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Comparative study of Zn deficiency in *L. sativa* and *B. oleracea* plants: NH_4^+ assimilation and nitrogen derived protective compounds

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ABSTRACT

Zinc (Zn) deficiency is a major problem in agricultural crops of many world regions. N metabolism plays an essential role in plants and changes in their availability and their metabolism could seriously affect crop productivity. The main objective of the present work was to perform a comparative analysis of different strategies against Zn deficiency between two plant species of great agronomic interest such as *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. For this, both species were grown in hydroponic culture with different Zn doses: 10 μM Zn as control and 0.01 μM Zn as deficiency treatment. Zn deficiency treatment decreased foliar Zn concentration, although in greater extent in *B. oleracea* plants, and caused similar biomass reduction in both species. Zn deficiency negatively affected NO_3^- reduction and NH_4^+ assimilation and enhanced photorespiration in both species. Pro and GB concentrations were reduced in *L. sativa* but they were increased in *B. oleracea*. Finally, the AAs profile changed in both species, highlighting a great increase in glycine (Gly) concentration in *L. sativa* plants. We conclude that *L. sativa* would be more suitable than *B. oleracea* for growing in soils with low availability of Zn since it is able to accumulate a higher Zn concentration in leaves with similar biomass reduction. However, *B. oleracea* is able to accumulate N derived protective compounds to cope with Zn deficiency stress.

1. Introduction

Zinc (Zn) is an essential micronutrient for living organisms found in a wide variety of metabolic processes such as protein synthesis, ribonucleases inhibition, maintaining the integrity and function of cell membranes and synthesis of auxin precursor tryptophan [1]. In addition Zn is part of carbonic anhydrase enzyme for starch synthesis, Cu-Zn-superoxide dismutase (Cu-Zn SOD), dehydrogenases and Zn-finger structural domains that mediate transcription factors binding to DNA [2].

It has been found that Zn deficiency is the most widespread micronutrient deficiency. Zn deficiency in plants occurs in soils with low concentration of available Zn, found in many world regions [3]. External symptoms occurring in plants with Zn deficit are observed mainly in leaves and usually consist of reduced biomass, internervial chlorosis, necrotic spots, browning, rosette disposal, small and deformed leaves, and growth delay [4]. As a result of Zn deficiency several changes in physiological processes occur: reduction in photosynthesis, glycolysis, starch synthesis, protein synthesis activity, membranes destabilization and also flowering and seed production are affected [5]. Besides these processes, under Zn deficiency nitrogen (N) metabolism is altered and it was found that NO_3^- absorption is reduced and therefore their concentration in the plant [6].

N metabolism plays an essential role in plants and changes in their availability and their metabolism could seriously affect crop productivity [7,8]. In this regard, several researchers have demonstrated a direct relationship between NO_3^- concentration and biomass production and the same relationship occurs between biomass and foliar N [9,10]. Furthermore, as described later, the degree of sensitivity to Zn deficiency can be correlated with the alteration of N reduction and assimilation and the formation of protective N compounds against stress conditions.

Abbreviations: AA, amino acid; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cu-Zn SOD, Cu-Zn-superoxide dismutase; GB, glycinebetaine; GDH, glutamate dehydrogenase; GGAT, glutamate: glyoxylate aminotransferase; Gln, glutamine; Glu, glutamate; Gly, glycine; GO, glyoxylate oxidase; GOGAT, glutamate synthase; GS, glutamine synthetase; His, histidine; HR, hydroxypyruvate reductase; NiR, nitrite reductase; NR, nitrate reductase; Pro, proline; ROS, reactive oxygen species; Ser, serine; Tyr, tyrosine.

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NO_3^- is the main N source for plants in most agricultural soils. It is absorbed by roots and transported to leaves, where it gives rise to assimilation products as amino acids (AAs) and proteins, needed for biomass production [11]. NO_3^- is reduced to NH_4^+ by nitrate reductase (NR) and nitrite reductase (NiR) enzymes. Photorespiration is a process that also produces significant amounts of NH_4^+ and is essential for maintaining the adequate N level in the plant [12]. It involves several enzymes from different organelles including glyoxylate oxidase (GO) (EC.1.2.3.5) and glyoxylate aminotransferase (GGAT) (EC.2.6.1.4) in peroxisomes and hydroxypyruvate reductase (HR) (EC.1.1.1.81) in mitochondria. In addition to providing NH_4^+, photorespiration provides metabolites for other processes, protects against photoinhibition and against different types of stress [13].

Once produced NH_4^+ is mainly assimilated into organic form by two enzymes: glutamine synthetase (GS) (EC6.3.1.2) and glutamate synthase (GOGAT) (EC1.4.1.13) that produce glutamine (Gln) and glutamate (Glu) respectively, and they are the precursors for the synthesis of other AAs, nucleic acids, polyamines and chlorophylls. On the other hand glutamate dehydrogenase (GDH) (EC1.4.1.2) can also assimilate NH_4^+ when it is high concentrated in plants [14].

