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Improvement of the physiological response of barley plants to both Zinc deficiency and toxicity by the application of calcium silicate

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ABSTRACT

An adequate availability of Zinc (Zn) is crucial for plant growth and development given the essentiality of this element. Thus, both Zn deficiency and Zn toxicity can limit crop yields. In plants, the responses to Zn imbalances involve important physiological aspects such as reactive oxygen species (ROS) accumulation, phytohormone balance, tricarboxylic acid cycle (TCA) metabolism, and organic acids (OAs) accumulation. However, a way to improve tolerance to stresses such as those produced by nutritional imbalances is the application of beneficial elements such as silicon (Si). In this study, we grew barley plants in hydroponics under Zn deficiency and toxicity conditions, applying Si in the form of $CaSiO_3$ in order to assess its effectiveness against Zn imbalances. Parameters related to plant growth, oxidative stress, TCA enzyme activities, phytohormones and OAs accumulation were analyzed. Both Zn deficiency and toxicity reduced leaf biomass, increased ROS accumulation, and affected phytohormone and OAs concentrations and TCA enzyme activities. $CaSiO_3$ treatment was effective in counteracting these effects enhancing Zn accumulation under Zn deficient conditions and limiting its accumulation under toxic conditions. In addition, this treatment decreased ROS levels, and improved ascorbate/glutathione and phytohormonal responses, citrate synthase activity, and malate/oxalate ratio. Therefore, this study enhanced the notion of the efficacy of $CaSiO_3$ in improving tolerance to Zn imbalances.

1. Introduction

An appropriate supply of Zinc (Zn) is crucial for plant growth and development. Zn is an essential element involved in basic metabolic processes such as carbohydrate metabolism, protein, and lipid synthesis, enzyme activation, gene expression, and regulation and also has a structural and functional role in cell membranes [1]. Thereby, crops grown under Zn deficient conditions present an impaired growth and a reduced yield. It should underlined that the deficiency of Zn is the most widespread micronutrient deficiency [2]. Alternatively, Zn toxicity is present in crops grown in areas polluted with Zn-rich wastes of industrial and agricultural origins. The excessive Zn accumulation inhibit growth, alters physiological processes, and in some cases causes cell death [1].

Zn imbalances, as other stresses, cause oxidative stress resulting in ROS accumulation [3,4]. ROS can alter DNA, induce cell damages and membrane destabilization through lipid peroxidation. Thus, $O_2^{\cdot-}$ and H_2O_2 concentrations are good indicators of stress in plants [5]. To counteract the oxidative stress, plants accumulate antioxidant compounds such as ascorbate (AsA) and glutathione (GSH) increase the activities of antioxidant enzymes such as superoxide dismutase (SOD) (that eliminate $O_2^{\cdot-}$), and catalase (CAT) and/or peroxidase that eliminate H_2O_2 [5]. Most of these antioxidant systems participate in the AsA/GSH cycle that recycles and detoxifies ROS, thus preserving the reduced state that is usually lost in cells during oxidative stress [6,7].

Phytohormones are other key elements involved in stress response.

Abbreviations: ABA, abscisic acid; ACC, 1-Aminocyclopropane 1-carboxylic acid; APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; CKs, cytokinins; CS, citrate synthase; FUM, fumarase; GAs, gibberellins; GR, glutathione reductase; GSH, glutathione; JA, jasmonic acid; iP, isopentenyl adenine; MDH, malate dehydrogenase; OAs, organic acids; PEPC, ROS, reactive oxygen species; SA, salicylic acid; TCA, tricarboxylic acid cycle; tZ, trans-zeatin.

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These compounds have different molecular structures and regulate many processes in plants. Phytohormones are crucial for stress adaptation/acclimation, by regulating adaptive responses of growth, development, senescence, and plant nutrition. Many compounds have been described as phytohormones, but the most studied belong to five groups: auxins, cytokinins (CKs), gibberellins (GAs), ethylene, and abscisic acid (ABA) [8]. Besides, phytohormones modulate responses that affect nutrient uptake and homeostasis, therefore nutrients such as Zn influence the synthesis and action of phytohormones [9]. Thus, Zn is involved in auxin synthesis because it is necessary to produce the precursor tryptophan. Similarly, Zn is part of the ethylene receptor so its availability can affect the response to this hormone [10]. Therefore, Zn imbalances disturb hormones' accumulation and regulation. For instance, lettuce and cabbage subjected to Zn deficiency showed a different phytohormone response compared to control plants [11].

Other elements within plant metabolism which are closely related to oxidative stress and Zn nutrition are tricarboxylic acid cycle (TCA) and organic acids (OAs). The TCA cycle occurs in mitochondria and fulfills a fundamental role in plant physiology, as it is central in energy production, providing precursors for amino acids; moreover, it is closely connected to ion balance and photorespiration [12]. The response of TCA enzyme activities to micronutrient imbalances depends on plant species analyzed and specific micronutrients [11,13,14]. OAs (e.g. malate, citrate, and oxalate) fulfill a role in the tolerance to metal toxicity, by solubilizating nutrients in the soil, and increasing their bioavailability under deficient conditions; furthermore OAs facilitate elements transport to the shoot, and their storage into vacuoles [15]. Thus, OAs act as chelates that sequester Zn ions in subcellular compartments to avoid toxicity or enhance Zn transport to cope with Zn deficiency [16–18].

