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**Comparative study of Zn deficiency in *L. sativa* and *B. oleracea* plants: NH4+ 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 NO3- reduction and NH4+ 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.

**Keywords** Zn deficiency · N metabolism · Proline · Glycinebetaine · *Lactuca sativa* · *Brassica oleracea*

**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.

**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 NO3- 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 NO3- 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.

NO3- 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]. NO3- is reduced to NH4+ by nitrate reductase (NR) and nitrite reductase (NiR) enzymes. Photorespiration is a process that also produces significant amounts of NH4+ and is essential for maintaining the adequate N level in the plant [12]. Photorespiration is a consequence of ribulose-1,5-bisphosphate (RuBP) oxygenation catalyzed RuBP carboxylase / oxygenase (Rubisco) (EC.4.1.1.39) producing glycolate, which is oxidized to glyoxylate in the peroxisome by glyoxylate oxidase (GO) (EC.1.2.3.5) enzyme. Subsequently glutamate: glyoxylate aminotransferase (GGAT) (EC.2.6.1.4) catalyzes the transamination of glyoxylate to form glycine (Gly) that is transported to the mitochondria. Gly formed becomes serine (Ser) by decarboxylase enzymes and glycine hydroxymethyltransferase. This reaction forms NH4+, which integrates into N assimilation pathway. Ser formed is transported to peroxisome where it becomes hydroxypyruvate and this is reduced to glycerate by hydroxypyruvate reductase (HR) (EC.1.1.1.81) closing the photorespiratory cycle. In addition to providing NH4+, photorespiration provides metabolites for other processes, protects against photoinhibition and against different types of stress [13].

Once produced NH4+ 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 NH4+ 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 NO3- reduction and they suggested that it was due to the lower NO3- 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, NH4+ 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 NH4+ 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 NH4+ accumulation occurred, probably as a result of photorespiration increase. In this work a reduction in protein content and biomass was observed, although the NH4+ 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 are 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 *L. sativa* and *B. 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.

In short, considering that according to the literature consulted Zn deficiency sensitivity can be correlated with N reduction and assimilation alteration and N derived protective compounds synthesis, we analyzed parameters related with these processes in plants. The main objective of this work was to perform a comparative analysis of different strategies against Zn deficiency between two plant species of great agronomic interest as lettuce (*Lactuca sativa* cv. Phillipus) and cabbage (*Brassica oleracea* cv. Bronco).

**2. Materials y methods**

*2.1. Plant material, growth conditions and tratments*

*L. sativa* cv. Phillipus, and *B. oleracea* cv. Bronco seeds were germinated and grown for 30 days in cell flats (cell size = 3 cm x 3 cm x 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 25/15ºC (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 3l. Throughout the experiment the plants received a growth solution composed of 4 mM KNO3, 3 mM Ca(NO3)2 •4 H2O, 2 mM MgSO4 •7 H2O, 6 mM KH2PO4, 1 mM NaH2PO4 •2 H2O, 2 μM MnCl2 •4 H2O, 10 μM ZnSO4, 0.25 μM CuSO4 •5 H2O, 0,1 μM Na2MoO4 •2 H2O 5ppm, Fe-chelate (Sequestrene;138FeG100) and 10 µM H3BO3. 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. Control plants received complete nutrient solution, while treatment of Zn deficiency received a Zn dose of 0.01 µM Zn as ZnSO4. 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 -80ºC for later performance of 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, NO3-, NH4+, total reduced N y 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 H2O2 by the method of Wolf [29], then Zn concentration was determined by ICP- MS.

NO3- 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 NO3- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO3- concentration by spectrophotometry as performed by Cataldo et al. [30]. NH4+ 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 37ºC 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 0ºC in 50 mM KH2PO4 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, 34, 35].

