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**Running title:** Carboxylate metabolism in Zn-deficient *L. sativa* and *B.oleracea* plants

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**Response of carboxylate metabolism to Zn deficiency in *Lactuca sativa* and *Brassica oleracea* plants**

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**Keywords** Zn deficiency, Zn use efficiency, organic acids, carboxylate metabolism, *Lactuca sativa*, *Brassica oleracea*

**Abstract**

Different species or genotypes differ in their Zn use efficiency (ZnUE) under low Zn availability in the soil. Organic acids (OAs) synthetised by plant carboxylate metabolism could play a role in Zn-deficiency tolerance. The main objective of the present work was to assess the response of two species of great agronomic interest such as *Lactuca sativa* and *Brassica oleracea* to Zn deficiency focusing on OAs and carboxylate metabolism. For this, *L. sativa* and *B. oleracea* plants were grown in hydroponic culture with different Zn-application rates: 10 µM Zn as control and 0.1 µM Zn as deficiency treatment. ZnUE parameters, OAs concentration and carboxylate metabolism enzymes were analysed. *L. sativa* accumulated more Zn and registered better Zn uptake efficiency (ZnUpE) while *B. oleracea* lost less biomass and it registered better Zn utilization efficiency (ZnUtE). In *L. sativa* citrate and oxalate concentrations and PEPC and CS activities increased while FUM and MDH activities declined. In *B. oleracea* no significant response were found in OAs concentration neither carboxylate metabolism except for a decrease in FUM activity. These results suggest that a possible factor that induces TCA cycle could be a low ZnUtE rather than a low Zn concentration under Zn-deficiency conditions. In *L. sativa* citrate, oxalate, PEPC and CS could play a key role to face Zn deficiency while in *B. oleracea* the higher ZnUtE can not be explained by a rise in OAs synthesis.

**Abbreviations** CS, citrate synthase; Cu-Zn SOD, Cu-Zn superoxide dismutase; FUM, fumarase; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; OAs, organic acids; PEPC, phosphoenolpyruvate carboxylase; TCA, tricarboxylic acid cycle; ZnUE, Zn use efficiency; ZnUpE, Zn uptake efficiency; ZnUtE, Zn utilization efficiency; LMWOAs, low-molecular weight organic acids.

**1 Introduction**

Zinc (Zn) is an essential micronutrient for living organisms which is present in a wide variety of metabolic processes such as protein synthesis, maintenance of cell membrane integrity, and synthesis of auxin precursor tryptophan (*Vallee and Auld*, 1990). Furthermore, Zn is part of carbonic anhydrase, Cu-Zn-superoxide dismutase (Cu-Zn SOD), dehydrogenases, and Zn-finger structural domains of DNA transcription factors (*Fox and Guerinot*, 1998). It has been found that Zn deficiency is the most widespread micronutrient deficiency. It occurs in plants growing in soils that have low total Zn concentrations or low plant-available Zn, located in many world regions (*Sillanpää*, 1982). Low Zn bioavailability in soils leads to a fall in Zn concentration in the shoot, often resulting in stunted growth (*Alloway*, 2008). However, different species or genotypes differ in their ability to produce biomass when they grow under similar nutrient concentrations in the soil, i.e. they differ in their use efficiency of the nutrient (*Rengel*, 2007). The so-called nutrient use efficiency parameters are useful to evaluate the nutrient efficiency. These show a direct relationship between biomass and micronutrient concentration. For instance, Zn uptake efficiency (ZnUpE) indicates the plant ability to uptake Zn from the soil (*Siddiqi and Glass*, 2008) and Zn utilization efficiency (ZnUtE) shows the plant capacity to transfer and utilize Zn within plant organs (*Elliott and Læuchli*, 1985). As a result of Zn deficiency several changes in physiological processes occur: a decline in processes such as photosynthesis, glycolysis, starch synthesis, protein synthesis, flowering, seed production, and also membrane destabilization (*Brown et al.*, 1993). Furthermore, as described later, Zn deficiency stress can induce changes in carboxylate metabolism and organic acids (OAs) concentration.