There are few studies about how Zn deficiency affects N metabolism described above. Harper and Paulsen [15] in an experiment in wheat plants grown under Zn deficiency observed a decrease in NO_3^- reduction and they suggested that it was due to the lower NO_3^- content in Zn deficient plants as these absorb it less than control plants. However, this effect on N metabolism did not result in a lower concentration of assimilation products neither proteins in the plant. Seethambaram and Das [16], in rice and millet plants, observed that Zn deficiency decreased NR activity in both species. Nevertheless, NH_4^+ assimilation (GS/GOGAT cycle) was reduced only in millet and therefore in this species a decrease in AAs and proteins concentrations and also in growth was produced. Furthermore, Zn deficiency increased photorespiration in rice plants. The authors suggest that GS/GOGAT cycle is maintained in rice plants to remove the NH_4^+ excess produced in photorespiration, which could be toxic to plants. This agrees with observations by Kitagishi and Obata [17] in rice meristems in which a NH_4^+ accumulation occurred, probably as a result of photorespiration increase. In this work a reduction in protein content and biomass was observed, although the NH_4^+ assimilation process was not affected as it increased the AAs concentration being the direct products of this process.

Besides its importance in primary metabolism, protective secondary compounds such as proline (Pro) and glycinebetaine (GB) can be synthesized from N, and they act as compatible organic solutes that are not normally toxic at high concentrations in the cell [18]. These compounds can protect plants against stress by adjusting osmotic potential, detoxifying reactive oxygen species (ROS), protecting membrane integrity and stabilizing enzymes and proteins [19]. However, depending on the species the accumulation of these compounds may be only indicative and not stress tolerance mechanism [20].

Pro is the most detectable osmolyte in plant cells in response to abiotic stress and is synthesized from Glu, so their synthesis is closely related to N metabolism [21]. Pro importance under Zn deficiency conditions seems to depend on the species studied. Thus, in red cabbage plants (*B. oleracea*) it has been found that Pro concentration increased under Zn deficiency. Although this did not increase plant tolerance to Zn deficit, as there is a considerable biomass reduction, but it increased the plant tolerance to drought stress [22]. In another experiment conducted in rice plants grown under Zn deficiency conditions Pro concentrations was five times higher than in control plants. This higher concentration of Pro improved in this case Zn deficit tolerance and authors postulated that this could be due to the enhancer Pro function of the plant antioxidant system [23]. Nevertheless in another study with *Phaseolus vulgaris* plants a lower Pro concentration was observed in Zn deficient leaves and this AA increases with increasing Zn concentration, so in this experiment Pro did not improve Zn deficiency tolerance [24].

It has been shown that both exogenous GB supply and enhanced plant biosynthesis by genetic engineering can increase the tolerance of plants to abiotic stress [25]. In GB synthesis Ser produced in photorespiratory cycle becomes ethanolamine and this forms choline, which is the basis for GB synthesis in the chloroplast [26]. It has been observed that GB levels in plants increases under various abiotic stresses [27]. However, there are no studies to establish the possible relationship between Zn deficiency and GB concentration in plants. Previous works by our research group have shown that *Lactuca sativa* and *Brassica oleracea* have different levels of tolerance to Zn toxicity and show effects on N metabolism and in the synthesis and accumulation of Pro and GB, being GB a good indicator of this stress type in *L. sativa* [28]. Although a comparative analysis of these species under Zn deficiency conditions has not yet been studied.

Lettuce (*L. sativa* cv. Phillipus) and cabbage (*B. oleracea* cv. Bronco) are two leafy vegetables of great agronomic interest with a large production in the last years. These are two leafy vegetables consumed worldwide as salads. The main producers are China and India (http://faostat.fao.org), countries where Zn deficiency is one of the major problems in their crops [4]. In short, the main objective of this work was to perform a comparative analysis of different strategies against Zn deficiency between these two species.

2. Materials and methods

2.1. Plant material, growth conditions and treatments

L. sativa cv. Phillipus and *B. oleracea* cv. Bronco seeds were germinated and grown for 30 days in cell flats (cell size = 3 cm × 3 cm × 10 cm) filled with perlite mixture, and flats were placed on benches in an experimental greenhouse in southern Spain (Granada, Motril, Saliplant S.L.). The 30-day-old seedlings were transferred to a growth chamber under controlled environmental conditions with a relative humidity of 60–80%, temperature of 22/18 °C (day/night) and 12/12-h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of plants 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 7 cm in height) with a volume of 3 l. Throughout the experiment the plants received a growth solution composed of 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4\,H_2O$, 2 mM $MgSO_4{\cdot}7\,H_2O$, 1 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; 138FeG100) and 10 μM H_3BO_3. This solution, with a pH of 5.5–6.0, was changed every three days. Treatments were initiated 30 days after germination and were maintained for 21 days. Plants were grown with different Zn doses: 10 μM of $ZnSO_4$ as control and 0.01 μM of $ZnSO_4$ as deficiency treatment. The shape of the experimental design consisted of randomized complete block with four treatments (*L. sativa*-control, *B. oleracea*-control, *L. sativa*-0.01 μM Zn, *B. oleracea*-0.01 μM Zn), eight plants per treatment and three replications each.