The NR assay followed the methodology of Kaiser and Lewis [34]. The NO2- 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 30ºC 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 KH2PO4 buffer (pH 7.5) with 200 mM NH4 sulfate, 0.15 mM 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 NH4Cl, 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 0ºC 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 KH2PO4 buffer (pH 7.5) with 4 mM EDTA, 1000 mM L-sodium glutamate, 450 mM MgSO4·7H2O, 300 mM hydroxylamine, 100 mM ATP, and enzyme extract. Two controls were prepared, one without glutamine and the other without hydroxylamine. After incubation at 28ºC 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 3,500 *× g* for 10 min and the supernatant was preserved at 4ºC 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 100ºC. 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 hours. Extract was filtered and added 2 ml of 2N H2SO4, the solution was incubated 16 h at 4°C and then centrifuged at 9,000 *× g* 15 min at 0 ºC. The pellet obtained by centrifugation was resuspended in 1, 2 dichloroethane. After 2 hours 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].

*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 NH4+: NO3- reduction and photorespiration*

NO3- 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 NH4 + concentration did not differ with respect to the plants without deficit Zn,but in *B. oleracea* plants foliar concentration of NH4 + 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. NH4+ 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 theN derived protective compoundsconcentration 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).

*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 [4]. Other studies in different species showed a reduction of biomass by Zn deficiency [45, 46]. The most obvious symptom in Zn deficient *L. sativa* plants is the reduction in biomass [47], as in *B. oleracea*, biomass showed reductions of up to 62% in Zn deficient leaves [48].

In our study the results suggest that under Zn deficiency conditions *L. sativa* is able to accumulate higher Zn concentrations in the shoot (Fig. 3A). 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 (Fig. 3A). However, this unequal reduction in leaf Zn concentration caused a similar reduction in biomass in both species studied (Fig. 3B), although there was a greater reduction in root biomass over control in L. *sativa* (Fig. 3C). 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 it 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. NH4+ production: NO3- reduction and photorespiration*

Several studies have shown that NO3- reduction to NH4+ 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 NO3- concentration increases slightly compared to control in Zn deficient *L. sativa* plants, whereas in *B. oleracea* plants subjected to the same treatment decreases (Fig. 3D). 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 (Fig. 3E). The lower NO3- concentration in Zn deficient *L. sativa* plants can be explained by the fact that NO3- 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 NO3- may occur due to the decreasing rate of reduction to NO2- [49]. Furthermore, it was found that NO3-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 NO3- absorption [50]. This could explain the decrease in NO3-concentration we observed in Zn deficient *B. oleracea* plants with respect to control plants (Table 2). In addition, that decrease in NO3- 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 NO3- concentration in these plants because its absorption was less than in control plants without Zn deficiency. In another study of 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].

NH4+ 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 (Fig. 3F). It has been found that when photorespiratory process is active can produce more NH4+ than by reducing NO3- 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 (Fig. 3H and 3I). 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 NH4+ should be increased on the plant, but this only happens in *B. oleracea* (Table 2). In a study conducted by Seethambaram et al. [51] similar results were observed in rice plants being that Zn deficiency caused an increase in the photorespiratory cycle and hence a higher release of NH4+ by decarboxylation of Gly could compensate the decrease in the NO3- reduction.

*4.3. NH4+ incorporation and assimilation products*

Once NO3- is reduced to NH4+ this is rapidly assimilated because if NH4+ accumulates it can produce toxicity symptoms [8]. The NH4+ assimilation is carried out mainly by the GS/GOGAT cycle that produces Glu as a result of primary assimilation of N [52]. 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 NH4+ from photorespiration must be detoxified and assimilated by these enzymes [17, 16].

According 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 (Fig. 3J). If increased levels of NH4+ from photorespiration is not linked to increased GS / GOGAT activity can produce a toxic buildup of NH4+ [8]. In *L. sativa* NH4+ 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 NO3- reduction and smaller increase in GGAT activity produced in this species (Fig. 3H), resulting in a lower production of NH4+ in *L. sativa* compared to *B. oleracea*. Furthermore, as observed in *Arabidopsis thaliana*, *L. sativa* plants could compartmentalize NH4+ itself or as urea in the root cells vacuoles to avoid toxic accumulation in leaves [53, 54].

GDH enzyme has a minor role in NH4+ assimilation. However, it has been found that this enzyme is more active when there is a higher NH4+ 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 (Fig. 3L). Although NH4+ 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 NH4+ 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 (Fig. 3M). This could be due to the lower GS/GOGAT activity which occurs as a result of Zn deficiency. However, we didn’t 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 plant are able to induce their GS and GOGAT activities in roots to detoxify the NH4+ excess produced due to B deficit stress [55]. 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 (Fig. 3N, O). In a study in *Phaseolus 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 (Fig. 3N, O). 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.