Several studies have demonstrated that tolerant genotypes to Zn and Fe deficiencies present an enhanced production and release of Zn ligands such as low-molecular weight organic acids (LMWOAs) (*Widodo et al.*, 2010; *Rose et al.*, 2011; *Covarrubias and Rombolà*, 2015). This is probably due to the role of OAs as chelants and metal ion transporters and they are necessary for Zn uptake, transport, and accumulation within the plant. Therefore, they can be crucial for the proper distribution of Zn to face deficiency conditions (*Liu et al.*, 2012). Among LMWOAs, citric, malic, and oxalic acids are the most important and they can influence both nutrient uptake and transport, thus they are usually accumulated under deficiency conditions (*Evangelou et al.*, 2007). Furthermore, in a study concerning *Brassica rapa* plants grown under Zn-deficiency conditions an increase in citrate concentration has been related to a higher Zn-solution extraction ability (*Blasco et al.*, 2015).

The main OAs are synthesized in tricarboxylic acid (TCA) cycle, known as Krebs cycle, that produces citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, and oxalate. TCA cycle plays a key role within plant physiology since it is closely related to other important processes such as N assimilation, amino acid biosynthesis, photorespiration, and energy metabolism. Besides, metabolites involved in TCA directly or indirectly regulate other metabolic pathways (*Foyer et al.*, 2011). TCA cycle takes part in the mitochondrial matrix and it consist of several enzymes. Citrate synthase (CS) catalyses the condensation of acetyl-CoA with a molecule of oxalate to produce citrate and it is the only enzyme to catalyze the formation of carbon-carbon bond (*Popova and Pinheiro de Carvalho*, 1998). Fumarase (FUM) catalyzes the formation of malate from fumarate. Malate dehydrogenase (MDH) catalyzes the reversible reaction of malate to oxaloacetate and it contributes to NADH oxidation as well to the exchange of different compounds across the mitochondrion membrane (*Araújo et al.*, 2012). MDH forms malate in the cytosol but it also catalyzes its degradation in the mitochondria, favoring the formation of oxaloacetate. Phosphoenolpyruvate carboxylase (PEPC) catalyzes the formation of oxaloacetate from phosphoenolpyruvate (PEP) and HCO3 (*Lance and Rustin*, 1979). PEPC in plant is an enzyme involved in several physiological processes and there is a PEPC C3 branching from glycolysis to replenish TCA intermediates within an anaplerotic pathway, and thereby providing precursors for amino acid synthesis (*Stitt*, 1999).

In a previous study by our research group concerning *Lactuca sativa* cv. Phillipusand *Brassica oleracea* cv. Bronco it was demonstrated that these species show differences in carboxylate metabolism and they are affected differently by Zn toxicity. While *L. sativa* accumulates mainly malate, *B. oleracea* accumulates mainly citrate. This work concludes that *B. oleracea* is more tolerant than *L. sativa* against Zn toxicity because it showed lower decline in shoot biomass despite it registered stronger Zn accumulation. In this case citrate could contribute to Zn homeostasis in leaves, thus this OA could be key in Zn toxicity tolerance (*Barrameda-Medina et al.*, 2014). Briefly, in this work we analyze the role of carboxylate metabolism in these two species grown under Zn-deficiency conditions to ascertain whether carboxylate metabolism is a key physiological process to select and/or generate plants with Zn-deficiency tolerance.

**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 x 3 cm x 10 cm) filled with a 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 22/18ºC (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 with hydroponic culture in lightweight polypropylene trays (60 cm top diameter, 60 cm bottom diameter, and 7 cm high) 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, 0.25 μM CuSO4 • 5 H2O, 0.1 μM Na2MoO4 • 2 H2O 5 ppm, 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 started 30 days after germination and were kept for 21 days. Plants were grown with different Zn-application rates: 10 µM of ZnSO4 as control and 0.1 µM of ZnSO4 as deficiency treatment. The experimental design consisted of randomized complete block with four treatments (*L. sativa*-control, *B. oleracea*-control, *L. sativa*-0.1 µM Zn, *B. oleracea*-0.1 µM Zn), eight plants per treatment and three replications each.