2.2. 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 −80 °C for later performance of

Table 1
Root and leaf biomass and Zn concentration in *L. sativa* and *B. oleracea* plants submitted to Zn deficiency.

		Zn foliar concentration (μg g^{-1} PS)	Leaf biomass (g DW/plant)	Root biomass (g DW/plant)
L. sativa	Control	44.58 ± 0.18	4.12 ± 0.07	0.16 ± 0.01
	0.01 μM Zn	35.80 ± 1.50	3.16 ± 0.03	0.07 ± 0.01
	p-value	**	***	***
	$LSD_{0.05}$	4.20	0.20	0.03
B. oleracea	Control	50.49 ± 3.17	3.61 ± 0.02	0.21 ± 0.01
	0.01 μM Zn	16.36 ± 4.36	2.91 ± 0.13	0.13 ± 0.00
	p-value	**	**	***
	$LSD_{0.05}$	14.97	0.34	0.03
Analysis of variance				
Doses (D)		***	***	***
Especies (E)		*	***	***
D × E		**	NS	NS
$LSD_{0.05}$		6.46	0.18	0.02

Values are means ± S.E. (n = 9) and differences between means were compared by Fisherś least-significance test (LSD; P = 0.05). The levels of significance were represented by p > 0.05: ns (not significant).
* p < 0.05.
** p < 0.01.
*** p < 0.001.

biochemical assays and the other half of the plan material sampled was lyophiliced to obtain the dry weight (DW) and the subsequent analysis of Zn, NO_3^-, NH_4^+, total reduced N and GB concentrations.

2.3. Analysis of Zn and N forms

For the Zn concentration determination, a sample of 150 mg dry material was subjected to a process of mineralization with sulfuric acid and H_2O_2 by the method of Wolf [29], then Zn concentration was determined by ICP-MS.

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. [30]. NH_4^+ was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom [31]. Total reduced N concentration was analyzed from digested samples. A 1-ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 μM 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 15 min, and organic N was measured by spectrophotometry according to the method of Baethgen and Alley [32].

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, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. 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, GOGAT, and GDH. The extraction medium was optimized for these enzyme activities so that they could be extracted together according to the same method [33–35].

The NR assay followed the methodology of Kaiser and Lewis [34]. 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 [36].

GOGAT activity was assayed spectrophotometrically at 30 °C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance [33] and Singh and Srivastava [35], always within 2 h of extraction. The decrease in absorbance was recorded for 5 min.

GDH activity was assayed by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance [33] and Singh and Srivastava [35]. The reaction mixture consisted of 50 mM KH_2PO_4 buffer (pH 7.5) with 200 mM NH_4 sulfate, 0.15 mM

Table 2
Response of NO_3^- reduction and NH_4^+concentration in *L. sativa* and *B. oleracea* leaves submitted to Zn deficiency.

		NO_3^- (mg g^{-1} DW)	NR (μM NO_2 mg prot^{-1} min^{-1})	NH_4^+ (mg g^{-1}DW)
L. sativa	Control	94.47 ± 1.15	0.49 ± 0.01	2.16 ± 0.05
	0.01 μM Zn	100.56 ± 0.96	0.23 ± 0.01	2.01 ± 0.07
	p-value	***	***	NS
	$LSD_{0.05}$	3.17	0.03	0.18
B. oleracea	Control	104.26 ± 1.48	1.71 ± 0.03	1.85 ± 0.09
	0.01 μM Zn	85.29 ± 0.94	1.30 ± 0.13	2.25 ± 0.05
	p-value	***	***	**
	$LSD_{0.05}$	3.72	0.12	0.21
Analysis of variance				
Doses (D)		***	***	NS
Especies (E)		*	***	NS
D × E		***	*	***
$LSD_{0.05}$		2.35	0.06	0.13

Values are means ± S.E. (n = 9) and differences between means were compared by Fisherís least-significance test (LSD; P = 0.05). The levels of significance were represented by p > 0.05: ns (not significant).
* p < 0.05.
** p < 0.01.
*** p < 0.001.

Table 3
Response of some photorespiration enzymes in *L. sativa* and *B. oleracea* leaves submitted to Zn deficiency.

		GO (ΔAbs mg prot^{-1} min^{-1})	GGAT (ΔAbs mg prot^{-1} min^{-1})	HR (ΔAbs mg prot^{-1} min^{-1})
L. sativa	Control	0.003 ± 0.00	0.01 ± 0.00	0.86 ± 0.03
	0.01 μM Zn	0.01 ± 0.00	0.03 ± 0.00	0.80 ± 0.03
	p-value	***	***	NS
	$LSD_{0.05}$	0.00	0.00	0.08
B. oleracea	Control	0.005 ± 0.00	0.02 ± 0.00	0.88 ± 0.01
	0.01 μM Zn	0.01 ± 0.00	0.05 ± 0.00	0.92 ± 0.02
	p-value	***	***	NS
	$LSD_{0.05}$	0.00	0.00	0.04
Analysis of variance				
Doses (D)		***	***	NS
Especies (E)		***	***	**
D × E		*	***	*
$LSD_{0.05}$		0.00	0.00	0.05

Values are means ± S.E. (n = 9) and differences between means were compared by Fisherís least-significance test (LSD; P = 0.05). The levels of significance were represented by p > 0.05: ns (not significant).
* p < 0.05.
** p < 0.01.
*** p < 0.001.

