Research Article

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

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Abstract

BACKGROUND: Excessive rates of nitrogen (N) fertilizers may result in elevated concentrations of nitrate (NO_3^-) in plants. Considering that many programs of biofortification with trace elements are being performed, it has become important to study how the application of these elements affects plant physiology and, particularly, N utilization in leaf crops. The main objective of the present study was to determine whether the NO_3^- accumulation and the nitrogen use efficiency was affected by the application of different doses of Zn in *Lactuca sativa* plants.

RESULTS: Zn doses in the range 80–100 μmol L^{-1} produced an increase in Zn concentration provoking a decrease of NO_3^- concentration and increase of the nitrate reductase, glutamine synthetase and aspartate aminotransferase activities, as well as the photorespiration processes. As result, we observed an increase in reduced N, total N concentration and N utilization efficiency. Consequently, at a dose of 80 μmol L^{-1} of Zn, the amino acid concentration increased significantly.

CONCLUSION: Adequate Zn fertilization is an important critical player in lettuce, especially at a dose of 80 μmol L^{-1} of Zn, because it could result in an increase in the Zn concentration, a reduction of NO_3^- levels and an increase the concentration of essential amino acids, with all of them having beneficial properties for the human diet.
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Keywords: *Lactuca sativa*; nitrate accumulation; nitrogen use efficiency; Zn biofortification

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.[1] Particularly, among micronutrients, Zn deficiency is occurring in both crops and humans.[2] More than 3 billion people worldwide suffer from micronutrient malnutrition, including Zn deficiency.[2] In this situation, Zn deficiency can cause growth retardation, delayed skeletal and sexual maturity, dermatitis, diarrhoea, alopecia and defects in immune function, with a resulting increase in the susceptibility to infection and maternal mortality in humans.[3]

Recently, a complimentary solution to mineral malnutrition was proposed, known as biofortification. Although this strategy for biofortification is unproven as yet, it has the potential to become both sustainable and cost effective and to reach remote rural populations.[4] Concern about Zn, and the impact of its application on increasing crop yields has been recorded for most crops, both under irrigated and rainfed conditions,[5] and the recent literature indicates that the combination of agronomic biofortification with breeding is an applicable and sustainable approach to the Zn-deficiency problem in humans.[6]

In addition to the nutritional quality of agricultural products destined for human consumption, another aspect to consider is the fact that the rapid population growth over recent decades has generated an increase in the demand for vegetable products. In this sense, further estimates suggest that agricultural production must increase by 70% in 2050 to feed over 9 billion people worldwide[5] and, because plants require nitrogen (N) in large amounts, N frequently limits plant development. For this reason, the application of N fertilizers is a common practice. However, high N applications are costly and may pollute surface and ground waters. Indeed, 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.[7] As a result, a high consumption of these plants could lead to serious pathological disorders in human health. That is, an excess of NO_3^- has been associated with respiratory problem, such as, the 'blue baby' syndrome, blood disorders (methemoglobinemia) and the formation of carcinogenic compounds in the human digestive tract.[8]

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Particularly, green leafy vegetables are among the vegetable species most susceptible to NO_3^- accumulation, such as lettuce, spinach, broccoli, cabbage, celery, radish and beetroot, etc. Especially, plants belonging to the Asteraceae family, such as lettuce, are among those accumulating more NO_3^- in their leaves[7] and, as a result of the health hazards arising from the human consumption of NO_3^- in lettuce, legislation has been introduced setting maximum limits on the nitrate contents of these crops.[9]

Because the consumption of these crops can harm human health, the development of a suitable strategy and the cultivation of edible crops with a low NO_3^- content are both very important. In this sense, N use efficiency (NUE) is an important environmental and social issue.[10] 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, optimizing N supply in correlation with the desired final produce quality,[11] or selecting and growing N-efficient crop genotypes, which, also has high commercial value because it can increase yields with lower N application rates, even in soils where N is a limiting element.[12]

In this respect, fortification of horticultural produce, and leafy vegetables in particular, could be a successful strategy for improving human diets, resulting in an increase in the value and quality of the produce itself.[11] In this sense, Hulagur and Dangarwala[13] demonstrated that an application of N influences in the Zn absorption by plants and vice versa. Indeed, 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, the N-nutritional status of plants is an important critical player in root uptake and the accumulation of Zn in plants and deserves special attention in the biofortification of food crops with Zn.[14] The main objective of the present study was to determine whether NO_3^- accumulation and NUE was affected by the application of different doses of Zn and to clarify the influence of this trace element in a biofortification program in lettuce plants. Accordingly, we analyzed the effect of Zn on nitrogen metabolism, photorespiration and the final products of those processes.

MATERIALS AND METHODS

Plant materials and experimental design

Seeds of *Lactuca sativa* cv. Phillipus were germinated and grown for 35 days in cell flats of $3 \times 3 \times 10$ cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant SL, Spain). After 35 days, the seedlings were transferred to a growth chamber under a 12:12 h photoperiod at a photosynthetic photon flux density 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) at a day/night temperature of 22/18 °C and 50% relative humidity. Under these conditions, the plants were grown in hydroponic culture in lightweight polypropylene trays (top diameter 60 cm, bottom diameter 60 cm, height 7 cm) 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 mmol L^{-1} KNO_3, 3 mmol L^{-1} Ca $(NO_3)_2 \cdot 4H_2O$, 2 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 6 mmol L^{-1} KH_2PO_4, 1 mmol L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 2 μmol L^{-1} $MnCl_2 \cdot 4H_2O$, 0.25 μmol L^{-1} $CuSO_4 \cdot 5H_2O$, 0.1 μmol L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 10 μmol L^{-1} Fe-ethylenediamine di-*O*-hydroxyphenyl acetic acid (Sequestrene-138 FeG100) and 10 μmol L^{-1} 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, 80 and 100 μmol L^{-1} of $ZnSO_4 \cdot 7H_2O$]. This solution, with a pH of 5.5–6.0, was changed every 3 days. The experimental design was a randomized complete block with six treatments arranged in individual trays with eight plants per treatment and three replications each, so that the total number of plants was 120.

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.

