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REGULAR ARTICLE

Elevated zinc concentrations did not induce thiols in spinach (*Spinacia oleracea*) and parsley (*Petroselinum crispum*)

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Abstract

Background: Plants evolved various mechanisms to cope with metal stress. Cadmium (Cd) exposure specifically induces the synthesis of thiol-rich substances such as phytochelatins. Due to the chemical similarity of Cd and zinc (Zn), similar detoxification mechanisms for both metals are under discussion.

Aim: We conducted a nutrient solution experiment to investigate thiol accumulation of parsley (*Petroselinum crispum)* and spinach (*Spinacia oleracea*) cultivars at different metal toxicity levels *in vivo*.

Methods: Three metal treatments were applied: 1 µM Zn (control), 10 µM Zn, and 1 µM Zn + 1 µM Cd. After 10 days, thiol accumulation in parsley and spinach cultivars, which differ in their Zn tissue tolerance, was measured.

Results: Spinach and parsley cultivars differed in metal uptake, translocation, and resistance. In spinach, Cd application induced more severe toxicity symptoms and biomass reduction than Zn. Cadmium toxicity was more pronounced in spinach than in parsley due to higher Cd translocation of spinach cultivars. Despite comparable Zn tissue concentrations, parsley did not show any Zn toxicity symptoms. Due to lower Cd tissue concentrations, only a slight browning of parsley roots was found after Cd treatment. Whereas Cd application induced thiol synthesis in both plant species, Zn excess did not.

Conclusion: As elevated Zn concentrations in plant tissues did not induce thiol synthesis, a contribution of phytochelatins to Zn homeostasis and detoxification was excluded.

KEYWORDS
cadmium, metal resistance, metal stress, parsley, phytochelatins, spinach, thiol-containing substances

1 | INTRODUCTION

To cope with metal stress plants evolved various mechanisms regarding enzymatic and non-enzymatic protection systems (Clemens, 2006; Viehweger, 2014; Song et al., 2017). Synthesis of thiol-containing substances is often discussed in the literature to be one important mechanism of plants to cope with metal stress (Clemens, 2019; Sofo et al., 2013; Stuiver et al., 2014; Wu et al., 2018). Especially, synthesis of phytochelatins, metallothioneins, and glutathione was reported in this context (Leitenmaier & Küpper, 2013). Glutathione, the precursor for phytochelatin synthesis, seemed to play a key role not only in metal detoxification but also in protecting plant cells from oxidative stress (Barrameda-Medina et al., 2014; Krężel & Maret, 2016; Noctor et al., 2012). Also, free cysteine was identified as metal ligand for detoxification and transport of metals in plants (Harris et al., 2012). In mammalian cells, metallothioneins were shown to be involved in cell redox status, metal detoxification, and zinc (Zn) homeostasis (Maret, 2000). Similar functions of thiol-containing metallothioneins

are assumed in plants. Phytochelatins are peptides with the general structure (γ-Glu–Cys)n–Gly (n = 2–11) (Grill et al., 1987). The role of phytochelatins in detoxification of arsenic (As) and cadmium (Cd) is a secured state of knowledge (Cobbett & Goldsbrough, 2002; Rauser, 1990; Rea, 2012; Schmöger et al., 2000).

Cadmium is a dangerous Zn mimic that is not essential for plants and does not provide any beneficial effects on plant behavior (Chmielowska-Bąk et al., 2013; EFSA, 2009). However, chemical similarity of Cd and Zn causes many problems in plant physiology (Cakmak et al., 2000) due to the displacement of Zn by Cd at binding sites of metabolites and peptides (Küpper & Andresen, 2016). Under Cd stress, phytochelatin synthesis is specifically induced in plants for detoxification (Cobbett & Goldsbrough, 2000; Loeffler et al., 1989). Under elevated Zn concentrations, similar interactions such as uncontrolled displacement of cations and binding to enzymes take place (Andresen et al., 2018; Colvin et al., 2010). Whereas previous literature indicated no involvement of phytochelatins in Zn resistance (Davies et al., 1991; Grill et al., 1988), recent reports assumed an induction of phytochelatin synthesis for detoxification and homeostasis of Zn in plants (Clemens & Peršoh, 2009; Kühnlenz et al., 2016; Tennstedt et al., 2009). Also in textbooks, it is written that "synthesis of phytochelatins is induced by the exposure to toxic heavy metals" or "to high concentrations of micronutrients" (Buchanan et al., 2015) and plays an important role in Zn resistance (Marschner, 2012). However, the involvement of thiol-containing substances in Zn detoxification and Zn homeostasis is still under discussion.