*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 [56]. In other experiments carried out in tomato [57] 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 was more effective promoting the plant grown and this complex is one of the most helped the Zn accumulation in the shoot [58, 59]. The lower arginine (Arg), Asn, Glu y Gln concentrations might be caused by the lower N assimilation activity in this species under Zn deficiency (Fig. 3J y 3K). 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* [60], 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 [61]. 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 NH4+ increase in leaves as we observed in our *B. oleracea* plants grown under Zn deficiency (Fig. 3F) to avoid NH4+ excess in leaves [62].

**5. Conclusions**

The results show that Zn deficiency negatively affects both NO3- reduction and NH4+ 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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**References**

[1] B.L. Vallee, D.S. Auld, Zinc coordination, function, and structure of zinc enzymes and other proteins, Biochemistry. 29 (1990) 5647–5659. doi:10.1021/bi00476a001.

[2] T.C. Fox, M. Lou Guerinot, Molecular biology of cation transport in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 669–696. doi:10.1146/annurev.arplant.49.1.669.

[3] M. Sillanpää, Micronutrients and the nutrient status of soils: A global study, Food & Agriculture Org., Rome, 1982.

[4] B.J. Alloway, Zinc in soils and crop nutrition, 3th ed., International Zinc Association Brussels, Belgium, 2008.

[5] P.H. Brown, I. Cakmak, Q. Zhang, Form and function of zinc plants, in: A.D. Robson (Ed.), Springer Netherlands, 1993: pp. 93–106.

[6] A.D. Johnson, J.G. Simons, Diagnostic indices of zinc deficiency in tropical legumes, J. Plant Nutr. 1 (1979) 123–149. doi:10.1080/01904167909362705.

[7] W.R. Ullrich, Salinity and Nitrogen Nutrition, in: A. Läuchli, U. Lüttge (Eds.), Salin. Environ., Springer Netherlands, 2002: pp. 229–248.

[8] P. j. Lea, R. a. Azevedo, Nitrogen use efficiency. 1. Uptake of nitrogen from the soil, Ann. Appl. Biol. 149 (2006) 243–247. doi:10.1111/j.1744-7348.2006.00101.x.

[9] C.T. MacKown, S.J. Crafts-Brandner, T.G. Sutton, Relationships among soil nitrate, leaf nitrate, and leaf yield of burley tobacco, Agron. J. 91 (1999) 613. doi:10.2134/agronj1999.914613x.

[10] J. Ruiz, L. Romero, Cucumber yield and nitrogen metabolism in response to nitrogen supply, Sci. Hortic. (Amsterdam). 82 (1999) 309–316. doi:10.1016/S0304-4238(99)00053-9.

[11] S. Sivasankar, A. Oaks, Nitrate assimilation in higher plants : The effect of metabolites and light, Plant Physiol. Biochem. 34 (1996) 609–620.

[12] A. Wingler, P.J. Lea, W.P. Quick, R.C. Leegood, Photorespiration: metabolic pathways and their role in stress protection, Philos. Trans. R. Soc. London. Ser. B Biol. Sci. 355 (2000) 1517–1529. doi:10.1098/rstb.2000.0712.

[13] S.-W. Guo, Y. Zhou, Y.-X. Gao, Y. Li, Qi-Rong, S., New insights into the nitrogen form effect on photosynthesis and photorespiration, Pedosphere. 17 (2007) 601–610. doi:10.1016/S1002-0160(07)60071-X.

[14] S.A. Robinson, A.P. Slade, G.G. Fox, R. Phillips, R.G. Ratcliffe, G.R. Stewart, The role of glutamate dehydrogenase in plant nitrogen metabolism, Plant Physiol. 95 (1991) 509–516. doi: 10.1104/pp.95.2.509.

[15] J.E. Harper, G.M. Paulsen, Nitrogen assimilation and protein synthesis in wheat seedlings as affected by mineral nutrition. II. Micronutrients, Plant Physiol. 44 (1969) 636–640. doi:10.1104/pp.44.5.636.