Zn concentration

For the determination of Zn concentration, a sample of 0.15 g of dry material was subjected to a process of mineralization. Mineral analysis was conducted using inductively coupled plasma mass spectrometry. Briefly, samples were further diluted 1:10 with milli-Q water (Millipore, Billerica, MA, USA) and analyzed using an inductively coupled plasma mass spectrometer (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, UK) included Zn, in the range 0–100 μg L^{-1}. The Zn concentration was expressed as μg g^{-1} DW.

Enzyme extractions and assays

Leaves were ground in a mortar at 0 °C in 50 mmol L^{-1} KH_2PO_4 buffer (pH 7.5) containing 2 mmol L^{-1} ethylenediaminetetracetic acid (EDTA), 1.5% (w/v) soluble casein, 2 mmol L^{-1} dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at $30\,000 \times g$ for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure enzyme activity of nitrate reductase (NR) and glutamate dehydrogenase (GDH).

The NR assay followed the methodology of Kaiser and Lewis.[15] The NO_2^- formed was colorimetrically determined at 540 nm after azocoupling with sulfanilamide and naphthylethylenediamine dihydrochloride.

Glutamine synthetase (GS) was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis.[15] Leaves were ground in a mortar at 0 °C in 50 mL of maleic acid-KOH buffer (pH 6.8) containing 100 mmol L^{-1} sucrose, 2% (v/v) β-mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at $30\,000 \times g$ 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 mmol L^{-1} KH_2PO_4 buffer (pH 7.5) with 4 mmol L^{-1} EDTA, 1000 mmol L^{-1} L-sodium glutamate, 450 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 300 mmol L^{-1} hydroxylamine, 100 mmol L^{-1} ATP and enzyme extract. Two controls were prepared: one without glutamine and

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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.

GDH activity was assayed by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance.[16] The reaction mixture consisted of 50 mmol L^{-1} KH_2PO_4 buffer (pH 7.5) with 200 mmol L^{-1} NH_4^+ sulfate, 0.15 mmol L^{-1} NADH, 2.5 mmol L^{-1} 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.

Aspartate aminotransferase (AAT) activity was assayed spectrophotometrically at 340 nm using the method published by González *et al.*[17] AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mmol L^{-1} Tris–HCl buffer (pH 8), 4 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} aspartic acid and enzyme extract. The decrease in absorbance was recorded for 3 min.

For determination of glutamate-glyoxylate aminotransferase (GGAT), serine-glyoxylate aminotransferase (SGAT) and hydroxypyruvate reductase (HPR), leaves were ground in a chilled mortar in 100 mmol L^{-1} Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mmol L^{-1} DTT. The homogenate was centrifuged at 20 000 × *g* for 10 min. The resulting extract was used to measure enzyme activity. 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 mmol L^{-1} Tris HCl (pH 7.3), 20 mmol L^{-1} glutamate, 1 mmol L^{-1} glyoxylate, 0.18 mmol L^{-1} NADH, 0.11 mmol L^{-1} pyridoxal-5-phosphate, 83 mmol L^{-1} NH_4Cl and 0.3 U of GDH in a final volume of 0.6 mL.[18]

SGAT activity was measured by determining the rate of glycine formation from glyoxylate.[18] The reaction was assayed in a mixture containing 100 mmol L^{-1} Tris–HCl (pH 7.3), 5 mmol L^{-1} serine, 1 mmol L^{-1} glyoxylate, 0.11 mmol L^{-1} pyridoxal-5-phosphate, 83 mmol L^{-1} NH_4Cl and 0.3 U of 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 HPR assay was performed with 100 mmol L^{-1} Tris–HCl (pH 7.3), 5 mmol L^{-1} hydroxypyruvate and 0.18 mmol L^{-1} NADH. Activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm.[18]

The protein concentration of the extracts was determined according to the method of Bradford,[19] using bovine serum albumin as the standard.

N forms analysis and nitrogen use efficiency

NO_3^- was analyzed from an aqueous extraction of 0.1 g DW in 10 mL of Millipore-filtered water. An aliquot of 100 μL 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.*[20] Ammonium (NH_4^+) was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom.[21] For the reduced N determination, a sample of 0.15 g DW was digested with sulfuric acid and H_2O_2. After dilution with deionized water, an aliquot (1 mL) of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 mmol L^{-1} 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.[22] 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.[23]

Amino acid analysis by oPA-high-performance liquid chromatography (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 Sep-Pak C18 Light Cartridges (Waters, Milford, MA, USA). 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 obtained Gilson Inc. (Middleton, WI, USA). The *o*PA derivatives were separated on a reverse-phase C18 ultrasphere column (250 × 4.6 mm). Solvent A consisted of 50 mmol L^{-1} NaOAc (pH 7) plus 1% tetrahydrofurane, and solvent B was absolute methanol (Carlo Erba, Milan, Italy). 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 Inc.). Quantification of single amino acids was made against a relative calibration curve and expressed as μmol g^{-1} FW.[24]

Statistical analysis

Data were subjected to a simple analysis of variance (ANOVA) at 95% confidence, using Statgraphics, version 5.1 (http://www.statgraphics.com). Means were compared by Fisher's least-significant differences (LSD). $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered statistically significant for both analyses, or as NS (not significant).

RESULTS

Biomass and Zn concentration

The present study clearly demonstrated that the application of increasing doses of Zn does not lead to a decrease in the biomass of the edible part of *L. sativa* with respect to the basal dose (Table 1). In addition, an increase in the dose of Zn supplemented in the nutrient solution was accompanied by a concomitant increase in the concentration of Zn in the edible part of *L. sativa* (Table 1).

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

Zn dose (μmol L^{-1})	Leaf biomass g DW)	Zn concentration (μg g^{-1} DW)
10	1.75 ± 0.31^a	75.47 ± 3.18^f
20	1.50 ± 0.24^a	115.74 ± 0.60^e
40	1.59 ± 0.12^a	131.64 ± 4.25^d
80	1.42 ± 0.12^a	232.77 ± 2.64^b
100	1.66 ± 0.01^a	265.25 ± 3.58^a
P-value	NS	***
$LSD_{0.05}$	0.60	9.80

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant. Means followed by the same superscript lowercase letter do not differ significantly.