**2.2 Plant sampling**

Plants from each treatment were divided into roots and leaves, washed with distilled water, dried on filter paper, and weighed for fresh weight (FW). Half of the roots and leaves from each treatment were frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure the dry weight (DW) and the Zn concentration was determined.

**2.3 Analysis of Zn and ZnUE parameters**

For Zn concentration determination, a sample of 150 mg dry material was subjected to a process of mineralization with sulfuric acid and H2O2, then Zn concentration was determined by ICP-MS (*Wolf*, 1982). Zn use efficiency paramaters (ZnUE) were calculated for each treatment as follow:

ZnUtE was calculated as leaf tissue dry weight (LDW) divided by Zn concentration (g2 LDW mg−1 Zn) (*Siddiqi and Glass*, 2008).

ZnUpE was calculated as total Zn accumulation divided by root dry weight (RDW) (mg Zn g−1 RDW) (*Elliott and Læuchli*, 1985).

**2.4 Extraction and analysis of organic acids**

Malic, citric and oxalic acids were analysed according to *Gómez-Romero et al.* (2010) with some modifications. Briefly, 75 mg of freeze-dried and ground leaves were dropped in 1 ml of cold (-20°C) extraction mixture of methanol/water/acetic acid (80/19.5/0.5, v/v/v). Solids were separated by centrifugation (14800 rpm, 15 min) and re-extracted for 30 min at 4ºC in additional 1 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridges (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40ºC under vacuum to near dryness. The residue was dissolved in 1 ml water/methanol/acetic acid (94.5/5/0.5, v/v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA). 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The analytes were separated using a Zorbax SB-C18 HPLC column (5 µm, 150 x 0.5 mm, Agilent Technologies, Santa Clara, CA, USA), maintained at 30 ºC. Mobile phase A, consisting of water/methanol/acetic acid (94.5/5/0.5), and mobile phase B, consisting of water/methanol/acetic acid (10/89.5/0.5), were pumped at a flow rate of 300 µl·min-1. The elution programme maintained 100% A for 5 min, then a linear gradient from 0 to 6% B in 10 min, followed by another linear gradient from 6 to 100% B in 5 min, and finally 100% B maintained for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 15 min before each analytical run. The mass spectrometer was operated in the negative mode with a capillary spray voltage of 2500 V. The sheath gas flow rate was set to 35 ml·min-1 whereas the auxiliary gas was set to a flow rate of 10 ml·min-1. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the organic acids, calibration curves were constructed for each analysed component (1, 2.5, 5, and 10 mg l-1).

**2.5 Enzyme extractions and assays**

Extracts for measuring enzyme activities were made following the method of *Li* (2000), modified by grinding 0.1 g of leaves in 1 ml of extraction buffer containing 1 mM EDTA-Na, 10% glycerol,1% TritonX-100, 5 mM DTT and 1% polyvinylpyrrolidone (PVP) in 100 mM Tris–HCl pH 8.0. The slurry was centrifuged for 5 min at 14700 rpm and 4 ºC, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 0.2 ml (final volume) of the media indicated below. CS (EC4.1.3.7) activity was assayed spectrophotometrically by monitoring the reduction of acetyl coenzyme A (CoA) to Co A with 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm (*Srere*, 1969). The reaction was carried out in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5mM oxalate and 100 mM Tris–HCl, pH8.1. PEPC (EC4.1.1.31) activity was measured in a coupled enzyme assay with the MDH in 2 mM phosphoenolpyruvate (PEP), 10 mM NaHCO3, 5 mM MgCl2, 0.16 mM NADH and 100 mM of N,N-bis[2-hydroxyethyl]glycine (Bicine)-HCl, pH 8.5 (*López-Millán et al.*, 2001). FUM (EC4.2.1.2) was assayed following the increase in optical density at 240 nm due to the formation of fumarate in 50 mM malate and 100 mM phosphate buffer, pH 7.4 (*Bergmeyer et al.*, 1974). Finally MDH (EC 1.1.1.37) activity was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (*Dannel et al.*, 1995). The reaction was carried out with 0.1 mM NADH, 0.4 mM oxalate and 46.5 mM Tris–HCl, pH 9.5. The protein concentration of the extracts was determined using bovine-serum albumin as the standard (*Bradford*, 1976).

