine aldehyde dehydrogenase (BADH) to GB (Chen and Murata 2002); therefore, changes in GB levels could be related to variations in BADH activity.

Lactuca sativa plants treated with 0.5 mM Zn showed higher levels of GB (+ 80 %) as previously reported for other plants upon abiotic stress (Cardi et al. 2015). Interestingly, a corresponding + 35 % increase in BADH activity with respect to untreated plants was observed. *L. sativa* is sensitive to Zn stress; thus our data contrast with the hypothesis that GB accumulation represents a symptom of an enhanced tolerance to abiotic stress (Sakamoto and Murata 2000).

On the contrary, in *B. oleracea*, Zn exposure led to a reduction of GB levels at 50 % of their initial value, leaving BADH activity substantially unaltered (Table 3).

It has been suggested that low levels of GB may preserve membranes and photosynthetic apparatus upon abiotic stress (Sakamoto and Murata 2000; Yang et al. 2006); salt stress triggers an increase in GB levels in barley (Cardi et al. 2015), and higher levels of GB were observed in saltsensitive barley cultivars with respect to the salt-tolerant cultivar (Chen et al. 2007). These authors hypothesized that a low GB concentration in salt-tolerant plants is required to alleviate stress toxic effects, while the accumulation in saltsensitive plants could represent a symptom of stress damage; anyway, the relationship between GB accumulation and enhanced stress tolerance is possibly species specific (Ashraf and Foolad 2007).

In plants, GABA, a non-protein amino acid, plays an important role as osmoprotectant (Rhodes and Hanson 1993); it is mainly synthesized through ‘‘the GABA shunt’’. This pathway originated from cytosolic glutamate decarboxylase (GAD) reaction, (Fait et al.

2007); GABA is produced from polyamine degradation by the coordinated action of diamine oxidase (DAO) and polyamine oxidase (PAO) (Alcàzar et al. 2010).

Table 3. Levels of glycine betaine and activities of betaine aldehyde dehydrogenase (BADH) in *L. sativa* and *B. oleracea* control plants, and plants exposed to 0.5 mM Zn.

Cultivar/Treatment	Glycine betaine (nmol g^{-1} FW)	BADH (ΔAbs mg^{-1} prot min^{-1})
***Lactuca sativa* cv. Phillipus**		
Control	80.78 ± 4.39	2.11 ± 0.19
0.5 mM	218.4 ± 35.7	1.43 ± 0.04
p-value	**	*
$LSD_{0.05}$	83.02	0.53
***Brassica oleracea* cv. Bronco**		
Control	61.43 ± 0.66	0.70 ± 0.06
0.5 mM	382.4 ± 60.6	0.61 ± 0.08
p-value	***	NS
$LSD_{0.05}$	139.86	0.29

FW; fresh weight Values are means (n = 9) ± standard error. $LSD_{0.05}$ Fisher's least significant difference (LSD) ($p > 0.05$), NS not significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In Zn-treated *L. sativa*, GABA levels remained unaltered, while *B. oleracea* showed a small (+ 10 %), but significant accumulation of GABA, possibly related to an increased synthesis (Table 4).

Actually, Zn-treated *B. oleracea* showed a significant 60 % increase of GAD activity; differently, GAD activity remained unchanged after Zn exposure in *L. sativa* (Table 4). Concomitantly, both *L. sativa* and *B. oleracea* did not show any significant changes in PAO and DAO activities in response to Zn supply (Table 4).

These results would suggest that in *L. sativa*, GABA does not play a key role in response to Zn stress.

Furthermore, it should be considered that GABA shunt plays a role in the provision of succinate and NADH to the respiratory chain; thus, it has been postulated that GABA levels could play a role to contrast ROS accumulation upon oxidative stress (Bouche`

and Fromm 2004). Therefore, it can be proposed that in *B. oleracea*, GABA may play a role to avoid the effects of Zn.

Table 4. Levels of ɣ-aminobutyric acid (GABA), and related enzymes, in *L. sativa* and *B. oleracea* control plants, and plants exposed to 0.5 mM Zn.

Cultivar/ Treatment	`GABA (nmol g^{-1} FW)	GAD (ΔAbs mg^{-1} prot min^{-1})	PAO (ΔAbs mg^{-1} prot min^{-1})	DAO (ΔAbs mg^{-1} prot min^{-1})
Lactuca sativa **cv. Phillipus**				
Control	0.98 ± 0.09	0.11 ± 0.02	6.69 ± 0.68	8.81 ± 0.95
0.5 mM	1.04 ± 0.08	0.09 ± 0.01	6.28 ± 0.51	10.82 ± 0.75
p-value	NS	NS	NS	NS
$LSD_{0.05}$	0.14	0.05	1.91	2.72
Brassica oleracea **cv. Bronco**				
Control	1.40 ± 0.07	0.08 ± 0.01	3.16 ± 0.45	7.05 ± 0.82
0.5 mM	1.53 ± 0.06	0.13 ± 0.01	2.01 ± 0.32	7.14 ± 0.52
p-value	*	**	NS	NS
$LSD_{0.05}$	0.11	0.03	1.64	2.31

FW; fresh weight, GAD; glutamate decarboxylate, DAO; diamine oxidase, PAO; polyamine oxidase. Values are means (n = 9) ± standard error. $LSD_{0.05}$ Fisher's least significant difference (LSD) ($p > 0.05$), NS not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.4. Conclusion

In conclusion, in *L. sativa* Zn toxicity is able to induce an increase in the levels of both Pro and GB. Since these plants showed a significant decrease in their biomass, it is assumed that these two compounds do not represent a signal of resistance to toxicity, but probably a symptom of Zn stress.

Differently, in *B. oleracea* Zn stress caused an increment of Pro degradation, catalysed by PDH activity, and an increase in GABA levels. These two processes resulted in an improved Zn tolerance in *B. oleracea* and suggest that ROS detoxification under Zn stress can be essential to provide metal tolerance. Therefore, these data support our previous work, demonstrating that the homoeostasis of GSH and its role in

oxidative metabolism are essential to improve the resistance of *B. oleracea* to Zn toxicity.

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Supplemental Table 1. Fresh weight in leaf and root of *L. sativa* and *B. oleracea* plants.

	Leaf FW (g)	Root FW (g)
***L. sativa* var. Phillipus**		
Control	30.93 ± 4.36	4.31 ± 0.40
0.5 mM	20.65 ± 1.30	1.14 ± 0.12
p-Value	*	***
LSD	9.75	0.90
***B. oleracea* var. Bronco**		
Control	6.48 ± 1.30	1.91 ± 0.14
0.5 mM	5.11 ± 0.55	0.55 ± 0.06
p-Value	NS	***
LSD	3.03	0.32

FW; Fresh weight. Values are means (n = 9) ± standard error. $LSD_{0.05}$ Fischer´s least significant difference (LSD) ($p > 0.05$), NS not significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4. Discusión general

Los mecanismos de tolerancia de las plantas han sido estudiados ampliamente. No obstante, el interés de estos estudios se ha focalizado en plantas hiperacumuladoras de metales pesados. Sin embargo, es de gran interés conocer los mecanismos de tolerancia en plantas de consumo diario. Por ello, el primer objetivo de esta Tesis Doctoral ha sido realizar un estudio comparativo del efecto tóxico del Zn a nivel agronómico y fisiológico en dos plantas hortícolas ampliamente cultivadas a nivel mundial, *L. sativa* y *B. oleracea* con el fin de incrementar aquellos mecanismos que a priori parecen promover la acumulación de Zn en la parte aérea, siendo esto de gran potencial en la mejora de programas de fitoextracción así como de biofortificación con este elemento.

Los datos obtenidos en este cuarto capítulo de Tesis Doctoral muestran como un incremento de Zn indujo una respuesta diferencial en plantas de *L. sativa* y *B. oleracea*. Nuestros resultados sugieren que tanto en programas de fitoextracción como de biofortificación con Zn, *L. sativa* es menos efectiva que *B. oleracea*:

En *L. sativa* un incremento de las actividades MDH y CS y elevados niveles de malato no parecen promover la tolerancia en esta especie. Así mismo, las actividades de las enzimas así como los compuestos antioxidantes no parecen ser un mecanismo potente involucrado en los mecanismos de tolerancia. Además, en esta especie la acumulación de PCs parece ser más bien un mecanismo de supervivencia que de tolerancia. Finalmente, la acumulación de compuestos tales como la Pro o la GB, parece ser un síntoma de toxicidad de Zn más que una inducción promovida para ofrecer tolerancia al Zn en esta especie. En base a nuestros resultados podemos definir a *L. sativa* como una

especie sensible a la toxicidad de Zn debido a que una acumulación de Zn en hojas resultó en una reducción de la biomasa de este órgano.

En *B. oleracea*, una disminución de la actividad MDH así como una elevada actividad de la enzima CS se vio acompañada de una elevada concentración del anión orgánico citrato, lo cual podría ser determinante en la tolerancia y mayor acumulación de Zn en hojas de *B. oleracea*. Además, la acumulación de GSH reducido y su uso en la detoxificación de ROS, a través de la inducción de enzimas, tales como APX, GST, y GPX, y la detoxificación de compuestos tóxicos, tales como metilglioxal, mediante un incremento de las actividades Gly I y Gly II, parecen ser un mecanismo fundamental para proporcionar tolerancia a Zn toxicidad en esta especie. Sin embargo, el estrés por Zn en *B. oleracea* provocó un incremento de la degradación Pro y un aumento en los niveles de GABA. Estos dos procesos resultaron en una mejora de la tolerancia de Zn en *B. oleracea* y sugieren que la detoxificación de ROS desintoxicación bajo estrés por Zn puede ser esencial para proporcionar tolerancia a los metales. En base a estos resultados, podemos definir a *B. oleracea* como una especie menos sensible que L. sativa a la toxicidad de Zn, ya que un incremento de Zn no afectó negativamente a la biomasa de la parte aérea gracias a la quelación y transporte de este elemento traza a la parte aérea, así como una potente detoxificación de ROS por una mayor producción de compuestos antioxidantes

En definitiva, podemos concluir que a la hora de realizar un programa de fitoextracción del Zn, *B. oleracea* será más eficiente que *L. sativa*, acumulando una mayor cantidad de este elemento en la parte aérea sin disminuir su biomasa. Además, estos datos revelan que tanto el metabolismo de los carboxilatos como del glutation son procesos claves en la tolerancia en plantas no hiperacumuladoras, como *B. oleracea*.

En cambio, tanto las enzimas de síntesis como los niveles de compuestos osmoprotectores no parecen estar relacionados con la resistencia a la toxicidad de Zn en ambas plantas hortícolas.

Capítulo 5

Biofortificación agronómica con Zn en plantas hortícolas

5.1. Zinc biofortification, antioxidant properties and ionomic profile in two leafy vegetables: Lactuca sativa and Brassica oleracea

Abstract

Zn deficiency is considered as a major risk factor for human health. Here, the effect of Zn biofortification a complementary solution for mineral malnutrition, were examined in two leafy vegetables, *Lactuca sativa* and *Brassica oleracea* in order to select Zn-efficient plants. Zn supply did not affected the biomass, but the concentration of malondialdehyde increased in both plants. Zn-use efficiency increased with respect to Zn concentration, total Zn accumulation (TZnA), and Zn utilization efficiency (ZnUtE). Nevertheless, Zn-use efficiency in *L. sativa* was lower than in *B. oleracea*. *L. sativa* showed lower levels of phenolic compounds, and reduced ascorbate, reduced glutathione and γ-aminobutyric acid than in *B. oleracea*. Furthermore, both plants had higher concentrations of Ca, Mg, Fe, and Mn, especially upon 20 μM Zn in *L. sativa* and 10-20 μM in *B. oleracea*. Our results indicate that the application of 20 μM Zn in *L. sativa* and 20-80 μM Zn in *B. oleracea* promoted the Zn accumulation and improved the leaf essential-nutrient quality for human health. Nevertheless, in *B. oleracea* higher Zn concentration, TZnA, phenolics, antioxidants and nutrients concentrations were observed. *B. oleracea* is emerging as a better choice and more efficient candidate in Zn-biofortification programs. The best approach to increase human Zn levels is to encourage greater consumption of both green leafy vegetables, specially *B. oleracea*.

Abbreviations: AsA, ascorbate; DMPD, N,N-dimethyl-p-phenylenediamine dihydrochloride; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing ability of plasma; GABA, γ-aminobutyric acid; GSH, reduced glutathione; MDA,

malondyaldehyde; O-dPh, ortho-diphenols; PPG, phenylpropanoid glycosides; TEAC, trolox equivalent antioxidant capacity; TPC, total phenolic compounds; TZnA, total Zn accumulation; TZnC, total Zn concentration; ZnUtE, Zn utilization efficiency.

Keywords*:* Zn deficiencies, biofortification, leafy vegetables, Zn-use efficiency

5.1.1. Introduction

Deficiencies in micronutrients such as zinc (Zn), iron (Fe), iodine (I), and vitamin A constitute critical issues in the developing industrialized countries, severely impairing human health and development (Cakmak 2002). Among micronutrients, particularly Zn deficiency is occurring in both crops and humans (Welch and Graham 2004). In fact, Zn deficiency is currently listed as a major risk factor for human health and cause of death globally. In this regard, more than 3 billion people worldwide suffer from micronutrient malnutrition, including Zn deficiency (Welch and Graham 2004; Cakmak 2008)**.**

In this respect, plant-derived foods provide a major source of proteins and dietary minerals but the concentrations of some minerals, especially Zn, Fe, I, and selenium (Se) are inherently low in plants (Waters and Sankaran 2011). For this reason, the contribution to human health through Zn fertilization of staple food crops seems to be an excellent way to alleviate Zn deficiency-related problems in humans worldwide (Cakmak 2009). In this sense, the concentration and accumulation of Zn in edible parts of plants is the basis for recommending Zn fertilization.

Recently, a complimentary solution to mineral malnutrition has been proposed, and this approach being known as biofortification. This term has defined by White and Broadley (2005) as the process of increasing the bioavailable concentrations of essential elements in edible portions of crop plants through agricultural intervention or genetic

selection. Hence, there is considerable interest in breeding mineral-efficient crops that produce high yields and accumulate minerals from infertile soils. Although this strategy for biofortification is unproven as yet, it has the potential to become sustainable and cost effective and to reach remote rural populations (Bouis et al. 2011).

However, a limitation in the biofortification programs is that the edible portion of the most crops appear to have considerable genetic variation in the mineral elements most frequently lacking in humans (White and Broadley 2005). In this regard, White and Broadley (2011) demonstrated that plant species can differ greatly in their tissue Zn concentrations when grown under comparable conditions. This work also observed that cereals and legumes have lower Zn concentrations. Consequently, the incidence of Zn-deficiency disorders has been increasing in populations that change their traditional diets based predominantly on vegetables, pulses, and fruits to diets predominated by cereals (Graham et al. 2001). As a result, biofortification programs should pay attention not only to these types of crops but also in other plant species, including commonly consumed fruits and vegetables (White and Broadley 2005; Müller 2013; Mao et al. 2014). However have been demonstrated as an increased of some trace elements was more correlated in leafy vegetables than fruits and the variation between this vegetables species was more pronounced specially in cabbage, lettuce or spinach plants (Müller 2013; Weng et al. 2013).

At present, another aspect of great interest is the relation between the consumption of fresh fruits and vegetables and their health benefits of lowering the risk of cancer and cardiovascular diseases (Liu 2004). These properties have been attributed to foods that are rich sources of numerous bioactive compounds such as phytochemicals with antioxidant properties known to confer health-promoting benefits (Cartea et al. 2011; Pérez-López et al. 2013). In this sense, the major antioxidants of vegetables are vitamin

E, phenolic compounds, and especially flavonoids, carotenoids, and ascorbate (AsA). These antioxidants scavenge radicals and inhibit chain initiation or break chain propagation (Podsedek 2007). For this reason, crude extracts of fruits and vegetables as well as other plant foods rich in antioxidant molecules have attracted growing interest because they delay oxidative degradation of lipids and thereby improve the nutritional value of food (Nicolle et al. 2004).

Among cultivated plant species, *Lactuca sativa* (family *Asteraceae*) is a major food crop within the European Union (Baslam et al. 2013). Lettuce is an important leafy vegetable that is primarily consumed fresh or in salad mixes and is consumed in increasing amounts due to their being perceived as healthy foods with high nutritive value (DuPont et al. 2000; Pérez-López et al. 2013). This health effect of lettuce includes their cholesterol-lowering properties (Huang 1980) and benefits against cardiovascular diseases (Serafini et al. 2002; Nicolle et al. 2004). The healthy properties are attributed to a large supply of antioxidant compounds (e.g. vitamin E, polyphenols, anthocyanins, carotenoids, AsA) and fibre content (Serafini et al. 2002; Nicolle et al. 2004; Li et al. 2010).

Other species of widely cultivated plants in Europe are those within the genus *Brassica*. The genus *Brassica* is the most important one within the tribe Brassiceae, which includes some crops and species of great worldwide economic importance such as *Brassica oleracea*. *Brassica* vegetables are consumed throughout the year as the ingredients of different salads or after cooking of raw and frozen vegetables (Podsedek 2007). The popularity and consumption of *Brassica* vegetable species is increasing because of the contribution to human nutrition and for other health benefits (Salunkhe and Kadam 1998) including cancer and cardiovascular disease-preventing properties (Cartea et al. 2011). Moreover, cabbage has been valued for medicinal purposes in

treating headaches, gout, diarrhoea, and peptic ulcers (Cheney 1950). These health-promoting properties are attributed to their antioxidant capacity (Podsedek 2007). Particularly, *Brassica* contains many antioxidants, including carotenoids, phenolics compounds, anthocyanin, AsA, and γ-aminobutyric acid (GABA) (Wang et al. 1996; Galati and O'Brien 2004).

There are many studies on Zn biofortification which, until today, have focused their efforts on increasing the concentration of this element in cereals, legumes, and tubers. However, rarely has attention been paid to the effect of Zn supplementation in horticultural plants of daily and global consumption. Therefore, the aim of our work is to examine the effect of Zn biofortification in edible part of two different leafy vegetables in order to select Zn-efficient plants in these types of programs. For this reason, we make a comparative analysis between *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco and determine parameters for improved growth, antioxidant capacity, and nutritional quality. The information provided by this study will be helpful for breeders to develop lettuce and cabbage cultivars with an higher accumulation of Zn, which could promote an optimal contents of health-promoting antioxidant phytochemicals through genetic manipulation and plant breeding efforts.

5.1.2. Material and methods

5.1.2.1. Plant materials and experimental design

Seeds of *L. sativa* cv. Phillipus and *B. oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 cm x 3 cm x 10 cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant S.L.). After 35 days, the seedlings were transferred to a growth chamber under the following con-trolled environmental

conditions, with relative humidity of 50%, day/night temperature of 22/18◦C, a photoperiod of 12/12 h at a photosynthetic photon flux density (PPFD) of 350 µmol $m^{-2}s^{-1}$(measured at the top of the seedlings with a 190 SB quantum sensor, LICOR Inc., Lincoln, NE, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) in a volume of 3 l. At 35 days after germination and throughout the experiment, control plants received a growth solution, which was composed of 4 mM KNO_3, 3 mM Ca $(NO_3)_2$ ·4 H_2O, 2 mM $MgSO_4$·7 H2O, 6 mM KH_2PO_4, 1 mM NaH_2PO_4·2 H_2O, 2 µM $MnCl_2$·4 H_2O, 0.25 µM $CuSO_4$·5 H_2O, 0.1 µM Na_2MoO_4·2 H2O, 5 ppm Fe-chelate (Sequestrene; 138 FeG100) and 10 µM H_3BO_3. At the same time, treatments were applied with the same growth solution amended with different supraoptimal Zn concentration (10 (control plants), 20, 40, 60, 80 and 100 µM of $ZnSO_4$·7 H_2O). This solution, with a pH of 5.5–6.0, was changed every three days. The experimental design was a randomized complete block with 12 treatments arranged in individual trays with eight plants per treatment and three replications each, so that the total number of plants was 288.

5.1.2.2. Plant sampling and lipid peroxidation

L. sativa and *B. oleracea* plants were sampled after 21 days further growth under these conditions. The edible leaves of each treatment washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the edible leaves from each treatment were frozen at -30◦C for later performance of biochemical assays and the other half of the plant material sampled dried in a forced air oven at 70◦C for 24 h to obtain the dry weight (DW) and the subsequent analysis of the concentration of Zn and macro and other micronutrients concentrations.