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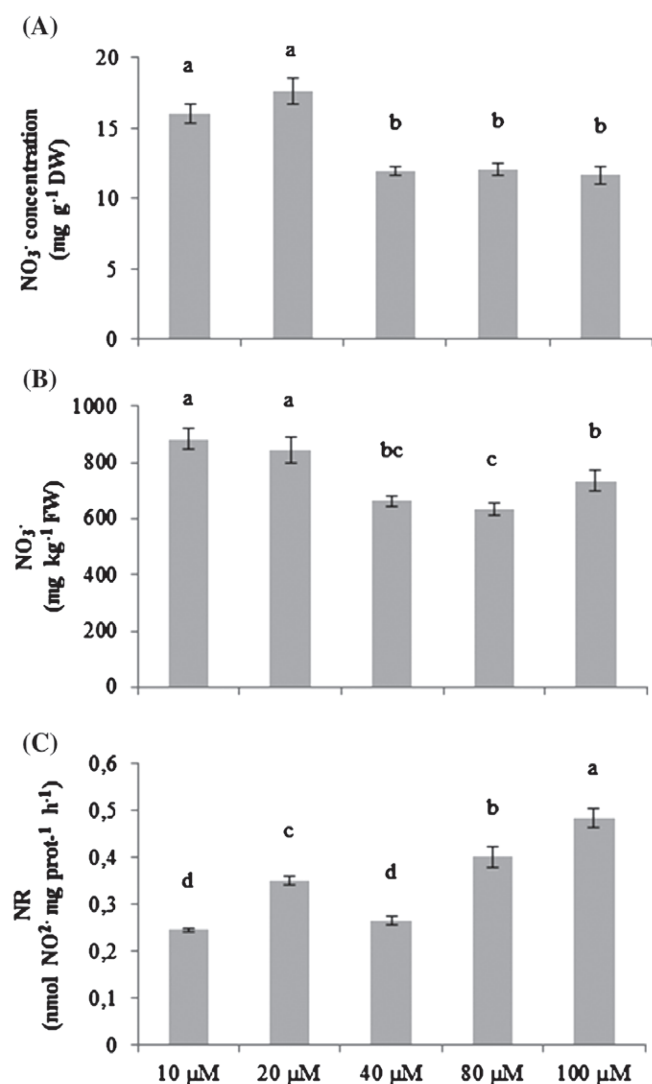

Figure 1. Response of NO_3^- concentration (A, B) and NR activity (C) in the edible part of *L. sativa* submitted to different Zn application. Values are means ($n = 9$) and differences between means were compared using Fisher's test least significant difference (LSD, $P = 0.05$).

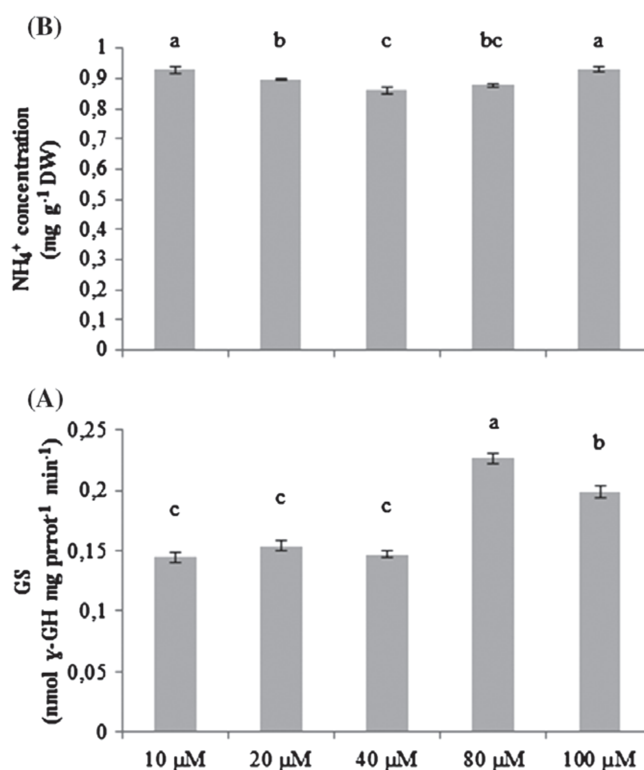

Figure 2. Response of NH_4^+ concentration (A) and enzymes responsible for NH_4^+ assimilation: GS in the edible part of *L. sativa* submitted to different Zn application (B). Values are means ($n = 9$) and differences between means were compared using Fisher's test least significant difference (LSD,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

Nitrate reduction

In general, when the dose of Zn was increased in the nutrient solution from 20 µmol L^{-1}, the NO_3^- concentration decreased significantly with respect to the control ($P < 0.001$) (Fig. 1A, B). Likewise, NR activity was positively affected when the dose of Zn was increased in the nutrient solution, reaching maximum values with a dose of 100 µmol L^{-1} with respect to control plants ($P < 0.001$) (Fig. 1C).

Ammonium assimilation

The NH_4^+ concentration decreased significantly when the doses of Zn applied were in the range 20–80 µmol L^{-1} ($p < 0.001$) (Fig. 2A). With respect to GS and AAT activities, both enzymes showed a similar pattern. With an increasing Zn dose, there was an increase in both activities, reaching maximum values when the dose applied was 80 µmol L^{-1} with respect to control plants ($P < 0.001$) (Figs 2B and 3B). Finally, GDH activity was affected negatively; except with a dose of 20 µmol L^{-1}, reaching minimum values when the dose applied was 100 µmol L^{-1} with respect to control plants ($P < 0.001$) (Fig. 3A).

Photorespiratory cycle

GGAT, SGAT and HPR activities were markedly increased when the Zn dose was increased in the solution (Table 2). GGAT increased significantly when the dose applied was in the range 20–80 µmol L^{-1} (Table 2). On the other hand, SGAT increased for all Zn treatments, reaching the higher value at a dose of 100 µmol L^{-1} of Zn with respect to 10 µmol L^{-1} (Table 2). Finally, the HPR activity increased strongly for all treatments, reaching maximum values when the dose applied was 80 µmol L^{-1} with respect to the basal Zn dose (Table 2).

Nitrogen use efficiency

The reduced N concentration increased as Zn supplementation was increased, reaching maximum values at a dose of 20, 80 and 100 µmol L^{-1} with respect to the basal dose of Zn ($P < 0.001$) (Fig. 4A). Likewise, TNC was not affected negatively, showing a significant increase when the doses applied were 20 and 100 µmol L^{-1} ($P < 0.05$) (Fig. 4B). Finally, NUtE was positively correlated with an increase of the dose of Zn applied in the nutrient solution, reaching maximum values when the dose applied was 100 µmol L^{-1} with respect to 10 µmol L^{-1} ($P < 0.001$) (Fig. 4C).