Therefore, we conducted nutrient solution experiments with various Zn and Cd concentrations to test the hypothesis that the detoxification by accumulation of thiols under Zn excess is similar to Cd in spinach and parsley. Both plants have been discussed as suitable species for Zn biofortification purposes, leading to the question of how spinach and parsley deal with excess Zn and whether thiols may be involved in its detoxification.

2 | MATERIALS AND METHODS

2.1 | Plant cultivation

Two spinach cultivars (*Spinacia oleracea* L. cv. Camaro and cv. Seychelles) and two parsley cultivars (*Petroselinum crispum* Mill. convar. *crispum* cv. Fidelio and cv. Gigante d'Italia) which differed in their Zn tissue tolerance in pre-experiments were chosen in this study. Spinach and parsley seeds were soaked in an aerated solution consisting of 1 mM $CaSO_4$ and 20 µM H_3BO_3 at room temperature for 24 h. Seeds were sown in sand in the climate chamber and molded with one-fourth concentrated nutrient solution for germination. Seeds were irrigated with 1 mM $CaSO_4$ and 20 µM H_3BO_3. Spinach seeds were grown for 10 days and parsley seeds for 24 days, respectively. These seedlings were transferred to one-fourth concentrated nutrient solution in 5 L pots. Each pot contained three plants. After further 3 and 6 days, the concentration of the nutrient solution was increased to half and full strength, respectively. The full-concentrated nutrient solution contained the following salt concentrations: 2 mM $CaNO_3$, 2 mM $CaCl_2$, 1 mM K_2SO_4, 0.75 mM $MgSO_4$, 0.3 mM $NH_4H_2PO_4$, 0.03 mM Na_2Fe-EDTA, 20 µM H_3BO_3, 2 µM $MnSO_4$, 0.3 µM $CuSO_4$, 0.1 µM $NiSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, and 1 µM $ZnSO_4$. The pH value of the fresh nutrient solution was about 5.4. The nutrient solution was renewed every 3 days. Aeration of the nutrient solution was provided until harvest. The day length was 16 h at 22°C and the dark period 8 h at 18°C. The light intensity was 400 µmol s^{-1} with a relative humidity of about 60%. Six days after transfer of spinach plants to nutrient solution and 45 days after transfer of parsley plants to nutrient solution, the three metal treatments were applied: 1 µM Zn (control), 10 µM Zn (Zn excess), 1 µM Zn + 1 µM Cd (Cd treatment). Zinc and Cd were applied as chloride salts. Four pots (replicates) with three plants each were cultivated per treatment. The Cd treatment served as positive control for the induction of thiol synthesis. Ten days after start of Cd and Zn application, spinach and parsley plants were harvested and shoots and roots were divided into two aliquots. One aliquot was immediately frozen in liquid nitrogen, ground with mortar and pestle, and stored at −80°C for thiol and disulfide determination. The other aliquot was dried at 90°C in a fan-forced oven (ED 720, Binder). After drying to constant weight, dry weights were determined and the dried samples were ground (≤ 1 mm) with a mill (MF-10 basic, IKA). Homogenized samples were wet-ashed for determination of Zn and Cd concentrations.

