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ORIGINAL ARTICLE

Calcium silicate ameliorates zinc deficiency and toxicity symptoms in barley plants through improvements in nitrogen metabolism and photosynthesis

Valeria Paradisone[1] · Eloy Navarro-León[2] · Juan M. Ruiz[2] · Sergio Esposito[1] · Begoña Blasco[2]

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Abstract

Zinc (Zn) deficiency causes serious issues to plant growth and development, negatively affecting crops in many world regions. On the other hand, Zn toxicity impairs plant growth, producing physiological alterations, and even cell death. In plants, two of the processes that most determine growth are nitrogen (N) metabolism and photosynthesis. In the last decades, several authors proved that silicon (Si) and calcium (Ca) mitigate the effects of various abiotic and biotic stresses in plants. The objective of this research is to study the effect of Si application to barley (*Hordeum vulgare* cv. Nure) plants grown under Zn deficiency and Zn toxicity. Hence, barley plants were grown in hydroponics and supplied with a low Zn dose (0.01 µM $ZnSO_4$) and a high Zn dose (100 µM $ZnSO_4$) and were supplied with $CaSiO_3$. Parameters related to Zn accumulation, N metabolism, and photosynthesis were measured. Zn stress affected leaf Zn concentration and reduced biomass in barley plants. Both Zn toxicity and deficiency inhibited N metabolism and enhanced photorespiration, increasing stress symptoms. $CaSiO_3$ mitigated Zn stress effects, probably regulating Zn levels in plant cells and enhancing N metabolism and photosynthesis. We conclude that $CaSiO_3$ could be beneficial to grow barley plants in soils with high or low availability of Zn.

Keywords *Hordeum vulgare* · N metabolism · Photosynthesis · Silicon · Zn deficiency · Zn toxicity

Abbreviations

AA	Amino acid
AAT	Aspartate aminotransferase
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
GB	Glycinebetaine
GDH	Glutamate dehydrogenase
GGAT	Glutamate: glyoxylate aminotransferase
GO	Glyoxylate oxidase
GOGAT	Glutamate synthase
GS	Glutamine synthetase
NiR	Nitrite reductase
NR	Nitrate reductase
NUE	N use efficiency
NUpE	N uptake efficiency
NUtE	N utilization efficiency

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✉ Eloy Navarro-León
enleon@ugr.es

[1] Dipartimento di Biologia, Università di Napoli "Federico II", Complesso Universitario di Monte Sant'Angelo, Via Cinthia, 80126 Napoli, Italy

[2] Department of Plant Physiology, Faculty of Sciences, University of Granada, 18071 Granada, Spain

Introduction

Plants require an optimal Zn supply to grow and to maintain a correct metabolism functioning. Thus, Zn is an essential micronutrient necessary for carbohydrate metabolism, enzyme activation, structural and functional integrity of membranes, gene expression and regulation, and protein and lipid synthesis (Broadley et al. 2007). Thereby, Zn deficiency represents a serious issue, and it is one of the most widespread micronutrient deficiencies (Kabir et al. 2014). Leaves are the plant organs more affected by Zn shortage, showing alterations in photosynthesis (Brown and Cakmak 1993). On the other hand, in the last decades, numerous terrestrial and aquatic environments have been contaminated by Zn-rich wastes. Plants accumulate the excess of Zn in their tissues, which alters physiological processes, inhibiting growth, and even cause cell death (Broadley et al. 2007).

In plants, two of the processes that most determine growth are N metabolism and photosynthesis (Erenoglu et al. 2011; Pfannschmidt and Yang 2012). Abiotic stresses, such as Zn imbalances, negatively affect these processes. Thus, stress reduces nitrogen (N) uptake and assimilation, photosynthesis efficiency, and chlorophyll content (Song et al. 2014). Zn is involved in N metabolism as a catalytic and structural constituent of enzymes (Broadley et al. 2007). Zn plays a role in N metabolism because it has a positive effect on N use efficiency (NUE), and subsequently in total reduced N concentration (Das and Green 2013). N metabolism determines crop productivity because N along with carbon is the basis of amino acids (AAs) and proteins, which are the main products of NO_3^- assimilation in leaves (Erenoglu et al. 2011). Another important process is photorespiration because it is necessary to ensure adequate N levels in plants, provide metabolites, and defends against photo-inhibition (Wingler et al. 2000). On the other hand, some nitrogenous compounds, such as glycinebetaine (GB) and proline (Pro), and protect plants against stress acting as stress-tolerance mechanisms (Ashraf and Foolad 2007).

Many researches proved that Si application is beneficial for plants suffering stress (Song et al. 2014; Guerriero et al. 2016). Indeed, Si is regularly used as a fertilizer, in spite of it is not an essential nutrient for plants (Epstein 2009). Si can be applied to plants as calcium (Ca) and magnesium silicates or other minerals. Plants uptake Si as orthosilicic acid, at pH below 9, and accumulate up to 10% of the total dry biomass. *Poaceae* plants such as barley have an active mechanism for Si uptake (Adrees et al. 2015; Rizwan et al. 2015). He et al. (2013) suggested that the main effects of Si supply involve the generation of an amorphous silica barrier within the apoplast that defends plant cells against biotic and abiotic stresses. Likewise, Si could mobilize the Zn stored in roots under micronutrient deprivation (Adrees et al. 2015; Hernández-Apaolaza 2014). Furthermore, Song et al. 2014 observed that 1.5 mM K_2SiO_3 alleviated Zn toxicity (2 mM) through the reduction of Zn phytoavailability in the growth medium. Furthermore, Ca is a plant macronutrient that is crucial in cell signaling processes and plays a structural role in cell walls and membranes (White and Broadley 2003). An increase in Ca concentration in the culture medium reduces Zn uptake and translocation, which could help to reduce Zn toxicity (Prasad et al. 2016).