NADH, 2.5 mM 2-oxoglutarate, and enzyme extract. The decrease in absorbance was recorded for 3 min.

For the GO determination, fresh leaf tissue (0.25 g) was ground in a chilled mortar with PVPP and 1 ml of 50 mM Tris–HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mM DTT. The homogenate was centrifuged at 30,000*g* for 20 min. The supernatant was decanted and immediately used for the enzyme assay. GO was assayed as described by Feierabend and Beevers [37] with modifications. A volume of assay mixture containing 50 mM Tris–HCl buffer (pH 7.8), 0.009% Triton X-100, 3.3 mM phenylhydrazine HCl (pH 6.8), 50 μl plant extract, and 5 mM glycolic acid (neutralized to pH 7 with KOH) was used to start the reaction. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm for 2 min after an initial lag phase of 1 min.

For determination of GGAT and HR, 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 [38].

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 [39].

HR 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 [38].

GS was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis [34]. 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 mM $MgSO_4{\cdot}7H_2O$, 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 [40].

The protein concentration of the extracts was determined according to the method of Bradford [41] using bovine-serum albumin as the standard.

2.5. Pro and GB determination

For the determination of the Pro concentration, leaves were homogenized in 5 ml of ethanol at 96%. Insoluble fraction was washed with 5 ml of ethanol at 70%. The extract was centrifuged at 3500*g* for 10 min and the supernatant was preserved at 4 °C for proline determination [42]: a 1 ml aliquot of the supernatant was taken and, after adding reactive ninhydrin acid reagent (ninhydrin, phosphoric acid 6 M, glacial acetic acid 60%) and glacial acetic acid at 99% (2.5 ml), was placed in a water bath at 100 °C. After 45 min, the tubes were cooled on ice, and 5 ml of benzene were added. After 5–10 min the absorbance of the organic phase was measured at 515 nm.

GB concentration was determined by the method of Grieve and Grattan [43]. GB was extracted from 38 mg of dry plant material in 1.5 ml of distilled water gently shaking for 24 h. Extract was filtered and added 2 ml of 2 N H_2SO_4, the solution was incubated 16 h at 4 °C and then centrifuged at 9000*g* 15 min at 0 °C. The pellet obtained by centrifugation was resuspended in 1,2 dichloroethane. After 2 h the GB content was measured by reading absorbance at 365 nm, and quantified using a standard curve of GB.

2.6. Amino acid determination

Soluble AAs were extracted in 1 ml of 80% ethanol, left for 30 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 AA analysis. Chromatographic equipment was from Gilson. The *o*PA derivatives were separated on a reverse-phase C18 ultrasphere column (250 mm × 4.6 mm). Solvent A consisted of 50 mM NaOAc (pH 7) plus 1% tetrahydro-furane 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 AAs was made against a relative calibration curve and expressed as μmol g^{-1} fresh weight [44].

Table 4
Response of enzymes responsible for NH_4^+ assimilation and concentration of total reduced N in *L. sativa* and *B. oleracea* leaves submitted to Zn deficiency.

		GS (μM glutamylhydroxamate mg prot^{-1} min^{-1})	GOGAT (ΔAbs mg prot^{-1} min^{-1})	GDH (ΔAbs mg prot^{-1} min^{-1})	Total reduced N (mg g^{-1} PS)
L. sativa	Control	15.04 ± 0.58	1.32 ± 0.06	1.00 ± 0.09	44.12 ± 2.07
	0.01 μM Zn	2.62 ± 0.31	0.56 ± 0.05	0.17 ± 0.01	44.98 ± 1.87
	p-value	***	***	***	NS
	$LSD_{0.05}$	1.39	0.16	0.19	5.91
B. oleracea	Control	11.07 ± 0.38	1.35 ± 0.06	4.25 ± 0.14	24.36 ± 1.40
	0.01 μM Zn	6.67 ± 0.21	0.45 ± 0.04	0.26 ± 0.03	17.07 ± 0.64
	p-value	***	***	***	***
	$LSD_{0.05}$	0.91	0.14	0.31	3.26
Analysis of variance					
Doses (D)		***	***	***	NS
Especies (E)		NS	NS	***	***
D × E		***	NS	***	*
$LSD_{0.05}$		0.8	0.1	0.17	3.24

Values are means ± S.E. (n = 9) and differences between means were compared by Fisherís least-significance test (LSD; P = 0.05). The levels of significance were represented by p > 0.05: ns (not significant). ** p < 0.01.
* p < 0.05.
*** p < 0.001.

2.7. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI program. A two-tailed ANOVA was applied to ascertain whether the doses of Zn and the species significantly affected the results and 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).