For the malondialddehyde (MDA) assay, 0.1 g of fresh leaves were homogenized with 3 ml of 50 mM solution containing 0.07% $NaH_2PO_4 \cdot 2H_2O$ and 1.6% $Na_2HPO_4 \cdot 12\ H_2O$ and centrifuged at 20,000 g for 25 min in a refrigerated centrifuge. For measurement of MDA concentration 3 ml of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added to a 1 ml aliquot of the supernatant. The mixture was heated at 95ºC for 30 min, quickly cooled in an ice bath and then centrifuged at 10,000 g for 10 min. The absorbance of the supernatant was read at A_{532} and A_{600} nm. The concentration of MDA was calculated using the MDA extinction coefficient of 155 $mM^{-1}cm^{-1}$ (Heath and Packer 1968). The result of MDA was expressed as µmol g^{-1} FW.

5.1.2.3. Zn concentration and Zn use efficiency

For the determination of Zn concentration, a sample of 0.15 g dry material was subjected to a process of mineralization by the method of Wolf (1982). Mineral analysis was conducted using ICP-MS. Briefly, samples were further diluted 1-in-10 with milli-Q water and analyzed using an ICP-MS (X-Series II; Termo FisherScientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml^{-1}) and Ir (5 ng ml^{-1}) in 2% TAG HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEXCerti-Prep Ltd, Stanmore, Middlesex, UK) included Zn, in the range 0–100 µg l^{-1}. Zn concentration was expressed as µg g^{-1}DW.

Total Zn accumulation (TZnA) was calculated by multiplying the total Zn concentration (TZnC) by the DW of total edible leaves (Sorgonà et al. 2006), the results being expressed as mg Zn in edible leaves.

Zn Utilization Efficiency (ZnUtE) was calculated as the quotient between edible leaf DW and TZnC (Siddiqi and Glass 1981). The results were expressed in g^2 DW mg^{-1} Zn.

$$ZnUtE = \text{Total edible leaf DW} / TZnC$$

5.1.2.4. Total antioxidant capacity

The total antioxidant capacity was measured using, ferric reducing ability of plasma (FRAP), the trolox equivalent antioxidant capacity (TEAC), the test of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging effect and N, N-dimethyl-p-phenylene diamine dihydrochloride (DMPD) method. For all assays, 0.1 g of fresh material was homogenized in a cold mortar with addition of 1 ml of metannol. The FRAP assay was made with FRAP reagent, i.e. 1 mM 2, 4, 6-tripyridyl-2-triazine (TPTZ) and 20 mM $FeCl_3$ in 0.25M CH_3COONa, pH 3.6. An aliquot of 100 µl of extract was added to 2 ml of FRAP reagent and mixed thoroughly. After the mixture was left at room temperature for 5 min, absorbance at 593 nm was measured. Calibration was against a standard curve (25–1600 mM Fe_3^+) using freshly prepared ammonium ferrous sulphate (Benzie and Strain 1996). The result of FRAP was expressed as mg g^{-1} FW. The TEAC was determined as described by Re et al. (1999) using 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonate) solution (ABTS) and 2, 20-azo-bis (2-methylpropionamidine) dihydrochloride, for the production of the ABTS radical ($ABTS^-$). The TEAC value of an extract represents the concentration of a Trolox solution that has the same antioxidant capacity as the extract. The result of TEAC was expressed as mg g^{-1} FW. In the DPPH assay, antioxidants reduce the free radical 2,2-diphenyl-1-picrylhydrazyl, which has an absorption maximum at A_{515}. Test for DPPH free-radical scavenging effect was performed according to Hsu et al. (2003). The absorbance of the reaction mixture at A_{517} was read with a spectrophotometer. Methanol (0.5 ml), replacing the extract, was used as the blank. The percentage of free-radical scavenging effect was calculated as follows: scavenging effect (% g^{-1}) = [1 − (A_{517} sample/A_{517} blank)] × 100 (Hsu et al. 2003). DMPD assay following the method described by Fogliano et al. (1999). 100 mM DTNB was prepared by dissolving 209 mg of DMPD in 10 ml of

deionized water; 1 ml of this solution was added to 100 ml of 0.1 M acetate buffer, pH 5.25, and the colored radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 ml of a solution of 0.05 M ferric chloride (final concentration 0.1 mM). At 5 µl of aliquot was added 800 µl of reagent mentioned above and its absorbance at 505 nm was measured.

5.1.2.5. TPC, O-dPh, PPGs, flavonoids and anthocyanins

The total phenolic compounds (TPC) concentrations of the plant material was extracted with methanol and was assayed quantitatively by A_{765} with Folin-Ciocalteau reagent (Rivero et al. 2001). The results were expressed as µg of caffeic acid g^{-1} FW. The ortho-diphenols (O-dPh) were determined by absorbance at 360 nm (Johnson and Schaal 1957; Giertych et al. 1999). The phenylpropanoid glycosides (PPGs) were determined according to a colorimetric method based on an *o*-dihydroxycinnamic derivate estimation, by the Arnow reaction. One ml of each sample was added to 2 ml of aqueous 0.5 M HCl, 2 ml of a 10% aqueous solution of NO_3Na and a 10% aqueous solution of $Na_2MoO_4 + 2H_2O$ (Arnow reagent), plus 2 ml of a aqueous 2 M NaOH. The solution was adjusted to 10 ml with water. After 10 min, the absorption was measured at A_{525} (Gálvez et al. 2005).

The total flavonoid concentration was measured by a colorimetric method (Kim et al. 2003) with minor modifications. Aliquots (0.5 ml) of appropriately diluted or standard solutions were pipetted into 15 ml polypropylene conical tubes containing 2 ml of double-distilled H_2O and mixed with 0.15 ml of 5% $NaNO_2$. After 5 min, 0.15 ml of 10% $AlCl_3 \cdot 6H_2O$ solution was added; the mixture was allowed to stand for another 5 min, and then 1 ml of the 1 M NaOH was added. The reaction solution was well mixed and kept for 15 min, and the absorbance was determined at A_{415}. The total flavonoid concentration was calculated using the standard rutin curve and expressed as

µg of rutin g^{-1} FW. Anthocyanins were determined according to Lange et al. (1971) with some modifications. The edible leaves were homogenized in propanol:HCl:H_2O (18:1:81) and further extracted in boiling water for 3 min. After centrifugation at 5,000 g for 40 min at 4ºC, the absorbance of the supernatant was measured at 535 and 650 nm. The absorbance due to anthocyanins was calculated as A= A_{535}-A_{650}. Finally, for the extraction of total carotenoids, 0.2 g of leaves were ground in semidarkness and resuspended in 1 ml of cold acetone at 80%. Immediately afterwards, the samples were centrifuged at 3,000 g and the absorbance of the supernatant was measured at 470 nm. The concentrations of total carotenoids were calculated following Wellburn (1994).

5.1.2.6. Antioxidant compounds

The determination of reduced AsA in leaf extracts was following the method of Law et al. (1983), modified from Okamura (1980). This method is based on the reduction of Fe_3^+ to Fe^{2+} by AsA in acid solution. Leaves material were homogenized in liquid N_2 with metaphosphoric acid at 5% (w/v) and centrifuged at 4◦C for 15 min. Absorbance was measured at A_{525} nm against a standard AsA curve that followed the same procedure as above. The result of reduced AsA were expressed as µg g^{-1} FW. Reduced glutathione (GSH) concentration was estimated following the method of Anderson (1985). Fresh leaves (0.2 g) were homogenized in 2 ml of 5 % (w : v) sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000 g for 10 min. To 0.5 ml of supernatant, 0.6 ml of 100 mM (pH 7.0) phosphate buffer and 40 µl of DTNB were added. After 2 min, the absorbance was read at 412 nm on spectrophotometer. A standard curve was prepared from varying concentrations of reduced GSH. GABA concentration was estimated by the method of Zhang and Bown (1997). Frozen leaves (0.2 g) were ground in liquid nitrogen to a fine powder, and 0.2 g of the ground tissue

was extracted in 0.1 ml methanol for 10 min at room temperature. The mixture was vacuum dried with a Vacufuge concentrator 5301 (Eppendorf, Hamburg, Germany). The dried sample was dissolved in 1 ml 70 mM lanthanum chloride, shaken for 15 min, and then centrifuged at 13,000 g for 5 min. The clear supernatant was transferred to a new tube, and 0.8 ml supernatant was mixed with 0.16 ml 1 M potassium hydroxide. The mixture was shaken for 5 min, and centrifuged at 13,000 g for 5 min. The supernatant was used for GABA determination. GABA was measured using GABase (Sigma, St. Louis, MO, USA). 1 ml assay mixture contained 0.6 mM $NADP^+$, 0.1 unit GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate and 550 µl sample. The reduction of $NADP^+$ to NADPH was monitored in a spectrophotometer at 340 nm after 10 min incubation at room temperature.

5.1.2.7. Nutrient concentration

The calcium (Ca), magnesium (Mg), iron (Fe), and manganese (Mn), were determinated as Zn concentration described above.

5.1.2.8. Statistical analysis

The data were analyzed by a simple variance analysis (ANOVA) and differences between the means were compared by Fisher's least-significant difference test (LSD). In addition, to ascertain whether the different plants and Zn-doses used in the experiment significantly influenced the results, a two-way ANOVA was used and the means were compared by Fisher's LSD test.

5.1.3. Results

5.1.3.1. Effect of Zn application on leaf biomass and lipid peroxidation

After 21 days in the presence of all supraoptimal Zn concentrations, the biomass of edible leaves was not affected in terms of DW in either plant (Fig. 1A). On the other hand, in *L. sativa* plants the MDA concentration increased only in the 100 µM treatment (Fig. 1B, $p < 0.01$), while in *B. oleracea* this peroxidation indicator increased from 20-µM doses and was more pronounced in the 100-µM treatment (Fig. 1B, $p < 0.001$). Fig. 1B shows that regardless of the treatments, *B. oleracea* accumulated a greater MDA concentration than did *L. sativa* for all treatments with respect to control plants (10 µM Zn).

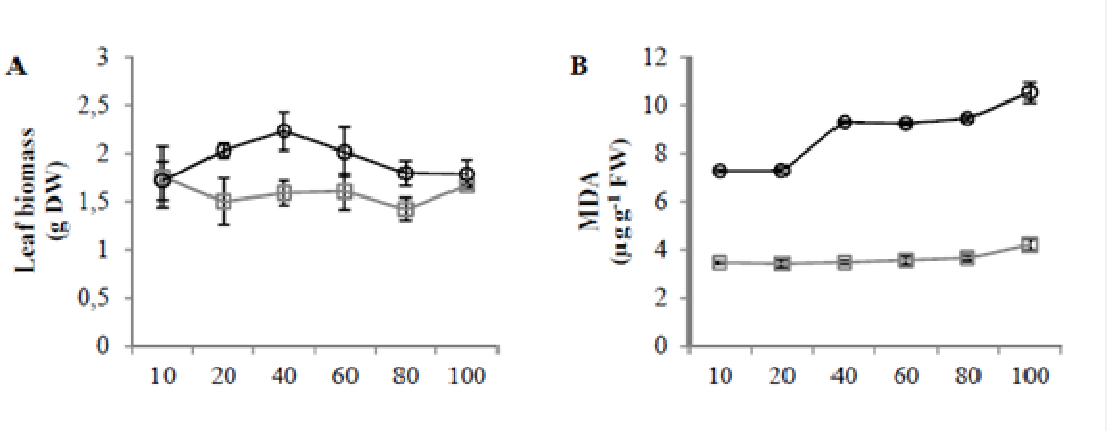

Fig 1. Effecct of Zn supplementation on leaf biomass (dry weight (DW)), and malondyaldehide (MDA) concentration, in edible part of *L. sativa* and *B. oleracea* plants. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

5.1.3.2. Zn concentration and Zn use efficiency

Zn concentration, TZnA and ZnUtE increased for all treatments in both plant species with respect to control, being more pronounced with the dose of 100 µM Zn with respect to the respective control plants (Fig. 2A, $p < 0.001$, Fig. 2B, $p < 0.001$, Fig. 2C,

$p < 0.001$). Regardless of Zn treatments applied, *B. oleracea* registered a higher Zn concentration, TZnA and ZnUtE (Fig. 2).

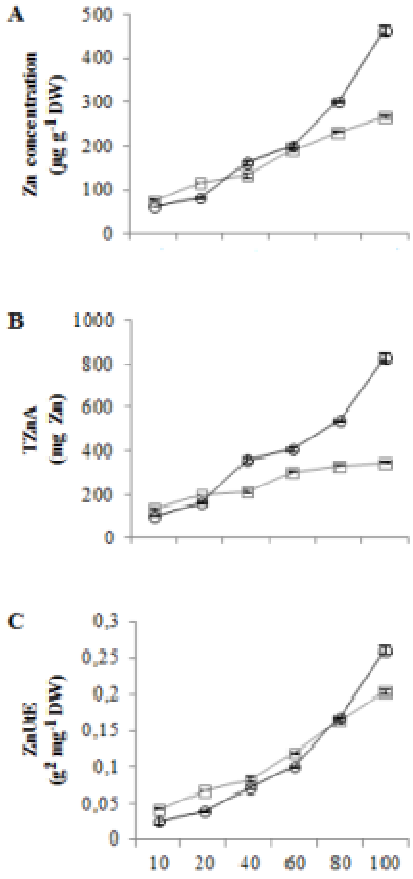

Fig 2. Zn concentration and Zn use efficiency in edible part of *L. sativa* (□) and *B. oleraccea* (○). Zn concentration, Zn accumulation (TZnA) and Zn utilization efficiency (ZnUtE). Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

5.1.3.3. Total antioxidant capacity

The total antioxidant capacity showed a similar pattern in both species, with lower FRAP values for all treatments compared to their respective controls (Table 1). In our study, the TEAC levels differed in both plants (Table 1). That is, this parameter significantly declined in *L. sativa* plants for all treatments with respect to control (Table 1), while in *B. oleracea* the TEAC test was not negatively affected compared with control (Table 1). The DPPH test indicated a marked decline for all treatments and in both plants with respect to control (Table 1). Finally, the DMPD test showed a similar

pattern in the two horticultural plants (Table 1). Exceptionally, we observed an increase in this parameter in *L. sativa* plants grown with the dose of 20 µM (Table 1). Nevertheless, the response of both plants with respect to the different specific methods differed significantly. That is, regardless of the Zn concentration in the nutrient solution, higher values were found in the TEAC and DMPD tests in *L. sativa* while in *B. oleracea* the maximum values were registered in the FRAP and DPPH tests (Table 1).

Table 1. Response of different antioxidants test in edible part of *L. sativa* and *B. oleracea* plants submitted to different applications of Zn: ferric reducing ability of plasma (FRAP), the Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging effect and N, N-dimethyl-p-phenylene diamine dihydrochloride (DMPD).

	FRAP (mg g^{-1} FW)	TEAC (mg g^{-1} FW)	DPPH (% g^{-1})	DMPD (Trolox equivalent antioxidant)
Lactuca sativa **cv. Phillipus**				
10 µM Zn	1.40 ± 0.02a	6.57 ± 0.02a	248.44 ± 6.25a	39.70 ± 0.57b
20 µM Zn	1.34 ± 0.02a	6.36 ± 0.03c	226.97 ± 7.82b	41.75 ± 0.56a
40 µM Zn	1.33 ± 0.10ab	6.38 ± 0.01c	202.04 ± 3.89c	39.87 ± 0.40ab
60 µM Zn	1.22 ± 0.00b	6.38 ± 0.01c	198.50 ± 5.22c	31.25 ± 1.16d
80 µM Zn	0.64 ± 0.00c	6.38 ± 0.01c	181.76 ± 5.61d	33.13 ± 0.57cd
100 µM Zn	0.59 ± 0.02c	6.47 ± 0.00b	180.86 ± 4.23d	33.30 ± 0.61c
p-Value	***	***	***	***
$LSD_{0.05}$	0.12	0.05	16.14	1.97
Brassica oleracea **cv. Bronco**				
10 µM Zn	1.63 ± 0.01a	6.33 ± 0.01ab	270.82 ± 2.79a	26.10 ± 1.17ab
20 µM Zn	1.45 ± 0.02b	6.34 ± 0.02a	244.54 ± 2.37b	22.59 ± 0.22cd
40 µM Zn	1.23 ± 0.06c	6.31 ± 0.01ab	239.89 ± 3.92b	24.98 ± 1.06bc
60 µM Zn	1.13 ± 0.01d	6.30 ± 0.01b	243.64 ± 5.92b	27.81 ± 1.19a
80 µM Zn	1.20 ± 0.01cd	6.30 ± 0.02b	240.71 ± 11.49b	22.39 ± 0.81d
100 µM Zn	1.04 ± 0.02e	6.29 ± 0.00b	232.44 ± 1.78b	19.44 ± 0.49e
p-Value	***	NS	***	***
$LSD_{0.05}$	0.08	0.04	15.38	2.58
Analysis of variance				
Plants (*P*)	***	***	***	***
Doses (*D*)	***	***	***	***
P x *D*	***	***	**	***

Significance levels are represented by $p > 0.05$. ns, not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Means followed by the same letter do not significantly differ.

5.1.3.4. TPC, O-dPh, PPGs, flavonoids, and anthocyanins

Fig. 3A shows that, in *L. sativa,* TPC significantly increased in the treatments of 20 and 40 µM with respect to the control ($p < 0.001$), while in *B. oleracea* only plants treated with 60 µM respect to 10 µM showed such an increase ($p < 0.001$). Furthermore, both plants declined in this compound in the 100-µM treatment with respect to control (Fig. 3A, $p < 0.001$). Similarly, O-dPh and PPGs concentration diminished, reaching minimum values at higher doses of Zn in both plants with regard to their respective controls (Fig. 3B, $p < 0.00$ and Fig. 3C, $p < 0.001$).

With regard to flavonoid concentration, in *L. sativa* an increase resulted with the dose of 20 µM, while this compound declined significantly after the treatment of 80 µM with respect to 10 µM (Fig. 3D, $p < 0.001$). In *B. oleracea* the flavonoid concentration of flavonoids fell with respect to control when the treatment applied was 20 and 100 µM (Fig. 3D, $p < 0.001$). Additionally, in both plants the minimum values resulted with the treatment of 100 µM (Fig. 3D). As for anthocyanins, while in *L. sativa* the anthocyanin concentration decreased after a dose of 40 µM (Fig. 3E, $p < 0.001$), *B. oleracea* registered an increase in this compound after the treatments of 20, 80, and 100 µM with respect to control plants (Fig. 3E, $p < 0.001$). The concentration of total carotenoids was significantly affected in both species (Fig. 3F, $p < 0.001$). Nevertheless, in *L. sativa*, increased Zn supplementation triggered a gradual decline in the carotenoids concentration, except for 40 µM, with respect to control (Fig. 3F, $p < 0.001$) whereas in *B. oleracea* the carotenoid concentration decreased for all treatments, reaching the minimum with the dose of 60 µM with respect to 10 µM Zn (Fig. 3F, $p < 0.001$).

In general, regardless of all the Zn doses provided, it should be noted that *B. oleracea* presented a higher concentration of all the compounds (TPC, O-dPh, PPGs, flavonoids, and anthocyanins) than did *L. sativa* (Fig. 3).