**2.6 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, Zn concentration, and ZnUE parameters**

Because of the important role of Zn within plants, when its concentration is low it constitutes a stress that can affect plant growth and development. Thus, the main symptom of plants suffering from Zn deficiency is a loss of biomass as it has been reported in crop plants such as carrot, lettuce, onion and tomato (*Alloway*, 2008). Likewise, it was found that Zn deficiency is the most widespread micronutrient deficiency in rice and its cause significant crop losses (*Neue and Lantin*, 1994). In the present study Zn deficiency treatment caused a fall in foliar biomass relative to control in both *L. sativa* and *B. oleracea* plants, although this decrease was greater in *L. sativa* (Fig. 1A). This reduction was caused by the lower Zn concentration we observed in both species submitted to Zn deficiency (Fig. 1B), however the drop in Zn concentration was greater in *B. oleracea.*

ZnUE parameters show a direct relationship between biomass and Zn concentration and they are useful to define the Zn uptake and utilization capacity within the plant (*Rengel*, 2007). ZnUtE showed no significant differences in comparison to control in *L. sativa* plants grown under Zn deficit but it was higher in *B. oleracea* plants submitted to Zn deficiency (Fig. 1C). Respecting ZnUpE, it was reduced by Zn deficiency in both species, although in greater extent in *B. oleracea* plants (Fig. 1D).

**3.2 Carboxylate metabolism and organic acids concentration**

The results showed that among all the OAs analysed, malate was the most important in the shoot of both species followed by citrate and oxalate as the least concentrated (Table 1). Nevertheless, malate concentration fall in *L. sativa* plants under Zn deficiency, contrasting with the increment in citrate and oxalate concentrations (Table 1). In *B. oleracea* plants grown under Zn deficiency we detected a decrease in oxalate concentration, while citrate and malate showed no significant differences with respect control (Table 1).

Respecting TCA enzymes, we found a rise in CS and PEPC activities and a decline in FUM and MDH activities in Zn-deficient *L. sativa* plants (Table 2). In *B. oleracea* plants no significant differences were found in comparison to control in all activities except for a reduction in FUM activity (Table 2).

**4 Discussion**

Several authors have reported a decline in both shoot biomass and Zn concentration in plants grown under Zn deficiency. *Hajiboland and Amirazad* (2010) grew *B. oleracea* plants without Zn in the nutritive solution for two weeks and their biomass declined by 62% in comparison to control plants. In another study various rice genotypes grown in hydroponics with 0.1 μM of Zn were stunted and their shoot Zn concentration declined by 75% (*Wissuwa et al.*, 2006). In the present study, Zn-deficiency treatment caused a foliar biomass reduction in both species, although this reduction was two-fold higher in *L. sativa* (44% lower than in control: Fig. 1A). The biomass loss was caused by a decrease in Zn concentration as we observed in both species; however this decrease was higher in *B. oleracea* plants (68% lower than in control: Fig. 1B). Regarding ZnUtE, there were no significant differences between Zn-deficiency and control treatments in *L. sativa* plants, while ZnUtE rose by a 154% in Zn-deficient *B. oleracea* respect to control (Fig. 1C). These results show that *B. oleracea* registered a better Zn efficiency and was able to keep its biomass despite its lower Zn concentration. However, the ZnUpE reduction was lower in *L. sativa* plants grown under Zn deficiency (47% lower) while in *B. oleracea* was 74% lower (Fig. 1D). These results are due to the higher Zn-uptake capacity of *L. sativa* which allows this species to keep its foliar Zn concentration under Zn-deficiency conditions.