3. Results

3.1. Biomass and Zn concentration

Zn deficiency treatment caused a decrease in foliar Zn concentration relative to control in both *L. sativa* and *B. oleracea*, although this decrease was greater in *B. oleracea* (Table 1). Plants subjected to Zn deficiency of both species showed a significant decrease in foliar and root biomass due to the lower concentration of Zn in the plant (Table 1). The shoot biomass was reduced equally in both species, although the reduction in root biomass was greater in *L. sativa* plants over control without Zn deficiency (Table 1).

3.2. Production of NH_4^+: NO_3^- reduction and photorespiration

NO_3^- concentration showed opposite trends in both species under Zn deficiency. While in *L. sativa* was increased relative to control in *B. oleracea* was reduced (Table 2). NR activity was lower compared to controls in both species under Zn deficiency (Table 2), being this reduction most important in *L. sativa*. In *L. sativa* plants NH_4^+ concentration did not differ with respect to the plants without Zn deficit, but in *B. oleracea* plants foliar concentration of NH_4^+ increased by Zn deficiency treatment (Table 2). Regarding photorespiration, both GO and GGAT activities increased in both species under Zn deficit compared to control plants (Table 3). There were no significant differences from controls in the HR activity in both species (Table 3).

3.3. NH_4^+ incorporation and assimilation products

GS, GOGAT and GDH activities diminished compared to controls in plants under Zn deficiency in both species (Table 4). Total reduced N concentration was not significantly affected by Zn deficiency treatment in *L. sativa*, whereas in *B. oleracea* was reduced compared to plants not subjected to Zn deficiency (Table 4).

3.4. N derived protective compounds

Zn deficiency had opposite effects on the N derived protective compounds concentration in the two species analyzed. Pro concentration was reduced by Zn deficiency treatment in *L. sativa*, while in *B. oleracea* increased compared to controls (Fig. 1). GB levels followed the same trend as those of Pro, decreasing in *L. sativa* and increasing in *B. oleracea* plants respect GB levels of control plants (Fig. 2).

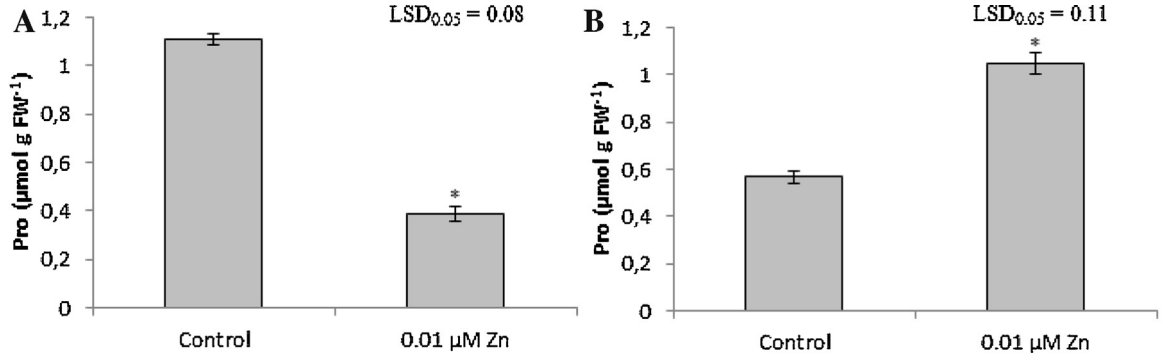

Fig. 1. Effect of Zn deficiency on Pro concentration in *L. sativa* (A) and *B. oleracea* (B) leaves. Columns are mean ± SE (n = 9) and differences between means were compared using Fisher's least-significant difference test (LSD; p = 0.05). Asterisk (*) indicates significant difference with control groups.

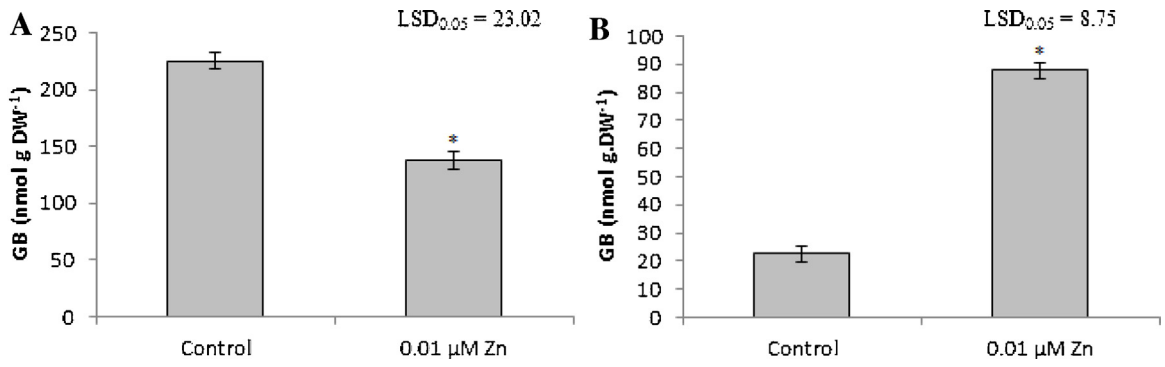

Fig. 2. Effect of Zn deficiency on GB concentration in *L. sativa* (A) and *B. oleracea* (B) leaves. Columns are mean ± SE (n = 9) and differences between means were compared using Fisher's least-significant difference test (LSD; p = 0.05). Asterisk (*) indicates significant difference with control groups.