2.2 | Determination of Zn and Cd concentrations

A homogenized aliquot of each sample was wet-ashed to determine total Zn and Cd concentrations of shoots and roots (modified after Rosopulo et al., 1976). For this purpose, 0.5 g dried and ground sample was weighed into 110 mL glass tubes and 2 mL trichlor ethylene were added as anti-foaming agent. For wet-digestion 10 mL acid mixture, consisting of concentrated HNO_3:$HClO_4$:H_2SO_4 (40:4:1; v:v:v; p.a.), were added, the tubes were closed (not sealed), and the samples were incubated overnight at room temperature. Wet digestion was started the next morning following a standardized temperature program in a heating block in which temperature was stepwise increased from 100 to 220°C every 2 h. For each wet digestion, two blanks and two plant standards (hay standard, institute-internal reference material) were prepared simultaneously.

2.3 | Determination of thiols and disulfides

Thiol and disulfide concentrations were determined according to Ellman (1959) and Thannhauser et al. (1984), respectively. Homogenized frozen samples (400 mg) were therefore mixed with ice-cold PBST-buffer (136 mM NaCl, 2.68 mM KCl, 10.0 mM Na_2HPO4, 1.98 mM KH_2PO_4, 3.0 mM Na_2EDTA, 0.05% Tween® 20) for 30 s and extracted for 2 × 2 min using sonication. For clarification, samples were centrifuged at 17,000 g at 0°C for 3 min in a micro-centrifuge (Hettich, EBA 12R). The supernatants were used for thiol and disulfide determination. Samples and extracts were always kept on ice

TABLE 1 Biomasses of spinach and parsley cultivars

		1 µM Zn (Control) 0 µM Cd		10 µM Zn 0 µM Cd		1 µM Zn 1 µM Cd	
Fresh weight (g plant^{-1})		Shoot	Root	Shoot	Root	Shoot	Root
Spinach	Camaro	11.14 ± 1.14^{A}	4.07 ± 0.55^{DE}	13.03 ± 0.32^{A}	4.52 ± 0.47^{D}	6.44 ± 0.49^{BC}	2.55 ± 0.15^{E}
	Seychelles	10.29 ± 1.42^{AB}	2.85 ± 3.85^{DE}	10.14 ± 1.27^{AB}	3.45 ± 0.25^{DE}	5.06 ± 0.22^{C}	1.52 ± 0.09^{F}
Parsley	Gigante	31.15 ± 2.15^{a}	23.82 ± 1.58^{b}	29.73 ± 1.41^{a}	30.65 ± 1.99^{a}	27.61 ± 1.25^{a}	24.00 ± 0.66^{b}
	Fidelio	26.54 ± 0.82^{a}	16.11 ± 1.32^{cd}	26.11 ± 0.77^{a}	17.30 ± 1.12^{cd}	26.68 ± 0.84^{a}	18.54 ± 0.96^{bc}

Root and shoot fresh weights of spinach and parsley cultivars after Zn and Cd treatment for 10 days in hydroponics; two cultivars of each plant species were grown for 24 days (spinach) or 45 days (parsley); means ($n = 4$) ± standard error; different letters indicate significant differences among spinach (capital letters) and parsley treatments (small letters).

to minimize enzyme activities. Thiol and disulfide concentrations were determined photometrically at 412 nm. Free thiols (-SH) were quantified using Ellman's reagent (5,5′-dithiobis–2-nitrobenzoic acid, DTNB) (Ellman, 1959). Thiol concentrations of samples were calculated from a glutathione (reduced) standard curve. For disulfide quantification (-S–S-) 2-nitro–5-thiosulfobenzoate (NTSB) was synthesized from DTNB as described by Chen and Liao (2008). Disulfide concentrations of samples were calculated from a glutathione (oxidized) standard curve. Total thiol concentrations were calculated as -SH + 2 -S–S-. Recovery for added glutathione was between 99% and 102%.

2.4 | Determination of total glutathione

Total glutathione concentrations of shoots and roots were determined spectrometrically (Griffith, 1980). Homogenized frozen samples (100 mg) were extracted with 4% sulfosalicylic acid and 5% insoluble polyvinyl-polypyrrolidone (PVPP) for 2 × 2 min using sonication. Samples were centrifuged and the supernatants were neutralized as described by Matthus et al. (2015). The reaction mix contained 0.6 mM 5,5′-dithiobs-(2-nitrobenzoic acid) (DTNB), 0.125 U glutathione reductase, and 20 µL of the neutralized extract. The reaction was started by adding NADPH (final concentration 0.3 mM) and was followed at 412 nm for 3 min. Total glutathione concentrations of samples were calculated from a glutathione (oxidized) standard curve.