In rice plants, a correlation was detected between ZnUpE and the exudation of OAs to the soil, which could help to Zn uptake (*Wissuwa et al.*, 2006). Different results have been obtained in studies relating OA concentration in leaves with micronutrient disorders but in most of them there was an increase in citrate concentration in plants grown under different mineral stress conditions: Fe deficiency and Zn toxicity in *Beta vulgaris* (*López-Millán et al*., 2001; *Sagardoy et al.*, 2011), and Zn deficiency and toxicity in *B. rapa* (*Blasco et al.*, 2015). This is consistent with our results in *L. sativa* where citrate concentration rose by 37%, however, in *B. oleracea* this OA was not important to face Zn deficiency (Table 1). *Barrameda-Medina et al.* (2014) reported similar results in the same species but grown under Zn toxicity conditions, thus it appears that citrate concentration is affected in the same way by both Zn toxicity and Zn deficiency in these species. Therefore, citrate could help *L. sativa* to accumulate more Zn under Zn shortage, or to store it under Zn toxicity conditions. This is supported by the fact that citrate is a strong chelator with high mineral-binding capacity and it could contribute to homeostasis in plants under mineral stress (*Sagardoy et al.*, 2011).

Malate is another OA that can be affected by Zn concentration, as reported in tolerant genotypes of rice to Zn deficiency in which this OA rose both their concentration and their efflux from roots (*Rose et al.*, 2011). Nevertheless, in other studies malate was not decisive for better tolerance to Zn stresses as observed in *B. rapa* plants where it declined (*Blasco et al.*, 2015). On the other hand, in leaves of *B. oleracea* plants submitted to Zn toxicity it has been reported an increase in malate concentration and it has been suggested that it could help to transport Zn to aerial tissues under Zn-toxicity conditions (*Barrameda-Medina et al.*, 2014). According the results of the present study malate did not play a transport role under Zn deficiency in both species assessed (Table 1).

There are no studies showing a relationship between oxalate and Zn-deficiency tolerance, but in a study by *Mathys* (1977) it was detected that applying Zn to the nutrient solution, the synthesis of oxalate was enhanced in Zn-resistant, but inhibited in Zn-sensitive ecotypes of *Silene cucubalus* and *Rumex acetosa*. By contrast, it has been observed in both *L. sativa* and *B. oleracea* that oxalate was not decisive for a greater tolerance under Zn toxicity conditions (*Barrameda-Medina et al.*, 2014). However, oxalate could be very important to face Zn deficiency in *L. sativa* plants since its concentration is sharply increased (Table 1). This is supported by a study that demonstrated that the reaction of Zn with oxalate forms a stable Zn-oxalate complex (*Sillen*, 1964), thus oxalate might join Zn in the soil forming Zn-oxalate complexes that would be absorbed by plants in soils with low Zn bioavailability.

Briefly, according our results ZnUtE was more important than Zn concentration within the plant to alter OA concentration. Because of *B. oleracea* plants have improved ZnUtE under Zn deficiency (Fig. 1C), they did not need to induce a response in OA concentration and their concentrations did not differ in comparison to control plants (Table 1). By contrast, *L. sativa* did not improve their ZnUtE under Zn deficiency (Fig. 1C) so OAs as citrate and oxalate would be accumulated in order to face Zn deficiency.

There are few studies about how micronutrient deficiencies affect TCA enzyme activities. The published literature show different results depending on the species and the micronutrient stress but most agree on a CS activity increase (*López-Millán et al.*, 2001; *Jelali et al.*, 2010; *Widodo et al.*, 2010; *Barrameda-Medina et al.*, 2014; *Blasco et al.*, 2015). Regarding PEPC activity, it was enhanced by Fe deficiency in tolerant genotypes of *Pisum sativum* (*Jelali et al.*, 2010) and in *Beta vulgaris* (*López-Millán et al.*, 2001) while it was not affected by Zn toxicity in *L. sativa* and *B. oleracea* plants (*Barrameda-Medina et al.*, 2014). In the present work Zn deficiency stimulates both CS and PEPC activities in *L. sativa* plants (Table 2); this can explain the rise in citrate and oxalate concentrations (Table 1) and it could be a strategy to synthetise more of these OAs in order to accumulate more Zn under deficiency conditions. CS and PEPC enzymes were not affected by Zn deficiency in *B. oleracea* as their activities were stabilized at control levels in plants grown under Zn deficit (Table 2).