3.5. AAs concentration

Zn deficiency treatment caused a great increase in Gly concentrations in *L. sativa* plants, but the rest of free AAs were decreased except Ser with no differences respect control plants (Table 5). Regarding *B. oleracea* plants, all the AAs analyzed increased their concentration by Zn deficiency treatment, except Ser and Gly whose values did not differ from control plants (Table 5).

4. Discussion

4.1. Biomass and Zn concentration

One of the most obvious symptoms of Zn deficiency treated plants is the loss of biomass as observed in crop plants like lettuce, tomato, potato, carrot and onion [4]. It was observed that Zn deficiency is the most widespread micronutrient deficiency in rice and its produces significant crop losses [45]. In an experiment with several rice genotypes grown in hydroponics with 0.1 μM of Zn plants were stunted and showed a 75% reduction in shoot Zn concentration [46]. In another study *B. oleracea* plants treated with 2 μM of Zn after two weeks without Zn in the nutritive solution, foliar biomass showed reductions of up to 62% from control plants [47]. In our study the results suggest that under Zn deficiency conditions *L. sativa* is able to accumulate higher Zn concentrations in the shoot (Table 1). In fact, in Zn deficient *L. sativa* plants, foliar Zn concentration reduced by 20% while *in B. oleracea* plants reduction was 68% compared to controls (Table 1). However, this unequal reduction in leaf Zn concentration caused a similar reduction in biomass in both species studied, although there was a greater reduction in root biomass over control in *L. sativa* (Table 1). Therefore, *L. sativa* is capable of storing Zn in the shoot greater extent than *B. oleracea*, nevertheless, deficiency effects are observed in this species manifested by a reduction of biomass. On the other hand, *B. oleracea* was unable to accumulate as much as Zn *L. sativa* but its reduction in biomass was similar to that of this specie, suggesting that *B. oleracea* is less sensitive to Zn deficiency than *L. sativa*.

4.2. NH_4^+ production: NO_3^- reduction and photorespiration

Several studies have shown that NO_3^- reduction to NH_4^+ has a direct impact on biomass production [9,10]. In our study this process is diminished in Zn deficient plants, which could be a reason why the biomass is reduced in the plants studied. We observed that NO_3^- concentration increases slightly compared to control in Zn deficient *L. sativa* plants, whereas in *B. oleracea* plants subjected to the same treatment decreases (Table 2). Furthermore, NR activity was lower in both species under Zn deficiency, being more significant the reduction in *L. sativa* with a 54% decrease compared to the control plants (Table 2). The lower NO_3^- concentration in Zn deficient *L. sativa* plants can be explained by the fact that NO_3^- absorption is not impaired and NR activity is diminished in these plants (Table 2), as it has been demonstrated that by inhibition of NR activity an accumulation of NO_3^- may occur due to the decreasing rate of reduction to NO_2^- [48]. Furthermore, it was found that NO_3^- concentration could decrease in Zn deficient plants because these absorb fewer this nutrient than control plants [15]. This could be caused by the impairment in cell membrane permeability and, therefore, the impairment in NO_3^- absorption [49]. This could explain the decrease in NO_3^- concentration we observed in Zn deficient *B. oleracea* plants with respect to control plants (Table 2). In addition, that decrease in NO_3^- levels could explain the lower NR activity in these plants (Table 2). Harper and Paulsen [15] observed the same results in wheat plants under Zn deficiency, they had lower NR activity and they suggested that it was due to the lower NO_3^- concentration in these plants because its absorption was less than in control plants without Zn deficiency. In another study with rice and millet plants under Zn deficiency the researchers observed that NR activity was reduced compared to the control plants, with the greatest reduction in rice. A possible explanation is the lack of NADH produced by reducing photosynthesis due to Zn deficiency, affecting lesser extent millet plants with C4 metabolism [16].

NH_4^+ levels showed no significant differences from the *L. sativa* control plants whereas in *B. oleracea* were increased by 22% in Zn deficient plants with respect control plants (Table 2). It has been found that when photorespiratory process is active can produce more NH_4^+ than by reducing NO_3^- and therefore is essential to maintain N metabolism [12]. In our experiment Zn deficiency causes an increase in photorespiratory cycle, manifested by a large increase in GO and GGAT activities regarding controls in the two species studied (Table 3). However, there were no significant differences from controls in the HR activity in both species (Table 3). Comparing both species, *B. oleracea* has higher GGAT and HR activities with respect to the controls that *L. sativa* plants (Table 3). This could be one reason for the *B. oleracea* greater resistance against Zn deficit, since greater photorespiration activity can help in ROS elimination [12]. As consequence of increased photorespiration rate levels of NH_4^+ should be increased on the plant, but this only happens in *B. oleracea* (Table 2). In a study conducted by Seethambaram et al. [50] similar results were observed in rice plants being that Zn deficiency caused an increase in the photorespiratory cycle and hence a higher release of NH_4^+ by decarboxylation of Gly could compensate the decrease in the NO_3^- reduction.