[16] Seethambaram Y., Das V.S.R., Effect of zinc deficiency on enzyme activities of nitrate reduction and ammonia assimilation of *Oryza sativa* L. and *Pennisetum americanum* L., Leeke, Proc. Indian Natn. Sci. Acad. 4 (1986) 491–496. doi: 005283382.

[17] K. Kitagishi, H. Obata, Effects of zinc deficiency on the nitrogen metabolism of meristematic tissues of rice plants with reference to protein synthesis, Soil Sci. Plant Nutr. 32 (1986) 397–405. doi:10.1080/00380768.1986.10557520.

[18] R. Serraj, T.R. Sinclair, Osmolyte accumulation: can it really help increase crop yield under drought conditions?, Plant. Cell Environ. 25 (2002) 333–341. doi:10.1046/j.1365-3040.2002.00754.x.

[19] H.J. Bohnert, R.G. Jensen, Strategies for engineering water-stress tolerance in plants, Trends Biotechnol. 14 (1996) 89–97. doi:10.1016/0167-7799(96)80929-2.

[20] C.F. de Lacerda, J. Cambraia, M.A. Oliva, H.A. Ruiz, J.T. Prisco, Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress, Environ. Exp. Bot. 49 (2003) 107–120. doi:10.1016/S0098-8472(02)00064-3.

[21] M. Ashraf, M.R. Foolad, Roles of glycine betaine and proline in improving plant abiotic stress resistance, Environ. Exp. Bot. 59 (2007) 206–216. doi:10.1016/j.envexpbot.2005.12.006.

[22] R. Hajiboland, F. Amirazad, Drought tolerance in Zn-deficient red cabbage (*Brassica oleracea* L. var. capitata f. rubra) plants, Hort. Sci.(Prague). 37 (2010) 88–98.

[23] Höller S, Hajirezaei M R, Wirén N, Frei M, Ascorbate metabolism in rice genotypes differing in zinc efficiency, Planta. 239 (2013) 367–379. doi:10.1007/s00425-013-1978-x.

[24] P.I. Michael, M. Krishnaswamy, The effect of zinc stress combined with high irradiance stress on membrane damage and antioxidative response in bean seedlings, Environ. Exp. Bot. 74 (2011) 171–177. doi:10.1016/j.envexpbot.2011.05.016.

[25] T.H.H. Chen, N. Murata, Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes, Curr. Opin. Plant Biol. 5 (2002) 250–257. doi:10.1016/S1369-5266(02)00255-8.

[26] S.D. McNeil, M.L. Nuccio, M.J. Ziemak, A.D. Hanson, Enhanced synthesis of choline and glycine betaine in transgenic tobacco plants that overexpress phosphoethanolamine N-methyltransferase, Proc. Natl. Acad. Sci. 98 (2001) 10001–10005. doi: 10.1073/pnas.171228998.

[27] R. Storey, N. Ahmad, R.G.W. Jones, Taxonomic and ecological aspects of the distribution of glycinebetaine and related compounds in plants, Oecologia. 27 (1977) 319–332. doi:10.1007/BF00345565.

[28] V. Paradisone, Y. Barrameda-Medina, D. Montesinos-Pereira, L. Romero, S. Esposito, J.M. Ruiz, Roles of some nitrogenous compounds protectors in the resistance to zinc toxicity in *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco, Acta Physiol. Plant. 37 (2015) 1–8. doi:10.1007/s11738-015-1893-9.

[29] B. Wolf, A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status, Commun. Soil Sci. Plant Anal. 13 (1982) 1035–1059. doi:10.1080/00103628209367332.

[30] D.A. Cataldo, M. Maroon, L.E. Schrader, V.L. Youngs, Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid, Commun. Soil Sci. Plant Anal. 6 (1975) 71–80. doi:10.1080/00103627509366547.

[31] M.D. Krom, Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate, Analyst. 105 (1980) 305–316. doi:10.1039/AN9800500305.

[32] W.E. Baethgen, M.M. Alley, A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests, Commun. Soil Sci. Plant Anal. 20 (1989) 961–969. doi:10.1080/00103628909368129.