A way to improve tolerance to nutritional stresses is the use of beneficial elements such as silicon (Si) [19,20]. Although Si is not included among the essential nutrients for higher plants, the importance of this element for plants is confirmed by the level of remarkable accumulation in vegetable tissues [20,21]. Indeed, gramineous species such as barley possess active mechanisms to uptake Si [22,23]. Si could ease Zn transport from the root under Zn deficiency but at the same time is able to limit Zn uptake from the medium, preventing Zn toxicity [19, 22,24,25]. Si usually is applied as potassium, magnesium, or calcium silicates [22,25]. Thus, several researches observed that Ca metasilicate ($CaSiO_3$) provides good Si bioavailability and can be used as a fertilizer [26–28]. Furthermore, $CaSiO_3$ application also provides an extra Ca supply, which was proved to have a positive effect in plants subjected to Zn stress [29]. Therefore, the present study aims to analyze the effect of $CaSiO_3$ on barley plants subjected to Zn imbalances and to study the physiological responses such as oxidative stress, phytohormones, TCA, and OAs.

2. Material and methods

2.1. Experiment conditions, plant material, and treatments

Barley seeds (*Hordeum vulgare*, cv. Nure) were used for the experiment. The seeds were acquired from the Semillas Columbia company (Valladolid, Spain) and were harvested in 2016. Throughout the experiment the seeds and then the plants were grown inside a growth chamber with controlled environmental conditions: 60–80% relative humidity, 28/19 °C day/night temperatures; 16 h/8 h light/dark photoperiod and a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1}. The germination of barley seeds was carried out on filter paper moistened with distilled water for 7 days. After, the seedlings were transferred to a hydroponic system. The composition of the nutritive solution was: 1 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4$, 0.05 mM KCl, 12.5 μM H_3BO_3, 10 μM Fe-HEDTA, 0.4 μM $MnSO_4$, 0.1 μM $CuSO_4$, and 0.1 μM MoO_3. The pH was adjusted to 6 using HCl or NaOH as required. Electric pumps were used to continuously aerate the solution.

The different treatments consisted of three Zn doses: 1 μM $ZnSO_4$ (control), 0.01 μM $ZnSO_4$ (deficiency) and 100 μM of $ZnSO_4$ (toxicity). The other factor was the application (+Si) or not (-Si) of 0.25 mM $CaSiO_3$. Thus, six different treatments were applied: 1 μM $ZnSO_4$, 1 μM $ZnSO_4$ + 0.25 mM $CaSiO_3$, 0.01 μM $ZnSO_4$, 0.01 μM $ZnSO_4$ + 0.25 mM $CaSiO_3$, 100 μM $ZnSO_4$, 100 μM $ZnSO_4$ + 0.25 mM $CaSiO_3$. Treatments were added to the nutritive solutions at the start of hydroponic culture (7 days after germination) and were continued for 14 days until the experiment was finished. The experimental design was comprised of a randomized complete block with 6 treatments, with eight plants per treatment and three replications each.

2.2. Plant sampling and biomass determination

Sampling was carried 21 days after the germination. Leaves were rinsed using distilled water, dried on filter paper, and weighed to determine the fresh weight (FW). A part of the barley leaves from each treatment were frozen at − 40ºC to later perform the biochemical analytics and the other part of the plant material was lyophilized to determine the dry weight (DW) and the mineral nutrients concentration.

2.3. Oxidative stress indicators (H_2O_2 and $O_2^{\cdot-}$)

Leaf H_2O_2 concentration was measured colorimetrically according to Junglee et al. [30]. The $O_2^{\cdot-}$ concentration in the leaves was determined colorimetrically as described by Barrameda-Medina et al. [31].

2.4. Determination of proteolytic activity

Proteolytic activity was determined by assessing the azocasein consumption and measuring absorbance at 440 nm following the method described by Coêlho et al. [32].

2.5. CAT, APX, and GR enzyme activities

To determine CAT activity the method described by Badiani et al. [33] was followed. This method registers the consumption of H_2O_2 at a wavelength of 240 nm for 3 min. The Rao et al. [34] method was used to determine APX and GR enzyme activities. APX activity was recorded as the change in absorbance at 290 nm for 3 min and GR following the oxidation of NADPH at 340 nm for 3 min. The protein concentration of the extracts was determined using the Bradford [35] method.

2.6. AsA and GSH determinations

The Law et al. [36] method was used to quantify AsA concentration. Dehydroascorbate (DHA) concentration was deduced from the difference between total AsA and reduced AsA. The Noctor and Foyer [37] method was used to determine GSH concentration. Reduced GSH levels were estimated as the difference between total GSH and GSSG. Redox states of AsA and GSH were calculated using the formula: [(Reduced form) X 100]/ [Reduced + Oxidized forms].

2.7. Hormones extraction and analysis

Hormones concentrations were analyzed using a U-HPLC-MS system as described by Navarro-León et al. [38]. Total CKs were calculated as the sum of isopentenyl adenine (iP) and trans-zeatin (tZ) concentrations. Total GAs were calculated as the sum of the three analyzed gibberellin forms concentrations.

2.8. TCA enzymes extractions and enzyme activities

The method of Li [39] with slight modification was used for TCA enzymes extraction. Citrate synthase (CS) activity was determined recording the reaction between DTNB and acetyl coenzyme A at 412 nm

according to López-Millán et al. [40]. Fumarase (FUM) activity was determined recording the generation of fumarate at 240 nm [41]. Malate dehydrogenase (MDH) activity was measured using oxalate as substrate and recording the enzymatic oxidation of NADH at 340 nm [42]. Phosphoenolpyruvate carboxykinase (PEPC) activity was measured according to López-Millán et al. [40]. The protein concentration of the extracts was determined using the Bradford [35] method.

2.9. Determination of OAs concentration

Citric, malic, and oxalic acids concentration were determined using a U-HPLC system as described in Navarro-León et al. [43].

2.10. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence to evaluate the differences between treatments. A two-tailed ANOVA was applied to ascertain whether the Ca doses (D), the mutants (M), or the interaction (D * M) significantly affected the results. 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). All statistical analyses were carried out employing the Statgraphics Centurion XVI software.