4.3. NH_4^+ incorporation and assimilation products

Once NO_3^- is reduced to NH_4^+ this is rapidly assimilated because if NH_4^+ accumulates it can produce toxicity symptoms [8]. The NH_4^+

assimilation is carried out mainly by the GS/GOGAT cycle that produces Glu as a result of primary assimilation of N [51]. In a study of millet plants grown under Zn deficiency conditions, it was observed that GS and GOGAT activities were decreased. The authors postulated that this effect was due to the lack of ATP for GS activity and the lack of reduced ferredoxin for GOGAT activity that occurs as a result of Zn deficiency [16]. In similar studies in rice plants no decrease was observed in GS and GOGAT activities because, according to the authors, the release of NH_4^+ from photorespiration must be detoxified and assimilated by these enzymes [17,16].

According to our results, Zn deficiency adversely affects GS/GOGAT enzyme activity in leaves of both species (Table 4). However for GS activity, the percentage reduction relative to control was higher in *L. sativa*, with 83% reduction activity with respect to plants without Zn deficiency (Table 4). If increased levels of NH_4^+ from photorespiration is not linked to increased GS/GOGAT activity can produce a toxic buildup of NH_4^+ [8]. In *L. sativa* NH_4^+ concentration was not affected despite GS and GOGAT activities were affected by Zn shortage (Table 2). This could be due to the greater decrease in NO_3^- reduction and smaller increase in GGAT activity produced in this species (Table 3), resulting in a lower production of NH_4^+ in *L. sativa* compared to *B. oleracea*. Furthermore, as observed in *Arabidopsis thaliana*, *L. sativa* plants could compartmentalize NH_4^+ itself or as urea in the root cells vacuoles to avoid toxic accumulation in leaves [52,53].

GDH enzyme has a minor role in NH_4^+ assimilation. However, it has been found that this enzyme is more active when there is a higher NH_4^+ concentration in cells [14]. In our experiment, GDH activity is heavily reduced in both species (Table 4), with 80% reduction in *L. sativa* and 90% in *B. oleracea* with respect to controls (Table 4). Although NH_4^+ concentration is higher in Zn deficient *B. oleracea* plants (Table 2), in these plants there is less GDH activity (Table 4). This is probably because Zn is necessary for normal activity of this enzyme [1].

The result of NH_4^+ assimilation can be quantified by analyzing the total reduced N, which is usually the product of assimilation of N and consists mainly of AAs and proteins. Therefore, it is an essential parameter to determine the plant nutritional status [10]. Our results show that total reduced N concentration is not affected significantly by Zn deficit in *L. sativa*, while is reduced by 30% compared to control in *B. oleracea* plants (Table 4). This could be due to the lower GS/GOGAT activity which occurs as a result of Zn deficiency. However, we did not observe a lower total N reduced concentration in Zn deficient *L. sativa* plants in spite of the lower GS/GOGAT activity (Table 4). A possible explanation could be that GS/GOGAT cycle in roots is not affected to the same extent as in leaves and therefore AAs produced in root would be transported to the shoot and total reduced N concentration would be maintained. It has been proved that Boron (B) deficient tobacco plants are able to induce their GS and GOGAT activities in roots to detoxify the NH_4^+ excess produced due to B deficit stress [54]. Further research would be needed in order to find out how Zn deficiency affects N metabolism in *L. sativa* roots.

4.4. N derived protective compounds

N derived protective compounds act as organic compatible solutes and normally they are not toxic at high concentrations in the cell [18]. These compounds can protect plants against stress by adjusting the osmotic potential, detoxifying ROS, protecting membrane integrity and stabilizing enzymes and proteins [19]. Among these compounds are Pro and GB [21].

According to our results, Zn deficiency causes a 65% decrease in Pro concentration and 39% decrease in GB concentration in *L. sativa* plants compared to controls (Figs. 1 A and 2 A). In a study in *P. vulgaris* plants under Zn deficit a lower concentration of Pro was also observed in Zn deficient leaves [24]. This could be because Zn affects some necessary process for Pro synthesis in this species. One possibility would be a lower synthesis of Glu as a result of the decrease in GS/GOGAT and GDH activities since Glu is a precursor in Pro synthesis. In *B. oleracea* we observe the opposite effect to that produced in *L. sativa* because in plants under Zn deficit Pro and GB concentration increase by 86% and 287% respectively compared to controls (Figs. 1 B and 2 B). This is consistent with what was observed in cabbage plants (*B. oleracea*) [22] and rice plants [23] in which Pro concentration increase over control in Zn deficient plants.

Considering the GB synthesis route in plants, if Ser accumulates it will enhance GB synthesis. According to our results, *B. oleracea* plants under Zn deficiency appropriate conditions for Ser building up exist: there is an increase in GO and GGAT activities and also HR activity is not increased (Table 3) so hydroxypyruvate accumulates and hence Ser would accumulate too. Therefore, in *B. oleracea* Pro and especially GB buildup seems to be a mechanism for Zn deficiency tolerance because despite having greater decrease on Zn concentration in leaves, compared with *L. sativa*, the biomass decrease is similar to that of this species.