[33] R.G. Groat, C.P. Vance, Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.) Developmental Patterns and response to applied nitrogen, Plant Physiol. 67 (1981) 1198–1203. doi:10.1104/pp.67.6.1198.

[34] J.J. Kaiser, O. a. M. Lewis, Nitrate reductase and glutamine synthetase activity in leaves and roots of nitrate-fedHelianthus annuus L., Plant Soil. 77 (1984) 127–130. doi:10.1007/BF02182818.

[35] R.P. Singh, H.S. Srivastava, Increase in glutamate synthase (NADH) activity in maize seedlings in response to nitrate and ammonium nitrogen, Physiol. Plant. 66 (1986) 413–416. doi:10.1111/j.1399-3054.1986.tb05944.x.

[36] Hageman R.H., Hucklesby D.P., Nitrate reductase, Meth Enzym. 23 (1971) 497–503.

[37] J. Feierabend, Beevers, H., Developmental studies on microbodies in wheat leaves, Planta. 123 (1972) 63–77. doi:10.1007/BF00388061.

[38] Hoder M, Rej R, Berjmeyer HU, Berjmeyer J, Alanine aminotransferase, in: Gral M, Weinhein, Chemie, 1983: pp. 444–456.

[39] D. Igarashi, H. Tsuchida, M. Miyao, C. Ohsumi, Glutamate:glyoxylate aminotransferase modulates amino acid content during photorespiration, Plant Physiol. 142 (2006) 901–910. doi:10.1104/pp.106.085514.

[40] R.M. Wallsgrove, P.J. Lea, B.J. Miflin, Distribution of the enzymes of nitrogen assimilation within the pea leaf cell, Plant Physiol. 63 (1979) 232–236. doi:10.1104/pp.63.2.232.

[41] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254. doi:10.1016/0003-2697(76)90527-3.

[42] J.J. Irigoyen, D.W. Einerich, M. Sánchez-Díaz, Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (Medicago sativd) plants, Physiol. Plant. 84 (1992) 55–60. doi:10.1111/j.1399-3054.1992.tb08764.x.

[43] C.M. Grieve, S.R. Grattan, Rapid assay for determination of water soluble quaternary ammonium compounds, Plant Soil. 70 (1983) 303–307. doi:10.1007/BF02374789.

[44] A. Rogato, E. D’Apuzzo, A. Barbulova, S. Omrane, A. Parlati, S. Carfagna, A. Costa, F.L. Schiavo, S. Esposito, M. Chiurazzi, Characterization of a developmental root response caused by external ammonium supply in *Lotus japonicus*, Plant Physiol. 154 (2010) 784–795. doi:10.1104/pp.110.160309.

[45] L. Ozturk, S. Karanlik, F. Ozkutlu, I. Cakmak, L. V. Kochian, Shoot biomass and zinc/cadmium uptake for hyperaccumulator and non-accumulator Thlaspi species in response to growth on a zinc-deficient calcareous soil, Plant Sci. 164 (2003) 1095–1101. doi:10.1016/S0168-9452(03)00118-3.

[46] M. Wissuwa, A.M. Ismail, S. Yanagihara, Effects of zinc deficiency on rice growth and genetic factors contributing to tolerance, Plant Physiol. 142 (2006) 731–741. doi:10.1104/pp.106.085225.

[47] P.C. Srivastava, U.C. Gupta, Trace elements in crop production., Science Publishers, Inc, 1996.

[48] R. Hajiboland, F. Amirazad, Growth, photosynthesis and antioxidant defense system in Zn-deficient red cabbage plants, Plant Soil Env. 56 (2010) 209–217.

[49] I. Bonilla, C. Cadahía, O. Carpena, V. Hernando, Effects of boron on nitrogen metabolism and sugar levels of sugar beet, Plant Soil. 57 (1980) 3–9. doi:10.1007/BF02139636.

[50] Welch RW, Webb MJ, Loneragan JF, Zinc in membrane function and its role in phosphorus toxicity, in: Scaife A. (Ed.), Warwick University, UK, 1982: pp. 710–715.