3. Results

3.1. Biomass and Zn, Si, and Ca concentrations

Barley plants subjected to Zn shortage (0.01 μM ZnSO4), and excess (100 μM ZnSO4) showed an evident reduction (roughly 40%) in leaf biomass compared to control (1 μM ZnSO4). The application of $CaSiO_3$ to barley plants supplied with both Zn supplies significantly enhanced leaf DW value. Conversely, $CaSiO_3$ application to the nutritive solution with control Zn dose, shoot biomass was reduced compared to barley plants without $CaSiO_3$ (Table 1).

As expected, the low Zn dose reduced Zn concentration in barley leaves. Moreover, the high Zn dose increased Zn accumulation in barley plants. The application of $CaSiO_3$ did not affect to Zn accumulation of control plants, although significantly augmented Zn absorption in plants subjected to Zn deficiency, showing a similar Zn concentration in comparison to control plants. Furthermore, $CaSiO_3$ supply reduced Zn accumulation in plants subjected to Zn toxicity. Besides, $CaSiO_3$ supply incremented Si accumulation, although in a major extent in control plants. Considering Ca, plants with $CaSiO_3$ showed lower values regardless of the Zn supply (Table 1).

3.2. Oxidative stress indicators

The not-optimal Zn doses induced an increment in H_2O_2 levels, and proteolytic activity in barley. However, $CaSiO_3$ supply decreased the levels of these parameters and also $O_2^{\cdot-}$ concentration in barley that received the not-optimal Zn doses but increased them in plants grown under optimal Zn dose (Fig. 1).

3.3. ASA/GSH cycle and antioxidant enzymes

Zn deficiency increased catalase activity in barley. Likewise, Zn toxicity enhanced CAT activity with a 35% increment in CAT activity compared to control plants. In barley grown under Zn deficiency, $CaSiO_3$ caused a significant reduction of CAT activity. In contrast, the supply of $CaSiO_3$ to Zn control plants slightly increased the CAT activity. Besides, $CaSiO_3$ did not affect the CAT activity of plants under Zn toxicity (Fig. 2A). APX activity was not significantly modified by Zn supply. However, $CaSiO_3$ application reduced APX activity in leaves (Fig. 2B). Finally, both high and low Zn doses enhanced GR activity in barley although $CaSiO_3$ application reduced this activity (Fig. 2C).

The Zn treatments did not change reduced AsA concentration. Nevertheless, $CaSiO_3$ application increased AsA levels of plants supplied with deficient and toxic Zn doses. Diversely, plants subjected to Zn toxicity showed a greater concentration of DHA in comparison to the other Zn doses. $CaSiO_3$ application reduced DHA levels in barley grown with high and low Zn concentrations. Plants subjected to Zn toxicity presented the lowest values for AsA/DHA and ascorbate redox status, whereas plants grown under Zn deficiency showed higher AsA/DHA compared to control plants. The application of $CaSiO_3$ enhanced these two parameters in plants supplied with both un-optimal Zn doses (Table 2).

Plants that received 100 μM Zn dose showed lower GSH concentrations in comparison to the rest of plants that presented similar GSH values. $CaSiO_3$ supply enhanced GSH accumulation of plants that received the highest and the lowest Zn doses. Both Zn un-optimal applications incremented GSSG levels in barley, compared to control Zn dose. $CaSiO_3$ supply decreased the GSSG concentration of these plants. Likewise, non-optimal Zn doses decreased the levels of GSH redox status and GSH/GSSG was lower in plants subjected to Zn toxicity. However, $CaSiO_3$ supply enhanced GSH/GSSG ratio in all plants analyzed and GSH redox status in plants supplied with deficient and toxic Zn doses (Table 3).

3.4. Phytohormones profile

Both Zn deficiency and toxicity reduced the concentration of indole-3-acetic acid (IAA), GA3, and total GAs of barley leaves compared to control plants. Nevertheless, $CaSiO_3$ application increased IAA levels in plants grown under both Zn stresses and also enhanced GA1, and total GAs concentrations in Zn-deficient plants. Interestingly, Zn toxicity enhanced tZ and total CKs accumulation and Zn deficiency increased iP levels of barley plants. $CaSiO_3$ application reduced tZ presence in barley grown with the Zn stresses and total CKs in plants grown under Zn excess. However, $CaSiO_3$ promoted iP accumulation in barley leaves. Furthermore, plants grown under Zn deficiency showed lower jasmonic acid (JA) and 1-aminocyclopropane 1-carboxylic acid (ACC)

Table 1
Effect of Zn supply and $CaSiO_3$ application on leaf DW, Zn, Si, and Ca concentrations in barley leaves.

	Leaf DW (mg DW)			Leaf Zn (μg g^{-1} DW)			Leaf Si (mg g^{-1} DW)			Leaf Ca (mg g^{-1} DW)		
μM Zn	0.01	1	100	0.01	1	100	0.01	1	100	0.01	1	100
- Si	24.10	41.12	26.61	13.50	22.51	123.18	0.78	1.57	1.26	0.95	1.05	0.81
+ Si	32.09	30.61	31.79	18.00	24.00	109.53	3.22	3.49	2.57	0.85	0.74	0.71
p-value	*	*	*	*	NS	*	*	*	*	*	***	*
0.01 Zn		28.10b			15.75c			2.00b			16.50c	
1 Zn		35.85a			20.25b			2.53a			39a	
100 Zn		29.21b			116.25a			1.91b			20b	
$LSD_{0.05}$		1.88			3.02			0.18			1.09	

Values are means (n = 9) and differences between means were compared by Fisher's least-significance test (LSD; P = 0.05). Plants without $CaSiO_3$ (- Si) and plants supplied with $CaSiO_3$ (+ Si). The upper part of the table shows the effect of $CaSiO_3$ application, and the lower part shows the effect of Zn doses. Values with different letters indicate significant differences. The levels of significance were represented by NS ($p > 0.05$), * ($p < 0.05$), and *** ($p < 0.001$).