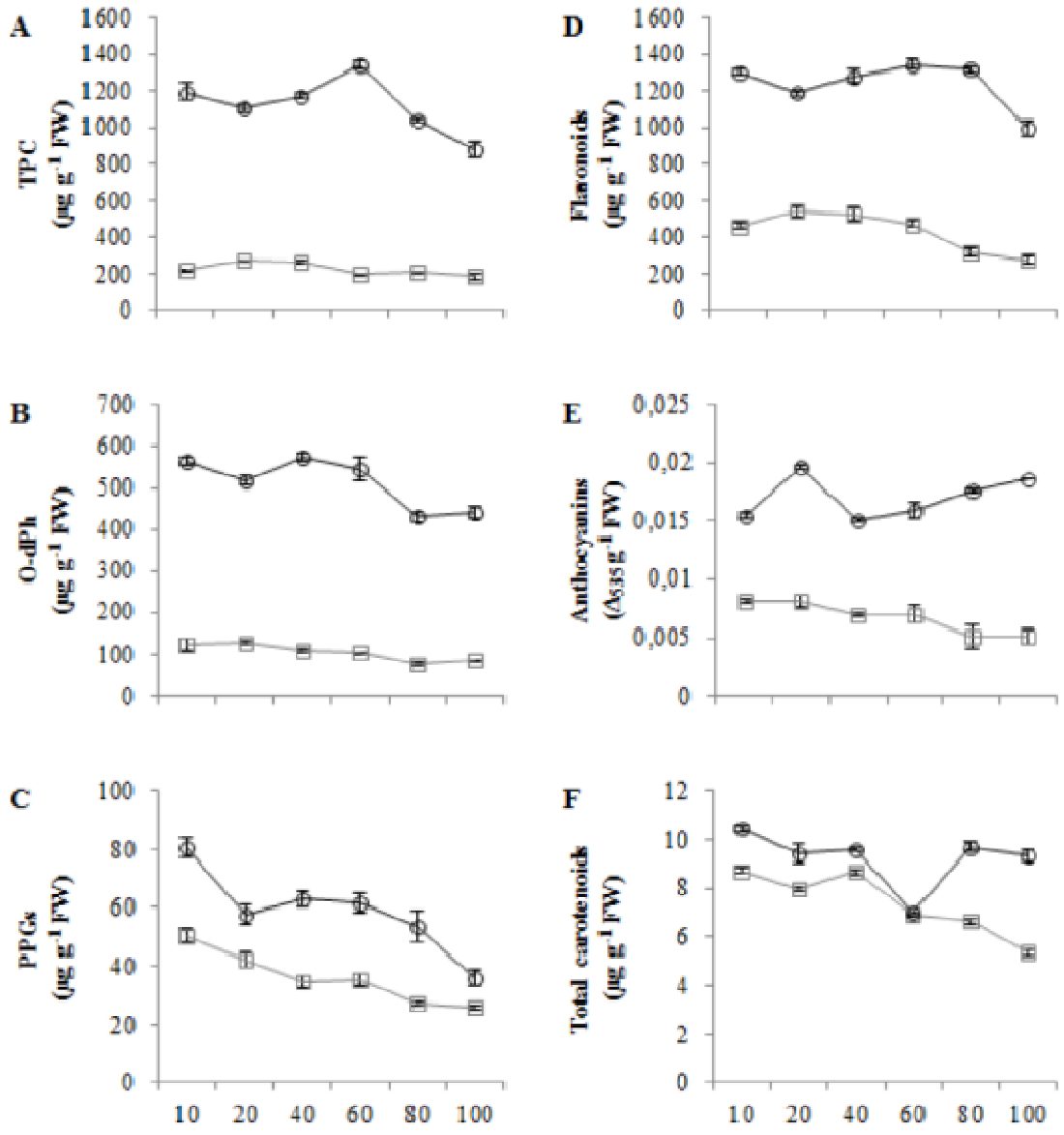

Fig 3. Effect of Zn application on phenolic compounds and pigments in edible part of *L. sativa* (□) and *B. oleraccea* (○). Total phenolic compunds (TNC), orthodiphenols (OdPh) phenylpropanoid glycosides (PPGs) and pigments concentration: flavonoids, anthocyanins and total carotenoids. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

5.1.3.5. Antioxidant compounds

In our study, reduced AsA decreased for all Zn applications in *L. sativa* compared to control plants (10 µM Zn) (Fig. 4A, $p < 0.001$). Instead, the AsA concentration was lowered only by the 80-µM treatment, but increased significantly in the treatments of 20 and 100 µM in *B. oleracea* (Fig. 4A, $p < 0.001$). Reduced GSH concentration was

adversely affected from the dose of 40 µM in *L. sativa* (Fig. 4B, $p < 0.001$), while in *B. oleracea* the concentration of this antioxidant compound significantly increased for all treatments compared to the control plants (Fig. 4B, $p < 0.001$). Finally, in *L. sativa* plants, GABA increased after the treatment of 80 µM (Fig. 4C, $p < 0.001$). Nevertheless, in *B. oleracea* plants this compound decreased, with the lowest value recorded at the dose of 100 µM compared to control dose (Fig. 4C, $p < 0.001$). In short, regardless of the Zn doses used in this study, *B. oleracea* showed a higher concentration of antioxidant compounds than did *L. sativa* (Fig 4).

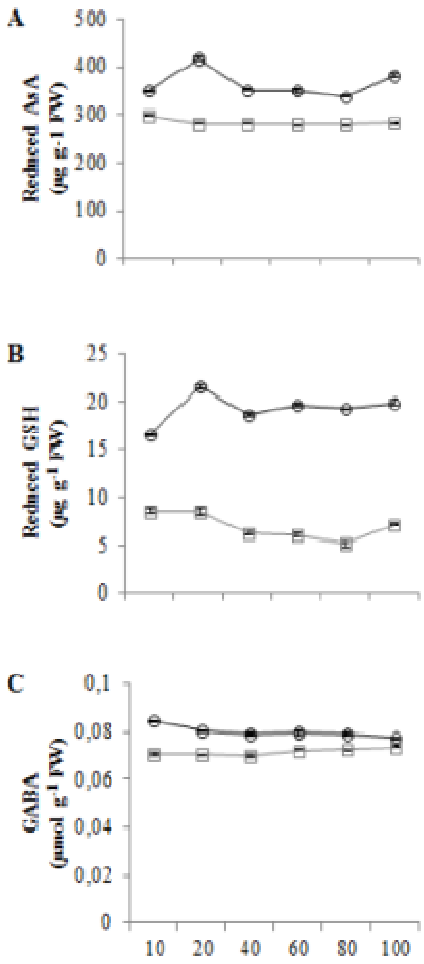

Fig 4. Antioxidant concentration under different Zn concentration in edible part of *L. sativa* (□) and *B. oleraccea* (○). Reduced ascorbate (AsA), reduced glutation (GSH) and γ aminobutyric acid (GABA). Values are means (n = 9) and differences between means were compared using Fisher's test least

5.1.3.6. Nutrient concentration

In general, differences were found in both plants regarding the interaction of Zn with macronutrient and micronutrient concentrations in the different treatments with respect to the control plants (Table 2).

Table 2. Effect of Zn supplementation on nutrient concentration in edible part of *L. sativa* and *B. oleracea* plants. Calcium (Ca), magnesium (Mg), iron (Fe) and manganese (Mn).

	Ca (mg g^{-1} DW)	Mg (mg g^{-1} DW)	Fe (µg g^{-1} DW)	Mn (µg g^{-1} DW)
Lactuca sativa cv. Phillipus				
10 µM Zn	7.61 ± 0.19c	4.84 ± 0.13b	91.45 ± 3.50b	53.55 ± 0.75d
20 µM Zn	8.62± 0.06a	5.41 ± 0.07a	118.27 ± 5.19a	64.11 ± 0.59a
40 µM Zn	7.16 ± 0.03d	4.69 ± 0.02b	76.45 ± 1.19c	50.38 ± 0.56e
60 µM Zn	8.23 ± 0.11b	4.88 ± 0.01b	76.85 ± 4.81c	56.48 ± 0.41c
80 µM Zn	7.99 ± 0.05b	4.40 ± 0.04c	72.74 ± 0.74c	54.06 ± 0.33d
100 µM Zn	8.54 ± 0.08a	5.38 ± 0.07a	112.03 ± 6.86a	61.59 ± 0.59b
p-Value	***	***	***	***
$LSD_{0.05}$	0.31	0.22	13.26	1.71
Brassica oleracea cv. Bronco				
10 µM Zn	32.53 ± 0.33a	10.39 ± 0.11bc	56.42 ± 15.03b	94.97 ± 0.73a
20 µM Zn	29.27 ± 0.43b	11.81 ± 135.40a	67.21 ± 1.92b	86.21 ± 1.07b
40 µM Zn	25.87 ± 0.91cd	10.32 ± 0.41bc	67.48 ± 3.19ab	77.03 ± 2.80c
60 µM Zn	24.63 ± 0.44d	10.13 ± 0.17c	61.56 ± 4.60b	78.31 ± 1.50c
80 µM Zn	27.72 ± 1.22bc	10.94 ± 0.37b	93.48 ± 12.76a	77.46 ± 2.15c
100 µM Zn	27.75 ± 0.62bc	9.92 ± 0.20c	56.22 ± 3.11b	80.77 ± 1.61bc
p-Value	***	**	NS	***
$LSD_{0.05}$	2.25	0.80	26.18	5.48
Analysis of variance				
Plants (*P*)	***	***	***	***
Doses (*D*)	***	***	**	***
P x *D*	***	***	***	***

Significance levels are represented by p > 0.05. ns, not significant. *p < 0.05, **p < 0.01, ***p < 0.001. Means followed by the same letter do not significantly differ.

In both plants, Ca and Mn showed a similar pattern with regard to their respective controls (Table 2). That is, in *L. sativa* both nutrients increased when the Zn dose applied was 20 µM, whereas this concentration exceptionally decreased with 40 µM with respect to the Zn basal dose (10 µM Zn) (Table 2). However, in *B. oleracea* both

Ca and Mn concentrations dipped significantly, reaching the minimum values in the treatment of 60 and 40 µM, respectively, compared to the treatment of 10 µM (Table 2). On the other hand, in *L. sativa* the Mg and Fe concentrations showed a marked increase in treatments of 20 and 100 µM but when the dose applied was 80 µM the negative effect was stronger than in control plants (Table 2). However, *B. oleracea* showed a marked increase in the Mg concentration with 20 µM, while Fe concentration was not affected by all doses respect to the basal dose (Table 2). Finally, regardless of the treatment of Zn, *B. oleracea* showed higher concentrations for all nutrients studied, except in the Fe concentration (Table 2).

5.1.4. Discussion

One of the limiting factors in the biofortification programs with trace elements, based on the application of increasing doses of the element, is to avoid reaching phytotoxicity. In the specific case of Zn, this trace element may accumulate in excess in plant tissues, interfering with important metabolic processes, such as photosynthesis, and by generating oxidative stress (Barrameda Medina et al. 2014). In this situation, the general symptoms of Zn stress include a decrease in chlorophyll content and even leaf chlorosis (Sagardoy et al. 2009), which contributes to the growth inhibition and weakens membrane integrity. Consequently, under Zn toxicity, some of the most reliable and widely used indicators of stress are plant biomass and lipid peroxidation, expressed as MDA concentration (Barrameda-Medina et al. 2014). In our study, this was demonstrated as increased Zn in the nutrient solution but did not involve a growth alteration in the two target species (Fig. 1A). Similar to our results, some studies have demonstrated that under a biofortification programs with different elements, the biomass of this organ was maintenance (in terms of DW) in lettuce and cabagge plants

(White and Broadley 2005; Müller, 2013; Mao et al., 2014; D´Imperio et al., 2015; Lawson et al., 2015). However, the MDA concentration rose, reaching maximum levels of up to 21% in *L. sativa* and 44% in *B. oleracea* when the dose applied was 100 µM (Fig. 1B). In this sense, a greater MDA concentration is indicative of plant stress. Nevertheless, in this study, a higher MDA concentration was not correlated with a concomitant lower production of the edible part in either plants (Fig. 1A and Fig. 1B), probably since the levels of this compound was not high enough to cause serious damage in both plants.

Improved soil fertility and the efficient use of mineral nutrients are of high ecological and economic importance (Cakmak 2002). Particularly, in edible crops, greater micronutrient density (or biofortification), including Zn, will greatly improve human nutrition on a global scale (Waters and Sankaran 2011; White and Broadley 2011). In this sense, biofortification strategies include the application of Zn fertilizers and the development of crop genotypes that acquire more Zn from the soil and accumulate it in edible portions (White and Broadley 2011). Based on our results, an application of Zn in the range of 10-100 µM proved sufficient to promote the normal development of both plants (Fig. 1A) with the concomitant increase in the Zn concentration in the edible part of *L. sativa* and *B. oleracea* (Fig. 2A). However, while *L. sativa* registered a maximum value for 100-µM treatment, representing up to 251% with respect to the control plants (Fig. 2A), in *B. oleracea* this increase was 687% for the same dose with respect to the basal dose (Fig. 2A). Similar to our results, White and Broadley (2011) have announced that the Zn concentration in leaves, stems, and root can be increased through the application of Zn-fertilizers. Furthermore, the leaf Zn concentration in a range of 100-700 µg g^{-1} DW (and perhaps higher) can be achieved without yield losses in Zn-tolerant crops when Zn fertilizers are applied to the soil (Fageria, 2009). In the present

experiment, by increasing the Zn dose of applied to the solution, both plants showed an increase in the concentration of this element in the edible part and the maximum Zn concentrations differed greatly in both plants (Fig. 2A). That is, while in while in *L. sativa* was reached accumulate a Zn concentration in the range of 75-265 µg g^{-1} DW (Fig. 2A), in *B. oleracea* this range was 59-465 µg g^{-1} DW (Fig. 1A) in comparison to control (Fig. 2A). As mentioned above, in the two plants the edible biomass was not negatively affected in any of the treatments (Fig. 1A), defining all the doses used in this study as optimal and supra-optimal (< 700 µg g^{-1} DW).

In terms of TZnA, the results were similar in Zn concentration, again with higher values in *B. oleracea*, since the increase in this nutrient accumulation reached 162% in *L. sativa* vs. 718% in *B. oleracea* in 100 µM in comparison to their respective controls (Fig. 2B). Similar to our results, numerous findings in biofortifications programs have shown that the concentration of the elements applied, included Zn, varies greatly among different edible crops (White and Broadley 2011; Müller 2013; Weng et al. 2013). According to our results, studies with a I-biofortification programs, have demonstrated as an increase on this element concomitant produce a higher concentration of I in both plants but the higher values were obtained in the edible portions of Brassica, defining this cultivar as more effective in this type of programs (Hong et al. 2008; Lawson et al. 2015). Also, the variation in TZnA in both species has been attributed to differences in the uptake ability of the roots. This data confirm the results obtained in other studies where the choice of appropriate species is important. That is, D´Imperio et al. (2015) demostrated as the better selection between some vegetables was the genus of Brassica, showing an increase of Silicon (Si) concentration and accumulation higher than the other species objetive of this study.

In this respect, Zn efficiency is the ability of plants to maintain growth and yield. In this sense, Rengel and Graham (1995) announced that the ZnUtE should be considered a potential Zn-efficiency mechanism. In our work, despite the observed increase of ZnUtE in both plants (Fig. 2C), in *L. sativa* this was 371% while that in *B. oleracea* reached 889% with the Zn dose of 100 µM compared to control plants (Fig. 2C). Our results are consistent with results from previous studies in which species such as cabagge, lettuce and spinach were shown to be the most efficient crop in a biofortification programs with several elements (Müller 2013). Moreover, Müller (2013) demonstrated as an increase of the nutrient concentration in the growth conditions promoted an increase of them in the leafy vegetables.

Several studies have been conducted on Zn biofortification and several transgenic crops have been created to have higher Zn concentrations in their edible tissues with respect to conventional varieties. These include the root of *Manihot esculenta* with about 40 µg g^{-1} DW (Sayre et al. 2011), *Oryza sativa* subsp. *indica* and *Oryza sativa* cv. Nipponbare with 56–95 µg g^{-1} DW (Vasconcelos et al. 2003; Johnson et al. 2011), and *Hordeum vulgare* with 85 µg g^{-1} DW (Ramesh et al. 2004). However, these Zn concentrations in no case exceeded those found in the present study, probably due to increased transport via xylem of this element in the species under study. Based on these results, the consumption of leafy vegetables could improve Zn deficiency in humans, it should also be noted that between our two plants, *B. oleracea* is emerging as a better candidate for biofortification programs with Zn, given the higher Zn concentration and TZnA, maintaining the biomass of the edible part. The similar results was obtained by Mao et al. (2014) in a Zn-biofortification program with *Brassica rapa*, but the concentration achieved of this element (35 mg kg^{-1} DW) was significantly lower than than those found in this experiment. In this sense, a study into wide species variation

regarding minerals in staple crops including rice, wheat, maize, beans, cassava and *B. oleracea* was reviewed by White and Broadley (2005), demonstrating that the Zn concentration in *B. oleracea* was higher than in any other crop except for spinach. In the present study, we confirm that *B. oleracea* has enormous natural potential to accumulate Zn in its edible part compared with cereals, legumes and others brassica species.

Epidemiological studies suggest the importance of high consumption of secondary plant products distributed in vegetables and fruits to prevent or ameliorate some common chronic diseases (Kinsella et al. 1993). These substances possess a high antioxidative potential and are counterparts to oxidative stress (Schlesier et al. 2002). In this regard, various methods have been described in the literature for measuring the total antioxidant capacity: FRAP, TEAC, DPPH, and DMPD, among others (Sharma et al. 2008). In the present experiment, the data reveal significant differences between the different Zn applications in comparison to the respective controls of both plants for the majority of the tests evaluated (Table 1).

In general, every test used reflected decreases in both plants, with the exception of the TEAC test in *B. oleracea* (Table 1). However, regardless of the treatments applied, *L. sativa* showed higher values only in the test DMPD, with higher values in this parameter when the dose applied was 20 µM with respect to control (Table 1), while *B. oleracea* showed a greater total antioxidant capacity with respect to the FRAP and DPPH tests (Table 1). These results suggest, that the FRAP, DPPH, and especially TEAC tests, could be strongly related to the total antioxidant activity in *B. oleracea* with the consequent increase in the Zn concentration without affecting the biomass of the edible part, while in *L. sativa* higher DMPD values could indicate that under 20 µM of Zn in this plant could promote greater concentrations of antioxidant compounds

(Table 1). In addition, based on our results, a dose greater than 100 µM could reduce the total antioxidant capacity in both plants (Table 1).

Interest in the role of free-radical-scavenging antioxidants in human health has prompted research in the fields of horticulture and food science to assess the antioxidant phytochemicals in vegetables and fruits (Singh et al. 2006). Moreover, there is growing evidence indicating the additive and synergetic effects of antioxidative compounds from vegetables and fruits on human health as they can diminish the risk of many pathologies related to oxidative stress (Blasco et al. 2008). Among phytochemicals, phenolic compounds, an extensive group with a wide range of chemical structures, are the most important ones (Blasco et al. 2008). Phenolic compounds possess many useful properties for human health, and the most important action of phenolics is their antioxidant activity, preventing enzymatic and non-enzymatic formation of ROS (Cartea et al. 2011). The results of our work show significant differences in TPC concentration for different Zn treatments and plants. That is, while in *L. sativa* an increase of this antioxidant compound was promoted when the doses applied were 20-40 µM respect to control plants (Fig. 3A), in *B. oleracea* the highest concentration of TPC was found with the 60-µM treatment with respect to the basal dose (Fig. 3A). However, in both plants the O-dPh and PPG concentration diminished in comparison to their respective control plants, this decrease being more pronounced in both plants when the treatments applied were 80 µM and 100 µM, respectively, with respect to 10 µM (Fig. 3B and Fig. 3C).

Another group of antioxidant compounds of great importance includes the flavonoids, which exhibit many biological functions such as anti-allergen, antitumour, antiviral, and antioxidant activities. Although they are usually found in low quantities in fruits and vegetables, they can have high rates of antioxidant activity (Shahidi et al. 1992). In this

case, the edible parts of both plants again showed significant differences in flavonoid concentration with respect to control (Fig. 3D), indicating that only an application of 20 µM increased the flavonoid concentration in *L. sativa* compared to basal dose (Fig. 3D) while, in *B. oleracea*, treatment in a range of 40-80 µM was sufficient to maintain high values of these antioxidant compounds (Fig. 3D). On the other hand, anthocyanins constitute one of the largest and most important groups of water-soluble pigments in most species in the plant kingdom (Nie et al. 2013). They are stress responsive compounds, but they are also important phytonutrients in a healthy diet (De Pascual-Teresa et al., 2010). The results of our experiment showed significant differences in both plant grown with different Zn applications (Fig. 3E). In general, while *L. sativa* showed a decline in these compounds when the doses applied exceeded 40 µM respect to 10 µM (Fig. 3E), in *B. oleracea* this pigment concentration increased for all doses with respect to control (Fig. 3E). Finally, carotenoids are naturally occurring lipophilic pigments found in plant-based foods. Carotenoids provide colour and a source of vitamin A, and are a component of biological metabolic processes (Thavarajah and Thavarajah 2012). In general, in *L. sativa* and *B. oleracea* the concentration of carotenoids declined when the dose of Zn was increased in the culture medium compared to their respective controls (Fig. 3F). Finally, the minimum values found in the most of the compounds studied were registered with the treatment of 100 µM in both species (Fig. 3).

AsA is an especially important dietary antioxidant, significantly diminishing the adverse effect of ROS and reactive nitrogen species (RNS) that can cause oxidative damage to macromolecules involved in chronic diseases (Halliwell and Gutteridge 1999). Also, reduced GSH is a crucial compound for the effective scavenging of ROS and for the maintenance of other antioxidants such as AsA and tocopherol (Yadav et al.

2005). In our study, the concentration of antioxidant compounds were affected conversely in both plants after the application of different Zn doses. In general, in *L. sativa* an increase in Zn dose greater than 20 µM lowered the concentration of reduced AsA and GSH with respect to control (Fig. 4A and Fig. 4B), while in *B. oleracea* an increase in the Zn dose was positively correlated with an increase in both antioxidant compounds, reaching maximum values with the dose of 20 µM (Fig. 4A and Fig. 4B).