Amino acid 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 the range

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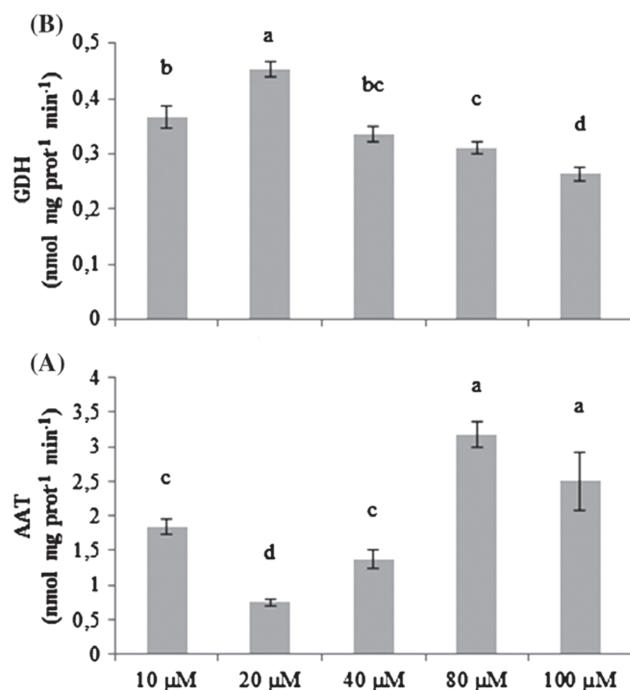

Figure 3. Response of GDH (A) and AAT (B) under different Zn supplementation in the edible part of *L. sativa*. Values are means ($n = 9$) and differences between means were compared using Fisher's test least significant difference (LSD, $P = 0.05$).

Zn dose (µmol L^{-1})	GGAT (nmol mg protein^{-1} min^{-1})	SGAT (nmol mg protein^{-1} min^{-1})	HPR (µmol mg protein^{-1} min^{-1})
10	4.55 ± 0.11^{b}	1.47 ± 0.03^{e}	0.27 ± 0.00^{c}
20	5.81 ± 0.20^{a}	2.41 ± 0.04^{c}	0.39 ± 0.01^{ab}
40	5.58 ± 0.19^{a}	1.83 ± 0.04^{d}	0.39 ± 0.01^{ab}
80	5.78 ± 0.14^{a}	2.73 ± 0.04^{b}	0.40 ± 0.01^{a}
100	5.02 ± 0.24^{b}	3.10 ± 0.13^{a}	0.37 ± 0.01^{b}
P-value	***	***	***
LSD$_{0.05}$	0.52	0.20	0.02

Table 2. Response of some photorespiration enzymes in the edible part of *L. sativa* submitted to different application of Zn

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant. Means followed by the same superscript lowercase letter do not differ significantly.

40–80 µmol L^{-1}. Moreover, an increase in the Zn concentration at a does of 80 µmol L^{-1} resulted in the maximum values of Leu and Ile with respect to the control plants. Finally, at a dose of 100 µmol L^{-1} of Zn, the amino acids Asp, Asn and Arg were increased with respect to the basal dose (Table 2). However, the amino acids Phe and His decreased when the plants were grown at doses higher than 10 µmol L^{-1} (Table 2).

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.[2] For this reason, among other essential parameters, an appropriate supply of micronutrients is also essential for the proper growth and yield of a crop. In this sense, the biofortification of crops has been proposed as a way of improving plant food nutritional quality, and is defined as the process of raising the concentration of bioavailable essentials, including Zn, in the edible parts of crops through agricultural practices or genetic selection.[4]

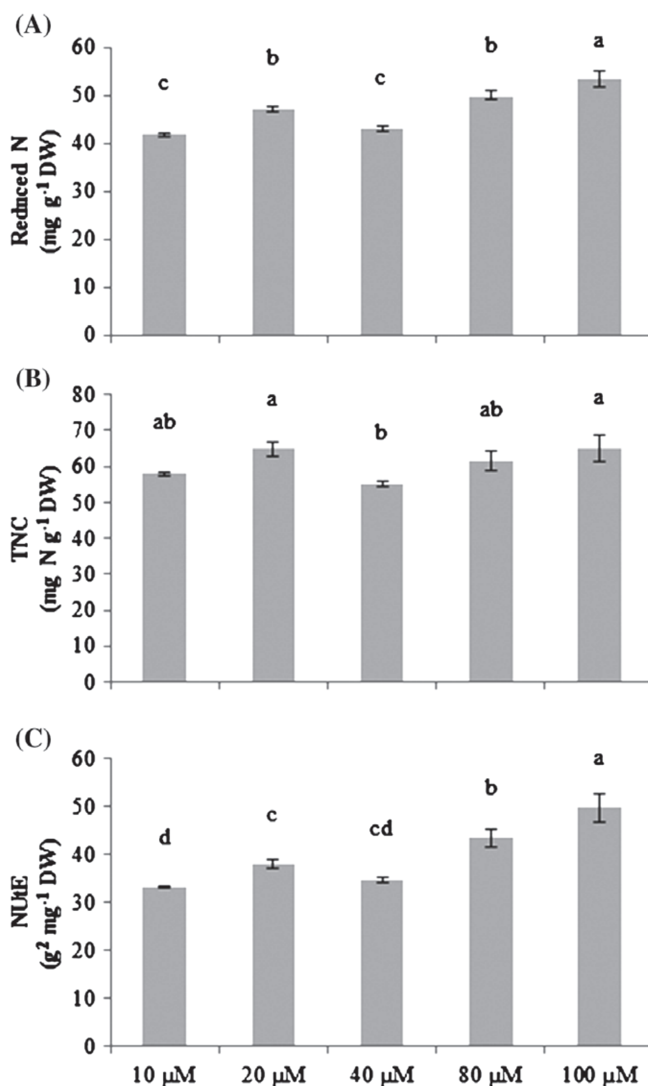

Figure 4. Effect of different Zn supplementations on the foliar concentration of reduced N (A) and TNC (B) and NUtE (C) in the edible part of *L. sativa*. Values are means ($n = 9$) and differences between means were compared using Fisher's test least significant difference (LSD, $P = 0.05$).