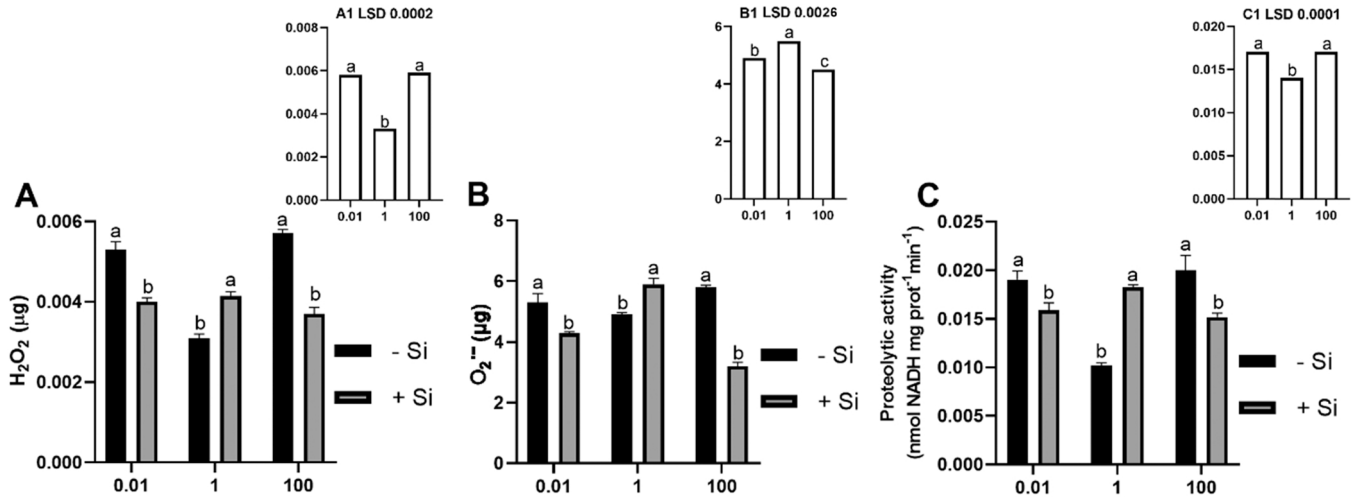

Fig. 1. Effects of Zn dose and the application (+Si) or not (-Si) of $CaSiO_3$ on H_2O_2 (A) and $O_2^{\cdot-}$ (B) concentrations, and Proteolytic activity (C) in barley leaves. The columns values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05). A1, B1, and C1 values are means (control +Si supply n = 18). Different letters indicate significant differences between plants supplied with $CaSiO_3$ and plants without $CaSiO_3$.

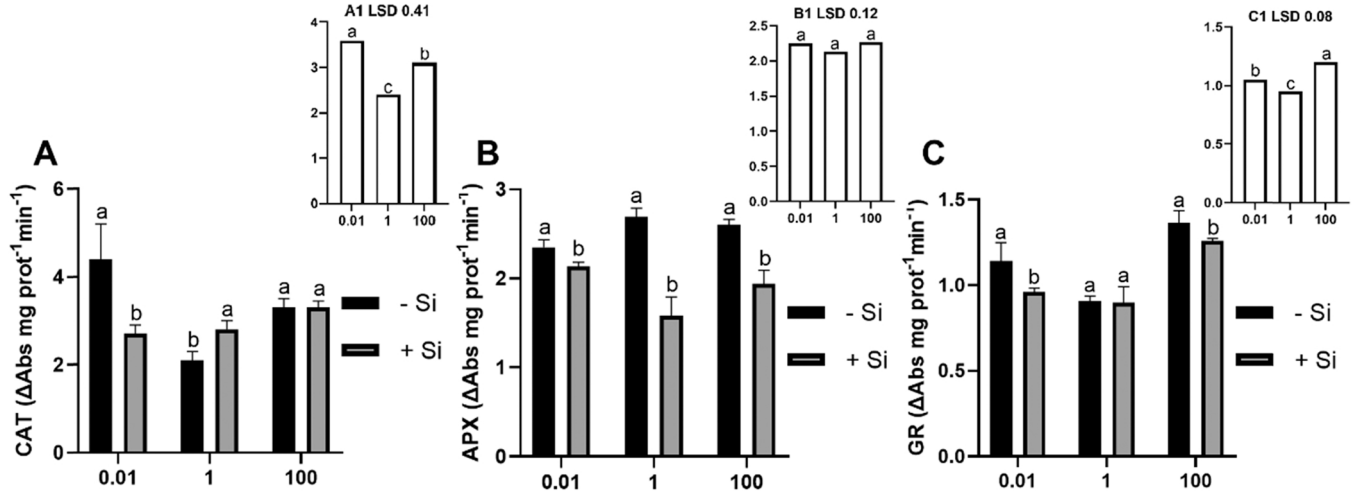

Fig. 2. Effects of Zn dose and the application (+Si) or not (-Si) of $CaSiO_3$ on CAT (A), APX (B), and GR (C) activities in barley leaves. The columns values are mean ± standard error (n = 9) and the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05). A1 and B1 values are means (control +Si supply n = 18). Different letters indicate significant differences between plants supplied with $CaSiO_3$ and plants without $CaSiO_3$.

Table 2
Effect of Zn supply and $CaSiO_3$ application on AsA forms, AsA/DHA ratio, and AsA redox status in barley leaves.