Respecting FUM and MDH activities, we found mixed results about their response under micronutrient deficiencies in published works. These two enzymes increased their activity in Fe-deficient *Beta vulgaris* (*López-Millán et al.*, 2001) and the enzyme activities returned to control levels 24 hours after the resupply with Fe. In another study, Fe deficiency caused a reduction in MDH activity in sensible grapevine genotypes although these activities were stabilized in tolerant genotypes. Thus MDH activity could be important in Fe-deficiency tolerance (*Covarrubias and Rombolà*, 2015). Regarding Zn deficiency, it has been reported that the MDH gene expression was higher in leaves of Zn-deficient rice plants but it was lower in the tolerant genotype, suggesting that this activity is not decisive in Zn-deficit tolerance (*Widodo et al.*, 2010). In another experiment concerning *B. rapa* plants grown under Zn deficiency it was noted that FUM activity dropped while MDH activity rose in comparison to controls (*Blasco et al.*, 2015). In our experiment we found an activity decline in the part of the TCA cycle carried out by FUM and MDH enzymes in *L. sativa* plants grown under Zn shortage (Table 2). The reduction in FUM activity could be the cause of malate concentration to fall by 35% (Table 1) and, in turn, this causes a fall by the same percentage in MDH activity (Table 2). Therefore in *L. sativa* plants submitted to Zn deficiency an increase in PEPC activity is essential to offset MDH decline and keep, even increase, oxalate concentration to continue the TCA cycle under lower Zn conditions. In both species assessed the decrease in FUM activity may be caused by a reduction in previous enzyme activities within the TCA cycle, or by an enhancement of divergent pathways leading to an exit of fumarate from the cycle, and thus reducing FUM activity. One of these divergent pathways leads to amino acid biosynthesis from 2-oxoglutarate. This pathway could be especially enhanced in *B. oleracea*, given that in a previous study we detected an increase in amino acids such as glutamine, aspartic acid, and arginine under Zn-deficiency conditions (*Navarro-León et al.*, 2016). This could be crucial to face Zn-deficiency stress in *B. oleracea* plants. Thus, a possibility in plant breeding could be the improvement of PEPC and CS activities and/or an inhibition of FUM activity to generate plants with better ZnUpE and ZnUtE indexes which are able to face Zn-deficiency stress.

**5 Conclusion**

The results obtained in this work show that both species assessed have different strategies to face Zn deficiency. *L. sativa* registered better capacity to accumulate Zn in the shoot, possibly through a rise in citrate and oxalate concentrations. Furthermore PEPC and CS activities could play a key role to face Zn deficiency in this species. *B. oleracea* had a high ZnUtE that can not be explained by an increment in OAs synthesis. Nevertheless, a decrease in FUM activity could indicate the enhancement of a divergent way of TCA cycle for increased amino-acid production that would help to face Zn deficit. Therefore, one possible factor that induces the TCA cycle could be a low ZnUE rather than a low Zn concentration under deficiency conditions. More research is needed to establish the role of OAs and carboxylate metabolism under Zn- deficiency conditions. However, a possible target for crop breeding may be the enhancement of CS and PEPC activities or the inhibition of FUM activity in order to generate plants with both higher Zn accumulation and higher ZnUE.

**Acknowledgments**

This work was financed by the PAI programme (Plan Andaluz de Investigación, Grupo de Investigación AGR161) and by a Grant from the FPU of the Ministerio de Educación y

Ciencia awarded to ENL

**References**

*Alloway, B.J.* (2008): Zinc in soils and crop nutrition, 3th ed. International Zinc Association Brussels, Belgium.

*Araújo, W.L., Nunes-Nesi, A., Nikoloski, Z., Sweetlove, L.J., Fernie, A.R.* (2012): Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. *Plant. Cell Environ.* 35, 1–21.

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

*Bergmeyer, H., Bernt, E., Schmidt, F.* (1974): 1n: Bergmeyer HU, ed. Methods of enzymatic analysis.

*Blasco, B., Graham, N.S., Broadley, M.R.* (2015): Antioxidant response and carboxylate metabolism in *Brassica rapa* exposed to different external Zn, Ca, and Mg supply. *J. Plant Physiol.* 176, 16–24.