Moreover, in plants, GABA could play numerous roles, i.e. osmoregulation, protection against oxidative stress, signal molecule, etc. (Bouché and Fromm 2004). In terms of the GABA concentration a differential response was again found in the two leafy vegetables (Fig. 4C). In *L. sativa*, there was an increase for doses 80 µM and 100 µM with respect to control (Fig. 4C), while in *B. oleracea* the GABA concentration declined as the Zn dose was increased Zn in the middle and the lowest value was registered with the treatment of 100 µM with respect to control plant (Fig. 4C). Some studies have reported that GABA levels in plant tissues are typically low, ranging from 0.03 to 2.00 µmol g^{-1} FW (Shelp et al. 1999), but the maximum level was found in plants subjected to different types of stress. In our plants, we found values of 0.07-0.085 µmol g^{-1} FW (Fig. 4C), indicating that none of our doses of Zn were phytotoxic in either plant. However, in *L. sativa* values were in all cases below those found in *B. oleracea* (Fig. 4C). In fact, GABA has been identified only in beans, spinach, potatoes, kale (Oh et al. 2003) and recently, in cabbage (Park et al. 2014).

In summary, regardless of the treatments, *B. oleracea* showed much higher values than did *L. sativa* in the concentration of TPC, O-dPh, PPGs, flavonoids, and anthocyanins (Fig. 3). In addition, in *L. sativa* the highest values found in terms of the concentration of TPC and flavonoids could explain why in this species the DMPD test increased when the dose applied was of 20 µM Zn (Table 2). Likewise, in *B. oleracea*

the high values found in this study, especially in a range of 40-80 µM Zn, could be explain the extremely high values in the FRAP, the DPPH, and especially the TEAC test (Table 2). Our results indicate that these tests are good indicators of the total antioxidant capacity in horticultural plants. On the other hands, a sharp drecease of the capacity and the fenolic compounds, in particular with of 100 µM Zn, could be explain as a higher MDA concentration in both plants was higher when the doses of Zn applied was increased (Fig. 1B). However, an increase of GABA in *L. sativa* and AsA and GSH in *B. oleracea* with the higher doses applied might explain as despite increasing the MDA content, the normal growth of the plant was unaffected negatively.

As described above, both plants can increase the Zn concentration in the edible part without the biomass of this organ being affected. Therefore the optimal dose in terms of antioxidants in Zn-biofortification programs in *L. sativa* appears to be 20 µM, and although the Zn concentration under these conditions (116 µg Zn g^{-1} DW) were lower than those found at higher doses, these values were higher than those found in other Zn-biofortification studies (Vasconcelos et al. 2003; Ramesh et al. 2004; Johnson et al. 2011; Sayre et al. 2011; Mao et al. 2014). Moreover, an increase in Zn application in *B. oleracea* not only augmented the concentration of this element, but also resulted in high efficiency values of this micronutrient and maintained high levels of antioxidant compounds. Although the Zn concentration and efficiency was superior in the treatment 100 µM, the concentration reached in this treatment became 465 µg Zn g^{-1} DW, and these values can alter long-term metabolism of the plant, as above 300 µg Zn g^{-1} DW doses are considered toxic. Therefore, a Zn-biofortification program in range of 20-80 µM (80-300 µg Zn g^{-1} DW) with *B. oleracea* could provide normal crop growth and enhance plant quality, potentially benefitting humans.

Plant species greatly differ in terms of the nutrient concentrations in their edible parts (Müller 2013; D´Imperio et al. 2015), and very little attention has been paid to nutritional quality and Zn concentrations in edible plant parts (Cakmak 2009). In addition, White and Broadley (2009) demonstrated that the concentrations of some mineral elements are often greater in leafy vegetables than in seed, fruit or tuber crops. However, the supply of one nutrient can affect the uptake and utilization of other nutrients in crop plants (Müller 2013; D´Imperio et al. 2015). Among other mineral elements, Ca, Mg, Fe, and Mn are critical for human health. With respect to the effect of supraoptimal intake of Zn on Ca concentrations, the data for the foliar Ca concentration showed a synergistic effect for all Zn treatments in *L. sativa* (except for 40 µM), the maximum values appearing with the Zn dose of 20 µM (Table 2). However, values for this macronutrient fell for all treatments in *B. oleracea*, reaching the minimum of 60 µM with respect to control (Table 2). Despite the differential response found in both plants, values in the *L. sativa* were two-fold lower than in *B. oleracea* under the same growth conditions (Table 2). Like Ca, others of the most abundant group II elements both in plants and in animals is Mg (Broadley et al. 2008). In our study, the Mg concentration rose when the dose applied was 20 µM in both plants (Table 2), although the values found were double in *B. oleracea* compared to *L. sativa* for all treatments. Similar to our results, findings in a review by White and Broadley (2009) showed Brassicaceae to have higher Ca and Mg values than those found in others species. Like Zn, Fe deficiency is critical in the developing industrialized countries, resulting in severe impairments of human health and development (Cakmak 2002). In our study, the effect of supraoptimal Zn on the Fe concentration was positive when the dose applied was 20 µM in *L. sativa* (Table 2). In this case, the concentration of Fe achieved with Zn biofortification was higher in *L. sativa* than in *B. oleracea*, perhaps because in *B.*

oleracea the Zn concentrations were much higher and may interfere with the Fe concentration. This results are in corcondance with the results obtained by Sagardoy et al. (2009) whrere an increase of the Zn concentration in leaves of *Beta vulgaris* promote a decrease of the Fe concentration. Finally, Mn deficiency, though less prevalent than Fe and Zn deficiency, can also lead to serious health problems such as birth defects (Bashir et al. 2013). In the present work, the Mn concentration rose in *L. sativa* when the dose applied was 20 µM with respect to 10 µM (Table 2), while the concentration of this micronutrient was negatively affected in *B. oleracea* for all treatments with respect to the basal dose (Table 2).

The addition of trace elements can affect the uptake of several essential nutrients from the plant. However, the results obtained in various programs of horticultural plants biofortification with varied enormously. That is, Mao et al. (2014) showed as an application of Zn, I or Se, not positively affected the concentration of macro or micronutrients, even in the genus of Brassica. Furthermore, in a Si-biofortification authors demonstrated as addiction of this element it did not promote changes in the concentration of Ca or Mg (D´Imperio et al. 2015).

5.1.5. Conclusions

In this study, we show that horticultural plants increase in efficiency with Zn-biofortification programs and that in these two horticultural species it is not be necessary to apply excessive concentrations of Zn to maintain the normal growth of their crops. On the basis of our results the application of 20 µM Zn in *L. sativa* and 20-80 µM Zn in *B. oleracea* boosts the nutritional quality in these species. Moreover, *B. oleracea* registered higher values for Zn concentration, for efficiency parameters, such as TZnA, phenolic compounds (TPC, O-dPh, PPGs, flavonoids, and anthocyanins) for

antioxidants compounds (reduced AsA and GSH, and GABA), and for nutrient concentration (Ca, Mg, and Mn). Because of the above results, *B. oleracea* is emerging as a better choice and more efficient candidate in Zn-biofortification programs. The best approach to increase human Zn levels is to encourage greater consumption of both green leafy vegetables, specially *B. oleracea*.

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5.2. Zn-biofortification enhanced nitrogen metabolism and photorespiration process in a green leafy vegetable: Lactuca sativa L.

Abstract

Background and aims Excessive rates of N fertilizers may result in elevated concentrations of nitrate (NO_3^-) and high NO_3^- concentrations are accumulated in the edible parts of leafy vegetables, as lettuce.

Methods The main objective of this work was to determine whether the NO_3^- accumulation and the nitrogen use efficiency (NUE) was affected by the application of different dosages of Zn, to ascertain the influence of this trace element in a biofortification programme in *Lactuca sativa* cv. Phillipus plants. For that, we analyzed the effect of Zn on NO_3^- assimilation, photorespiration, and the final products of those processes.

Results Zn-doses in a range of 80-100 μM produced an increase on Zn concentration, provoking a decrease of NO_3^- since an increase of this element were sufficient to promote the nitrogen assimilation by an increase of the nitrate reductase (NR), glutamine synthetase (GS), aspartate aminotransferase (AAT), and the photorespiration processes. As result, we observed an increase on reduced N, total N concentration (TNC) and N utilization efficiency (NUtE). Consequently, under 80 μM-Zn the amino acid concentration increased significantly, include the concentration of amino acid essential for human health, ie; Leu, Ile and Thr.

Conclusions Adequate Zn fertilization is an important critical player in lettuce, making it possible to increase lettuce productivity and quality, especially with 80 μM Zn, and

could decrease the level of Zn deficiency such as the toxic level of NO_3^- in human health.

Keywords *Lactuca sativa* • Nitrate • Nitrogen use efficiency • Zn deficiency

5.2.1. Introduction

Micronutrient deficiencies such as, zinc (Zn), iron (Fe), iodine (I) and vitamin A deficiencies are critical issues in the developing industrialized countries, and result in severe impairments of human health and development (Cakmak 2002). Particularly, among micronutrients, Zn deficiency is occurring in both crops and humans (Hotz and Brown 2004; Welch and Graham 2004). In fact, more than 3 billion people worldwide suffer from micronutrient malnutrition, include Zn deficiency (Welch and Graham 2004; Cakmak 2008). In this situation, Zn deficiency can cause growth retardation, delayed skeletal and sexual maturity, dermatitis, diarrhoea, alopecia and defects in immune function with resulting increase in susceptibility to infection and maternal mortality in human (Welch 2002).

Recently, has been proposed a complimentary solution to mineral malnutrition and this complementary programme is known as biofortification (White and Broadley 2005). Although this strategy for biofortification is unproven as yet, it has the potential to become sustainable, cost effective and reach remote rural populations (Bouis et al. 2011). Concern to Zn, impact of it application on increasing crop yields has been recorded on most crops, both under irrigated and rainfed conditions (Das and Green 2013), and recent literature indicates that the combination of agronomic biofortification with breeding is an applicable and sustainable approach to the Zn-deficiency problem in humans (Cakmak 2008).

In addition to the nutritional quality of agricultural products destined for human consumption, another aspect to consider at present is the fact that the rapid population growth over recent decades has generated an increase in the demand for vegetable products. In this sense, it further estimates that agricultural production must increase by 70% by 2050 to feed over 9 billion people worldwide (Das and Green 2013) and since plants require N in large amounts, N frequently limits plant development and for this reason the application of N-fertilizers is a common practice (Azcón et al. 1992). However, high N applications are costly and may pollute surface and ground waters (Vitosh and Jacobs 1990). In fact, excessive rates of N fertilizers may result in elevated concentrations of nitrate (NO_3^-) (Santamaria 2006) and high NO_3^- concentrations are accumulated in the edible parts of leafy vegetables (Prasad and Chetty 2008). As a result, a high consume of these plants could lead serious pathological disorders in human health (Mesinga et al. 2003). That is, excess of NO_3^- has been associated to respiratory problem, such as, the "blue baby" syndrome, blood disorder (methemoglobinemia) and to the formation of the carcinogenic compound in human digestive track (Mensinga et al. 2003; Santamaria 2006).

Particularly, green leafy vegetables are among the vegetable species most susceptible to NO_3^- accumulation, such as lettuce, spinach, broccoli, cabbage, celery, radish, beetroot, etc. (Prasad and Chetty 2008). Specially, plants belonging to the Asteraceae family, like lettuce, are among those accumulating more NO_3^- in their leaves (Gent 2003; Santamaria 2006) and due to the health hazards arising from human consumption of NO_3^- in lettuce, have been introduced the legislation of setting maximum limits on the nitrate contents of these crops (European Commission, 2011).

Because consuming these crops can harm human health, developing a suitable strategy and cultivating edible crops with low NO_3^- content are very important (Xu et al.

2012). In this sense, the nitrogen use efficiency (NUE) is an important environmental and social issue (Hirel et al. 2007; Xu et al. 2012; van Bueren et al. 2014). Maximizing N use and N utilization efficiency (NUtE) of crop production can be achieved by optimizing the supply of N to meet the requirements of a crop during growth and development (Hirel et al. 2007; Xu et al. 2012), optimizing N supply in correlation with the desired final produce quality (Stefanelli et al. 2010), or by selecting and growing N-efficient crop genotypes (Maranville and Madhavan 2002; Broadley et al. 2003), which, also has high commercial value, as it can increase yields with lower N application rates, even in soils where N is a limiting element (Svečnjak and Rengel 2006).

In this respect, fortification of horticultural produce, and leafy vegetables in particular, could be a successful strategy for improving human diets (Neeser et al. 2007), resulting in an increase in the value and quality of the produce itself (Stefanelli et al. 2010). In this sense, Hulagur and Dangarwala (1983) demonstrated that an application of N influences in the Zn absorption by plants and vice versa. In fact, N and Zn may show synergistic effects and the best yield could be obtained with the optimum combination of both nutrients. It is therefore of great importance for growers to combine optimum N and Zn rates to achieve optimum sustainable yields, improve product quality, and minimize adverse environmental effects. For this reason, N-nutritional status of plants is an important critical player in root uptake and accumulation of Zn in plants and deserves special attention in the biofortification of food crops with Zn (Erenoglu et al. 2011). The main objective of this work was to determine whether NO_3^- accumulation and NUE was affected by the application of different dosages of Zn, to ascertain the influence of this trace element in a biofortification programme in lettuce plants. For that, we analyzed the effect of Zn on nitrogen metabolism, photorespiration, and the final products of those processes.

5.2.2. Materials and Methods

5.2.2.1. Plant materials and experimental design

Seeds of *L. sativa* cv. Phillipus were germinated and grown for 35 days in cell flats of 3 cm x 3 cm x 10 cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant S.L.). After 35 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions, with relative humidity of 50%, day/night temperature of 22/18◦C, a photoperiod of 12/12 h at a photosynthetic photon flux density (PPFD) of 350 µmol $m^{-2}s^{-1}$(measured at the top of the seedlings with a 190 SB quantum sensor, LICOR Inc., Lincoln, NE, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) in a volume of 3 l. At 35 days after germination and throughout the experiment, control plants received a growth solution, which was composed of 4 mM KNO_3, 3 mM Ca $(NO_3)_2 \cdot 4\ H_2O$, 2 mM $MgSO_4 \cdot 7\ H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2\ H_2O$, 2 µM $MnCl_2 \cdot 4\ H_2O$, 0.25 µM $CuSO_4 \cdot 5\ H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2\ H_2O$, 5 ppm Fe-chelate (Sequestrene; 138 FeG100) and 10 µM H_3BO_3. At the same time, treatments were applied with the same growth solution amended with different supra-optimal Zn concentration (10 (control plants), 20, 40, 60, 80 and 100 µM of $ZnSO_4 \cdot 7\ H_2O$). This solution, with a pH of 5.5–6.0, was changed every three days. The experimental design was a randomized complete block with 6 treatments arranged in individual trays with eight plants per treatment and three replications each, so that the total number of plants was 144.

5.2.2.2. Plant sampling

Edible lettuce leaves were sampled after 21 days further growth under these conditions. The edible leaves of each treatment washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the edible leaves from each treatment were frozen at - 30◦C for later performance of biochemical assays and the other half of the plant material sampled dried in a forced air oven at 70◦C for 24 h to obtain the dry weight (DW) and the subsequent analysis of the concentration of Zn, NO_3^-, NH_4^+, reduced N, and total N.

5.2.2.3. Zn concentration

For the determination of Zn concentration, a sample of 0.15 g dry material was subjected to a process of mineralization by the method of Wolf (1982). Mineral analysis was conducted using ICP-MS. Briefly, samples were further diluted 1-in-10 with milli-Q water and analyzed using an ICP-MS (X-Series II; Termo FisherScientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml^{-1}) and Ir (5 ng ml^{-1}) in 2% TAG HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEXCerti-Prep Ltd, Stanmore, Middlesex, UK) included Zn, in the range 0–100 µg l^{-1}. Zn concentration was expressed as µg g^{-1} DW.

5.2.2.4. Enzyme Extractions and Assays

Leaves were ground in a mortar at 0◦C in 50 mM KH_2PO_4 buffer (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVP). The homogenate was filtered and then centrifuged at 30,000 g for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure enzyme activity of NR and GDH.

The NR assay followed the methodology of Kaiser and Lewis (1984). The NO_2^- formed was colorimetrically determined at 540 nm after azocoupling with sulfanilamide and naphthylethylenediamine dihydrochloride according to the method of Hageman and Hucklesby (1971).

GS was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis (1984). Leaves were ground in a mortar at 0◦C in 50 ml maleic acid-KOH buffer (pH 6.8) containing 100 mM sucrose, 2% (v/v) *β*-mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000 g for 20 min. The resulting extract was used to measure enzyme activity of GS. The reaction mixture used in the GS assay was composed of 100 mM KH_2PO_4 buffer (pH 7.5) with 4 mM EDTA, 1000 mM L-sodium glutamate, 450 m $MgSO_4 \cdot 7H_2O$, 300 mM hydroxylamine, 100 mM ATP, and enzyme extract. Two controls were prepared, one without glutamine and the other without hydroxylamine. After incubation at 28◦C for 30 min, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove et al. 1979).

GDH activity was assayed by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986). The reaction mixture consisted of 50 mM KH_2PO_4 buffer (pH 7.5) with 200 mM NH_4^+ sulfate, 0.15 mM NADH, 2.5 mM 2-oxoglutarate, and enzyme extract. Two controls, without ketoglutarate and NH_4^+ sulfate, respectively, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 3 min.

AAT activity was assayed spectrophotometrically at 340 nm using the method published by González et al. (1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 4 mM $MgCl_2$,

10 mM aspartic acid, and enzyme extract. The decrease in absorbance was recorded for 3 min.

For determination of GGAT, SGAT, and HPR, leaves were ground in a chilled mortar in 100 mM Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20,000 g for 10 min. The resulting extract was used to measure enzyme activity. The extraction medium was optimized for the enzyme activities such that they could be extracted together using the same method (Hoder and Rej 1983).

GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH. The reaction was assayed in a mixture containing 100 mM Tris HCl (pH 7.3), 20 mM glutamate, 1 mM glyoxylate, 0.18 mM NADH, 0.11 mM pyridoxal-5-phosphate, 83 mM NH_4Cl, and 0.3 U GDH in a final volume of 0.6 ml (Igarashi et al. 2006).

SGAT activity was measured by determining the rate of glycine formation from glyoxylate. The reaction was assayed in a mixture containing 100 mM Tris–HCl (pH 7.3), 5 mM serine, 1 mM glyoxylate, 0.11 mM pyridoxal-5-phosphate, 83 mM NH_4Cl, and 0.3 U GDH in a final volume of 0.6 ml. After 20 min of incubation, the reaction was terminated by adding four volumes of absolute ethanol and heating at 80◦C. The rate of glycine was determined by the method of Yemm et al. (1955).

HPR assay was performed with 100 mM Tris–HCl (pH 7.3), 5 mM hydroxypyruvate, and 0.18 mM NADH. Activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder and Rej 1983).

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

5.2.2.5. N forms analysis and nitrogen use efficiency

NO_3^- was analyzed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100 µl aliquot was taken for NO_3^- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO_3^- concentration by spectrophotometry as performed by Cataldo et al. (1975). Ammonium (NH_4^+) was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom (1980). For the reduced N determination, a sample of 0.15 g DW was digested with sulfuric acid and H_2O_2 (Wolf 1982). After dilution with deionized water, a 1 ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 mM sodium phosphate, and 5.4% w/v sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37◦C for 45 min, and reduced N was measured by spectrophotometry according to the method of Baethgen and Alley (1989). Total N concentration (TNC) was assumed to represent the sum of reduced N and NO_3^-.

NUtE was calculated as leaves tissue DW divided by TNC (Siddiqui and Glass 1981).

5.2.2.6. Amino acid analysis by oPA-HPLC

Soluble amino acids were extracted in 0.85 ml of 80% ethanol, left for 15 min at 4°C, and centrifuged. The supernatant was filtered through Waters Sep-Pak C18 Light Cartridges. An aliquot (50 µl) of the extract was derivatized for 1 min with *o*PA and separated by HPLC for amino acid analysis. Chromatographic equipment was from Gilson. The *o*PA derivatives were separated on a reverse-phase C18 ultrasphere column (250 mm x 4.6 mm). Solvent A consisted of 50 mM NaOAc (pH 7) plus 1% tetrahydrofurane, and solvent B was absolute methanol (Carlo Erba). A sample (20 µl)

of the mixture was injected and eluted at a flow rate of 1 ml min^{-1}. The eluted *o*PA derivatives were detected by a fluorometer detector (model 121; GILSON). Quantification of single amino acids was made against a relative calibration curve and expressed as µmol g^{-1} FW (Rogato et al. 2010).