[51] Y. Seethambaram, A.N. Rao, V.S.R. Das, The levels of carbonic anhydrase and of photorespiratory enzymes under zinc deficiency in *Oryza sativa* L.and *Pennisetum americanum* L.Leeke, Biochem. Und Physiol. Der Pflanz. 180 (1985) 107–113. doi:10.1016/S0015-3796(85)80062-7.

[52] B. Hirel, P.J. Lea, Ammonia assimilation, in: Lea, JP, J.-F. Morot-Gaudry (Eds.), Springer Berlin Heidelberg, 2001: pp. 79–99.

[53] B. Li, G. Li, H.J. Kronzucker, F. Baluška, W. Shi, Ammonium stress in Arabidopsis: signaling, genetic loci, and physiological targets, Trends Plant Sci. 19 (2014) 107–114. doi:10.1016/j.tplants.2013.09.004.

[54] A. Bittsánszky, K. Pilinszky, G. Gyulai, T. Komives, Overcoming ammonium toxicity, Plant Sci. 231 (2015) 184–190. doi:10.1016/j.plantsci.2014.12.005.

[55] V.M. Beato, J. Rexach, M.T. Navarro-Gochicoa, J.J. Camacho-Cristóbal, M.B. Herrera-Rodríguez, A. González-Fontes, Boron deficiency increases expressions of asparagine synthetase, glutamate dehydrogenase and glutamine synthetase genes in tobacco roots irrespective of the nitrogen source, Soil Sci. Plant Nutr. 60 (2014) 314–324. doi:10.1080/00380768.2014.881706.

[56] M.T. Rose, T.J. Rose, J. Pariasca-Tanaka, T. Yoshihashi, H. Neuweger, A. Goesmann, M. Frei, M. Wissuwa, Root metabolic response of rice (*Oryza sativa* L.) genotypes with contrasting tolerance to zinc deficiency and bicarbonate excess, Planta. 236 (2012) 959–973. doi:10.1007/s00425-012-1648-4.

[57] J. Possingham, The effect of mineral nutrition on the content of free amino acids and amides in tomato plants I A comparison of the effects of deficiencies of copper, zinc, manganese, iron, and molybdenum, Aust. J. Biol. Sci. 9 (1956) 539–551. doi:10.1071/BI9560539.

[58] S. Ghasemi, A.H. Khoshgoftarmanesh, M. Afyuni, H. Hadadzadeh, The effectiveness of foliar applications of synthesized zinc-amino acid chelates in comparison with zinc sulfate to increase yield and grain nutritional quality of wheat, Eur. J. Agron. 45 (2013) 68–74. doi:10.1016/j.eja.2012.10.012.

[59] P. Mohammadi, A.H. Khoshgoftarmanesh, The effectiveness of synthetic zinc (Zn)-amino chelates in supplying Zn and alleviating salt-induced damages on hydroponically grown lettuce, Sci. Hortic. (Amsterdam). 172 (2014) 117–123. doi:10.1016/j.scienta.2014.03.047.

[60] M.J. Haydon, C.S. Cobbett, Transporters of ligands for essential metal ions in plants, New Phytol. 174 (2007) 499–506. doi:10.1111/j.1469-8137.2007.02051.x.

[61] H. Holländer-Czytko, J. Grabowski, I. Sandorf, K. Weckermann, E.W. Weiler, Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in Arabidopsis under stress conditions, J. Plant Physiol. 162 (2005) 767–770. doi:10.1016/j.jplph.2005.04.019.

[62] F. Potel, M.-H. Valadier, S. Ferrario-Méry, O. Grandjean, H. Morin, L. Gaufichon, et al., Assimilation of excess ammonium into amino acids and nitrogen translocation in *Arabidopsis thaliana*– roles of glutamate synthases and carbamoylphosphate synthetase in leaves, FEBS J. 276 (2009) 4061–4076. doi:10.1111/j.1742-4658.2009.07114.x.