*Bradford, M.M.* (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

*Brown, P.H., Cakmak, I., Zhang, Q.* (1993): Form and Function of Zinc Plants, in: Robson, A.D. (ed.): . Springer Netherlands, pp. 93–106.

*Covarrubias, J.I., Rombolà, A.D.* (2015): Organic acids metabolism in roots of grapevine rootstocks under severe iron deficiency. *Plant Soil* 394, 165–175.

*Dannel, F., Pfeffer, H., Marschner, H.* (1995): Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. *J. Plant Physiol.* 146, 273–278.

*Elliott, G.C., Læuchli, A.* (1985): Phosphorus efficiency and phosphate-iron interaction in maize. *Agron. J.* 77, 399.

*Evangelou, M.W.H., Ebel, M., Schaeffer, A.* (2007): Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. *Chemosphere* 68, 989–1003.

*Fox, T.C., Guerinot, M. Lou* (1998): Molecular biology of cation transport in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 669–696.

*Foyer, C.H., Noctor, G., Hodges, M.* (2011): Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. *J. Exp. Bot.* 62, 1467–82.

*Gómez-Romero, M., Segura-Carretero, A., Fernández-Gutiérrez, A.* (2010): Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. *Phytochemistry* 71, 1848–64.

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

*Jelali, N., Wissal, M., Dell’orto, M., Abdelly, C., Gharsalli, M., Zocchi, G.* (2010): Changes of metabolic responses to direct and induced Fe deficiency of two *Pisum sativum* cultivars. *Environ. Exp. Bot.* 68, 238–246.

*Lance, C., Rustin, P.* (1979): The central role of malate in plant metabolism. *Physiol. végétale* 22, 625–641.

*Li, X.F.* (2000): Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123, 1537–1544.

*Liu, D., Liu, A.-H., He, C., Wang, J.-H., Wang, Y.-A.* (2012): Response of organic acids to zinc homeostasis in zinc-deficient and zinc-toxic apple rootstock roots. *Pedosphere* 22, 803–814.

*López-Millán, A.F., Morales, F., Abadía, A., Abadía, J.* (2001): Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris*) leaves. *Physiol. Plant.* 112, 31–38.

*Mathys, W.* (1977): The role of malate, oxalate, and mustard oil glucosides in the evolution of zinc-resistance in herbage plants. *Physiol. Plant.* 40, 130–136.

*Navarro-León, E., Barrameda-Medina, Y., Lentini, M., Esposito, S., Ruiz, J.M., Blasco, B.* (2016): Comparative study of Zn deficiency in *L. sativa* and *B. oleracea* plants: NH4+ assimilation and nitrogen derived protective compounds. *Plant Sci.* 248, 8–16.

*Neue, H.U., Lantin, R.S.* (1994): Micronutrient toxicities and deficiencies in rice, in: Yeo, A.R., Flowers, T.J. (eds.): Soil Mineral Stresses: Approaches to Crop Improvement. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 175–200.

*Popova, T.N., Pinheiro de Carvalho, M.Â..* (1998): Citrate and isocitrate in plant metabolism. *Biochim. Biophys. Acta - Bioenerg.* 1364, 307–325.

*Rengel, Z.* (2007): Genotypic differences in micronutrient use efficiency in crops. *Commun. Soil Sci. Plant Anal.* 32, 1163–1186.

*Rose, M.T., Rose, T.J., Pariasca-Tanaka, J., Widodo, Wissuwa, M.* (2011): Revisiting the role of organic acids in the bicarbonate tolerance of zinc-efficient rice genotypes. *Funct. Plant Biol.* 38, 493–504.

*Sagardoy, R., Morales, F., Rellán-Álvarez, R., Abadía, A., Abadía, J., López-Millán, A.F.* (2011): Carboxylate metabolism in sugar beet plants grown with excess Zn. *J. Plant Physiol.* 168, 730–3.

*Siddiqi, M.Y., Glass, A.D.M.* (2008): Utilization index: A modified approach to the estimation and comparison of nutrient utilization efficiency in plants. *J. Plant Nutr.* 4, 289–302.

*Sillanpää, M.* (1982): Micronutrients and the Nutrient Status of Soils: A Global Study. Food & Agriculture Org., Rome.