5.2.2.7. Stadistical analysis

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics 5.1 program. Means were compared by Fisher's least-significant differences (LSD). The significance levels for both analyses were expressed as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ or NS (not significant).

5.2.3. Results

5.2.3.1. Biomass and Zn concentration

Our study clearly demonstrated as the application of increasing doses of Zn not led to a decrease in the biomass of the edible part of *L. sativa* regard to the basal dose (Table 1). In addition, an increase in the dose of Zn supplemented to the nutrient solution was accompanied by a concomitant increase in the concentration of Zn in the edible part of *L. sativa* (Table 1).

5.2.3.2. Nitrate reduction

In general, when the dose of Zn was increased in the nutrient solution from 20 µM, the NO_3^- concentration decreased significantly as compared to the control (Fig. 1A, $p < 0.001$ and Fig. 1B, $p < 0.001$). Likewise, NR activity was positively affected when the dose of Zn was increased in the nutrient solution, reaching maximum with the treatment of 100 µM with respect to control plants (Fig. 1C, $p < 0.001$).

Table 1. Effect of Zn supplementation on biomas (dry weight (DW)) and Zn concentration in the edible part of *L. sativa*.

	Leaf biomass (g DW)	Zn concentration (μg g^{-1} DW)
Zn Doses		
10 μM	1.75 ± 0.31a	75.47 ± 3.18f
20 μM	1.50 ± 0.24a	115.74 ± 0.60e
40 μM	1.59 ± 0.12a	131.64 ± 4.25d
60 μM	1.60 ± 0.19a	190.29 ± 0.94c
80 μM	1.42 ± 0.12a	232.77 ± 2.64b
100 μM	1.66 ± 0.01a	265.25 ± 3.58a
p-Value	NS	***
$LSD_{0.05}$	0.59	8.83

Significance levels are represented by $p > 0.05$. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ.

5.2.3.3. Ammonium assimilation

The NH_4^+ concentration decreased significantly when the doses of Zn applied in the range of 20-80 μM (Fig. 2A, $p < 0.001$). With respect to GS and AAT activities, both enzymes showed a similar pattern. That is, as is increment Zn dose, there was an increase of both activities reaching maximum values when the dose applied was 80 μM compared with control plants (Fig. 2B, $p < 0.001$ and Fig. 3B, $p < 0.001$). Finally, GDH activity was affected negatively; except with the treatment of 20 μM, reaching minimum values when the dose applied was 100 μM with respect to control plants (Fig. 3A, $p < 0.001$).

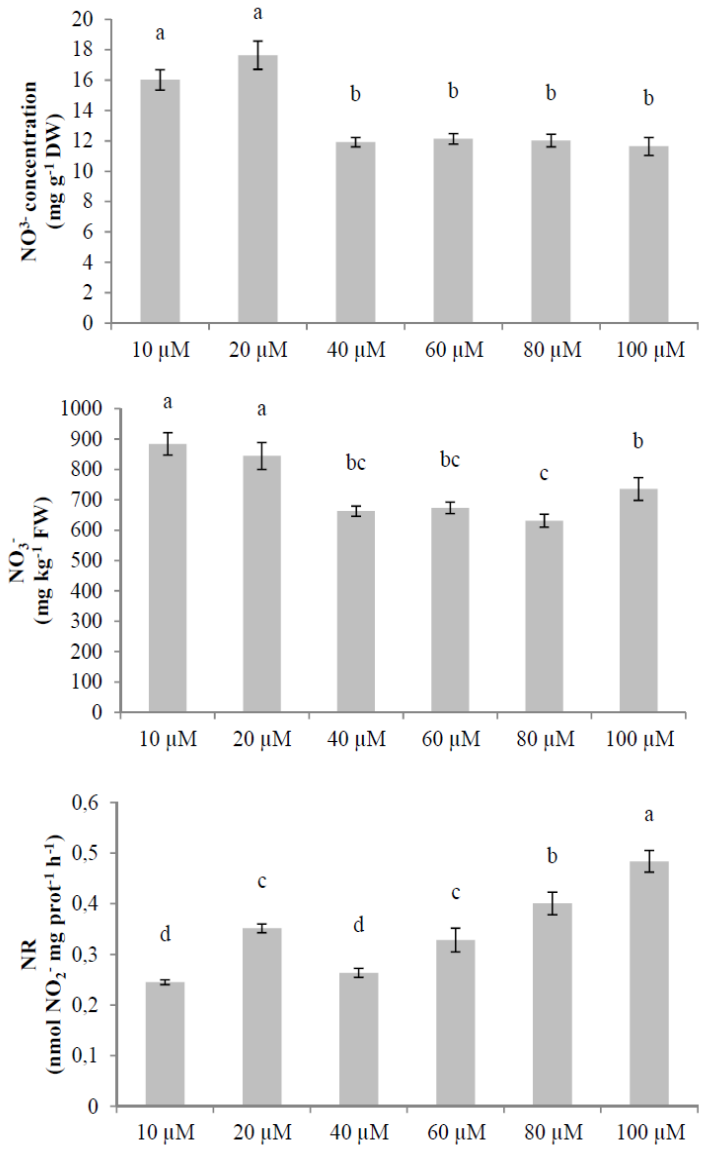

Fig 1. Response of NO_3^- reduction and nitrate reductase activity (NR) in the edible part of *L. sativa* submitted to different Zn application. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

5.2.3.4. Photorrespiratory cycle

GGAT, SGAT and HPR activities were marked increased by the Zn dose was increased in the solution (Table 2). That is, GGAT increased significantly when the dose applied were 20, 40 and 80 µM (Table 2). On the other hand, SGAT increased for all Zn-treatment reaching the higher value at 100 µM-Zn respect to 10 µM (Table 2). Finally,

the HPR activity increased strongly for all treatment reaching the maximum values with the treatment of 80 µM with respect to the basal Zn dose (Table 2).

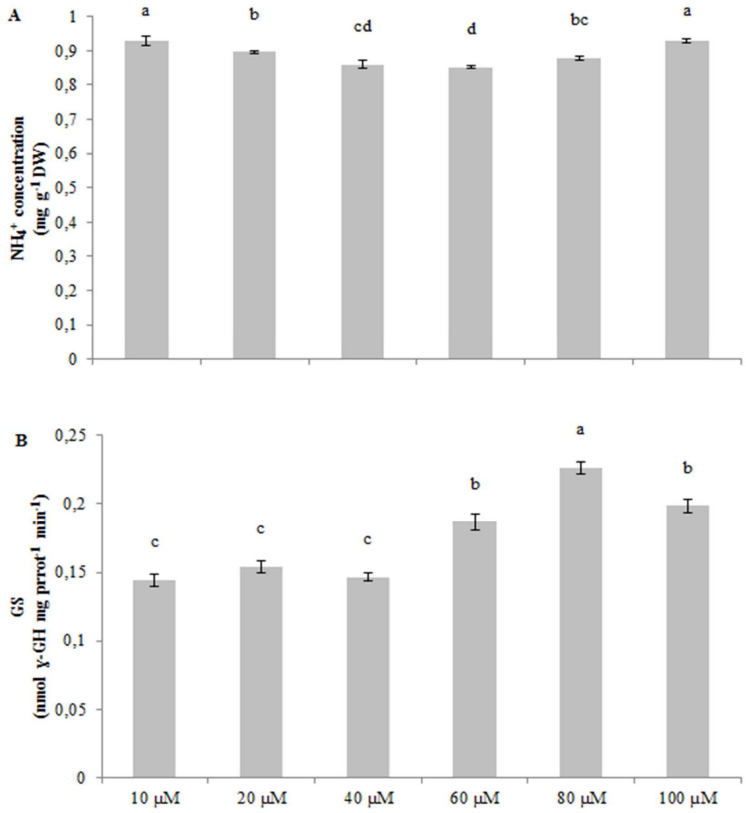

Fig 2. Response of NH_4^+ concentration and enzymes responsible for NH_4^+ assimilation: Glutamine synthetase (GS) in the edible part of *L. sativa* submitted to different Zn application. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

5.2.3.5. Nitrogen use efficiency

The reduced N concentration increased as Zn supplementation was increased, reaching maximum values with 20, 80 and 100 µM with respect to the basal dose of Zn (Fig. 4A, $p < 0.001$). Likewise, TNC was not affected negatively, showing a significant increase when the dose applied were 20 and 100 µM (Fig. 4B, $p < 0.05$). Finally, NUtE was positively correlated with increasing Zn applied in the nutrient solution, reaching maximum values with treatment the 100 µM respects to 10 µM (Fig. 4C, $p < 0.001$).

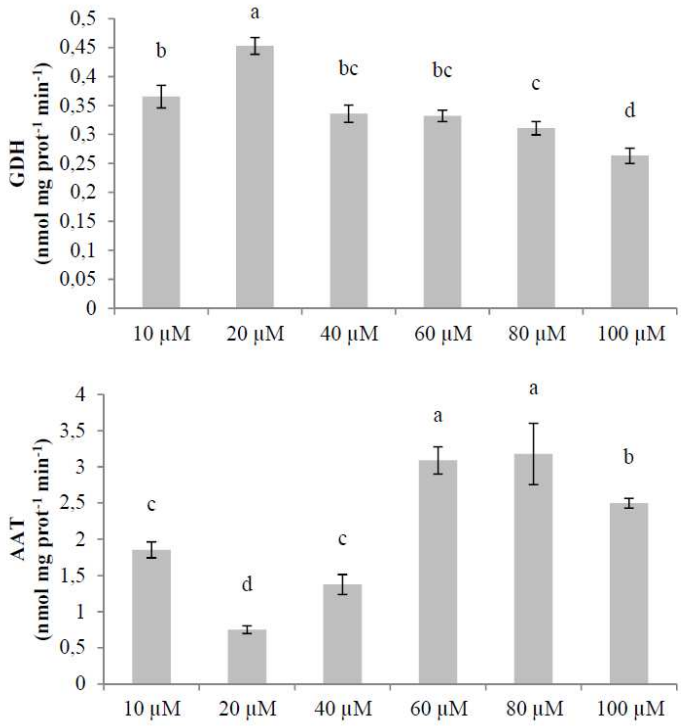

Fig 3. Response of glutamate dehydrogenase (GDH) and aspartate aminotransferase (AAT) under different Zn supplementation in the edible part of *L. sativa*. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

Table 3. Response of some photorespiration enzymes in the edible part of *L. sativa* submitted to different application of Zn.

	GGAT (nmol mg prot^{-1} min^{-1})	SGAT (nmol mg prot^{-1} min^{-1})	HPR (µmol mg prot^{-1} min^{-1})
Zn Doses			
10 µM	4.55 ± 0.11b	1.47 ± 0.03e	0.27 ± 0.00d
20 µM	5.81 ± 0.20a	2.41 ± 0.04c	0.39 ± 0.01ab
40 µM	5.58 ± 0.19a	1.83 ± 0.04d	0.39 ± 0.01ab
60 µM	4.56 ± 0.07b	1.79 ± 0.02d	0.30 ± 0.01c
80 µM	5.78 ± 0.14a	2.73 ± 0.04b	0.40 ± 0.01a
100 µM	5.02 ± 0.24b	3.10 ± 0.13a	0.37 ± 0.01b
p-Value	***	***	***
$LSD_{0.05}$	0.48	0.03	0.03

Significance levels are represented by $p > 0.05$. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ.

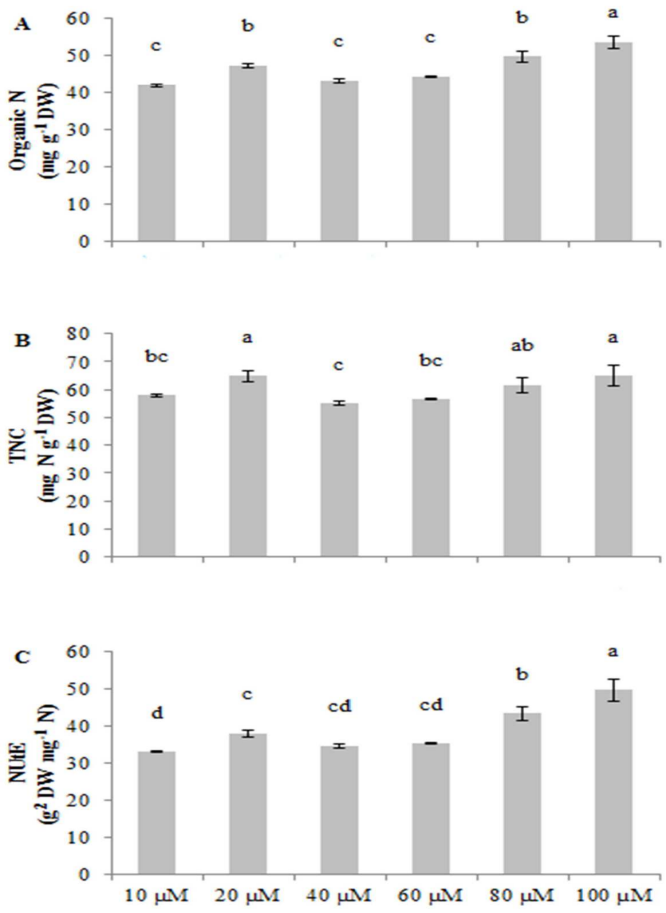

Fig 4. Effect of different Zn supplementations on the foliar concentration of reduced and total N (TNC) and nitrogen utilization efficiency (NUtE) in the edible part of *L. sativa*. Values are means (n = 9) and differences between means were compared using Fisher's test least significant difference (LSD, $p = 0.05$).

5.2.3.6. Amino acids profile

In general, glutamine (Gln), aspartate (Asp), asparagine (Asn), leucine (Leu), isoleucine (Ile), threonine (Thr), tyrosine (Tyr), Lysine (Lys), serine (Ser), glycine (Gly), alanine (Ala), arginine (Arg), and tryptophan (Trp) increased when the Zn-doses applied in the nutrient solution were higher than the control treatment (Table 2). The amino acids Gln, Asn, Thr, Tyr and Ser were increased in a range of 40-80 µM. Moreover, an increase on the Zn concentration in a range of 60-80 µM registered the maximum values of Leu and Ile respect to the control plants. Finally, under 100 µM-Zn the amino acids Asp, Asn,

Arg were increased respect to the basal dose (Table 2). However, the amino acids Phe and His decreased when the plants were grown under all doses higher than 10 μM (Table 2).

5.2.4. Discussion

Zn has emerged as the most widespread micronutrient deficiency in soils and crops worldwide resulting in severe yield losses and deterioration in nutritional quality (Hotz and Brown 2004; Welch and Graham 2004). For this reason, among other essential parameters, appropriate supply of micronutrients is also essential for proper growth and yield of crop. In this sense, the crops biofortification has been proposed as a way of improving plant food nutritional quality, and defined as the process of raising the concentration of bioavailable essential; include Zn, in the edible parts of crops through agricultural practices or genetic selection (Bouis 2003; White and Broadley, 2005). In fact, field tests have shown that micronutrients, have a significantly positive effect on crop yield and quality (Malakouti and Tehrani 2005; Malakouti 2008, Samreen et al. 2013).

Regarding to the essential parameters in research on nutrient efficiency, the biomass determination is one of them, especially in lettuce plants, where the growth of the aerial part is a determining factor in the agricultural value of this crop (Blasco et al. 2012). Our results with respect to the biomass of the edible parts reveal that the dosages of Zn applied in this study remained the biomass of the edible parts of *L. sativa* plants in all treatment in comparison with respect to the control plants (Table 1). Similarly to our results, recent reports have demonstrated as adequate application of Zn-dosage can improve the nutritional quality and production in pea, canola and mungbeans plants (Stoyanova and Doncheva 2002; Malakouti et al. 2005; Samreen et al. 2013).

Table 2. Impact of Zn supplementation on amino acid concentration in the edible part of *L. sativa*.

Amino acids (mmol g^{-1} FW)	Zn doses (μM)						*p*-Value	$LSD_{0.05}$
	10	20	40	60	80	100		
Glu	7.50 ± 0.00b	5.77 ± 0.07d	7.59 ± 0.17b	8.23 ± 0.14a	6.55 ± 0.02c	4.54 ± 0.12e	***	0.34
Gln	0.59 ± 0.00e	1.70 ± 0.61d	2.04 ± 0.06c	2.53 ± 0.03a	2.41 ± 0.20b	1.69 ± 0.05d	***	0.10
Asp	2.64 ± 0.21c	3.75 ± 0.03a	2.40 ± 0.08c	2.87 ± 0.09c	3.61 ± 0.04b	4.34 ± 0.30a	***	0.49
Asn	0.47 ± 0.01c	0.54 ± 0.00bc	0.67 ± 0.01b	0.70 ± 0.00b	0.67 ± 0.01b	1.54 ± 0.15a	***	0.19
Leu	0.56 ± 0.02b	0.64 ± 0.02a	0.59 ± 0.00b	0.65 ± 0.00a	0.66 ± 0.01a	0.44 ± 0.00c	***	0.04
Ile	0.52 ± 0.02c	0.58 ± 0.01b	0.58 ± 0.00b	0.61 ± 0.00a	0.62 ± 0.01a	0.59 ± 0.01ab	***	0.03
Thr	0.87 ± 0.00d	0.81 ± 0.00e	0.94 ± 0.00b	1.05 ± 0.01a	0.90 ± 0.01c	0.68 ± 0.01f	***	0.02
Tyr	0.09 ± 0.00c	0.09 ± 0.00c	0.12 ± 0.00ab	0.12 ± 0.01a	0.11 ± 0.00b	0.10 ± 0.00c	***	0.01
Lys	4.71 ± 0.12b	5.55 ± 0.02a	5.70 ± 0.04a	5.60 ± 0.10a	5.52 ± 0.03a	3.76 ± 0.01c	***	0.21
Ser	1.74 ± 0.02c	1.41 ± 0.02e	2.37 ± 0.02a	2.17 ± 0.01b	2.08 ± 0.01b	1.60 ± 0.01d	***	0.13
Gly	0.09 ± 0.00d	0.12 ± 0.00c	0.37 ± 0.01a	0.37 ± 0.00a	0.17 ± 0.00b	0.13 ± 0.01c	***	0.02
Ala	2.26 ± 0.01c	2.28 ± 0.00c	4.06 ± 0.02a	4.06 ± 0.33a	3.40 ± 0.00b	3.87 ± 0.04a	***	0.42
Phe	0.82 ± 0.06a	0.61 ± 0.00b	0.63 ± 0.00b	0.63 ± 0.01b	0.64 ± 0.01b	0.45 ± 0.00c	***	0.08
Trp	0.39 ± 0.01d	0.67 ± 0.01a	0.51 ± 0.01b	0.45 ± 0.00c	0.49 ± 0.001c	0.36 ± 0.00e	***	0.02
His	3.74 ±0.01a	nd	0.36 ± 00b	0.36 ± 0.01b	nd	0.24 ± 0.01c	***	0.03
Arg	0.48 ± 0.00d	0.65 ± 0.01b	0.54 ± 0.00c	0.64 ± 0.01b	0.35 ± 0.00e	1.31 ± 0.01a	***	0.04
Val	0.26 ± 0.01ab	0.25 ± 0.00b	0.25 ± 0.00b	0.29 ± 0.01a	0.24 ± 0.01b	0.23 ± 0.02b	*	0.03

Significance levels are represented by $p > 0.05$. ns, not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Means followed by the same letter do not significantly differ.

On the other hand, we showed as an increased of Zn in the nutrient solution was correlated with a higher concentration of this element in the edible parts of *L. sativa*, registered a maximum value for the treatment of 100 µM-Zn, representing up to 251% with respect to the control plants without reaching levels considered to be toxic (> 300 µg g^{-1} DW) (Table 1). Similar effects have been demonstrated in plants of *Thlaspi caerulescens*, *Vigna radiata*, *Brassica oleracea* and *Lactuca sativa* plants (Zhao et al. 1998; Samreen et al. 2013; Barrameda-Medina et al. 2014). However, high doses of Zn in *Vigna radiata* (> 70 µM) and *Lactuca sativa* (500 µM) resulted in a decrease of the shoot biomass (Samreen et al. 2013; Barrameda-Medina et al. 2014). Based on these results, we can define all the doses used in this study as optimal and supra-optimal (< 700 µg g^{-1} DW).