Regarding the essential parameters in research on nutrient efficiency, biomass determination is one where, especially in lettuce plants, the growth of the aerial part is a determining factor in the agricultural value of this crop.[25] The results obtained in the present study with respect to the biomass of the edible parts reveal that the doses of Zn applied maintained the biomass of the edible parts of *L. sativa* plants in all treatments with respect to the control plants (Table 1). Similar to our results, recent studies have demonstrated that an adequate application of Zn can improve nutritional quality and production in pea plants.[26]

On the other hand, we showed that an increase in Zn in the nutrient solution was correlated with a higher concentration of Zn in the edible parts of *L. sativa*, reaching maximum values for a dose of 100 µmol L^{-1} of Zn, representing values of up to 251% with respect to the control plants without reaching the levels

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considered to be toxic (>300 μg g^{-1} DW) (Table 1). Similar effects have been demonstrated in plants of *Thlaspi caerulescens*, *Brassica oleracea* and *Lactuca sativa* plants.[27,28] Based on these results, we can define all of the doses used in the present study as optimal and supra-optimal (<700 μg g^{-1} DW).

N plays a pivotal role in the plant metabolism and hence in determining growth. The inorganic nutrition of plants has been dominated by the N because it improves both quantity and quality of the produce.[29] However, an excessive use of N fertilizers has generated serious environmental contamination. Indeed, a 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, and therefore diminishes the nutritional quality of crops.[7] Thus, great efforts have been made with respect to reducing the NO_3^- foliar content in lettuce, such as by partial substitution of NH_4^+ for NO_3^- in pots filled with an organic substrate,[30] and, in recirculating nutrient solution, an application of different doses of N and biofortification programs with minerals including selenium and iodine.[11,31,32]

Figure 1A shows the results for the foliar concentration of NO_3^-, with a significant decline when the application of Zn was higher than 20 μmol L^{-1}. Moreover, the European Commission[9] has set upper NO_3^- limits in lettuce leaves of between 3500 and 4500 mg kg^{-1} FW, and it should be noted that the NO_3^- concentrations in the present study were below the imposed limit, regardless of the rate Zn applied (Fig. 1B), suggesting that an adequate supplementation of Zn increases the quality of lettuce. Similar to our results, Stefanelli *et al.*[11] have demonstrated that normal fertilization with N will increase lettuce quality, reducing the NO_3^- content and increasing essential elements such as Zn, as well as increasing antioxidant capacity. However, the effect of Zn biofortification on the NO_3^- concentration in *L. sativa* has not yet been studied. In the present study, we confirm that an adequate application of Zn is a powerful tool in these horticultural plants.

It is well known that Zn is required as a structural and catalytic component of enzymes for normal growth and development. Indeed, Zn is very closely involved in the N metabolism of plants.[33] With respect to this metabolism, the enzyme NR plays a key role in the reduction of NO_3^-.[34] The present data reveal that an increase of Zn in the nutrient solution promoted a sharp increase of NR activity of up to 63% (80 μmol L^{-1}) and 97% (100 μmol L^{-1}) (Fig. 1C). Some studies have demonstrated a high inhibition of NR under Zn deficient conditions in plants of *Oryza sativa* and *Pennisetum americanum* such as under Zn toxicity in *Triticum aestivum*.[35,36] However, in leaves of *L. sativa*, an increase of the Zn dose 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 study reporting that an increase of Zn was correlated with NR activity in *Cicer arietinum*.[37] Likewise, an increase in the NR activity explains the drastic decline of the NO_3^- concentration (Fig. 1A, C), 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) occurs through the cooperative activity of GS and glutamine oxoglutarate aminotransferase (GOGAT). Because 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 N metabolism and has been considered as the major assimilatory enzyme for ammonia produced from N fixation, as well as NO_3^- or NH_4^+ nutrition.[38] In the present study, we show that the NH_4^+ concentration in the leaves does not increase, or even decrease, with an increase in the Zn dose (Fig. 2A). Additionally, GS activity in *L. sativa* plants was strongly affected positively at Zn doses higher than 40 μmol L^{-1} and it should be noted that an application of 80 μmol L^{-1} resulted in up to 57% higher activity with respect to the control plants (Fig. 2B). In accordance with the results of the present study, Thomsem *et al.*[39] have demonstrated a strong correlation between the levels of NH_4^+ and GS activity. Thomsem *et al.*[39] concluded that an overexpression of GS in leaves could be advantageous for efficient NH_4^+ assimilation and also the export of amino acids via the phloem, promoting plant growth. Indeed, the overexpression of this enzyme promoted a decrease of NH_4^+ of up to seven-fold less than that in tobacco plants.[40] Regarding Zn, a decline of the GS activity was noted in *P. americanum* under Zn deficiency.[35] Furthermore, in the present study, we observed a positive correlation for NO_3^-–NR–GS (Figs 1A, C and 2B). For this reason, the Zn doses applied were adequate for 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 μmol L^{-1}.

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 K_m for NH_4^+, indicating that it is unlikely to act in favor of biosynthesis. In *L. sativa* leaves, the activity of GDH decreased at the same time that the Zn dose was higher than 60 μmol L^{-1} (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.[38] In this sense, Frechilla *et al.*[41] 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 that toxic levels of NH_4^+ in plants can reduce GS activity. According to the results of the present study, we can define the Zn doses applied as being appropriate in a biofortification program with this element because an increase of this element promoted a higher activity of the key enzyme of the N metabolism (NR, GS) improving NO_3^- reduction, such as via NH_4^+ assimilation. Finally, the enzyme AAT plays a key role in the metabolic regulation of C and N metabolism in all organisms. In general, but particularly under C shortage, the action of AAT ensures that the different keto-acids needed by the plant are available.[38] In this respect, *L. sativa* showed a similar effect on AAT activity with respect to NR and GS, in that an increase of the application of Zn induced a concomitant increase of AAT activity, with the maximum values obtained at 80 μmol L^{-1}, reaching values of up to 109% with respect to the basal dose (Fig. 3B). An increase of NR and GS could promote a higher use of the Gln and Glu produced from the cycle GS/GOGAT and, subsequently, the formation of aspartate (Asp) and other essential amino acids required for development by AAT activity. As noted above, the biomass of the edible part of *L. sativa* was not affected negatively (Table 1) and this could be the result of 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 generally referred to as secondary NH_4^+ assimilation and include amino acid and protein catabolism, phenylpropanoid biosynthesis and, especially, photorespiration.