*Sillen, L.* (1964): Stability constants of metal-ion complexes. Section 1: inorganic ligands. Section 2: organic ligands. .

*Srere, P.A.* (1969): Citric acid cycle, Methods in Enzymology. Elsevier.

*Stitt, M.* (1999): Nitrate regulation of metabolism and growth. *Curr. Opin. Plant Biol.* 2, 178–86.

*Vallee, B.L., Auld, D.S.* (1990): Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 29, 5647–5659.

*Widodo, J.A., Broadley, M.R., Rose, T., Frei, M., Pariasca-Tanaka, J., Yoshihashi, T., Thomson, M., Hammond, J.P., Aprile, A., Close, T.J., Ismail, A.M., Wissuwa, M.* (2010): Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity. *New Phytol.* 186, 400–414.

*Wissuwa, M., Ismail, A.M., Yanagihara, S.* (2006): Effects of zinc deficiency on rice growth and genetic factors contributing to tolerance. *Plant Physiol.* 142, 731–741.

*Wolf, B.* (1982): A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. *Commun. Soil Sci. Plant Anal.* 13, 1035–1059.

**Table 1** Organic acid concentrations in *L. sativa* and *B. oleracea* plants submitted to Zn deficiency

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | | Citrate  (mg g-1DW) | Malate  (mg g-1 DW) | Oxalate  (μg g-1DW) | |
| *L. sativa* | Control  0.1 µM Zn  *p*-value  LSD0.05 | 1.67 ± 0.04  2.28 ± 0.06  \*\*  0.20 | 25.36 ± 0.32  16.76 ± 0.05  \*\*\*  0.90 | 0.90 ± 0.50  11.11 ± 0.90  \*\*\*  2.85 | |
| *B. oleracea* | Control  0.1 µM Zn  *p*-value  LSD0.05 | 4.28 ± 0.08  4.40 ± 0.06  NS  0.27 | 8.54 ± 0.19  8.75 ± 0.23  NS  0.83 | 15.51 ± 0.19  12.27 ± 0.33  \*\*  1.06 | |
| Analysis of variance | | | | |  |
| Doses (D)  Species (S)  D x S  LSD0.05 | | \*\*\*  \*\*\*  \*\*  0.14 | \*\*\*  \*\*\*  \*\*\*  0.50 | \*\*\*  \*\*\*  \*\*\*  1.26 | |

Values are mean ± 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<0.01 (\*\*) and p<0.001 (\*\*\*)

**Table 2** Activities of carboxylate metabolism enzymes in *L. sativa* and *B. oleracea* plants submitted to Zn deficiency

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | | CS (ΔAbs mg prot-1min-1) | PEPC (ΔAbs mg prot-1min-1) | FUM (ΔAbs mg prot-1min-1) | MDH (ΔAbs mg prot-1 min-1) | | |
| *L. sativa* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 0.10 ± 0.01  0.16 ± 0.02  \*\*  0.04 | 0.36 ± 0.01  0.40 ± 0.01  \*  0.039 | 0.20 ± 0.03  0.08 ± 0.01  \*\*  0.07 | 11.46 ± 0.61  7.26 ± 0.79  \*\*  2.22 | |
| *B. oleracea* | Control  0.01 µM Zn  *p*-value  LSD0.05 | 0.07 ± 0.01  0.08 ± 0.01  NS  0.03 | 0.11 ± 0.01  0.10 ± 0.01  NS  0.03 | 0.15 ± 0.01  0.08 ± 0.01  \*\*\*  0.03 | 16.16 ± 0.77  14.74 ± 1.09  NS  2.97 | |
| Analysis of variance | | |  |  | |  | | |
| Doses (D)  Species (S)  D x S  LSD0.05 | | NS  \*\*\*  NS  0.02 | \*\*  \*\*\*  \*  0.02 | \*\*\*  NS  NS  0.03 | \*\*  \*\*\*  NS  1.74 | | |

Values are mean ± 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 leaf biomass (A), Zn concentration (B), ZnUtE (C) and ZnUpE (D) in *L. sativa* and *B. oleracea* plants. Values are expressed as means ± standard error of the mean.