	AsA (µg g^{-1} FW)			DHA (µg g^{-1} FW)			AsA/DHA			Redox status		
µM Zn	0.01	1	100	0.01	1	100	0.01	1	100	0.01	1	100
-Si	203	234	219	93.07	75.21	94.87	2.21	3.24	2.32	68.65	76.39	69.80
+Si	221	198	221	58.01	74.58	75.11	4.64	2.70	2.95	79.15	72.77	74.68
p-value	*	***	*	***	NS	***	***	***	***	***	***	***
0.01 Zn		212a			75.54b			3.55a			75.08a	
1 Zn		216a			74.90b			2.97b			74.58a	
100 Zn		220a			84.99a			2.64c			72.24b	
p-value		NS			**			* **			* *	
$LSD_{0.05}$		0.06			4.63			0.14			1.36	

Values are means (n = 9) and differences between means were compared by Fisher's least-significance test (LSD; P = 0.05). Plants without $CaSiO_3$ (-Si) and plants supplied with $CaSiO_3$ (+Si). The upper part of the table shows the effect of $CaSiO_3$ application, and the lower part shows the effect of Zn doses. Values with different letters indicate significant differences. The levels of significance were represented by NS ($p > 0.05$) and * ($p < 0.05$).

concentrations, but Zn toxicity increased JA accumulation compared to control conditions. Moreover, plants suplied with $CaSiO_3$ and high and control Zn doses presented lower ABA levels. ACC and salicylic acid (SA) concentrations were higher in plants supplied with $CaSiO_3$ and control dose, whereas all barley plants that received $CaSiO_3$ treatment showed lower JA concentrations (Fig. 3; Table S1).

Table 3
Effect of Zn supply and $CaSiO_3$ application on GSH forms, GSH/GSSG ratio, and GSH redox status in barley leaves.

	GSH (mg g^{-1}FW)			GSSG (mg g^{-1}FW)			GSH/GSSG			Redox status		
µM Zn	0.01	1	100	0.01	1	100	0.01	1	100	0.01	1	100
- Si	0.055	0.078	0.066	0.074	0.059	0.089	0.63	0.91	0.73	43.22	56.66	42.61
+ Si	0.097	0.071	0.106	0.060	0.060	0.064	1.73	1.31	1.06	61.59	53.82	62.01
p-value	***	NS	***	***	NS	***	***	*	***	***	NS	***
0.01 Zn	0.076a			0.067b			1.18a			52.41b		
1 Zn	0.075a			0.060c			1.11a			55.24a		
100 Zn	0.086b			0.077a			0.89b			52.31b		
P-value	**			***			***			*		
$LSD_{0.05}$	0.006			0.002			0.09			2.03		

Values are means (n = 9) and differences between means were compared by Fisher's least-significance test (LSD; P = 0.05). Plants without $CaSiO_3$ (- Si) and plants supplied with $CaSiO_3$ (+ Si). The upper part of the table shows the effect of $CaSiO_3$ application, and the lower part shows the effect of Zn doses. Values with different letters indicate significant differences. The levels of significance were represented by NS ($p > 0.05$) and * ($p < 0.05$)

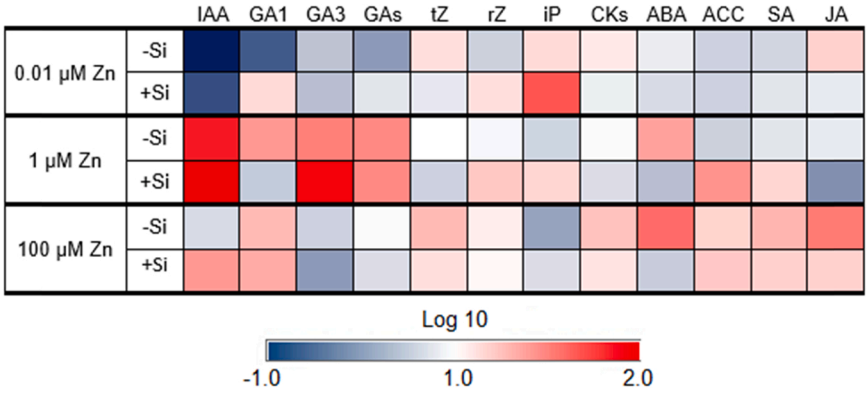

Fig. 3. Heat map presenting the effect of the different Zn doses and the $CaSiO_3$ supply on phytohormone concentrations. Color scale refers to the logarithmic transformation (log10) of measured values (higher values are shown in red, lower values in blue, and intermediate values in white colors). Phytohormone names are specified on the top, Zn doses and application (+Si) or not (-Si) of $CaSiO_3$ on the right side of clusters. For the interpretation of the color-code, refer to Supplementary tables (Table S1).(For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

3.5. TCA and OAs

Both un-optimal Zn doses increased citrate and oxalate accumulation of barley leaves in comparison to control conditions. Although, malate concentration only was higher in plants supply with the highest Zn dose. In barley plants grown under Zn deficiency conditions, $CaSiO_3$ enhanced malate and oxalate accumulations. Under control conditions, $CaSiO_3$ reduced citrate and oxalate accumulation but increased malate concentration. Besides, in plants subjected to Zn toxicity, $CaSiO_3$ reduced citrate accumulation but increased oxalate levels in comparison to plants without $CaSiO_3$ (Table 4).

Zn deficiency decreased CS and FUM activities but increased MDH and PEPC activities. In addition, plants subjected to Zn excess registered lower CS activity but higher FUM, MDH, and PEPC activities in comparison to control plants. $CaSiO_3$ had a positive effect on CS, FUM, MDH activities but reduced PEPC activity in plants subjected to low Zn dose. Under control Zn dose, $CaSiO_3$ reduced CS and FUM, whereas under Zn toxicity plants showed higher CS activity but lower PEPC activity compared to plant without $CaSiO_3$ (Fig. 4).