Rao et al. (2019), Murillo-Amador et al. (2007), and Anitha et al. (2019) proved that Ca meta-silicate ($CaSiO_3$) can be used as fertilizer and to mitigate plant stress. Indeed, the silicate from $CaSiO_3$ is more bioavailable than silicate from Ca_2SiO_4. There are no studies about the effect of $CaSiO_3$ on Zn imbalances. Thereby, taking into account the potential beneficial effects of Si and Ca, the hypothesis to test in this study is that $CaSiO_3$ application will improve tolerance to Zn deficiency and toxicity of barley plants. In this study, we focus on the effects on photosynthesis and N metabolism because of the relevance of these processes in plant growth.

Materials and methods

Plant material, growth conditions, and treatments

Barley seeds (*Hordeum vulgare*, Nure) were germinated on moistened filter paper for 7 days. The seedlings were grown in a growth chamber under the following controlled environmental conditions: day/night temperatures of 28/19 °C; Relative humidity 60–80%; 16/8 h light/dark photoperiod at a photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, Nebraska, USA). The plants were placed in a hydroponic system, under these conditions and after 7 days from germination. The composition of the nutritive solution was: 1 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.25 $MgSO_4$, 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 solution was continuously aerated with electric pumps and was renewed daily, adjusting the pH to 6 using HCl and NaOH as required. Treatments were initiated 7 days after germination and were maintained for 14 days. Plants were supplied with three different Zn doses: 1 µM $ZnSO_4$ as control, 0.01 µM $ZnSO_4$ as deficiency treatment and 100 µM of $ZnSO_4$ as toxicity treatment. Besides, barley plants were supplied with 0.25 mM $CaSiO_3$ (+ Si) or without $CaSiO_3$ (− Si). 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 $ZnSO_4$ + 0.25 mM $CaSiO_3$, 100 µM $ZnSO_4$ + 0.25 mM $CaSiO_3$. The use of $CaSiO_3$ and the utilized doses were selected based on a previous experiment growing barley plants with several $ZnSO_4$ and $CaSiO_3$ doses (data not shown) and in other studies (Murillo-Amador et al. 2007; Song and Jeong 2014; Anitha et al. 2019). The experimental design consisted of two factors, Zn dose applied and 0.25 mM $CaSiO_3$ application. Treatments were added in nutrient solutions at the beginning of hydroponic growth. The experimental design consisted of a randomized complete block with six treatments, arranged in individual benches with twenty plants per treatment and three replications each.

Plant sampling

Plant shoots were washed with distilled water, dried on filter paper, and weighed to determine the fresh weight (FW). Half of the shoots from each treatment were frozen at − 80 °C for later biochemical determinations and the other half was

lyophilized to measure the dry weight (DW) and the ions concentration.

Relative growth rate (RGR) determination

To determine the relative growth rate (RGR), the shoots from three plants were sampled 7 days after germination. The shoots were dried for 24 h in a forced-air oven at 70 °C, and DW was registered. Treatments were started 7 days after germination and plants were sampled 21 days after germination ($T = 14$ days). RGR was calculated from the increase in leaf DW at the beginning and the end of the treatments, using the formula:

$$RGR = (\ln DW_{final} - \ln DW_{initial})/(T).$$

Ions concentration determination

Zn, Si, and Ca concentrations were measured using a sample of 150 mg dry material that was mineralized by wet digestion according to Wolf (1982). Dry leaves were ground and mineralized with a mixture of nitric acid (HNO_3)/perchloric acid ($HClO_4$) and H_2O_2. After, 20 ml of milli-Q H_2O was added and the concentrations of elements were measured by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA).

Extraction and N forms determination and NUE parameters

The methodology described by Cataldo et al. (1975) was used to determine NO_3^- concentration. First, 0.1 g of DW was mixed with 10 ml of Milli-Q water and subjected to aqueous extraction. After, 100 µl aliquot was taken and mixed with 10% (w/v) salicylic acid in 96% sulfuric acid. Finally, NO_3^- concentration was measuring by spectrophotometry.

The Krom (1980) method was used to determine total reduced N. First, a sample of 0.1 g DW was digested using H_2SO_4 and H_2O_2. After, the samples were diluted using Milli-Q water. Then, a 1 ml aliquot was mixed with sodium silicate/sodium nitroprusside and sodium hydroxide and sodium dichloroisocyanurate. Finally, total reduced N was determined by spectrophotometry. To determine the concentration of soluble NH_4^+, the same aqueous extracts as for NO_3^- analysis were processed following the Krom (1980) method.

NUE parameters were calculated as follow:

N uptake efficiency (NUpE) was calculated as total N accumulation divided by root DW (mg N g^{-1} RDW).

N utilization efficiency (NUtE) was calculated as leaf DW divided by N concentration (g^2 LDW mg^{-1} N).

N metabolism and photorespiration enzyme extractions and assays

To determinate nitrate reductase (NR) (EC 1.7.1.1), glutamate dehydrogenase (GDH) (EC 1.4.1.2), and glutamate synthase (GOGAT) (EC 1.4.1.13) activities, leaves were ground at 4 ºC in 50 mM KH_2PO_4 buffer (pH 7.5) containing 2 mM dithiothreitol (DTT), 2 mM EDTA, and 1% (w/v) PVPP. The mix was centrifuged for 20 min at 30,000 × *g*. The resulting supernatant was used to determine enzymatic activities. NR activity was obtained as in Kaiser and Lewis (1984). GOGAT and GDH activities were determined by measuring the oxidation of NADH at 340 nm, as described by Singh and Srivastava (1986) and Groat and Vance (1981), respectively.