**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  0.01 µM Zn  *p*-value  LSD0.05 | 44.58±0.18  35.80±1.50  \*\*  4.20 | 4.12±0.07  3.16±0.03  \*\*\*  0.20 | 0.16±0.01  0.07±0.01  \*\*\*  0.03 |
| *B. oleracea* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 50.49±3.17  16.36±4.36  \*\*  14.97 | 3.61±0.02  2.91±0.13  \*\*  0.34 | 0.21±0.01  0.13±0.00  \*\*\*  0.03 |
| Analysis of variance | | | | |
| Doses (D)  Especies (E)  D x E  LSD0.05 | | \*\*\*  \*  \*\*  6.46 | \*\*\*  \*\*\*  NS  0.18 | \*\*\*  \*\*\*  NS  0.02 |

Values are means ± S.E. (n=9) and differences between means were compared by Fisher´s 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 (\*\*) and p<0.001 (\*\*\*).

**Table 2** Response of NO3- reduction and NH4+concentration in *L. sativa* and *B. oleracea* leavessubmitted to Zn deficiency.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | | NO3-  (mg g-1 DW) | NR  (µM NO2 mg prot-1 min-1) | NH4+  (mg g-1DW) | |
| *L. sativa* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 94.47±1.15  100.56±0.96  \*\*\*  3.17 | 0.49±0.01  0.23±0.01  \*\*\*  0.03 | 2.16±0.05  2.01±0.07  NS  0.18 | |
| *B. oleracea* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 104.26±1.48  85.29±0.94  \*\*\*  3.72 | 1.71±0.03  1.30±0.13  \*\*\*  0.12 | 1.85±0.09  2.25±0.05  \*\*  0.21 | |
| Analysis of variance | | | | |  |
| Doses (D)  Especies (E)  D x E  LSD0.05 | | \*\*\*  \*  \*\*\*  2.35 | \*\*\*  \*\*\*  \*  0.06 | NS  NS  \*\*\*  0.13 | |

Values are means ± S.E. (n=9) and differences between means were compared by Fisher´s 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 (\*\*) and p<0.001 (\*\*\*).

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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | | GO | GGAT | HR |
| (ΔAbs mg prot-1 min-1) | | |
| *L. sativa* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 0.003±0.00  0.01±0.00  \*\*\*  0.00 | 0.01±0.00  0.03±0.00  \*\*\*  0.00 | 0.86±0.03  0.80±0.03  NS  0.08 |
| *B. oleracea* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 0.005±0.00  0.01±0.00  \*\*\*  0.00 | 0.02±0.00  0.05±0.00  \*\*\*  0.00 | 0.88±0.01  0.92±0.02  NS  0.04 |
| Analysis of variance | | | |  |
| Doses (D)  Especies (E)  D x E  LSD0.05 | | \*\*\*  \*\*\*  \*  0.00 | \*\*\*  \*\*\*  \*\*\*  0.00 | NS  \*\*  \*  0.05 |

Values are means ± S.E. (n=9) and differences between means were compared by Fisher´s 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 (\*\*) and p<0.001 (\*\*\*).

**Table 4** Response of enzymes responsible for NH4+ assimilation and concentration of total reduced N in *L. sativa* and *B. oleracea* leavessubmitted to Zn deficiency.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | | GS  (µM glutamylhydroxamate mg prot-1 min-1) | GOGAT  (ΔAbs mg prot-1min-1) | GDH  (ΔAbs mg prot-1 min-1) | Total reduced N  (mg g-1 PS) |
| *L. sativa* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 15.04±0.58  2.62±0.31  \*\*\*  1.39 | 1.32±0.06  0.56±0.05  \*\*\*  0.16 | 1.00±0.09  0.17±0.01  \*\*\*  0.19 | 44.12±2.07  44.98±1.87  NS  5.91 |
| *B. oleracea* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 11.07±0.38  6.67±0.21  \*\*\*  0.91 | 1.35±0.06  0.45±0.04  \*\*\*  0.14 | 4.25±0.14  0.26±0.03  \*\*\*  0.31 | 24.36±1.40  17.07±0.64  \*\*\*  3.26 |
| Analysis of variance | | |  |  |  |
| Doses (D)  Especies (E)  D x E  LSD0.05 | | \*\*\*  NS  \*\*\*  0.8 | \*\*\*  NS  NS  0.1 | \*\*\*  \*\*\*  \*\*\*  0.17 | NS  \*\*\*  \*  3.24 |

Values are means ± S.E. (n=9) and differences between means were compared by Fisher´s 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 (\*\*) and p<0.001 (\*\*\*).