N plays a pivotal role in the plant metabolism a nd hence in determining growth. The inorganic nutrition of plants has been dominated by the N, as it improves both quantity and quality of the produce (Fageria 2001; Lea and Azevedo 2006). However, an excessive use of N fertilizers has generated serious environmental contamination (Hirel et al. 2007). In fact, high NO_3^- concentration is important in plants grown for human consumption because a high intake of this nutrient can be harmful to human health (Santamaria 2006), and therefore diminishes nutritional quality of crops (Santamaria et al, 1999; Prasad and Chetty 2008). Thus, great efforts for reducing the NO_3^- foliar content in lettuce have been devoted, such as, a partial substitution of NH_4^+ for NO_3^- in pots filled with an organic substrate (Scaife et al. 1986), and in recirculating nutrient solution (Van der Boon et al. 1990), an application of different doses of N (Stefanelli et al. 2011) and biofortification programs with minerals, include, selenium (Se) (Ríos et al. 2010) and I (Blasco et al. 2011). Fig. 1A shows the results for the foliar concentration of NO_3^-, with a significant decline when the application of Zn was higher than 20 µM.

Moreover, the European Commission (2011) has set upper NO_3^- limits in lettuce leaves of between 3,500 and 4,500 mg kg^{-1} FW, and it should be mentioned that NO_3^- concentrations in our work that were below the limit imposed by the regardless of the rate Zn applied (Fig. 1B), suggest that the adequate supplementation of this element increases the quality of lettuce. Similar to our results, Stefanelli et al. (2011) have demonstrated as a normal fertilization with N will increase lettuce quality reducing the NO_3^- content, increasing essential elements such as Zn, and increasing antioxidant capacity. However, the effect of Zn-biofortification on NO_3^- concentration in *L.* sativa has not yet been studied. Here, we can confirm that an application adequate of Zn is a powerful tool in these horticultural plants.

It´s know that Zn is required as structural and catalytic components enzymes and enzymes for normal growth and development (Broadley et al. 2007). In fact, Zn is very closely involved in the N metabolism of plants (Mengel and Kirkby 1987). Respect to this metabolism, the enzyme NR plays a key role in the reduction of NO_3^- (Tischner 2000). Present data revealed as an increase of Zn in the nutrient solution promoted a sharp increase of NR activity achieving up 63% (80 µM) and 97% (100 µM) (Fig1. C).

In this respect, some studies have demonstrated a high inhibition of NR under Zn deficiency conditions in plants of *Oryza sativa* and *Pennisetum americanum* (Seethambaram and Das 1986) such as under Zn toxicity in: *Silene cucubalus, Deschampsia cespitosa* and *Triticum aestivum* (Mathys 1975; Smirnoff and Stewart 1987; Luna et al. 2000). However, in leaves of *L. sativa* an increase of Zn doses resulted in a higher concentration of this element with a concomitant increase of the NR activity (Fig. 1C). Our data are in concordance with a recent publication which an increase of Zn was correlated with NR activity in *Cicer arietinum* (Siddiqui et al. 2015). Likewise, an increase in the NR activity explains the drastic decline of NO_3^- concentration (Fig.

1A and Fig. 1C) encouraging the normal growth of the edible parts of *L. sativa* (Table 1).

In most higher plants, the primary assimilation of NH_4^+ into glutamine (Gln) through the cooperative activity of GS and GOGAT (Igarashi et al. 2006). In fact, since NH_4^+ is highly toxic in plants, this is quickly absorbed by the GS/GOGAT cycle. In this respect, GS plays a central role in the N metabolism and has been considered as the major assimilatory enzyme for ammonia produced from N fixation, and NO_3^- or NH_4^+ nutrition (Miflin and Habash 2002). In our results, we showed that NH_4^+ concentration in the leaves part do not increase, or even decrease, with an increase of the Zn doses (Fig. 2A). Additionally, GS activity in *L. sativa* plants was strongly affected positively with Zn-doses higher than 40 µM and it should be noted as an application of 80 µM was higher up to 57% than the control plants (Fig 2B). According to our results, Thomsem et al. (2014), have demonstrated a strongly correlation between the levels of NH_4^+ and the GS activity. These authors concluded that an overexpression of GS in leaves could be advantageous for the efficient NH_4^+ assimilation and the export of amino acids via phloem promoting the plant growth. In fact, an overexpression of this enzyme promoted a decrease of NH_4^+ up to seven fold less in tobacco plants (Oliveira et al. 2002). Regarding to Zn, in *Pennisetum americanum* was denoted a decline of the GS activity under Zn deficiency (Seethambaran and Das 1986). Furthermore, in the present study, we observed a positive correlation NO_3^--NR-GS (Fig. 1A, Fig. 1C and Fig. 2B). For this reason, the Zn-doses applied were adequate inducing the effective reduction of NO_3^- and NH_4^+ assimilation in the edible parts of *L. sativa*, especially when the Zn-doses applied was 80 µM.

Additionally, GDH can catalyze the reversible reaction of NH_4^+ and 2-oxoglutarate to glutamate (Glu). However, this double physiological function has recently stimulated

considerable debate, because GDH has been found to have a low Km for NH_4^+ indicating that it is improbable that it would act in favor of biosynthesis. In fact, in *L. sativa* leaves the activity of GDH decreased at the same time that the Zn dose was higher than 60 µM (Fig. 3A). Some studies have revealed that GDH levels also increase under various stress conditions and again the plant may well need to give priority to carbon metabolism and keto-acid production over N metabolism (Miflin and Habasch 2002). In this sense, Frechilla et al. (2002) have indicated that GDH also participates in the assimilation of N in the presence of GS when the concentration of NH_4^+ is elevated. On the other hand, some studies have demonstrated as toxic levels of NH_4^+ in plants can reduce the GS activity. According to our results, we can defined the Zn doses applied in this study as appropriate in a biofortification program with this element, since an increase of this element promoted a higher activity of the key enzyme of the N metabolism (NR, GS) improving the NO_3^- reduction such as the NH_4^+ assimilation. Finally, the enzyme AAT plays a key role in the metabolic regulation of C and N metabolism in all organisms (Torre et al. 2014). In general, but particularly under C shortage, the operation of AAT will ensure that the different keto-acids needed by the plant will be available (Miflin and Habasch 2002). In this respect, *L. sativa* showed a similar patron of the AAT activity respect to NR and GS, that is, an increase of the Zn application induced a concomitant increase of the AAT activity with the maximum values obtained with 80 µM, reaching values up to 109% respect to basal dose (Fig. 3B). An increase of NR and GS could promote a higher use of Gln and Glu produced from the cycle GS/GOGAT and subsequently the formation of aspartate (Asp) and others essential amino acids required for development by the AAT activity. As mentioned about, the biomass of the edible part of *L. sativa* was not affected negatively

(Table 1) and this fact could be due to an efficient amino acid synthesis by AAT activity.

In addition, NH_4^+ is continuously formed during various metabolic processes in the tissues of higher plants. These processes are in general called secondary NH_4^+ assimilation and include, amino acid and protein catabolism, phenylpropanoid biosynthesis, and, especially, photorespiration.

In fact, N metabolism is complicated in leaves of C3 plants by fluxes of NH_4^+ around the photorespiratory cycle, which can be 20-fold more NH_4^+ than is generated by the reduction of NO_3^- (Hirel and Lea 2001). For this reason, the photorespiration has been considered a wasteful process (Igarashi et al. 2006). In this regard, the photorespiration suppression has negative effects on plants, producing a decrease in the CO_2 assimilation rate, poor vegetable growth, and alterations in chloroplast structure. In this sense, Shi-Wei et al. (2007) indicated that this process can provides protection against photoinhibition and increases protection against different types of stress. Likewise, the photorespiration appears to be an essential process in the control of amino acid biosynthesis and metabolism (Igarashi et al. 2006). In this regard, Igarashi et al. (2006) have demonstrated as the peroxisomal glyoxylate aminotransferases (GGAT and SGAT) play a central role within the photorespiratory pathway. Regard to this, we observed as, in general, under an increase on Zn-doses in the nutrient solution, the enzymes GGAT and SGAT showed a higher activities than the control plants, reaching values up 27% (80 µM) for GGAT activity and 86% (80 µM) or 111% (100 µM) (Table 2). On the other hand, the enzyme HPR complete this cycle. In our work, this enzyme exhibited a similar patron than GGAT and SGAT (Table 2). That is, in a range of 20-100 µM was promoted an increased in the HPR activity, especially with in doses of 80 µM, reaching values up to 37% compared to basal doses (Table 2). In this sense, Lu et

al. (2014) have demonstrated as in GO-suppressed plants, the activities of GGAT, GGAT and HPR, were not suppressed and even increased. An explanation of this fact has been reported recently by Xu et al. (2009) and Yu et al. (2010) in *Oryza sativa* plants. These authors conclude that the glyoxylate cycle may serve as anaplerotic reactions to compensate for the glyoxylate formation, particularly when the glycolate oxidation pathway is disrupted. On the other hand, the photorespiratory nitrogen cycle contributes to the metabolism of certain amino acids (Gln, Glu, Ser and Gly). In this way, GGAT, SGAT (Igarashi et al. 2006) and HPR could function cooperatively in the production of amino acids. For this reason, in *L. sativa* plants an increase of GGAT, SGAT and HPR activities (Table 2) can promote the integration of carbonated skeletons from photorespiration into the Calvin cycle, promoting the reduction and assimilation of N. Based on these results, we can conclude that Zn-doses applied in these study, especially 80 µM-Zn, could be sufficient for maintenance an suitable balance C/N promoting the amino acid synthesis and the normal growth of the edible parts of *L. sativa* (Table 1).

Zn deficiency in plants decreases yield and quality, and results in sub-optimal nutrient-use efficiency (Das and Green 2013). In addition, it known that an improvement of NUE is a major goal of crop, in which the selecting and growing N-efficient crops is a special issue (Miflin and Habash 2002; Van Bueren et al. 2014). However, still lack of sufficient experimental data to conclude micronutrients effects on uptake and utilization of other essential plant nutrients (Fageria 2001). Likewise, Erenoglu et al. (2011) indicated that N-nutritional status of plants is an important critical player in root uptake and accumulation of Zn in plants and deserves special attention in the biofortification of food crops with Zn. Furthermore, the possible involvement of this element in NUE, and consequently the concentration of total

reduced N, offers some idea of the interaction between Zn and N and therefore of the potential repercussions for plant growth and development. Fig. 4A shows that the value for reduced N was maintenance or include higher when the Zn-doses applied was higher, reaching a maximum values up to 27% (100 μM) respect to control plants. Respect to this fact, Smirnnoff and Stewart (1987) demonstrated as Zn deficiency can cause a decrease in the concentrations of reduced N compounds and the development of shoot. Additionally, the leaf N concentration not only represents N uptake and nutrition status in plant but is also highly correlated with plant growth and biomass (Kusano et al. 2011). Moreover, TNC was also determined as indexes of the NO_3^- nutrition status of pure hydroponic grown (Hernandez et al. 1995). Respect to TNC, we showed a similar patron of this index that the reduced N concentration (Fig. 4B).

Similar to our results, Hernandez et al. (1995) demonstrated a positive correlation in the TNC and NR respect to NO_3^- status in plants. Finally, other index used for NUE definitions, is NUtE (Siddiqi and Glass 1981). In this respect, Fig. 4C show as an increase of Zn-doses rise an increase of this parameter reaching a maximum values with higher doses, especially 100 μM (50% higher than basal dose). On the basic of our results, these data suggest that an adequate fertilization with Zn can promote the NUE in *L. sativa* plants. Similar results were obtained in different studies in cereals, corn, beans, forages, and oil seeds (Malakouti and Tehrani 2005; Malakouti 2007).

N metabolism process involves uptake, transport, assimilation, and utilization for amino acid biosynthesis and ultimately for growth (Nunes-Nesi et al. 2010) and all of these steps are crucial for NUtE (Hirel et al. 2007; Masclaux-Daubresse et al. 2010; Xu et al. 2012). Some different studies have demonstrated as an increase of enzymatic N activities are strongly implicate in a NUtE, ie; NR, GS and GGAT (Hernandez et al. 1995; Miflin and Habash 2002; Igarashi et al. 2006; Thomsen et al., 2014). In the

present study, the activities of NR, GS, AAT, GGAT, SGAT and HPR were strongly correlated with NUE in the edible parts of lettuce, promoting the accumulation of N compounds and the normal growth of this organ.

Finally, the amino acid metabolism is tightly linked to carbohydrate metabolism, NH_4^+ (absorbed and synthesized from NO_3^-), and demand for protein synthesis and secondary metabolism. For this reason, the regulation of amino acid content, fluxes, and transport through the plant is thus critical for plant adaptation to carbon and nitrogen status, development, and defence (Patrelli and Pilot 2014). In general, on the basic of our results we can confirm as a Zn-biofortification program in a range of 40-100 µM can promote an increased in the amino acid concentration (Table 2). That is, in a range of 40-80 µM the amino acids Gln, Asn, Thr and Tyr were increased and this data could be explain since to the higher activities of the enzyme involved in the N metabolism, such as GS and AAT (Table 2, Fig 2B and Fig 3B). Moreover, an increase of both enzymes was accompanied with an increase of Asp and Ile when the doses applied was 80 µM respect to the basal dose (Table 2). On the other hand, in the treatment of 100 µM we observed as an increase of the amino acid Asp was correlated with an increase of the amino acid Asn, and this fact could be explain for an increase of the AS activity, which could explain the decrease of certain amino acid transport, such as Thr an Lys in this treatment (Table 2). Finally, was promote an increase of the Ser and Gly concentrations compared to the control plants (Table 2). These results are in relation with the higher activities observed in some enzymes involved in the secondary assimilation of NH_4^+, such as GGAT, SGAT and HPR (Table 3). Our results are in concordance with Samreen et al. (2013). These authors demonstrated as the Zn contents and N compounds concentration were higher when greater supply of Zn doses was applied. On the basic of our results, *L. sativa* grown under a Zn-biofortification

programme can promote the NUtE through the synthesis of primary metabolites necessary for normal growth of the plant, some of which are essential (or semi-essential) for the human health, such as Thr, Leu, Ile, Gln, and Tyr.

5.2.5. Conclusions

Considering that many programmes of biofortification with trace elements are being performed, it has become extremely important to study how the application of these elements affects plant physiology and, particularly, N utilization in leaf crops. As far as we know, the present work is the first one to have studied the effect of Zn application on N accumulation and utilization in lettuce, and its results, apart from being revealing, have practical implications. The results presented here indicate that an application of Zn in a range of 80-100 μM produce an increase on Zn concentration, provoking a decrease of NO_3^- since an increase of this element was sufficient to promote the N metabolism (NR, GS and AAT) and photorespiration process (GGAT, SGAT and HPR). As result, we observed an increase on reduced N, TNC and NUtE. Consequently, under 80 μM-Zn the amino acid concentration increased significantly, include the concentration of amino acid essential for human health, ie; Leu, Ile and Thr. In conclusion, adequate Zn fertilization is an important critical player in lettuce, making it possible to increase lettuce productivity and quality, especially with 80 μM Zn, and could decrease the level of Zn deficiency such as the toxic level of NO_3^- in human health.

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5.3. Improved of amino acid profile and phytochemical content in Brassica oleracea cv. Bronco under a Zn-biofortification programme.

Abstract

Millions of hectares of cropland are affected by Zinc (Zn) deficiency and approximately one third of the human population suffers from an inadequate intake of Zn. The main aim of the current study was to determine the potential effect of a Zn-biofortification programme on the Zn concentration, the amino acid profile and the phytochemicals content in a green leafy vegetable, such as *Brassica oleracea* cv. Bronco. Our results indicate that supplementation of 80-100 μM Zn is optimal for maintaining the normal growth of plants and to promote the Zn concentration in the edible part of *B. oleracea*. Moreover, an increase of the Zn doses can induce an accumulation for the total amino acid concentration in the same range, such as of the glucosinolates (GSLs) levels (aliphatic, indolic and total GSLs), and the phenolic concentration (sinapic acid derivatives, flavonols and total phenols). On the basic of our results, an intake of *B. oleracea* grown under 80-100 μM Zn may increase the intake of this micronutrient and other elements beneficial for the human health.

Keywords: Zn deficiency, biofortification, *Brassica oleracea*, amino acids, phytochemicals

5.3.1. Introduction

Zinc (Zn) is essential for both plants and animals since this micronutrient is a structural constituent and regulatory co-factor in enzymes and proteins involved in many biochemical pathways. However, millions of hectares of cropland are affected by Zn deficiency and approximately one third of the human population suffers from an inadequate intake of Zn (Alloway 2008). Moreover, the intake of essential nutrient, secondary metabolites and antioxidant compounds are very low in the human diet under minerals deficiency conditions.

In terms of avoid the problem of micronutrient deficiency, include Zn, in the last years have been proposed many programmes in the agricultural sciences (White and Broadley 2011). In this sense, the ``biofortification´´ have been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of crop plants through agronomic intervention or genetic selection (White and Broadley 2005). This programme is the most cost effective and sustainable and can be carried out in both developed and developing countries (Gibson and Ferguson 1998; White and Broadley 2011).

Moreover, the dietary diversification/modification (DDM) can alleviate the mineral malnutrition (Gibson and Ferbuson 1998), since a diet based in cereal and legumes can accentuate the Zn deficiency in human (White and Broadley 2011). In this sense, a higher consume of fresh fruits and vegetables have been attributed to higher intake of phytochemical compounds, which can promote the healthier properties, reducing the risk of multiple diseases in human health (WHO 2006).

In this respect, the genus *Brassica* is the most important one within the tribe Brassiceae, which includes some crops and species of great worldwide economic importance such as *Brassica oleracea* L., *Brassica napus* L. and *Brassica rapa* L.

Brassicaceae vegetables represent an important part of the human diet worldwide, are consumed by people all over the world and are considered important food crops in China, Japan, India, and European countries and the main vegetable species is *B. oleracea* (Cartea et al. 2011). Moreover, *Brassica* vegetables are consumed all over the year as the ingredients of different salads or after cooking of raw and frozen vegetables (Podsędek 2007). In addition, food plants with apparent cancer and cardiovascular disease-preventing (and antimicrobial) properties include several varieties of *B. oleracea* (Beecher 1994; Podsędek 2007). For this reason, there is renewed interest in the use of *Brassica* species as a fresh food because of their relatively high protein, phytochemicals contents and high dry matter digestibility (Jung et al. 1979). Moreover, *Brassica* vegetables contain a variety of bioactive compounds and it is well know that *B. oleracea* contains phytochemicals such as amino acids (AAs), glucosinolates (GSLs), phenolic compounds, minerals, vitamins and anthocyanins like all other crucifers (Park et al. 2014).

On The other hand, the exploiting natural concentration of phytonutrients through crop breeding, increased production and consumption may better serve human health, than the addition of reduced bio-available elements in supplements or salts (Moreno et al. 2007). In fact, a recently published WHO/FAO report recommended the consumption of a minimum of 400 g of fruit and vegetables per day to prevent chronic diseases such as heart disease, cancer, diabetes and obesity, and prevent micronutrient deficiencies, especially in less developed countries (WHO 2006).

To date, the possibility of increasing the nutritional quality of grains by a Zn biofortification program has been widely investigated (White and Broadley 2011). However, improving the nutritional quality of fruits and vegetables with adequate Zn fertilization have not been extensively discussed.

For this reason, the main aim of the current study was to determine the potential effect of a Zn-biofortication programme on the Zn concentration and the phytochemicals content in a green leafy vegetable, such as *B. oleracea*. As cabbage, plants can be cultivated in soilless growing systems; we tested a different fertilization approach by adding $ZnSO_4$ to the nutrient solution used to grow cabbage plants hydroponically. In this experiment, the effects of different supra-optimal Zn supplementation were evaluated in the edible tissues.

5.3.2. Material and methods

5.3.2.1. Plant material and experimental design

Seeds of *B. oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 cm x 3 cm x 10 cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant S.L.). After 35 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions, with relative humidity of 50%, day/night temperature of 22/18◦C, a photoperiod of 12/12 h at a photosynthetic photon flux density (PPFD) of 350 µmol $m^{-2}s^{-1}$(measured at the top of the seedlings with a 190 SB quantum sensor, LICOR Inc., Lincoln, NE, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) in a volume of 3 l. At 35 days after germination and throughout the experiment, control plants received a growth solution, which was composed of 4 mM KNO_3, 3 mM $Ca(NO_3)_2 \cdot 4 H_2O$, 2 mM $MgSO_4 \cdot 7 H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2 H_2O$, 2 µM $MnCl_2 \cdot 4 H_2O$, 0.25 µM $CuSO_4 \cdot 5 H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2$ H2O, 5 ppm Fe-chelate (Sequestrene; 138 FeG100) and 10 µM H_3BO_3. At the same time, treatments were applied with the same growth solution

amended with different supraoptimal Zn concentration (10 (control plants), 20, 40, 80 and 100 µM of $ZnSO_4 \cdot 7\ H_2O$). This solution, with a pH of 5.5–6.0, was changed every three days. The experimental design was a randomized complete block with 5 treatments arranged in individual trays with eight plants per treatment and three replications each, so that the total number of plants was 120.