N metabolism is complicated in the leaves of C3 plants by fluxes of NH_4^+ around the photorespiratory cycle, which can involve 20-fold more NH_4^+ than that generated by the reduction of NO_3^-.[42] For this reason, photorespiration has been considered as a wasteful process.[43] In this regard, photorespiration suppression

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Table 3. Impact of Zn supplementation on amino acid concentration in the edible part of *L. sativa*

Amino acids (mmol g^{-1} FW)	Zn dose (μmol L^{-1}) 10	20	40	80	100	*P*-value	$LSD_{0.05}$
Glu	7.50 ± 0.00^{a}	5.77 ± 0.07^{c}	7.59 ± 0.17^{a}	6.55 ± 0.02^{b}	4.54 ± 0.12^{d}	***	0.32
Gln	0.56 ± 0.00^{d}	1.70 ± 0.61^{c}	2.04 ± 0.06^{b}	2.41 ± 0.20^{a}	1.69 ± 0.05^{c}	***	0.10
Asp	2.64 ± 0.21^{c}	3.75 ± 0.03^{b}	2.40 ± 0.08^{c}	3.61 ± 0.04^{b}	4.34 ± 0.30^{a}	***	0.54
Asn	0.47 ± 0.01^{b}	0.54 ± 0.00^{b}	0.67 ± 0.01^{b}	0.67 ± 0.01^{b}	1.54 ± 0.15^{a}	***	0.21
Leu	0.56 ± 0.02^{b}	0.64 ± 0.02^{a}	0.59 ± 0.00^{b}	0.66 ± 0.01^{a}	0.44 ± 0.00^{c}	***	0.04
Ile	0.52 ± 0.02^{c}	0.58 ± 0.01^{b}	0.58 ± 0.00^{b}	0.62 ± 0.01^{a}	0.59 ± 0.01^{ab}	***	0.04
Thr	0.87 ± 0.00^{c}	0.81 ± 0.00^{d}	0.94 ± 0.00^{a}	0.90 ± 0.01^{b}	0.68 ± 0.01^{e}	***	0.02
Tyr	0.09 ± 0.00^{c}	0.09 ± 0.00^{c}	0.12 ± 0.00^{a}	0.11 ± 0.00^{b}	0.10 ± 0.00^{c}	***	0.01
Lys	4.71 ± 0.12^{b}	5.55 ± 0.02^{a}	5.70 ± 0.04^{a}	5.52 ± 0.03^{a}	3.76 ± 0.01^{c}	***	0.18
Ser	1.74 ± 0.02^{c}	1.41 ± 0.02^{d}	2.37 ± 0.02^{a}	2.08 ± 0.01^{b}	1.60 ± 0.01^{c}	***	0.14
Gly	0.09 ± 0.00^{d}	0.12 ± 0.00^{c}	0.37 ± 0.01^{a}	0.17 ± 0.00^{b}	0.13 ± 0.01^{c}	***	0.02
Ala	2.26 ± 0.01^{d}	2.28 ± 0.00^{d}	4.06 ± 0.02^{a}	3.40 ± 0.00^{c}	3.87 ± 0.04^{b}	***	0.07
Phe	0.82 ± 0.06^{a}	0.61 ± 0.00^{b}	0.63 ± 0.00^{b}	0.64 ± 0.01^{b}	0.45 ± 0.00^{c}	***	0.09
Trp	0.39 ± 0.01^{d}	0.67 ± 0.01^{a}	0.51 ± 0.01^{b}	0.49 ± 0.001^{c}	0.36 ± 0.00^{e}	***	0.03
His	3.74 ± 0.01^{a}	ND	0.36 ± 00^{b}	ND	0.24 ± 0.01^{c}	***	0.04
Arg	0.48 ± 0.00^{d}	0.65 ± 0.01^{b}	0.54 ± 0.00^{c}	0.35 ± 0.00^{e}	1.31 ± 0.01^{a}	***	0.04
Val	0.26 ± 0.01^{a}	0.25 ± 0.00^{a}	0.25 ± 0.00^{a}	0.24 ± 0.01^{a}	0.23 ± 0.02^{a}	NS	0.03

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ND, not detected; NS, not significant. Means followed by the same superscript lowercase letter do not differ significantly.

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.*[44] indicated that this process can provide protection against photoinhibition and increase protection against different types of stress. Likewise, photorespiration appears to be an essential process in the control of amino acid biosynthesis and metabolism.[43] Igarashi *et al.*[43] have demonstrated that the peroxisomal glyoxylate aminotransferases (GGAT and SGAT) play a central role within the photorespiratory pathway. In the present study, in general, we observed that, with an increase in Zn doses in the nutrient solution, the enzymes GGAT and SGAT showed higher activities than the control plants, reaching values of up 27% (80 μmol L^{-1}) for GGAT activity and 86% (80 μmol L^{-1}) or 111% (100 μmol L^{-1}) (Table 2). On the other hand, the enzyme HPR completes this cycle. In the present study, this enzyme exhibited a similar effect as GGAT and SGAT (Table 2). A dose in the range 20–100 μmol L^{-1} promoted an increase in HPR activity, especially at doses of 80 μmol L^{-1}, reaching values of up to 37% with respect to basal doses (Table 2).

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 and HPR could function cooperatively in the production of amino acids.[43] 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 the Zn doses applied in the present study, especially 80 μmol L^{-1} of Zn, could be sufficient for maintaining an suitable balance of C/and N and promoting the amino acid synthesis and 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.[5] In addition, an improvement of NUE is a major goal for crops, in which the selection and growth of N-efficient crops is a special issue.[10–38] However, there is still a lack of sufficient experimental data to enable conclusions to be made about the effects of micronutrients on the uptake and utilization of other essential plant nutrients. Likewise, Erenoglu *et al.*[14] indicated that the N-nutritional status of plants is an important critical player in root uptake and the accumulation of Zn in plants and deserves special attention with respect to 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. Figure 4A shows that the value for reduced N was maintained or even higher when the dose of Zn applied was higher, reaching maximum values of up to 27% (100 μmol L^{-1}) respect to control plants. Smirnnoff and Stewart[45] demonstrated that Zn deficiency can cause a decrease in the concentrations of reduced N compounds and the development of shoots. Additionally, the leaf N concentration not only represents N uptake and nutrition status in plants, but also is highly correlated with plant growth and biomass. Moreover, TNC was also determined as indices of the NO_3^- nutrition status of pure hydroponic grown.[46] With respect to TNC, we showed a similar effect of this index on the reduced N concentration (Fig. 4B).