Glutamine synthetase (GS) (EC 6.3.1.2) activity was analyzed using the hydroxamate synthetase assay described by Kaiser and Lewis (1984). First, leaves were ground at 0 ºC in 50 ml maleic acid–KOH buffer (pH 6.8) containing 20% (v/v) ethylene glycol, 2% (v/v) β-mercaptoethanol, and 100 mM sucrose. Then, the mix was centrifuged at 20 min for 30,000 × *g*. Finally, GS activity was recorded by spectrophotometry.

Aspartate aminotransferase (AAT) (EC 2.6.1.1) activity was measured reading absorbance at 340 nm following the Gonzalez et al. (1995) method. The extraction methodology was the same as for GS enzyme. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 10 mM aspartic acid, 4 mM $MgCl_2$, and enzyme extract.

The methodology described by Feierabend and Beevers (1972) was followed to determine glyoxylate oxidase (GO) (EC 1.2.3.5) activity. First leaves were ground with 50 mM Tris–HCl buffer (pH 7.8) with 5 mM DTT, 0.01% Triton X-100 and PVPP. The mix was centrifuged for 20 min at 30,000 × *g*. The supernatant was used for enzyme reaction and the production of glyoxylate phenylhydrazone was registered at 324.

For glutamate-glyoxylate aminotransferase (GGAT) (EC 2.6.1.4) activity analysis, the method described by Igarashi et al. (2006) was followed. First, leaves were ground with 100 mM Tris–HCl buffer (pH 7.3) containing 10 mM DTT and 0.1% (v/v) Triton X-100. The mix was centrifuged for 10 min at 20,000 × *g*. The resulting extract was used to determine GGAT activity by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH.

The protein concentration of the extracts was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Soluble AAs analysis and glycine betaine (GB) determination

Soluble AAs concentration was determined as in Navarro-León et al. (2019) using Waters Acquity® UPLC System equipped with an Acquity fluorescence detector.

Glycine betaine (GB) concentration was measured as described by Grieve and Grattan (1983) using dichloroethane and measuring absorbance at 365 nm.

Leaf pigment concentration and SPAD value

Photosynthetic pigments concentration was obtained using the Wellburn et al. (1994) method with some modifications. First, 0.1 g of frozen samples was ground in 1 ml of methanol; then the extract was centrifuged for 5 min at 5000 × *g*. The absorbance was registered at 653 nm, 666 nm, and 470 nm. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids concentrations were determined using the Lichtenthaler equation:

$$\text{Chl } a = 15.65 \times \text{A666nm} - 7.34\ \text{A653nm}$$

$$\text{Chl } b = 27.05 \times \text{A653nm} - 11.21 \times \text{A666nm}$$

$$\text{Carotenoids} = (1000 \times \text{A470nm} - 2.86 \times \text{Chl } a - 129.2 \times \text{Chl } b)/221$$

Total Chl was calculated as the sum of Chl *a* and Chl *b*.

The chlorophyll content in the leaves was measured using the chlorophyll meter SPAD-502 (Konica Minolta Sensing Inc, Japan). Three measurements were made per each leaf, nine leaves were analyzed for each treatment and the average of the measurements was calculated.

Chl *a* fluorescence analysis

A leaf clip holder was used to kept leaves in the dark for 30 min before the measurements. Chl *a* fluorescence kinetics was measured using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK). The fluorescence curves were induced by red light (650 nm) with 3000 µmol photons m^{-2} s^{-1} light intensity and registered by the instrument. The data were analyzed using JIP test. Measurements were performed in fully expanded leaves of six plants at mid-stem position (Strasser et al. 2004). Parameters analyzed to study the energy flow and photosynthetic activities were: initial fluorescence value (*F*o), maximum fluorescence value (*F*m), variable fluorescence ($Fv = Fm - Fo$), maximum quantum yield for primary photochemistry (*F*v/*F*m), a general index of photosynthesis performance (PI_{ABS}), the proportion of active reaction centers (RCs) (RC/ABS), efficiency/probability with which a PSII trapped electron is transferred from Q_A–Q_B (Ψo), the maximum quantum yield of electron transport (ΦE_o), the time to reach *F*m (*t* for *F*m), area above the fluorescence curve (Area), and fluorescence value at 300 µs (K step). Besides, electron fluxes per reaction centers were considered: the photon flux absorbed by antenna pigments (ABS/RC), the trapped flow in reaction centers (TRo/RC), and the flow after Q_A (ET_o/RC) (Strasser et al. 2004).

Statistical analysis

Data were analyzed with a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI program. A two-way ANOVA was applied to ascertain whether the doses of Zn and $CaSiO_3$ supply 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).

Results

Biomass, RGR and Zn, Si, and Ca concentrations

Plants supplied with the low Zn (0.01 µM $ZnSO_4$), and high Zn (100 µM $ZnSO_4$) doses registered an evident diminution (about 40%) of leaf DW and RGR compared to control plants (1 µM $ZnSO_4$). The application of $CaSiO_3$ to plants grown under both Zn supplies significantly increased leaf DW and RGR values. In contrast, plants grown under control Zn dose and $CaSiO_3$ showed a reduction in shoot biomass and RGR in comparison to plants without $CaSiO_3$ (Table 1).

Zn deficiency decreased leaf Zn concentration. On the other hand, 100 µM $ZnSO_4$ caused a respective Zn accumulation in plant tissues. $CaSiO_3$ supply did not change Zn concentration in control plants but enhanced Zn uptake in barley plants supplied with the lowest Zn dose, presenting similar Zn concentration than control plants. In addition, $CaSiO_3$ application in plants supplied with the high Zn dose slightly decreased Zn concentration. Unsurprisingly, the plants that received $CaSiO_3$ treatment presented higher leaf Si concentration. However, control plants showed higher Si concentration in comparison to the other plants (Table 1). Regarding Ca, plants with $CaSiO_3$ registered higher Ca concentration regardless of the Zn application (Fig. S1).