4. Discussion

A clear symptom of stresses such as Zn deficiency and toxicity is biomass reduction [1,44]. Likewise, in our study, *H. vulgare* plants subjected to both toxic and deficient Zn doses reduced their biomass, although $CaSiO_3$ application mitigated this loss. These results showed the positive effect of $CaSiO_3$ application in limiting the reduced growth caused by Zn imbalances. The fact that Ca^{2+} concentration was not affected by $CaSiO_3$, could suggest that Si was the main element responsible for the positive effect on growth. Besides, $CaSiO_3$ application augmented Zn concentration in Zn-deficient plants and diminished it in plants subjected to Zn-toxicity. Similar positive results were observed by Pascual et al. [45] in rice subjected to Zn toxicity and in soybean subjected to Zn deficiency that received Si application [19,45]. However, in our study, $CaSiO_3$ decreased growth in control plants, suggesting that high Si intracellular levels might be negative under

Table 4
Effect of Zn supply and $CaSiO_3$ application on OAs concentrations in barley leaves.

	Citrate (µmol g^{-1} FW)			Malate (µmol g^{-1} FW)			Oxalate (µmol g^{-1} FW)		
µM Zn	0.01	1	100	0.01	1	100	0.01	1	100
-Si	4.69	4.17	5.09	8.87	9.04	10.78	0.10	0.08	0.10
+Si	4.66	3.56	4.24	10.14	9.63	10.33	0.16	0.05	0.13
p-value	NS	***	***	***	*	NS	***	**	*
0.01 Zn	4.69a			9.51b			0.13a		
1 Zn	4.17b			9.33b			0.06b		
100 Zn	5.09a			10.55a			0.12a		
p-value	**			***			***		
$LSD_{0.05}$	0.06			0.21			0.01		

Values are means (n = 9) and differences between means were compared by Fisher's least-significance test (LSD; P = 0.05). Plants without $CaSiO_3$ (-Si) and plants supplied with $CaSiO_3$ (+Si). The upper part of the table shows the effect of $CaSiO_3$ application, and the lower part shows the effect of Zn doses. Values with different letters indicate significant differences. The levels of significance were represented by NS ($p > 0.05$) and * ($p < 0.05$).

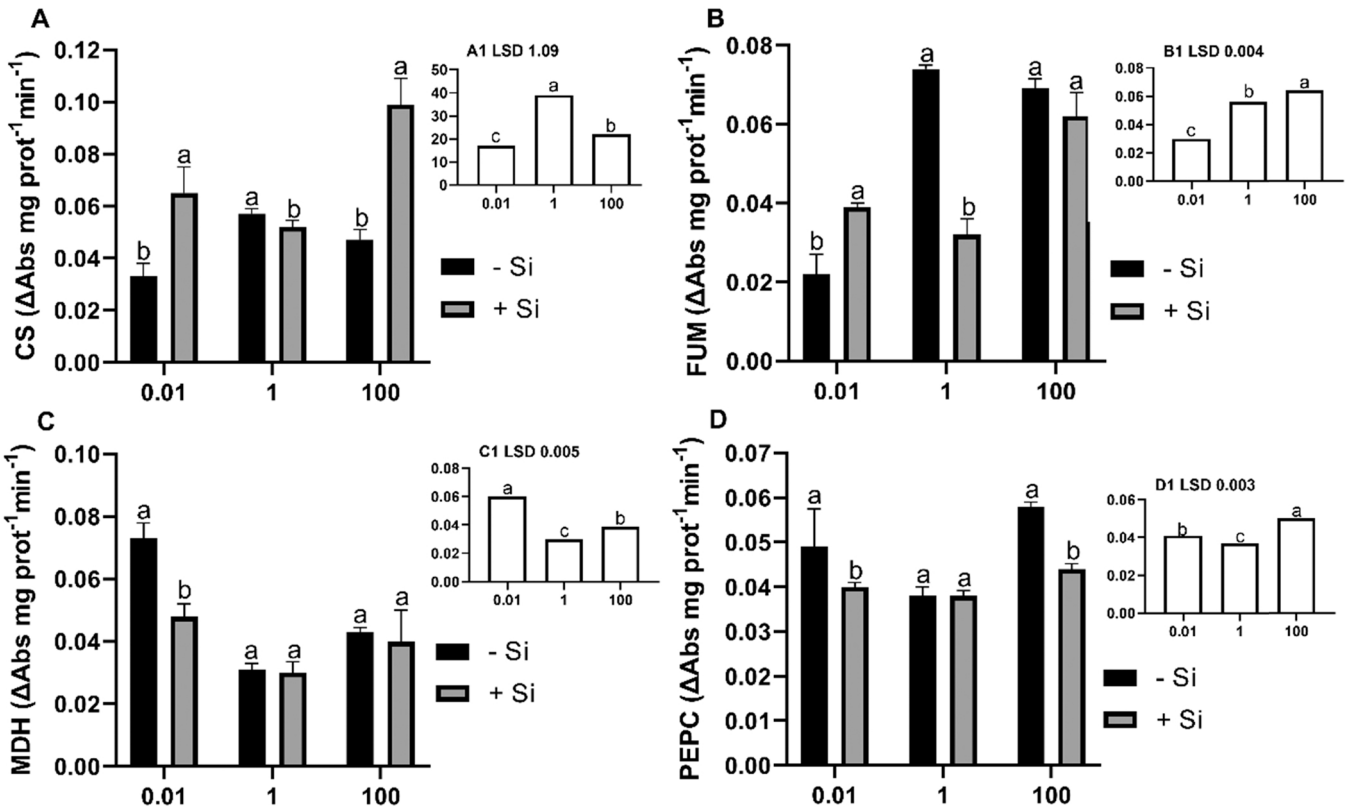

Fig. 4. Effects of Zn dose and the application (+Si) or not (-Si) of $CaSiO_3$ on CS (A), FUM (B), MDH (C), and PEPC (D) enzyme activities in barley leaves. The columns values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05). A1 and B1 values are means (control +Si supply n = 18). Different letters indicate significant differences between plants supplied with $CaSiO_3$ and plants without $CaSiO_3$.

control conditions.