Similar to our results, Hernandez *et al.*[46] demonstrated a positive correlation in the TNC and NR wiith respect to NO_3^- status in plants. Finally, another index used for NUE definitions is NUtE.[23] In this regard, Fig. 4C shows that an increase of Zn dose leads to an increase of this parameter, reaching maximum values with higher doses, especially 100 μmol L^{-1} (50% higher than basal dose). On the basis of our results, these data suggest that adequate fertilization with Zn can promote NUE in *L. sativa* plants.

N metabolism process involves uptake, transport, assimilation and utilization for amino acid biosynthesis and, ultimately, for growth, and all of these steps are crucial for NUtE.[47] Some different studies have demonstrated that an increase of enzymatic N activities are strongly implicate in a NutE (i.e. NR, GS and GGAT).[39,43,46]

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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, amino acid metabolism is tightly linked to carbohydrate metabolism, NH_4^+ (absorbed and synthesized from NO_3^-) and the 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 and development. In general, on the basic of our results, we can confirm that a Zn-biofortification program at a dose in the range 40–100 μmol L^{-1} can promote an increase in the amino acid concentration (Table 2). That is, in the range 40–80 μmol L^{-1}, the amino acids Gln, Asn, Thr and Tyr were increased and these data could be explained by the higher activities of the enzymes involved in N metabolism, such as GS and AAT (Figs 2B and 3B and Table 2). Moreover, an increase of both enzymes was accompanied by an increase of Asp and Ile when the dose applied was 80 μmol L^{-1} with respect to the basal dose (Table 2). On the other hand, at a dose of 100 μmol L^{-1}, we observed that an increase of the amino acid Asp was correlated with an increase of the amino acid Asn, and this could explain the increase of the asparagine synthetase activity, which in turn could explain the decrease of the transport of certain amino acids, such as Thr and Lys in this treatment (Table 2). Finally, there was an increase of Ser and Gly concentrations with respect to control plants (Table 2). These results are related to the higher activities observed in some of the enzymes involved in the secondary assimilation of NH_4^+, such as GGAT, SGAT and HPR (Table 3). Our results are in concordance with those of Samreen *et al.*,[48] who demonstrated that Zn content and N compound concentrations were higher when a higher dose of Zn was applied. On the basic of the results of the present study, *L. sativa* grown under a Zn-biofortification programme can promote NUtE through the synthesis of the primary metabolites necessary for normal growth of the plant, some of which are essential (or semi-essential) for human health, such as Thr, Leu, Ile, Gln and Tyr.

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 study is the first to have studied the effect of Zn application on NO_3^- accumulation and utilization in lettuce, and the results, apart from being revealing, have practical implications. The results reported here indicate that an application of Zn in the range 80–100 μmol L^{-1} produces an increase in Zn concentration, provoking a decrease of NO_3^- because an increase of this element was sufficient to promote N metabolism (NR, GS and AAT) and the photorespiration process (GGAT, SGAT and HPR). As result, we observed an increase in reduced N, TNC and NUtE. Consequently, at a dose of 80 μmol L^{-1} of Zn, the amino acid concentration increased significantly, including the concentration of amino acids essential for human health (i.e. 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 μmol L^{-1} of Zn because it could result in an increase in the Zn concentration, a reduction of NO_3^- levels and an increase in the concentration of essential amino acids, with all of these compriisng beneficial properties for the human diet.

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REFERENCES

1 Cakmak I, Plant nutrition research: Priorities to meet human needs for food in sustainable ways. *Plant Soil* **247**:3–24 (2002).
2 Welch RM and Graham RD, Breeding for micronutrients in staple food crops from a human nutrition perspective. *J Exp Bot* **55**:353–364 (2004).
3 Welch RM, The impact of mineral nutrients in food crops on global human health. *Plant Soil* **247**:83–90 (2002).
4 White PJ and Broadley MR, Biofortifying crops with essential mineral elements. *Trends Plant Sci* **10**:586–593 (2005).
5 Das S and Green A, Importance of zinc in crops and human health. *J SAT Agric Res* **11**:1–7 (2013).
6 Cakmak I, Enrichment of cereal grains with zinc: agronomic or genetic biofortification? *Plant Soil* **302**:1–17 (2008).
7 Santamaria P, Nitrate in vegetables: toxicity, content, intake and EC regulation. *J Sci Food Agric* **86**:10–17 (2006).
8 Mensinga TT, Speijers GJ and Meulenbelt J, Health implications of exposure to environmental nitrogenous compounds. *Toxicol Rev* **22**:41–51 (2003).
9 European Union, Commission Regulation (EU) No. 420/ 2011 of 29 April. Amending Regulation (EC) No 1881/2006. Setting maximum levels for certain contaminats in foodstuffs. *OJEU* **L111**:30 April 2011 (2011).
10 Van Bueren L, Thorup-Kristensen K, Leifert C, Cooper JM and Becker HC, Breeding for nitrogen efficiency: concepts, methods, and case studies. *Euphyta* **199**:1–2 (2014).
11 Stefanelli D, Goodwin I and Jones R, Minimal nitrogen and water use in horticulture: effects on quality and content of selected nutrients. *Food Res Int* **43**:1833–1843 (2010).
12 Svečnjak Z and Rengel Z, Nitrogen utilization efficiency in canola cultivars at grain harvest. *Plant Soil* **283**:299–307 (2006).
13 Hulagur BF and Dangarwala RT, Effect of zinc, copper, and phosphorus fertilization on the content and uptake of nitrogen and secondary nutrients by hybrid maize. *Madras Agric J* **70**:88–91 (1983).
14 Erenoglu EB, Kutman UB, Ceylan Y and Yildiz B, Cakmak I, Improved nitrogen nutrition enhances root uptake, root-to-shoot translocation and remobilization of zinc ((65) Zn) in wheat. *New Phytol* **189**:438–448 (2011).
15 Kaiser JJ and Lewis OAM, Nitrate reductase and glutamine synthetase activity in leaves and roots of nitrate-fed *Helianthus annuus* L. *Plant Soil* **77**:127–130 (1984).
16 Groat RG and Vance CP, Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). *Plant Physiol* **67**:1198–1203 (1981).
17 González EM, Gordon AJ, James CL and Arrese-Igor C, The role of sucrose synthase in the response of soybean nodules to drought. *J Exp Bot* **46**:1515–1523 (1995).
18 Sánchez-Rodríguez E, Romero L and Ruiz JM, Role of grafting in resistance to water stress in tomato plants: ammonia production and assimilation. *J Plant Growth Reg* **32**:832–842 (2013).
19 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).
20 Cataldo DA, Maroon M, Schrader LE and Youngs VL, Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun Soil Sci Plant Anal* **6**:71–80 (1975).
21 Krom MD, Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. *Analyst* **105**:305–316 (1980).
22 Baethgen WE and Alley MM, A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. *Commun Soil Sci Plant Anal* **20**:961–969 (1989).
23 Siddiqi MY and Glass ADM, Utilization index: a modified approach to the estimation and comparison of nutrient utilization efficiency in plants. *J Plant Nutr* **4**:289–302 (1981).
24 Rogato A, D'Apuzzo E, Barbulova A, Omrane S, Parlati A, Carfagna S *et al.*, Characterization of a developmental root response caused by external ammonium supply in *Lotus japonicus*. *Plant Physiol* **154**:784–795 (2010).
25 Blasco B, Ríos JJ, Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Leyva R, Romero L *et al.*, Study of the interactions between iodine and