N efficiency parameters

Plants reduced their NUpE values when supplied with both low and high Zn doses. This reduction was more important in plants subjected to Zn deficiency. However, $CaSiO_3$ application was positive for NUpE in plants grown with Zn toxicity and deficiency, although $CaSiO_3$ decreased NUpE under control conditions (Fig. 1A). Considering NUtE, both non-optimal Zn conditions increased the values of this parameter in comparison to control

Table 1 Effects of different Zn doses and the presence (+Si) or absence (−Si) of $CaSiO_3$ supply on leaf biomass, leaf RGR, and Zn and Si concentrations in barley leaves

	Leaf DW (mg DW)			Leaf RGR (mg/day)			Leaf Zn (μg g^{-1} DW)			Leaf Si (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	0.08	0.14	0.07	13.50	22.51	123.18	0.78	1.57	1.26
+ Si	32.09	30.61	31.79	0.09	0.08	0.09	18.00	24.00	109.53	3.22	3.49	2.57
p-value	*	*	*	*	*	*	*	NS	*	*	*	*
0.01 μM Zn		28.10b			0.091b			15.75c			2.00b	
1 μM Zn		35.85a			0.098a			20.25b			2.53a	
100 μM Zn		29.21b			0.082c			116.25a			1.91b	
$LSD_{0.05}$		1.88			0.001			3.02			0.18	

Values are means ($n=9$) and differences between means were compared by Fisher's least-significance test (LSD; $P=0.05$). 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 *($P<0.05$)

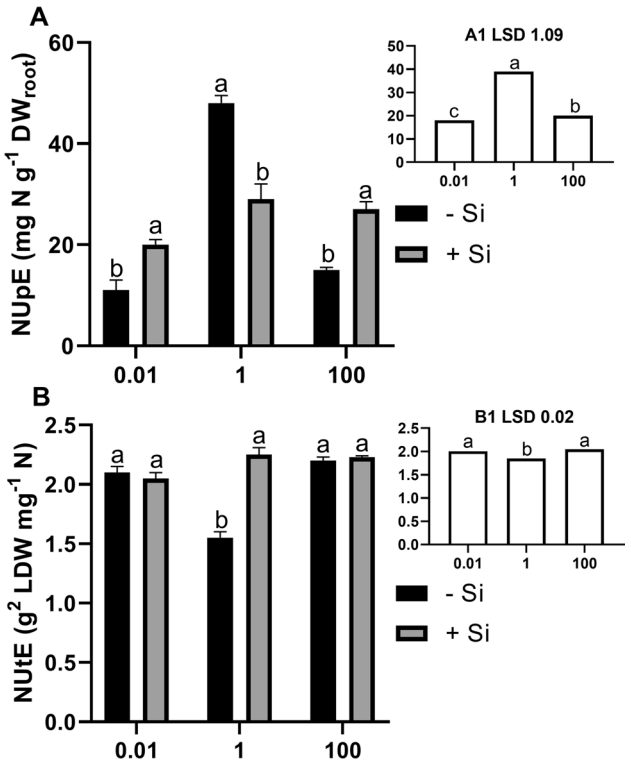

Fig. 1 Effects of Zn dose and the presence (+Si) or absence (− Si) of $CaSiO_3$ supply on NUpE (**A**), and NUtE (**B**) in barley leaves. The columns values are mean ± standard error ($n=9$), the differences between means were compared with the minimum significant difference of Fisher's test (LSD; $P=0.05$). A1 and B1 values are means (control + Si supply $n=18$). Different letters indicate significative differences between plants supplied with $CaSiO_3$ and plants without $CaSiO_3$

conditions. NUtE value was higher in plants grown under control conditions with $CaSiO_3$ than in plants without $CaSiO_3$. In contrast, $CaSiO_3$ application did not modify NUtE value in barley plants supplied with non-optimal Zn doses (Fig. 1B).

NH_4^+ production: NO_3^- reduction and photorespiration

Zn deficiency caused an increment of NO_3^- concentration in barley plants. Likewise, $CaSiO_3$ increased NO_3^- in barley grown under control and Zn-deficient conditions. Similarly, but to a lesser extent, $CaSiO_3$ supply increased NO_3^- concentration in Zn-toxicity plants. Interestingly, Zn dose did not affect to NR activity, although $CaSiO_3$ application raised NR activity in plants grown under control and deficient Zn doses. Nevertheless, a significant diminution was detected in plants subjected to Zn toxicity compared to plants that did not receive $CaSiO_3$. Besides, free NH_4^+ levels increased in plants supplied with 0.01 μM $ZnSO_4$ and 100 μM $ZnSO_4$. However, $CaSiO_3$ enhanced NH_4^+ concentration in plants supplied with and 1 μM $ZnSO_4$, 100 μM $ZnSO_4$, whereas reduced it in plants grown with 0.01 μM $ZnSO_4$. Total reduced N concentration was lower in plants supplied with non-optimal Zn doses compared to control plants. In contrast, $CaSiO_3$ application was positive to increased total reduced N in these plants (Table 2). Considering photorespiratory enzymes, GO and GGAT showed higher activities in plants grown under non-optimal Zn doses, compared to control conditions (Fig. 2). $CaSiO_3$ application increased GO activity in control plants, whereas caused a reduction in plants that received the rest of Zn doses (Fig. 2A). Differently, $CaSiO_3$ effects were unequally on GGAT activity depending on the Zn dose: it was lower in Zn-deficient plants but increased in control plants, whereas it did not cause effects in plants grown with 100 μM $ZnSO_4$ (Fig. 2B).