Zn ions fulfill an important role protecting enzymes and cell membranes against ROS by binding to phospholipids and sulfhydryl groups and being part of enzymatic defenses [46]. However, deficient or toxic Zn levels produce ROS accumulation [47]. In the present work, plants grown under non-optimal Zn concentration showed higher oxidative stress indicators although CaSiO3 application significantly reduce these indicators. The relief of oxidative stress may explain the increase in barley growth. Alternatively, proteolytic activity could be an indicator of tolerance to Zn toxicity in plants because under Zn toxicity this parameter increased [48]. In addition, Zn supplementation reduced proteolytic activity in Picea embryos, so Zn deficiency also could lead to higher proteolytic activity [49]. In our experiment, CaSiO3 reduces protein induced by Zn stresses. Nevertheless, this positive effect was not observed in control plants.

Enzymes such as APX, GR, and CAT are efficient H2°2 scavengers in plants [5]. Tewari et al. [50] proved the enhancement of CAT as a consequence of Zn un-optimal nutrition in mulberry plants. Accordingly, in our experiment, low and high Zn applications affected positively CAT activity. This enzyme activity could be correlated with the concentration of its substrate (H2°2) in plants supplied with CaSiO3. Thus, CAT was lower in Zn deficient plants in contrast to the higher levels in Zn control plants. This correlation is not produced in barley subjected to Zn toxicity, that showed a decrease in H2°2 levels but not a decline of CAT activity.

Previous studies showed that APX activity present different responses to changes in Zn supply depending on the species. Blasco et al. [14] noted an enhancement of APX activity in plants grown under Zn toxicity and deficiency conditions. However, Barrameda-Medina et al. [31] results showed no variations in APX activity in L. sativa grown under Zn toxicity, in agreement with our results. Furthermore, a correlation was observed between APX activity and H2O2 concentration, which were lower in plants subjected to Zn stresses that were supplied with CaSiO3. These results make sense considering that H2O2 is the substrate of APX enzyme [6]. Besides APX enzyme, GR is another effective H2°2 scavenger that takes part in AsA regeneration and NADP+, which receives electrons from ferredoxin and thereby prevent photoreduction of O2 to $O_2^{\cdot-}$ [7]. The increased levels of GR activity here observed might contribute to enhance the tolerance against stress by maintaining the pool of reduced GSH under toxic Zn conditions [51,52].

Other plant elements that contribute to stress tolerance are non-enzymatic antioxidants such as GSH and AsA, which are indicators of the redox state. Indeed, the AsA/GSH cycle represents an important antioxidant system being crucial in ROS equilibrium [5]. Subba et al. [53] study showed that mandarin leaf tissues suffering Zn shortage and excess produced AsA at a lower proportion. Moreover, Tsuji et al. [54] proved that *Duniella tertiolecta* plants suffering Zn stress increased GSH synthesis. Likewise, in Barrameda-Medina et al. [55] study the response of AsA and GSH to high Zn supply was higher presence of AsA and GSH oxidized forms. In the present study, the reduction in the redox state of these compounds suggests an enhancement of the AsA/GSH cycle also shown by the not inhibition of APX activity and the increment of GR activity that occurred at high Zn application.

Bityutskii et al. [56], proved a positive effect of Si against Zn deficiency through the enhancement of antioxidant defenses preventing ROS accumulation. In the present study, $CaSiO_3$ reduced APX activity in plants supplied with non-optimal Zn, probably by the reduction in H_2O_2 whereas in control plants H_2O_2 detoxification probably occurs through CAT enzyme. Hence, the decrease in APX activity was correlated with a minor DHA accumulation in Zn stressed plants. In Zn-deficient barley supplied with $CaSiO_3$, the higher concentrations of reduced forms of AsA and GSH could be positive for antioxidant protection. Furthermore, the

decline in GSSG form could be caused by the lower GR activity. These data were confirmed by the positive effect of $CaSiO_3$ in recovering the redox state of both AsA and GSH which could be positive for barley plants to face Zn imbalances.

Phytohormones are fundamentally involved in nutrient homeostasis in plants. Thus, phytohormones concentration usually varies in plants subject to nutrient deficit or excess to regulate plant responses to those situations [9,57,58]. In the present study, the deficient and toxic Zn doses lead to a lower accumulation of important growth promoter hormones such as IAA and GAs. The decline in IAA, especially under Zn deficiency could be due to Zn is necessary for the synthesis of auxin precursor [59]. Sekimoto et al. [60] also observed a GAs levels reduction in maize plants and proved that this loss has an important effect on biomass reduction. In our study, the application of $CaSiO_3$, lead to higher IAA and GAs in stress plants. Thus, the higher accumulation of these growth-promoting hormones could promote the higher leaf DW showed by these plants. In addition, the recovery in Zn levels could promote auxin synthesis in Zn deficient plants supplied with $CaSiO_3$.

Ethylene and ABA are stress-related hormones that usually are accumulated in plants suffering nutrient disbalances [61,62]. Moreover, Zn is involved in ethylene response because it forms part of ethylene receptor, so Zn also affects the sensibility of this phytohormone [10]. In the present study, only JA concentration incremented in plants subject to Zn disbalances. $CaSiO_3$ has a positive influence being that it reduced ABA and JA accumulation, especially in plants subjected to Zn toxicity. The higher ACC and SA under control conditions could be an indicator of the higher stress response as suggested by DW results and oxidative stress results. A previous study also suggested that a diminution in JA levels could contribute to improve Zn homeostasis in cabbage plants [11].