**Table 5** Response of foliar amino acids concentration in *L. sativa* and *B. oleracea* leavessubmitted to Zn deficiency.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | Arg | Asn | | Asp | | | Gln | | Glu | | Gly | | His | | Ser | | Tyr | |
|  | | Amino acids concentration (μmol g-1 FW) | | | | | | | | | | | | | | | | | |
| *L. sativa* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 2.52±0.03  1.05±0.09  \*\*\*  0.25 | 2.93±0.06  1.16±0.07  \*\*\*  0.26 | | 3.05±0.09  2.75±0.15  NS  0.50 | | 5.11±0.10  3.31±0.22  \*\*  0.68 | | | 4.85±0.12  3.97±0.12  \*\*  0.46 | | 0.23±0.00  1.93±0.02  \*\*\*  0.05 | | 0.36±0.02  0.35±0.02  NS  0.07 | | 1.70±0.09  1.76±0.08  NS  0.33 | | 0.18±0.00  0.11±0.01  \*\*\*  0.02 | |
| *B. oleracea* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 0.39±0.04  0.6±0.03  \*  0.13 | 0.94±0.01  1.07±0.03  NS  0.28 | | 1.81±0.1  2.24±0.08  \*  0.36 | | 2.37±0.01  3.07±0.16  \*\*  0.45 | | | 3.87±0.17  4.57±0.19  NS  0.71 | | 0.23±0.00  0.20±0.02  NS  0.04 | | 0.23±0.02  0.43±0.02  \*\*  0.08 | | 3.58±0.08  3.04±0.21  NS  0.62 | | 0.34±0.00  0.78±0.04  \*\*\*  0.11 | |
| Analysis of variance | | | |  | |  | | |  | |  | |  | |  | |  | |  |  |
| Doses (D)  Especies (E)  D x E  LSD0.05 | | \*\*\* | \*\*\* | | NS | | \*\* | | | NS | | \*\*\* | | \*\* | | NS | | \*\*\* | |
| \*\*\* | \*\*\* | | \*\*\* | | \*\*\* | | | NS | | \*\*\* | | NS | | \*\*\* | | \*\*\* | |
| \*\*\* | \*\*\* | | \* | | \*\*\* | | | \*\*\* | | \*\*\* | | \*\*\* | | \* | | \*\*\* | |
| 0.12 | 0.16 | | 0.26 | | 0.34 | | | 0.35 | | 0.03 | | 0.04 | | 0.29 | | 0.05 | |

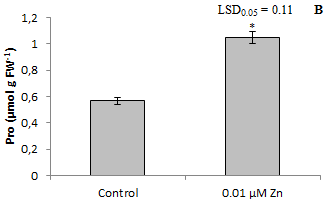
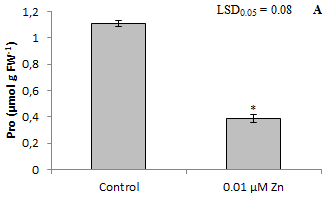
Values are means ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P=0.05). The levels of significance were represented by p>0.05: ns (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

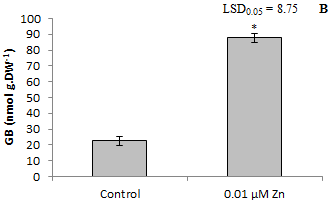
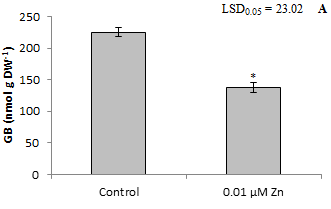
**Figures**

**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.

**Fig. 3.** Zn deficiency treatment percentages relatives to control in *L. sativa* and *B. oleracea* for all the parameters analyzed: Leaf Zn concentration (A), shoot biomass (B), root biomass (C), NO3- concentration (D), NR activity (E), NH4+ concentration (F), GO activity (G), GGAT activity (H), HR activity (I), GS activity (J), GOGAT activity (K), GDH activity (L) and total reduced N (M), Pro (N) and GB (O) concentrations. Horizontal line at 100% represents the mean value of controls and bars represent mean values of percentages relative to control ± SE (n = 9). Bars followed by the same letter are not significantly different using LSD (P = 0.05).

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