NH_4^+ incorporation and assimilation products

GDH, GOGAT, and GS activities increased in comparison to controls in plants supplied with under both Zn toxicity and deficiency doses. Conversely, $CaSiO_3$ application caused inhibition of these activities in Zn-stressed plants. AAT activity increased almost two-fold in plants supplied with 0.01 and 100 µM $ZnSO_4$ compared to controls. Moreover, $CaSiO_3$ decreased AAT activity in Zn-deficient plants, did not change in Zn-toxicity barley plants, whereas in control plants $CaSiO_3$ increased AAT activity (Table 3).

N derived protective compounds concentration

Pro and GB concentrations increased with Zn stress in barley leaves. Conversely, control plants showed low levels of both compounds compared to the other treatments. Pro levels decreased in plants grown with $CaSiO_3$ and non-optimal Zn doses, although in control conditions, $CaSiO_3$ application increased Pro concentration. In contrast, the $CaSiO_3$ application did change GB content compared to plants without $CaSiO_3$ (Fig. 3).

AAs concentration

Zn toxicity increased total free AAs, whereas $CaSiO_3$ application reduced total AAs regardless of the Zn dose applied. However, Zn deficiency did not affect AAs profile except by an increment in Tyr concentration. Furthermore, Zn toxicity increased the majority of AAs concentration except for Cys, Arg, Tyr, and Gly that did not change compared to control Zn supply. $CaSiO_3$ application reduced the concentration of most AAs, especially in barley grown under 1 µM $ZnSO_4$ dose. $CaSiO_3$ increased Pro concentration in plants supplied with 0.01 µM $ZnSO_4$ dose and did not change Asp, Glu, and Tyr concentrations. In control plants, Asp and Arg did not change, whereas Tyr concentration increased in plants with $CaSiO_3$. Finally, in barley grown under Zn toxicity, Glu, Cys, His and Gly concentrations did not change when $CaSiO_3$ was applied, whereas Tyr concentration increased (Fig. 4A; Table S1).

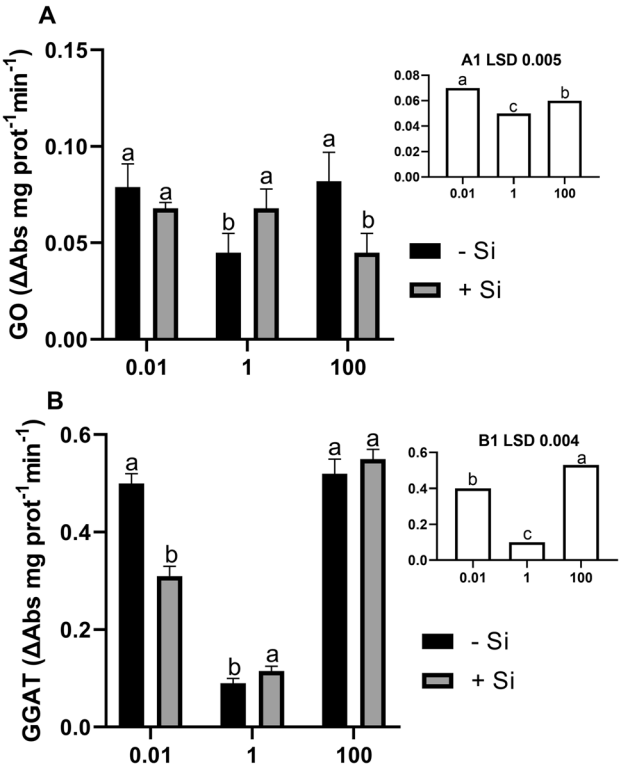

Fig. 2 Effects of Zn dose and the presence (+Si) or absence (− Si) of $CaSiO_3$ supply on GO (**A**), and GGAT (**B**) in barley leaves. The column's values are mean ± standard error ($n=9$), the differences between means were compared with the minimum significant difference of Fisher's test (LSD; $P=0.05$). A1 and B1 values are means (control + Si supply $n=18$). Different letters indicate significative differences between plants supplied with $CaSiO_3$ and plants without $CaSiO_3$

Table 2 N forms and NR activity in barley plants grown under different Zn doses, and with the presence (+Si) or absence (− Si) of $CaSiO_3$

	NO_3^- (mg g^{-1} DW)			NR (µM NO_2 mg $prot^{-1}$ min^{-1})			NH_4^+ (mg g^{-1} DW)			Total reduced N (mg g^{-1} DW)		
µM Zn	0.01	1	100	0.01	1	100	0.01	1	100	0.01	1	100
− Si	2.42	1.89	2.37	0.27	0.27	0.33	1.96	1.44	1.68	7.60	22.01	10.59
+Si	3.86	3.03	2.96	0.32	0.32	0.25	1.68	1.52	1.91	10.42	13.73	14.98
p-value	*	*	*	*	*	*	*	*	*	*	*	*
0.01 Zn		3.14a			0.29a			1.82a			9.01b	
1 Zn		2.46b			0.30a			1.49b			17.87a	
100 Zn		2.67b			0.29a			1.79a			10.39b	
$LSD_{0.05}$		0.25			0.01			0.05			1.85	

Values are means ($n=9$) and differences between means were compared by Fisher's least-significance test (LSD; $P=0.05$). 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 *($P<0.05$)

SPAD value and photosynthetic pigments

Both low and high Zn doses reduced SPAD value of barley plants compared to control conditions. Although, $CaSiO_3$ supply substantially restored SPAD values to control plant levels. Likewise, photosynthetic pigments concentration was significantly affected by Zn dose. Thus, low and high Zn concentrations decreased Chl *a* and Chl *b* concentrations. When $CaSiO_3$ was added, Chl levels increased in both control and Zn-deficient plants, whereas $CaSiO_3$ did not produce effects in plants supplied with the highest Zn dose. The Chl *a*/*b* results presented no significant changes comparing the three Zn doses. Nevertheless, in barley plants grown with $CaSiO_3$ and the high Zn dose, the Chl *a*/*b* values were higher than in plants without $CaSiO_3$. In contrast, $CaSiO_3$ did not modify Chl *a*/*b* values. Regarding controls, carotenoids levels increased in plants supplied with 0.01 µM $ZnSO_4$ and did differ in plants subjected to Zn excess. $CaSiO_3$ application did not produce effects in carotenoids concentration in Zn-deficient plants but induced a significant increase in both control and Zn toxicity plants (Table 4).