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mineral nutrients in lettuce plants. *J Plant Nutr* **35**:1958–1969 (2012).

26 Stoyanova Z and Doncheva S, The effect of zinc supply and succinate treatment on plant growth and mineral uptake in pea plant. *Braz J Plant Physiol* **14**:111–116 (2002).

27 Zhao FJ, Shen ZG and McGrath SP, Solubility of zinc and interactions between zinc and phosphorus in the hyperaccumulator *Thlaspi caerulescens*. *Plant Cell Environ* **21**:108–114 (1998).

28 Barrameda-Medina Y, Montesinos-Pereira D, Romero L, Blasco B and Ruiz, JM, Role of GSH homeostasis under Zn toxicity in plants with different Zn tolerance. *Plant Sci* **227**:110–121 (2014).

29 Lea PJ and Azevedo RA, Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. *Ann Appl Biol* **149**:243–247 (2006).

30 Scaife A, Ferreira MES and Turner MK, Effect of nitrogen form on the growth and nitrate concentration of lettuce. *Plant Soil* **94**:3–16 (1986).

31 Ríos JJ, Blasco B, Cervilla LM, Rubio-Wilhelmi MM, Rosales MA, Sanchez-Rodriguez E *et al.*, Nitrogen-use efficiency in relation to different forms and application rates of Se in lettuce plants. *J Plant Growth Reg* **29**:164–170 (2009).

32 Blasco B, Rios JJ, Cervilla LM, Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Rosales MA *et al.*, Iodine application affects nitrogen-use efficiency of lettuce plants (*Lactuca sativa* L.). *Acta Agric Scand B* **61**:378–383 (2011).

33 Broadley MR, White PJ, Hammond JP, Zelko I and Lux A, Zinc in plants. *New Phytol* **173**:677–702 (2007).

34 Tischner R, Nitrate uptake and reduction in higher and lower plants. *Plant Cell Environ* **23**:1005–1024 (2000).

35 Seethambaram Y and Das VSR, Effect of zinc deficiency on enzyme activities of nitrate reduction and ammonia assimilation of *Oryza sativa* L. and *Pennisetum americanum* L. Leeke. *Proc Indian Natl Sci Acad* **52**:491–494 (1986).

36 Luna CM, Casano LM and Trippi VS, Inhibition of wheat nitrate reductase activity by zinc. *Biol Plant* **43**:257–262 (2000).

37 Siddiqui SN, Umar S and Iqbal M, Zinc-induced modulation of some biochemical parameters in a high- and a low-zinc-accumulating genotype of *Cicer arietinum* L. grown under Zn-deficient condition. *Protoplasma* **252**:1335–1345 (2015).

38 Miflin BJ and Habash DZ, The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J Exp Bot* **53**:979–987 (2002).

39 Thomsen HC, Eriksson D, Møller IS and Schjoerring JK, Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? *Trends Plant Sci* **19**:656–663 (2014).

40 Oliveira IC, Brears T, Knight TJ, Clark A and Coruzzi GM, Overexpression of cytosolic glutamine synthetase. Relation to nitrogen, light, and photorespiration. *Plant Physiol* **129**:1170–1180 (2002).

41 Frechilla S, Lasa B, Aleu M, Juanarena N, Lamsfus C and Aparicio-Tejo PM, Short-term ammonium supply stimulates glutamate dehydrogenase activity and alternative pathway respiration in roots of pea plants. *J Plant Physiol* **159**:811–818 (2002).

42 Hirel B and Lea PJ, Ammonia assimilation, in *Plant Nitrogen*, ed. by Lea PJ and MorotGoudry JF. Springer, Heidelberg, pp. 79–99 (2001).

43 Igarashi D, Tsuchida H, Miyao M and Ohsumi C, Glutamate:glyoxylate aminotransferase modulates amino acid content during photorespiration. *Plant Physiol* **142**:901–910 (2006).

44 Shi-Wei G, Yi Z, Ying-Xu G, Yong L and Qi-Rong S, New insights into the nitrogen from effect on photosynthesis and photorespiration. *Phedosphere* **17**:601–610 (2007).

45 Smirnoff N and Stewart GR, Nitrogen assimilation and zinc toxicity to zinc-tolerant and non-tolerant clones of *Descampsia cespitosa*. *New Phytol* **107**:671–680 (1987).

46 Hernandez LE, Ramon AM, Carpena-Ruiz RO and Garate A, Evaluation of nitrate nutrition indexes in maize leaves: Metabolic nitrate, total nitrate content and nitrate reductase activity. *J Plant Nutr* **18**:869–887 (1995).

47 Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L and Suzuki A, Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann Bot* **105**:1141–1157 (2010).

48 Samreen T, Humaira Shah HU, Ullah S and Javid M, Zinc effect on growth rate, chlorophyll, protein and mineral contents of hydroponically grown mungbeans plant (*Vigna radiata*). *Arabian J Chem* doi:10.1016/j.arabjc.2013.07.005 (2013).

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