5.3.2.2. Plant sampling

B. oleracea plants were sampled after 21 days further growth under these conditions. The edible leaves of each treatment washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the edible leaves from each treatment were frozen at - 30◦C for later performance of AAs concentration and biochemical assays and the other half of the plant material sampled dried in a forced air oven at 70◦C for 24 h to obtain the dry weight (DW) and the subsequent analysis of the concentration of Zn, forms of sulfur (S), GSLs, and phenolic compounds.

5.3.2.3. Zn and different forms of S concentration

For the determination of Zn and total S concentration, a sample of 0.15 g dry material was subjected to a process of mineralization by the method of Wolf (1982). Mineral analysis was conducted using ICP-MS. Briefly, samples were further diluted 1-in-10 with milli-Q water and analyzed using an ICP-MS (X-Series II; Termo FisherScientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml^{-1}) and Ir (5 ng ml^{-1}) in 2% TAG HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEXCerti-Prep Ltd, Stanmore, Middlesex, UK) in the range 0–100 µg l^{-1}. Zn concentration was expressed as µg g^{-1}DW.

5.3.2.4. Amino acids determination by oPA-HPLC

Soluble amino acids were extracted in 0.85 ml of 80% ethanol, left for 15 min at 4°C, and centrifuged. The supernatant was filtered through Waters Sep-Pak C18 Light Cartridges. An aliquot (50 µl) of the extract was derivatized for 1 min with *o*PA and separated by HPLC for amino acid analysis. Chromatographic equipment was from Gilson. The *o*PA derivatives were separated on a reverse-phase C18 ultrasphere column (250 mm x 4.6 mm). Solvent A consisted of 50 mM NaOAc (pH 7) plus 1% tetrahydrofurane, and solvent B was absolute methanol (Carlo Erba). A sample (20 µl) of the mixture was injected and eluted at a flow rate of 1 ml min^{-1}. The eluted *o*PA derivatives were detected by a fluorometer detector (model 121; GILSON). Quantification of single amino acids was made against a relative calibration curve and expressed as µmol g^{-1} FW (Rogato et al. 2010).

5.3.2.5. Form and metabolism of S

S*ulfur metabolism.* ATP-sulphurylase (ATP-S) activity was measured by the method of Lappartient and Touraine (1996) with some modifications. First, 0.1 g of cabbage leaf were homogenized in 0.5 ml phosphate buffer containing 10 mM EDTA-Na_2 and 2 mM 1,4-Dithio-DL-therit(ol) (DTT), and then 50 µl extract were added to 250 µl reaction buffer. The mixture was incubated at 37°C for 10 min, and the reaction was stopped by adding 0.5 ml acetate buffer 0.5 M pH 4. After the addition of starting buffer, and after a lapse of 60 min, the samples were measured at 660 nm, on a pattern curve of inorganic phosphorus. The serine acetyltransferase (SAT) activity was performed following Tripati et al. (2012). The reaction mixture contained 63 mM Tris–HCl (pH 7.6), 1.25 mM EDTA, 1.25 mM DTNB (5,5′-dithiobis-2-nitrobenzoic acid), 0.1 mM acetyl-CoA, 1 mM and l-Serine. The rate of reaction was followed at A_{412} nm. Finally, *O*-

acetylserine(thiol)lyase (*O*AS-TL) activity was measured following Nakamura et al. (1987), and modified by Stuvier and De Kok (2001). First, 0.1 g of cabbage leaf was homogenized in 1 ml of phosphate buffer pH 8, with 10 mM 2 mercaptoethanol and 0.5 mM EDTA; the homogenate was ground with a mortar and pestle on ice, and centrifuged at 9500 rpm for 10 min. The samples were measured at 560 nm, using a pattern curve of cysteine.

5.3.2.6. Phenolic metabolism

For determination of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP; DS-Mn, DS-Co) and phenylalanine ammonia lyase (PAL) activities, 0.2 g whole fresh leaf was homogenized in 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifugated at 15000 g for 15 min at 4°C. DAHP activity was assayed using a modified method of Morris et al. (1989). For determination of shikimate dehydrogenase (SKDH) activity, whole fresh leaf was homogenized in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 15000 g for 15 min at 4°C. Then, the SKDH activity was determined according to Ali et al. (2006). PAL activity was measured by a method of Tanaka et al. (1974). For determination of cinnamate 4-hydroxylase (C4H), fresh sample of leaf was homogenized in 200 mM potassium phosphate buffer (pH 7.5) containing 2 mM of 2-mercaptoethanol. Homogenates were centrifuged at 10000 g for 15 min at 4°C. C4H activity was assayed by using the method described by Lamb and Rubery (1975). For 4-coumarate coenzyme A ligase (4CL) activity was performed. The extract buffer was 0.05 M Tris–HCl (pH 8.8) containing 14 mM mercaptoethanol and 30% glycerol. The activity was determined with the spectrophotometric method, using caffeic acid as the preferred phenolic substrate (Knobloch and Hahlbrock 1975). Finally, cinnamyl alcohol

dehydrogenase (CAD) enzyme activity was determined by measuring the increase in absorbance at 400 nm when coniferyl alcohol was oxidized to coniferaldehyde (Wyrambik and Grisebach 1975). The assay was performed for 10 min at 30°C in a total volume of 0.5 ml containing 100 mM Tris–HCl (pH 8.8), 100 µM coniferyl alcohol, 200 µM NADP and 50 µl enzyme extract.

5.3.2.7. Analysis of glucosinolates and phenolic compounds by HPLC-DAD-ESI-MS^n

The HPLC gradient for glucosinolate and phenolic analyses is a multipurpose chromatographic method that simultaneously separates glucosinolates and phenolics (Bennett et al. 2003) and it was recently applied to Galician turnip tops and greens (Francisco et al. 2009). Briefly, a portion of 150 mg of each sample were extracted in 4 mL of 70% MeOH at 70°C for 30 min with vortex mixing every 5 min to facilitate the extraction. The samples were centrifuged (13000g, 15 min), and 1 ml of supernatant was collected to completely remove methanol using a sample concentrator (DB-3D, Techne, UK) at 70 °C. The dry material obtained was redissolved in 1 mL of ultrapure water and filtered through a 0.20 µm syringe filters (Acrodisc® Syringe Filters, Pall Life Sciences). Chromatographic analyses were carried out on a Luna C18 column (250 mm × 4.6 mm, 5 µm particle size; Phenomenex, Macclesfield, UK). The mobile phase was a mixture of: (A) ultrapure water/trifluoro acetic acid (TFA) (99.9:0.1) and (B) methanol/TFA (99.9:0.1). The flow rate was 1 mL min^{-1} in a linear gradient starting with 0% B at 0–5 min, reaching 17% B at 15–17 min, 25% B at 22 min, 35% B at 30 min, 50% B at 35 min, 99% B at 50 min and at 55–65 min 0% B. The injection volume was 20 µL and chromatograms were recorded at 330 nm for phenolics derivatives and 227 nm for GLS in a Model 600 HPLC instrument (Waters) equipped with a Model 486 UV tunable absorbance detector (Waters). Glucosinolates were

quantified using sinigrin (sinigrin monohydrate from Phytoplan, Diehm and Neuberger GmbH, Heidelberg, Germany) as standard. Caffeoyl-quinic and p-coumaroyl-quinic acids derivatives were quantified as chlorogenic acid (5-caffeoyl-quinic acid, Sigma–Aldrich Chemie GmbH, Steinheim, Germany), flavonoids as kaempferol 3-rutinoside (Extrasynthese, Genay, France) and sinapic acid and derivatives as sinapic acid (Sigma).

5.3.2.8. Statistical analysis

The data were analyzed by a simple variance analysis (ANOVA) and differences between the means were compared by Fisher's least-significant difference test (LSD). In addition, to ascertain whether the different plants and Zn-doses used in the experiment significantly influenced the results, a two-way ANOVA was used and the means were compared by Fisher's LSD test.

5.3.3. Results and discussion

5.3.3.1. Effect of Zn supplementation on biomass and Zn concentration

In recent years, Zn biofortification has taken a special interest because it has increased this micronutrient deficiency in both crops and human health. In this regard, several studies in different plant species have been conducted in order to increase the concentration of this element in the edible part of plants (White and Broadley 2011). In this respect, in terms of selecting the appropriate dose of Zn in crop, the biomass in the aerial part it has been selected as a good indicator of the utilization efficiency of this micronutrient. On the basis of our results, all doses applied of Zn in this experiment can be considered as optimal or supra-optimal for normal plant growth since the biomass of the aerial part was not adversely affected (Table 1). Moreover, an increase of the Zn application to nutrient solution was powerful correlated with an increase of Zn

concentration in the aerial part of *B. oleracea* (Tabla 1). That is, this species registered a maximum value for 100-µM treatment, representing up to 687% with respect to the control plants (Table 1). Similar to our results, Broadley et al. (2007) have demonstrated as the Brassicaceae species have an enormous potential to Zn accumulate in the shoot without produce a decrease in the biomass of this organ. In this sense, a study into wide species variation regarding minerals in staple crops including rice, wheat, maize, beans, cassava and *B. oleracea* was reviewed by White and Broadley (2005), demonstrating that the Zn concentration in *B. oleracea* was higher than in any other crop except for spinach. In addition, have been demonstrated as in a iodine (I) and silicon (Si) biofortification programmes, an increase of these element supposed a concomitant increase on these element in the aerial part of the leafy vegetables being the higher values obtained in the edible portions of *Brassica*, defining this cultivar as more effective in this type of programs (Hong et al. 2008; Lawson et al. 2015; D´Imperio et al. 2015).

Table 1. Effect of Zn supplementation on biomass and Zn concentration in leaf of *B. oleracea*

Zn treatment (µM)	Leaf biomass (g DW)	Zn concentration (µg g^{-1} DW)
10	1.71 ± 0.20 b	59.03 ± 0.19 e
20	2.02 ± 0.07 ab	79.65 ± 1.68 d
40	2.23 ± 0.20 a	160.56 ± 5.58 c
80	1.79 ± 0.13 ab	300.45 ± 1.62 b
100	1.78 ± 0.15 ab	464.70 ± 10.37 a
p-Value	NS	***
$LSD_{0.05}$	0.50	16.92

Significance levels are represented by p > 0.05. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ

5.3.3.2. Impact of Zn supplementation on the amino acid concentration

The compositions of ingested foods have relationships to positive health effects, such as cardiovascular disorders, cancers, diabetes, hypertension and obesity (World Health Organization, 2003; World Cancer Research Fund/American Institute for Cancer Research, 2007). For this reason, it should pay especial attention on the food selection. Regard to this, is well recognized that the vegetables are important components of the diet, supplying a multitude of health-related phytochemicals compounds (Podsędek 2007; Park et al. 2014).

In this sense, humans are unable to synthesize methionine (Met), threonine (Thr), lysine (Lys), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr) and histidine (Hys) and therefore these so-called essential amino acids must be supplied in sufficient amounts in the diet to meet their metabolic needs (Mathews et al. 2012). In this study, we observe as the total of amino acids were higher when the doses applied were 80 (32%) and 100 µM (42%) of Zn with respect to the control plants (Table 2) and these data are in concordance with an increase of the concentration of the most of essential amino acids concentration, ie; Phe, Trp, Ile, Leu, His and Lys, in plants growth under these Zn-doses (Table 2). Moreover, the Zn supplementation in *B. oleracea* leaves produce an increase of others amino acids, such as, arginine (Arg) and glutamine (Gln) (require for human under certain pathologies conditions), alanine (Ala), aspartate (Asp) and glutamate (Glu) (Table 2). Many studies have been carried about the amino acids content in *B. oleracea* under normal conditions or in inbred lines of this species , but in almost cases the concentration amino acid concentration were lower than the obtained in our experiment (Park et al. 2014).

Table 2. Impact of Zn supplementation on amino acid concentration in the edible part of *B. oleracea*

Amino acids (mmol g^{-1} FW)	Zn doses (µM)					*p*-Value	$LSD_{0.05}$
	10	20	40	80	100		
Ser	2.61 ± 0.26a	0.97 ± 0.07b	0.64 ± 0.01b	0.99 ± 0.01b	0.73 ± 0.01b	***	0.38
Phe	1.00 ± 0.31b	0.60 ± 0.11b	0.82 ± 0.06b	0.83 ± 0.01b	1.63 ± 0.08a	**	0.48
Trp	1.35 ± 0.03bc	1.71 ± 0.03a	1.19 ± 0.11c	1.41 ± 0.03b	1.78 ± 0.05a	***	0.19
Tyr	0.39 ± 0.01b	0.16 ± 0.01d	0.21 ± 0.02c	0.52 ± 0.02a	0.15 ± 0.01d	***	0.05
Ala	5.49 ± 0.08a	4.86 ± 0.23b	4.82 ± 0.14b	4.92 ± 0.11b	5.62 ± 0.18a	***	0.49
Ile	0.32 ±0.02c	0.52 ± 0.03b	0.32 ±0.01c	0.79 ± 0.02a	0.87 ±0.07a	***	0.12
Leu	0.42 ±0.00b	0.36 ± 0.00c	0.39 ± 0.01bc	nd	0.59 ± 0.02a	***	0.04
Val	1.12 ±0.01a	0.12 ± 0.00d	0.14 ±0.00d	0.16 ± 0.00c	0.20 ± 0.01b	***	0.02
His	5.82 ± 0.06d	8.37 ± 0.03c	12.24 ± 0.33b	16.50 ± 1.32a	16.96 ± 0.80a	***	2.23
Lys	1.45 ± 0.13c	1.81 ± 0.10b	1.81 ± 0.01b	1.60 ± 0.03bc	2.59 ± 0.15a	***	0.31
Thr	0.31 ± 0.01a	0.24 ± 0.01b	0.16 ± 0.01c	0.24 ± 0.01b	0.34 ± 0.01b	***	0.03
Arg	1.14 ± 0.01d	1.26 ± 0.02d	2.92 ± 0.07b	3.70 ± 0.09a	1.59 ± 0.03c	***	0.17
Gly	2.88 ± 0.07a	0.67 ± 0.01b	0.39 ± 0.02c	0.65 ± 0.02b	0.65 ± 0.00b	***	0.11
Asp	6.48 ± 0.03d	6.77 ± 0.03c	6.55 ±0.01cd	7.02 ± 0.09b	8.16 ± 0.14a	***	0.24
Asn	0.56 ± 0.01a	0.51 ± 0.06a	0.24 ± 0.01b	0.33 ± 0.00b	0.31 ± 0.00b	***	0.09
Glu	2.64 ± 0.09c	3.68 ± 0.10b	2.44 ± 0.09c	3.66 ± 0.11b	4.17 ± 0.06a	***	0.29
Gln	1.37 ± 0.04d	1.50 ± 0.07cd	1.89 ± 0.05c	3.43 ± 0.26b	4.02 ± 0.16a	***	0.45
Total	35.38 ± 0.37cd	34.12 ± 0.59d	37.18 ± 0.35c	46.77 ± 1.54b	50.40 ± 1.11a	***	2.89

Significance levels are represented by $p > 0.05$. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ

It should be noted that among the essential amino acids, the aromatic amino acids (AAA) essential, Phe, Trp and Tyr, have a crucial role not only for the protein synthesis, but also serve as precursors for a wide range of secondary metabolites that are important for plant growth as well as for human nutrition and health (Tzin and Galili 2010 a). In this sense, the biosynthetic pathway of these amino acids and their regulation will be key for increase the beneficial properties of these nitrogenous compounds in the human health (Tzin and Galili 2010b). In this regard, the amino acid metabolism is closely related to other metabolic processes as; sulfur assimilation and the shikimate, phenylpropanoid and aspartate pathways among many others.

5.3.3.3. Synthesis and accumulation of glucosinolates under Zn-biofortification

To date, there have carried out several biofortification programs with various elements and have been observed as the supplementation with one or more elements may influence synergistically or antagonistically with the absorption and accumulation of other nutrients (Hafeez et al. 2013). Specially, under heavy metals can be produce a positive effect on the S asimilation (Jahangir et al. 2008; Sytar et al. 2016). Table 3 demonstrated as an increase of Zn doses (20-100 µM) increase the accumulation of the total S since an increase of the reduced S in this organ respect to 10 µM of Zn in the edible part of *B. oleracea*. This data are in concordance with the results obtained for the enzymes involved in the S assimilation. That is, under all supra-optimal doses of Zn used in this study (20-100 µM) were produced an increase in the activity of ATP-S, SAT and OAS-TL reaching a maximum values in general when the doses applied were 80 and 100 µM with an increase up 72 % and 77% for the ATP-S, 17% and 13% for SAT and 27% and 31% for OAS-TL (Figure 1 A-C). Similar to our results, have been demonstrated the positive effect of the application of supra-optimal Se doses in the S

assimilation in leafy vegetable as lettuce and broccoli plants (Ríos et al. 2008). However, the effect of Zn-biofortification on the S metabolism has not been extensity revealed, but it is well known that under heavy metals exposure the plants must regulate the cellular homeostasis. In this sense, the synthesis of some S-compounds such as methionine (Met), cysteine (Cys) and glutathione (GSH) have been demonstrated. In this regard, Na and Salt (2011) have demonstrated as an increase of heavy metals, include Zn, can promote an increase in the S assimilation in plants, due to an increase in the activities of ATP-S, SAT and OAS-TL.