Effects of Zn nutrition and $CaSiO_3$ supply on Chl *a* fluorescence parameters

No significant differences in *F*o, *F*m, *F*v/*F*m, PI_{ABS}, RC/ABS, and K step parameters were observed between the different Zn supplies. However, non-optimal Zn levels reduced the values of Ψ_{Eo}, *t* for *F*m, Area, ABS/RC, and ET/RC. Furthermore, Φ_{Eo} suffered a reduction lower in plants grown under 100 µM $ZnSO_4$ Zn, whereas TR/RC decreased in Zn-deficient plants. $CaSiO_3$ supply lowered *F*o value in plants grown with 0.01 µM $ZnSO_4$ but enhanced it in plants supplied with 1 and 100 µM $ZnSO_4$. In Zn-deficient plants, $CaSiO_3$ increased *F*m value but decreased it in control plants. $CaSiO_3$ application incremented *F*v/*F*m in plants grown under non-optimal Zn supplies. Irrespective of the Zn dose applied, $CaSiO_3$ increased the values of PI_{ABS}, RC/ABS, Ψ_{Eo}, Φ_{Eo}, *t* for *F*m, and Area, whereas it reduces the K step value compared to plants without $CaSiO_3$. Finally, $CaSiO_3$ also increased electron fluxed in control and Zn-toxicity plants but not in Zn-deficient ones that presented lower ABS/RC and TR/RC values (Fig. 4B; Table S2).

Discussion

Plant growth is related to leaf DW and RGR that represents the best parameters to estimate stress effects on plants (Gong et al. 2013). Hajiboland and Amirazad (2010) observed a decrease in leaf biomass of up to 62% in Zn-deficient *Brassica oleracea* plants. Furthermore, the exposition to toxic Zn concentration causes its accumulation in tissues reducing plant biomass (Gisbert et al. 2006). Likewise, in our study, barley plants suffering from Zn deficiency and Zn toxicity reduced their biomass and growth. However, the $CaSiO_3$ application mitigated the loss of biomass in Zn-deficient and Zn-toxicity plants. These results suggest a positive effect of $CaSiO_3$, which mitigates the biomass loss caused by low or high Zn presence. Among the elements provided by $CaSiO_3$, Si could be responsible for this positive effect, but not Ca because it did not increase its accumulation in barley plants. Thus, $CaSiO_3$ improved Zn accumulation in Zn-deficient barley plants and reduced Zn concentration in Zn-toxicity plants. A similar positive effect of Si was observed in rice plants grown under high Zn doses and in soybean subjected to Zn shortage (Song et al. 2014; Pascual et al. 2016). However, in the present study, $CaSiO_3$ reduced the growth in control plants, so a higher Si accumulation may be unfavorable in control conditions. Besides, the lower N uptake may reduce growth in these plants.

Smirnnoff and Stewart (1987) proved that low Zn reduces the presence of reduced N compounds. Accordingly, NUpE decreased in our barley plants grown under non-optimal Zn supplies indicating that Zn stress affects N uptake. However, NUtE increased in these plants, suggesting that Zn stress could balance the reduction in N availability by an increase of N utilization efficiency. Moreover, NUpE values suggested that $CaSiO_3$ reduced the effects of Zn deficiency and toxicity through a higher N accumulation. In control plants, despite the lower NUpE, a higher NUtE was observed, probably as a result of the higher NO_3^- and NH_4^+ concentrations and the increment in NR activity. Previous studies described inhibition of NR in Zn-deficient (Seethambaram and Das 1986) and plants grown under Zn toxicity (Luna et al. 2000). In our experiment, NO_3^- concentration increased under Zn stress, despite NR activity did not show changes and it was a lower N uptake. A diversion of reductants from basal metabolism to stress response could be a possible explanation (Esposito 2016). On the other hand, in plants affected by Zn deficiency, $CaSiO_3$ enhanced NR activity probably through an increase of NO_3^- concentration.

Besides from NO_3^- reduction, NH_4^+ is continuously produced by photorespiration (Wingler et al. 2000). In the present experiment, Zn shortage and excess enhanced photorespiratory enzyme activities, suggesting that an increased photorespiration helps in ROS elimination to counteract the oxidative burst under Zn stress (Wingler et al. 2000). Nevertheless, the results in barley plants suggest that $CaSiO_3$ could inhibit photorespiration in the Zn-stressed plants, which suggests a lower stress in these plants. Furthermore, NH_4^+ assimilatory enzymes showed higher activities in plants grown under non-optimal Zn doses. An increment of GS activity could

Table 3 Activities of N metabolism enzymes in barley plants grown under different Zn doses, and with the presence (+Si) or absence (− Si) of $CaSiO_3$

	GS (μM glutamyl hydroxamate mg prot^{-1} min^{-1})			GOGAT (ΔAbs mg prot^{-1} min^{-1})			GDH (ΔAbs mg prot^{-1} min^{-1})			AAT (ΔAbs mg prot^{-1} min^{-1})		
μM Zn	0.01	1	100	0.01	1	100	0.01	1	100	0.01	1	100
− Si	516.75	310.69	816.07	1.80	0.96	1.52	0.14	0.08	0.36	7.60	22.01	10.59
+Si	370.61	368.54	441.71	1.42	1.01	1.19	0.39	0.20	0.49	10.42	13.73	14.98
p-value	*	*	*	*	NS	*	*	*	*	*	*	*
0.01 Zn	443.68b			1.61a			0.27b			9.01c		
1 Zn	339.62c			1.04c			0.14c			17.87a		
100 Zn	628.89a			1.29b			0.43a			12.76b		
$LSD_{0.05}$	36.09			0.11			0.01			0.93		