Table 5
Response of foliar amino acids concentration (μmol g^{-1} FW) in *L. sativa* and *B. oleracea* leaves submitted to Zn deficiency.

		Arg	Asn	Asp	Gln	Glu	Gly	His	Ser	Tyr
L. sativa	Control	2.52 ± 0.03	2.93 ± 0.06	3.05 ± 0.09	5.11 ± 0.10	4.85 ± 0.12	0.23 ± 0.00	0.36 ± 0.02	1.70 ± 0.09	0.18 ± 0.00
	0.01 μM Zn	1.05 ± 0.09	1.16 ± 0.07	2.75 ± 0.15	3.31 ± 0.22	3.97 ± 0.12	1.93 ± 0.02	0.35 ± 0.02	1.76 ± 0.08	0.11 ± 0.01
	p-value	***	***	NS	**	**	***	NS	NS	***
	$LSD_{0.05}$	0.25	0.26	0.50	0.68	0.46	0.05	0.07	0.33	0.02
B. oleracea	Control	0.39 ± 0.04	0.94 ± 0.01	1.81 ± 0.1	2.37 ± 0.01	3.87 ± 0.17	0.23 ± 0.00	0.23 ± 0.02	3.58 ± 0.08	0.34 ± 0.00
	0.01 μM Zn	0.6 ± 0.03	1.07 ± 0.03	2.24 ± 0.08	3.07 ± 0.16	4.57 ± 0.19	0.20 ± 0.02	0.43 ± 0.02	3.04 ± 0.21	0.78 ± 0.04
	p-value	*	NS	*	**	NS	NS	**	NS	***
	$LSD_{0.05}$	0.13	0.28	0.36	0.45	0.71	0.04	0.08	0.62	0.11
Analysis of variance										
Doses (D)		***	***	NS	**	NS	***	**	NS	***
Especies (E)		***	***	***	***	NS	***	NS	***	***
D × E		***	***	*	***	***	***	***	*	***
$LSD_{0.05}$		0.12	0.16	0.26	0.34	0.35	0.03	0.04	0.29	0.05

Values are means ± S.E. (n = 9) and differences between means were compared by Fisherís least-significance test (LSD; P = 0.05). The levels of significance were represented by p > 0.05: ns (not significant).

* p < 0.05.
** p < 0.01.
*** p < 0.001.

4.5. AAs concentration

Previous studies show that free AAs profile change in plants submitted to Zn deficiency, in this sense, an increase in aspartic acid (Asp), asparagine (Asn) and Gln was observed in rice plants and this increase was lower in Zn efficient genotypes [55]. In other experiments carried out in tomato [56] and rice [17] grown under Zn deficiency Ser, Asn and Gln were also accumulated. In our experiment *L. sativa* plants grown under Zn deficiency showed a 728% increase in Gly concentration respect control plants (Table 5) and this could be one reason why *L. sativa* can accumulate a higher Zn concentration than *B. oleracea*. In two independent experiments carried out in lettuce plants treated with different Zn-AA complexes, the results showed that $Zn(Gly)_2$ complexes were more effective promoting the plant growth and this complex is one of the most helped the Zn accumulation in the shoot [57,58]. The lower arginine (Arg), Asn, Glu and Gln concentrations might be caused by the lower N assimilation activity in this species under Zn deficiency (Table 4). Respecting *B. oleracea* plants we did not observe differences in Gly concentration in Zn deficient plants with respect to control, but in this case histidine (His) increased by 63% (Table 5). This AA has high Zn affinity and it is important for Zn homeostasis in another Brassicaceae species like *Thlaspi caerulescens* [59], although in our experiment His did not help to accumulate more Zn. Furthermore in these plants tyrosine (Tyr) increased by 94% (Table 5) and this can be explained by an activation of Tyr metabolism caused by Zn deficiency to synthesise tocopherol antioxidants [60]. Finally Gln, Asp and Arg were increased by Zn deficiency in *B. oleracea* plants (Table 5). These are transport AAs and they are often produced actively as a result of NH_4^+ increase in leaves as we observed in our *B. oleracea* plants grown under Zn deficiency (Table 2) to avoid NH_4^+ excess in leaves [61].

5. Conclusions

The results show that Zn deficiency negatively affects both NO_3^- reduction and NH_4^+ assimilation, enhance photorespiration and change the free AAs profile in the two species studied. According to our results, *L. sativa* would be more suitable than *B. oleracea* for growing in soils with low concentration and/or availability of Zn since it is able to accumulate a higher Zn concentration in leaves likely due to a Gly accumulation and presents similar biomass reduction. However, *B. oleracea* is able to accumulate N derived protective compounds to cope with Zn deficiency stress. Therefore a possibility in plant breeding could be performing genetic manipulation techniques to induce greater production of such compounds. These techniques would be particularly useful in species such as *L. sativa* able to accumulate more Zn under deficiency conditions as they would reduce the biomass loss produced by Zn deficit.

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