Table 3. Effect of Zn biofortification on the different forms of S

Zn treatment (μM)	SO_4^{2-} (mg g^{-1} DW)	Reduced S (mg g^{-1} DW)	Ratio (Reduced S/SO_4^{2-})	Total S (mg g^{-1} DW)
10	1.06 ± 0.00 a	14.50 ± 0.25 c	13.70 ± 0.24 c	15.56 ± 0.25 c
20	1.06 ± 0.00 a	17.40 ± 0.39 a	16.45 ± 0.36 a	18.46 ± 0.38 a
40	1.06 ± 0.00 a	16.14 ± 0.61 b	15.25 ± 0.58 b	17.20 ± 0.61 b
80	1.06 ± 0.00 a	16.66 ± 0.15 ab	16.45 ± 0.36 ab	17.72 ± 0.15 ab
100	1.06 ± 0.00 a	17.22 ± 0.42 ab	16.28 ± 0.39 ab	18.28 ± 0.42 ab
p-Value	NS	**	**	**
$LSD_{0.05}$	0.00	1.25	1.18	1.25

Significance levels are represented by p > 0.05. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ

On the other hand, an increase of ATP-S and SAT activities produce the necessary substrate for the OAS-TL activity and this enzyme is crucial to cysteine (Cys) and Met synthesis require for the aliphatic GSLs synthesis. Moreover, an increase of certain amino acids, ie; Ala, Leu, Ile or Val, can contribute to the synthesis of these GSLs (Johnson et al. 2016). In this sense, we observed an increase of the aliphatic GSLs with a similar pattern of the three enzymatic activities mentioned about (Fig 1 and Table 4). That is, the GSL glucoiberin and the total of aliphatic GSLs were increased when the

doses applied was increased in the nutrient solution, reaching a maximum values up 76% and 93% in the treatment of 80 and 100 µM, respectively, respect to the control dose (Table 4). However, the aliphatic GSL sinigrin only increased when the dose applied was 20 µM (14%) (Table 4), and this increase could be explain for the increase of the activity OAS-TL in this treatment with respect to 10 µM of Zn (Figure 1C). Finally, an increase of Ile can promote the synthesis of the aliphatic amino acids in the treatment of 20, 80 and 100 µM and Ala and Leu on the 100 µM Zn dose (Table 2 and Table 4). Similar to our results, Asad et al. (2015) demonstrated as in plant of *Noccaea caerulescens* the non-toxic level of Zn can promote the synthesis of GSLs. Moreover, Coolong and Randle (2004) conclude that the Zn fertility can influence changes in GSL that may affect flavor or medicinal attributes associated with the GSL and their breakdown products, as well as elevate the nutritional status of Zn in the leaves of *B. rapa.*

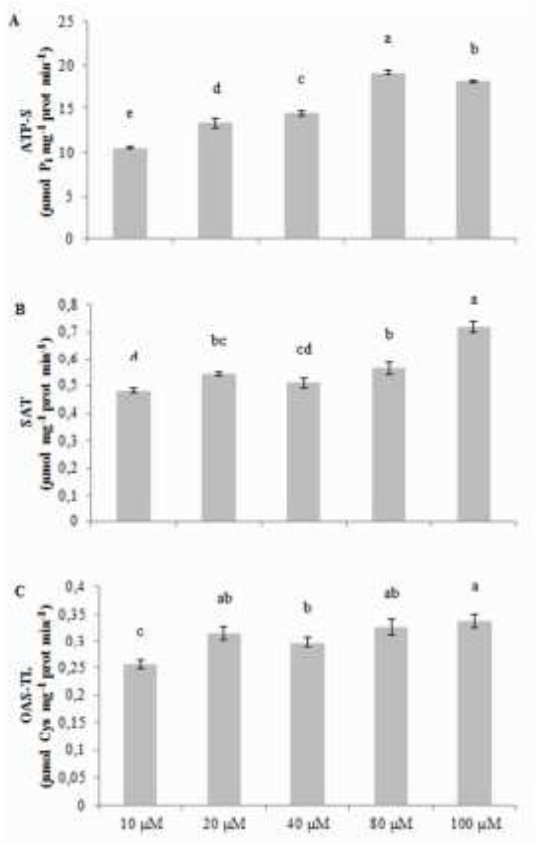

Fig 1. Influence of different doses of Zn on the assimilation of S in leaves of *B. oleracea*. ATP-Sulfurylase (ATP-S) (A), serine acetyltransferase (SAT) (B) and O-acetylserine tiol liasa (OAS-TL). Values are means (n = 9) and differences between means were compared using Fisher's test least signif cant difference (LSD, $p = 0.05$)

Table 4. The effect of Zn biofortification on glucosinolates (mg g^{-1} DW) content in the edible part of *B. oleracea*

Zn treatment (μM)	Glucoiberin	Sinigrin	Total aliphatic	Total indolic (4-hydroxyglucobrassicin)	Total glucosinolates
10	3.08 ± 0.03 c	6.80 ± 0.22 b	11.01 ± 0.11 d	0.27 ± 0.00 c	11.31 ± 0.12 c
20	4.22 ± 0.10 b	7.76 ± 0.19 a	12.05 ± 0.27 b	0.26 ± 0.01 c	13.14 ± 0.22 a
40	4.71 ± 0.20 b	6.89 ± 0.10 b	11.59 ± 0.10 c	0.29 ± 0.00 b	11.87 ± 0.11 b
80	5.43 ± 0.31 a	6.92 ± 0.02 b	12.91 ± 0.05 a	0.32 ± 0.00 a	13.20 ± 0.00 a
100	5.96 ± 0.06 a	7.21 ± 0.04 b	13.13 ± 0.03 a	0.32 ± 0.00 a	13.44 ± 0.05 a
p-Value	***	**	***	***	***
$LSD_{0.05}$	0.55	0.43	0.44	0.02	0.40

Significance levels are represented by $p > 0.05$. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ

As mentioned about, the AAA, Phe, Trp and Tyr, are central molecules in plant metabolism. Besides their function as building blocks of proteins, the three AAA serve as precursors for a variety of plant hormones, such as auxin and salicylate, as well as for a very wide range of aromatic secondary metabolites with multiple biological functions and biotechnological value in the health promoting, medical and food industries (Tzin and Galili 2010b) and nowadays it is well known the close relationship between the regulation of the AAA biosynthesis and the shikimate pathway (Tzin and Galili 2010a).

In this sense, here we showed as under a Zn-biofortification programme the activity of the enzymes involved in the shikimate pathway, such as, DAHP and SKDH increased strongly when the doses applied were 80 (123%) and 100 µM (171%) with respect to the control plants (Figure 2C and Figure 2D). Moreover, the higher activity of the isoform DAHP-Mn in the treatment of 80 (149%) and 100 (181%) can explain the total activity of this enzyme (Figure 2A). In adittion, in terms of the ratio DAHP (DS-Mn/DS-Co), despite the fact that we observed an increase of these for all Zn treatment respect to 10 µM, this increase was highly potent when the doses applied was 100 µM (226%) (data not shown).

This data are in concordance with the values obtained for the AAA (Table 2). That is, the concentration of Phe only increased strongly only under a 100 µM-Zn supplementation reaching values up 63%, Trp increased 32% at the same dose, and the amino acid Tyr increased 33% in the treatment of 80 µM respect to the basal dose (Table 2). Moreover, an increase of Trp in the treatment of 20 µM (27%) could be explained by an increase of the DAHP and SKDH with respect to 10 µM (Table 2 and Figure 2C-D). In addition, an increase of both activities when the doses applied were 80 and 100 µM explain the powerful increase on the His concentration (183% and 191% respectively) respect to the basal dose (Table 2).

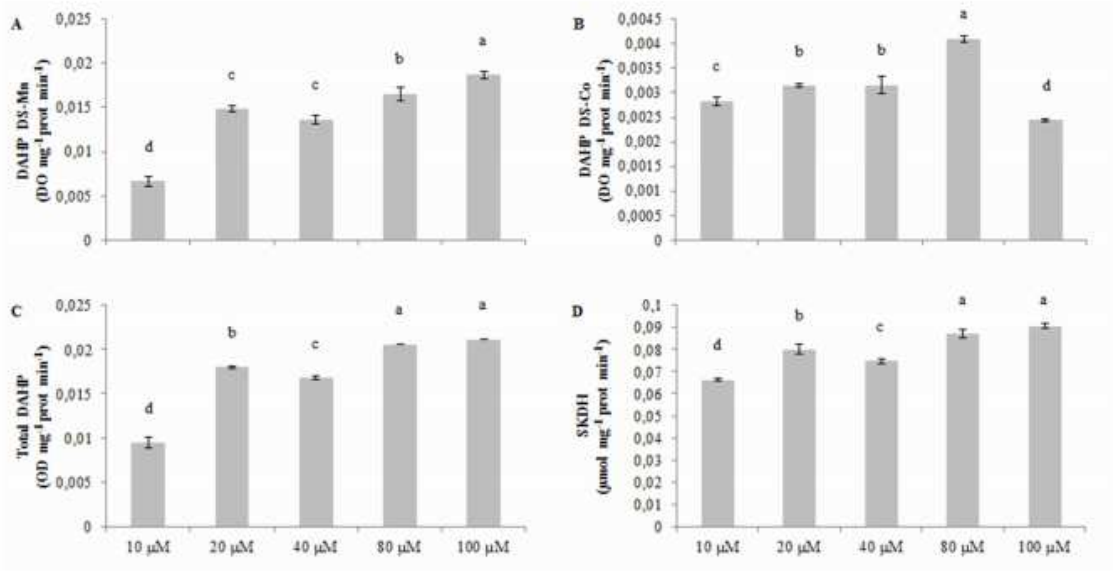

Fig 2. Effect of Zn supplementation in the shikimate pathway in the edible part of *B. oleracea*. 3-deoxy-o-arabino-heptulosonate 7-phosphate (DAHP) isoforms (A-C) and shikimate dehydrogenase (SKDH) (D). Values are means (n = 9) and differences between means were compared using Fisher's test least signif cant difference (LSD, $p = 0.05$)

Moreover, Phe Thr and Tyr are potential substrates for the synthesis of GLS aromatic, include the indolic GLSs. In this sense, in the treatment of 80 and 100 µM were produced an increase in the indolic GSLs, represented as 4-Hidroxyglucobrassicin (Table 4). In this respect, Tolrà et al. (2001) demonstrated as in the Zn-hyperaccumulator Brassicacea species, such as *Thalspi caerulescens*, the concentration of the indolic GSL decreased when the doses of Zn were increased in the nutrient solution. In this respect, we can deduce, that the Zn doses applied were optimal or supra-optimal; since the concentration of Zn applied in the nutrient solution was accompanied with an increase in the Zn and GSL concentration without promote negative effect on the edible part of *B. oleracea* (Table 1 and Table 4).

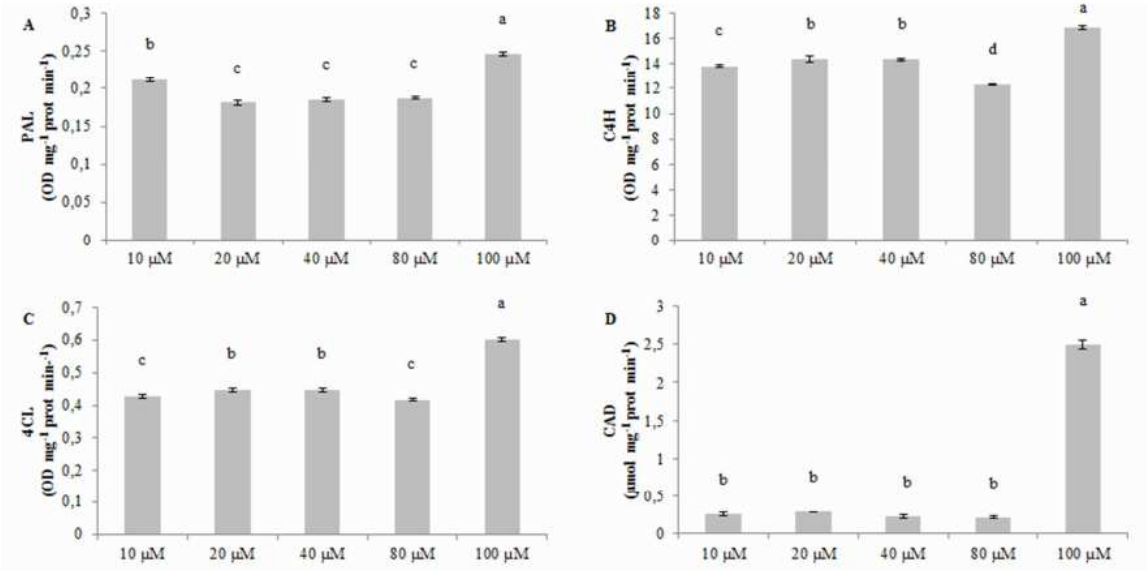

Fig 3. Impact of Zn on the phenylpropanoid metabolism in *B. oleracea* leaves. Phenylalanine ammonia lyase (PAL) (A), cinnamate-4-hydroxylase (C4H) (B), coumaroyl-CoA ligase (C4L) (C), cinnamyl-alcohol dehydrogenase (CAD) (D).Values are means (n = 9) and differences between means were compared using Fisher's test least signif cant difference (LSD, $p = 0.05$)

5.3.3.4. Synthesis and accumulation of phenolic compounds under Zn-biofortification

The amino acid Phe is the initial substrate required for the phenylpropanoid pathway. In this regard, the activity of PAL, a key enzyme in phenolic biosynthesis, increased its activity when the dose applied was 100 µM, while in the others treatment (20-80 µM) were maintained or decreased respect to the basal dose (Figure 3A). Additionally, with respect to the enzymes C4H, 4CL and CAD all treatment showed a similar pattern of the first enzyme implied in this pathway (Figure 3 B-D). Our results are in concordance with the results obtained in some different works. That is, the mineral fertilization (such as, Ca, P and N) have been used to increase the content of phenolic compounds, as maintenance the mineral is a prerequisite to provide cofactors for many enzymes in the phenylpropanoid pathway in plants of *Prumus callus*, *Helianthus annuus* or *Tobacco*, between others species (Treutter 2010).

Table 5. The effect of Zn biofortification on phenolic (mg g^{-1} DW) content in the edible part of *B. oleracea*

Zn treatment (μM)	Caffeic acid derivatives	Sinapic acid derivatives	Flavonols	Total phenols
10	4.08 ± 0.15 b	441.53 ± 15.26 c	57.90 ± 0.01 d	539.93 ± 37.45 c
20	5.23 ± 0.21 a	436.47 ± 14.52 c	66.62 ± 1.90 b	544.07 ± 45.28 c
40	5.25 ± 0.03 a	490.81 ± 3.14 b	77.46 ± 0.97 b	590.96 ± 18.01 bc
80	4.12 ± 0.11 b	492.30 ± 5.81 b	58.26 ± 1.98 a	636.39 ± 2.30 ab
100	4.34 ± 0.54 b	628.14 ± 3.60 a	87.16 ± 0.54 a	720.15 ± 5.28 a
p-Value	***	***	***	**
$LSD_{0.05}$	0.40	31.33	4.17	86.99

Significance levels are represented by p > 0.05. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means followed by the same letter do not significantly differ

The final step of the phenylpropanoid pathway results in the formation of the compound *p*-coumaroyl CoA, producing a great number of secondary products derived from phenylpropanoids in plants, such as flavonoids and isoflavonoids, coumarins, lignins, hydroxycinnamic acids, esters, and phenolic compounds (Jones 1984). In this respect, we observed an increase of the caffeic acid derivatives concentrations only with the treatment of 20 and 40 μM to respect to the control plants (Table 5). However, the sinapic acid derivatives increased when the dose applied were in a range of 40-100 μM, reaching a maximum values up 42% in the treatment of 100 μM respect to 10 μM (Table 5). Respect to the flavonols and total phenols concentration, in both cases were maintenance or increased when the doses applied were higher than the control plants, reaching the maximum values in plants growing under 80 and 100 μM (Table 5).

Our data reveal that an adequate supplementation of Zn, especially at the rates of 80 and 100 μM, promote a higher concentration of Zn, an induced higher activity in the

synthesis pathway of amino acids, GSLs and phenolic compounds without adversely effect on the biomass of the edible part of *B. oleracea*. On the basic of these results, the present study reveals that the quality of *B.oleracea* can be improved in a Zn-biofortification programme, it would provide this crop added nutritional value for the human diet, consisting of intake of primary and secondary metabolites and the micronutrient element Zn.

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Abbreviattion used

4CL, 4-coumarate coenzyme A ligase; AA, amino acid; AAA, aromatic amino acid; ATP-S, ATP-sulphurylase; CAD, cinammyl alcohol dehydrogenase; C4H, cinnamate 4-hydrolase; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; GSL, glucosinolate; *O*AS-TL, *O*-acetylserine (thiol) lyase; PAL, phenylananine ammonium lyase; SAT, serine acetyltransferase; SKDH, shikimate dehydrogenase.

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5.4. Discusión general

La biofortificación es una herramienta es una herramienta excelente, para disminuir la deficiencia de Zn en los cultivos, disminuyendo de este modo la aplicación excesiva con este micronutriente así como la deficiencia de este nutriente esencial en humanos. Sin embargo, la mayoría de los estudios realizados han centrado su atención en la realización de este tipo de programas en cereales o legumbres. No obstante, es bien conocido que estos tipos de cultivos tienen una capacidad inherentemente baja para acumular este elemento además de presentar ciertos compuestos como los fitatos que disminuyen la biodisponibilidad de nutrientes en el ser humana. Por ello, el objetivo de esta segunda parte de Tesis Doctoral ha sido estudiar el efecto de una suplementación supra-óptima en dos plantas hortícolas de consumo diario y de fácil acceso. Además, es bien sabido que las frutas y verduras poseen una gran cantidad de compuestos beneficiosas para la salud humana. Esto nos permitirá seleccionar que planta es la más apropiada para la realización de este tipo de programas, incrementando tanto la concentración de este micronutriente en la parte aérea como su calidad.

En términos generales podemos definir las dosis utilizadas en este estudio como óptimas o supra-óptimas para ambas plantas, debido a que un incremento en la aplicación de Zn no produjo efectos deletéreos en la parte comestible de ambas plantas manteniendo su calidad en términos de antioxidantes así como del balance de nutrientes, especialmente con la dosis de 20 μM en *L. sativa* y 20-80 μM en *B. oleracea*. Sin embargo, de manera global podemos definir a *B. oleracea* como la especie más eficiente en este tipo de programas. Estos datos se encuentran en concordancia con los datos

obtenidos en la primera parte de esta Tesis Doctoral, donde comprobamos que esta especie era capaz de acumular más Zn en condiciones de toxicidad que *L. sativa*.

Además debido a un incremento en la necesidad de aumentar la producción en los últimos años, se ha fertilizado en exceso con nitrógeno pudiendo resultar este tóxico para el ser humano por su acumulación en forma de NO_3^- en la parte comestible de las plantas. En este sentido, y sabiendo que *L. sativa* es hiperacumuladora de este compuesto tóxico, en este estudio se comprobó si una fertilización adecuada con Zn podría promover la eficiencia en el uso del N. Nuestros datos revelan que *L. sativa* es capaz de responder eficientemente en un programa de biofortificación con Zn, incrementando la concentración de este micronutriente en la parte aérea promoviendo el metabolismo del nitrógeno y la fotorrespiración, así como una mayor acumulación de aminoácidos esenciales para la salud humana, especialmente en plantas suplementadas con 80 μM de Zn.

Por otra lado, el Zn puede interaccionar sinérgica o antagónicamente con otros macro o micronutrientes. En este sentido, y sabiendo que *B. oleracea* es una importante fuente de compuestos bioactivos, se llevó a cabo un estudio sobre el efecto del Zn el perfil aminoacídico y el metabolismo de los glucosinolatos y fenoles debido a las propiedades beneficiosas que estos compuestos pueden ofrecer a la salud humana. En base a nuestros resultados, el Zn claramente puede promover la síntesis de compuestos primarios como los aminoácidos, así como secundarios como los glucosinolatos y compuestos fenólicos, sobre todo cuando la dosis aplicada de Zn en esta especie se encuentre en un rango de 80-100 μM.

En definitiva podemos concluir que un consumo de ambas especies hortícolas cultivadas bajo un programa de biofortificación con Zn podría promover un incremento de la ingesta de este micronutriente así como de otros elementos beneficiosos para la salud humana. Sin embargo desde un punto de vista de calidad, un consumo de *B. oleracea* podría aportar una mayor cantidad de elementos beneficiosos. Mientras que un cultivo de *L. sativa* bajo estas condiciones, podría suponer una disminución de la fertilización nitrogenada manteniendo su producción así como incrementar la síntesis de aminoácidos esenciales para el ser humano.

Capítulo 6

Conclusiones

1. According to our results we conclude that *L. sativa* was more sensitive to Zn toxicity than *B. oleracea*, making the latter more effective in a programne of Zn phytoextraction. The mechanisms that allow greater accumulation of Zn without a significant impact on the biomass of the aerial part in *B. oleracea* plants were:

- Increased synthesis and accumulation of organic acid citrate, through induction of citrate synthase enzyme.
- Greater use of reduced glutathione in the detoxification of reactive oxygen species and toxic compounds such as methylglioxal, mainly through the induction of enzymes such as ascorbate peroxidase, glutathione transferase, glutathione peroxidase, glyoxalase I, and glyoxalase II.
- More accumulation of osmoprotector compounds such as γ-aminobutyric acid, by inducing its enzyme of synthesis glutamate decarboxylase and direct detoxification of ROS mediated by proline dehydrogenase.

In short, the possible quelation of Zn with the organic acid citrate together with the detoxification of reactive oxygen species derived from Zn toxicity could be the determining mechanisms of greater tolerance of *B. oleracea*.

2. Adequate Zn fertilization can improve the quality and crop production studied maintaining and increasing the concentration of this micronutrient in plants. The results obtained confirm that both *L. sativa* and *B. oleracea* plants will be effective for its utilization in biofortification programs, although it is noted that the use of *B. oleracea* could be more effective since the characteristics mencioned about were induced more effectively. Moreover, from a nutritional standpoint, the recommended doses in a Zn-biofortification program depend on the plant species and the physiological process studied, so that:

- In *L. sativa*, given all the nutritional characteristics analyzed, the Zn dose recommended would be 20 µM of Zn. However, in terms of reduction of the foliar accumulation of NO_3^- an application of 80 µM Zn appears more effective since the induction of nitrogen and photorespiration metabolisms, which results in a improved of N efficiency and the synthesis of essential amino acids.

- In *B. oleracea*, given all the nutritional characteristics analyzed, the recommended dose of Zn would be in the range of 20-80 µM Zn. However, in terms of increasing the concentration of amino acids and phytochemicals compounds such as glucosinolates and phenols, an application in the range 80-100 µM of Zn may increase the accumulation of these compounds, especially glucoiberin, 4-hydroxyglucobrassicin, sinapic acid, and derivatives flavonols, consumption providing beneficial effects to human health.

Anexo I

Curricullum vitae

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FORMACIÓN ACADÉMICA

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PUBLICACIONES MAS RELEVANTES

Barrameda-Medina, Y., Montesinos-Pereira, D., Romero, L., Ruiz, J.M., Blasco, B (2014) Comparative study of the toxic effect of Zn in *Lactuca sativa* and *Brassica oleracea* plants: I. Growth, distribution, and accumulation of Zn, and metabolism of carboxylates. *Environmental and Experimental Botany* 107, 98–104.

PARTICIPACIÓN EN CONGRESOS

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