Values are means ($n=9$) and differences between means were compared by Fisher's least-significance test (LSD; $P=0.05$). 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 * ($P<0.05$)

induce a higher use of Glu and Gln generated from the GS/GOGAT cycle and afterward the formation of aspartate (Asp) and other essential AAs by the AAT activity (De la Torre et al. 2014). However, this increased AAs synthesis was not enough to counteract Zn stress. In addition, $CaSiO_3$ reduced GS activity to control values. These results suggest a decrease of NH_4^+ recycling through photorespiration, and therefore a reduction of stress symptoms. Besides, GDH also participates in NH_4^+ assimilation when is present in a high concentration in plant cells (Robinson et al. 1991). Thus, in our experiment, $CaSiO_3$ enhanced GDH activity, which probably contributes to NH_4^+ assimilation.

Under stress, plants usually increase total AAs concentration because of an increment of the protein degradation/synthesis ratio (Atilio and Causin 1996). Thus, specific stress-related AAs, such as Pro, are accumulated in plant tissues (Ashraf and Foolad 2007). This increase was observed in our experiment in barley plants subjected to Zn excess. Indeed, these plants presented a remarkable increment of Asp and Ser, probably as a response to the increased photorespiration activity. On the other hand, $CaSiO_3$ reduced AAs levels, suggesting lower proteolysis due to reduced stress symptoms. However, plants did not reduce basic AAs involved in N metabolism, such as Asp and Glu. Likewise, AAs composing GSH did not decrease under Zn toxicity, probably to synthesize more GSH to improve Zn homeostasis. In addition, $CaSiO_3$ application only increased Tyr concentration, which might be due to an enhancement of Tyr metabolism produced by Zn imbalance to synthesize tocopherol and related antioxidant compounds (Holländer-Czytko et al. 2005). Regarding Pro, other experiments showed that Zn toxicity increases this AA (Höller et al. 2013; Paradisone et al. 2015), which agree with our results. Likewise, Navarro-León et al. (2016) observed higher GB levels in

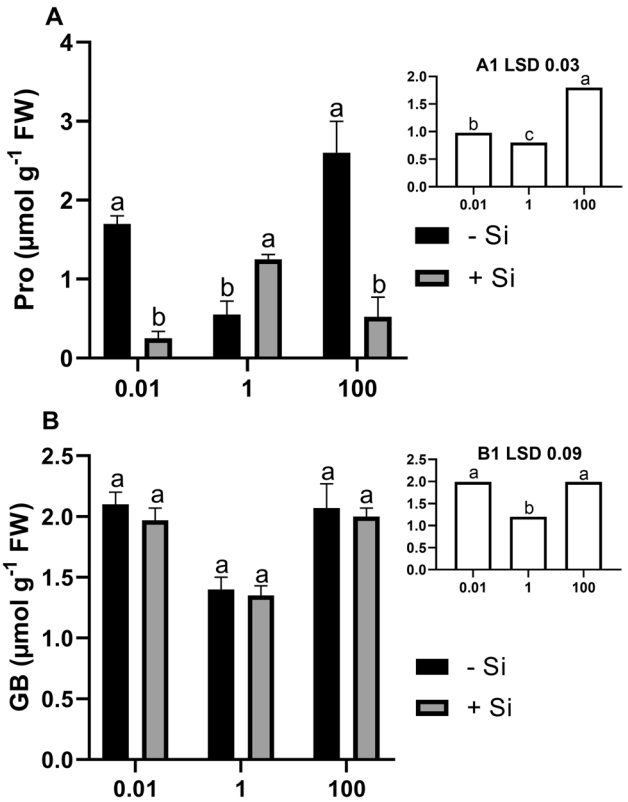

Fig. 3 Effects of Zn dose and the presence (+Si) or absence (− Si) of $CaSiO_3$ on Pro (**A**), and GB contents (**B**) in barley leaves. The columns values are mean ± standard error ($n=9$), the differences between means were compared with the minimum significant difference of Fisher's test (LSD; $P=0.05$). A1 and B1 values are means (control + Si supply $n=18$). Different letters indicate significative differences between plants supplied with $CaSiO_3$ and plants without $CaSiO_3$

Zn-deficient cabbage plants. Similarly, in the present experiment, GB levels increased in Zn-deficient barley plants.

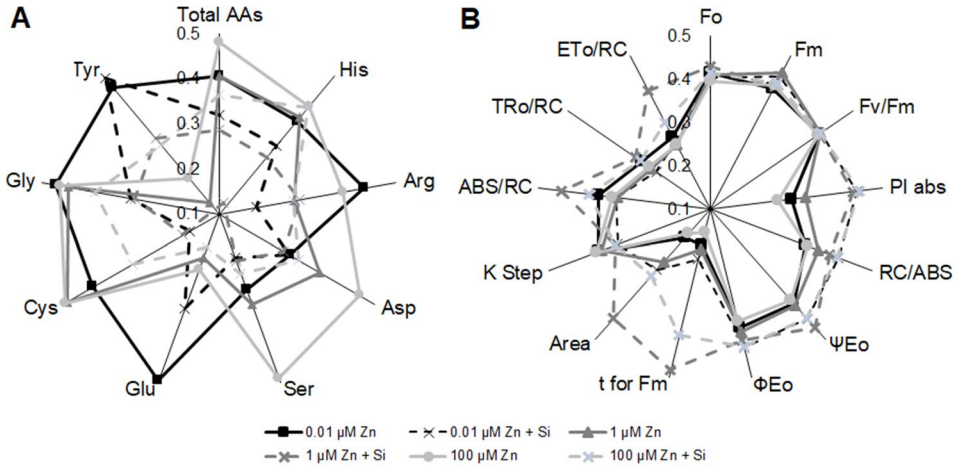

Fig. 4 Radar chart showing the AAs profile (**A**) and analyzed parameters of Chl *a* fluorescence (**B**) of barley plants subjected to the six different treatments. Actual values were normalized to show in the same range