Another process that several studies related to nutrient homeostasis in plants is TCA cycle [12]. Previous studies provided mixed results in plants grown under micronutrient deficiencies. Thus, a decline of MDH activity and an increase in CS and PEPC activities were registered in lettuce plants supplied with low Zn dose [43] and in the same species subjected to Zn toxicity that also showed higher MDH activity [31]. Blasco et al. [14] observed a decline in FUM activity whereas MDH activity increased *B. rapa* plants subjected to Zn deficiency. Similarly, in our study, barley plants exposed to high and low Zn doses registered greater MDH and lower FUM activities under Zn deficiency. The reduction in previous TCA enzyme activities or the enhancement of divergent pathways leading to an exit of fumarate from the cycle could be the cause of the lower FUM activity. Considering PEPC, Zn deficiency and toxicity stimulated its activity suggesting a strategy to produce more oxalate and citrate to chelate and make Zn more available under deficient conditions.

Considering Si application, the supply of this element enhanced TCA activities in rice stressed plants [63]. In this experiment, $CaSiO_3$ supply recovered CS activity, which could be related to the positive effect of Si. This fact is important given that the CS enzyme is the main regulator of the cycle [12] and in our study, it is strongly affected by Zn imbalances. The low Zn dose was the one that most changed to TCA enzyme activities. Thus, plants showed an increment of FUM and CS activities, that in combination with the lower MDH activity could promote malate accumulation. The $CaSiO_3$ effects, suggest that Si has not a decisive role in TCA cycle regulation under un-optimal Zn conditions.

OAs can chelate and transport Zn and they are crucial for the uptake, transport, accumulation, and storage within the plant. Therefore, they are necessary to ensure a proper Zn distribution and to detoxify Zn under high concentration of this elements [18]. Citrate, malate, and oxalate are the most important OAs in plants produced in the TCA [12]. Mixed results have been provided regarding OAs levels in leaves of plants under micronutrient imbalances, but more generally an increase in citrate concentration was registered in *Beta vulgaris* subjected to Zn toxicity [64], and in Zn-stressed *B. rapa* plants [14]. These data agree with our results in barley since citrate levels increased by roughly 20% under the un-optimal Zn supplies. Thus, citrate could promote Zn accumulation under low Zn conditions but also helps in its sequestration under toxic Zn conditions. Indeed, citrate presents a high mineral-binding capacity, which could help to nutrient homeostasis in plants suffering mineral stresses [64].

Malic acid is closely related to Zn nutrition as suggested by Rose et al. [65] in which tolerant rice genotypes to Zn shortage increased both malate accumulation and its efflux from roots. However, in another study in Zn-deficient *B. rapa* plants malate concentration declined so this OA was not determinant for better tolerance [14]. Conversely, *B. oleracea* plants grown under Zn toxicity showed an increment in malate concentration, which could contribute to transport Zn to the shoot or to retain it vacuoles avoiding toxicity [31]. Accordingly, malate was not determinant for Zn deficiency tolerance in barley plants. Although, Zn toxicity enhanced malate concentration in barley plants, which could be caused by a greater MDH activity, not necessarily participating in the TCA, but increasing the conversion of malate to oxaloacetate to fulfil the higher reductant demand under un-optimal Zn conditions.

Oxalate could enhance Zn toxicity tolerance as observed in a study by Mathys [66] where Zn tolerant ecotypes of *Rumex acetosa* and *Silene cucubalus* showed greater oxalate accumulation. By contrast, in Barrameda-Medina et al. [31] study oxalate was not determinant for better tolerance to toxic Zn conditions. Nevertheless, the malate/oxalate ratio could be related to Zn homeostasis in barley. Mostly in plants under Zn deficiency, the increment in MDH activity that catalyze the conversion of malate to oxaloacetate could explain the higher oxalate levels. In this process NAD(P)H is generated, which could help in counteracting stress. Moreover, the higher oxalate levels are supported by the Ferreira et al. [67] study, which proved that the reaction of this OA with Zn forms stable complexes. Hence, these complexes could be promoted in barley plants to increase Zn uptake in low Zn bioavailability soils.

Other studies also showed the positive effect of Si on OAs accumulation e.g. for malate accumulation in rice and citrate and oxalate in cucumber [56,63]. Our results support the beneficial role of Si in tolerance to non-optimal Zn doses. Thus, in Zn-deficient plants, $CaSiO_3$ application increased oxalate and malate accumulation probably contributing to Zn absorption and transport to the leaves. In plants of Zn toxicity treatment, $CaSiO_3$ decreased citrate accumulation which might reduce Zn uptake. In addition, $CaSiO_3$ supply induced a remarkable increase in oxalate accumulation lowering the malate/oxalate ratio, which may increase reductants production to counteract the oxidative stress caused by Zn imbalances. Indeed, $CaSiO_3$ supply reduced malate/oxalate by roughly 35% in plants grown under both Zn un-optimal.

5. Conclusions

In conclusion, both Zn deficiency and toxicity produced negative effects on barley plants such as reduced biomass, increased oxidative stress, and imbalance in phytohormone and OAs concentrations and TCA enzyme activity. However, the application of $CaSiO_3$ was effective in counteracting these effects as the plants showed increased growth and better control of Zn accumulation. In addition, this treatment decreased oxidative stress levels, improved AsA/GSH and phytohormonal responses, CS activity, and malate/oxalate ratio. Therefore, the efficacy of $CaSiO_3$ in improving tolerance to Zn imbalances is proven, although further research is needed to elucidate its mechanisms of action in plants.

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CRediT authorship contribution statement

J.M.R., B.B., and S.E. conceived the idea and designed the experiment. V.P, E.N.L., and A.A. performed the experiment. V.P., and E.N.L. analyzed the data. V.P., and E.N.L. wrote the manuscript. J.M.R., B.B., and S.E. revised the manuscript. All authors read and approved the final draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111259.

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