Table 4 Photosynthetic pigments and SPAD value in barley plants grown under different Zn doses, and with the presence (+Si) or absence (− Si) of $CaSiO_3$

	SPAD			Chl *a* (mg g^{-1} FW)			Chl *b* (mg g^{-1} FW)			Carotenoids (mg g^{-1} FW)		
μM Zn	0.01	1	100	0.01	1	100	0.01	1	100	0.01	1	100
− Si	26.63	44.40	27.42	0.19	0.19	0.16	0.08	0.08	0.07	0.03	0.02	0.01
+Si	43.27	43.73	37.71	0.20	0.20	0.16	0.09	0.09	0.07	0.03	0.03	0.02
p-value	*	NS	*	*	*	NS	*	*	NS	NS	*	*
0.01 Zn		34.95b		0.195b			0.088a			0.027a		
1 Zn		44.08a		0.201a			0.088a			0.024b		
100 Zn		32.56c		0.166c			0.073b			0.021c		
$LSD_{0.05}$		0.57		0.003			0.002			0.001		

Values are means ($n=9$) and differences between means were compared by Fisher's least-significance test (LSD; $P=0.05$). 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 * ($P<0.05$)

Because barley is sensitive to Zn imbalances, our results suggest that GB and Pro accumulations represent a stress indicator, instead of a tolerance mechanism. Accordingly, the lower Pro accumulation in $CaSiO_3$ plants suggests lower stress in these plants.

Photosynthesis is a sensitive process to stress (Pfannschmidt and Yang 2012). Thus, plants grown under non-optimal Zn doses presented lower Chls and SPAD levels, which agree with previous research (Song et al. 2014; Pascual et al. 2016). Our SPAD results suggested that $CaSiO_3$ supply protected barley plants against the chlorosis originated by the Zn imbalances. Accordingly, other studies also noted this positive effect of $CaSiO_3$ on photosynthetic pigments (Song et al. 2014; Pascual et al. 2016). Furthermore, carotenoids play a central role as antioxidant compounds removing ROS (Havaux 1998). Low Zn application promoted the accumulation of these pigments, probably as a response to stress, which was also observed by Hajiboland and Amirazad (2010) in *B. oleracea* plants subjected to Zn deficiency. Besides, $CaSiO_3$ has a positive effect on carotenoids concentration in barley plants, as also observed by Anwaar et al. (2015) in cotton plants. A high carotenoid content could be useful to increase antioxidant properties of food (Nicolle et al. 2004).

Chl *a* fluorescence is important to dissipate the energy excess in photosynthetic tissues (Strasser et al. 2004). The effects of Zn imbalances on Chl *a* fluorescence parameters vary depending on the species, although photosynthesis performance is usually negatively affected (Hajiboland and Amirazad 2010; Zhao and Wu 2017). Fluorescence parameters suggest that neither Zn deficiency nor toxicity caused remarkable effects on PSII performance, as indicative parameters, such as PI_{ABS} and RC/ABS, were unaffected by these Zn doses. However, parameters, such as Ψ_{Eo}, *t* for *F*m, Area, and electron fluxes, were negatively affected. In addition, Zn deficiency affected to a greater extent to photosynthesis performance compared to Zn toxicity as shown by the values of *t* for *F*m, and ABS/RC and TR/RC parameters. Moreover, Zn toxicity reduced Ψo and Φ_{Eo}, which suggests lower electron transport activity due to the accumulation of reduced Q^{-}.

Regarding the $CaSiO_3$ application, other authors observed that Si enhance *Fv*/*Fm* and electron transport (Sivanesan et al. 2011; Song et al. 2014). In our experiment, $CaSiO_3$ had a positive effect on PSII performance as it increased almost all parameters. Therefore, $CaSiO_3$ increased the proportion of active RCs, the energy flux through PSII, the pool of Q^-, and thus, the energy for photosynthesis. Likewise, $CaSiO_3$ reduced K step values, which suggest better electron flux between the OEC and the PSII.

Conclusion

Barley plants subjected to Zn shortage and Zn excess presented an inhibition of growth that could be caused by lower N uptake capability and higher stress as suggested by their increased photo-respiratory activity and their lower photosynthesis. On the other hand, $CaSiO_3$ increased tolerance to both Zn deficiency and toxicity because contributed to maintained plant growth. This could be related to $CaSiO_3$ enhanced Zn accumulation in Zn-deficient plants and reduced Zn accumulation in Zn-toxicity plants. Likewise, $CaSiO_3$ increased N uptake capacity, NH_4^+ assimilation by GDH and AAT activities, and N reduction under Zn deficiency. In addition, results suggest lower stress as plants presented lower photorespiration and AAs concentration, higher pigments concentration, and better photosynthesis performance. Briefly, the results suggest that $CaSiO_3$ increases tolerance of plants to both low and high Zn doses, although further research is required to reveal its mechanism of action.

Author contribution statement JMR, BB, and SE conceived the idea and designed the experiment. VP, and ENL performed the experiment. VP, and ENL analyzed the data. VP, and ENL wrote the manuscript. JMR, BB, and SE revised the manuscript. All authors read and approved the final draft.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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