2.5 | Statistical analysis

Data were processed using the statistics program IBM SPSS Statistics (Version 20). In cases of homogeneity of variance significant differences among Zn and Cd treatments were evaluated with variance analysis (ANOVA). In cases of inhomogeneity of variances, data were transformed [log10 (x)] to obtain homogenous variances before evaluation of significant differences. Multiple comparisons among metal treatments were conducted with the Tukey HSD test and were considered to be significant when $p \leq 0.05$. Homogeneity of variance was tested with the Levene test and was found to be $p \geq 0.05$. The variation is indicated by standard error (error bars in the figures).

FIGURE 1 Spinach cultivars with Zn and Cd toxicity. Zn and Cd toxicity symptoms at the leaf surface of 24-day-old spinach plants after Zn and Cd treatment for 10 days

3 | RESULTS

3.1 | Biomasses of parsley and spinach

Shoot and root fresh weights did not differ between spinach and parsley cultivars in any Zn treatment. Cadmium application decreased shoot and root fresh weights of spinach cultivars whereas parsley biomasses were not affected in comparison to control plants (Table 1).

3.2 | Obvious toxicity symptoms

For spinach, Zn toxicity symptoms such as chlorosis were only visible for cultivar Seychelles in the high-Zn treatment (10 µM Zn). However, both spinach cultivars showed Cd toxicity symptoms at shoots (Figure 1) and roots (not shown). First symptoms of Zn and Cd toxicity for spinach were obvious after 7 days of treatment. Parsley cultivars did show neither Zn nor Cd toxicity of shoots (not shown). Only parsley cultivar Fidelio showed slight browning of roots after Cd treatment for 9 days (Figure 2).

FIGURE 2 Parsley cultivar Fidelio with Cd toxicity symptoms. Browning of roots of 60-day-old parsley plants after Cd treatment for 10 days

3.3 | Zn and Cd concentrations

For both plant species, Cd concentrations were only measured in roots and shoots after Cd treatment (1 µM Cd). Except for parsley cultivar Fidelio, Zn concentrations of shoots and roots significantly increased in spinach and parsley in comparison to control plants (1 µM Zn) after application of 10 µM Zn (Table 2).

3.4 | Shoot:root ratios of Zn and Cd

In the high-Zn treatment (10 µM Zn), spinach cultivar Seychelles showed a significantly reduced Zn translocation from root to shoot (shoot:root ratio < 4) in comparison to control plants with a shoot:root ratio > 4. Zinc translocations of parsley cultivars were only half as high than those of spinach cultivars (shoot:root ratio < 2). Zinc translocation to the shoot was also reduced in the high-Zn treatment (10 µM Zn) in relation to control plants (1 µM Zn) in both parsley cultivars. In parsley, Cd was predominantly retained in roots with a shoot:root ratio < 1. In contrast, spinach accumulated Cd in the shoot (shoot:root ratio > 2) (Figure 3).

3.5 | Thiol and disulfide concentrations

Thiol and disulfide concentrations of spinach shoots and roots were significantly increased after Cd treatment (1 µM Cd). However, thiol and disulfide concentrations were unchanged in both Zn treatments (1 µM or 10 µM Zn). No differences were observed between spinach cultivars (Figure 4). For parsley, increased thiol and disulfide concentrations were only observed in roots after Cd treatment (1 µM Cd). Thiol and disulfide concentrations were not affected by a high-Zn application (10 µM Zn) in parsley plants. No differences were observed between parsley cultivars (Figure 5).

3.6 | Total glutathione concentrations

For parsley, no differences in glutathione concentrations were observed after Zn (10 µM) or Cd (1 µM) treatment in comparison to control plants. Glutathione concentrations were also not affected in spinach roots. However, for both spinach cultivars increased concentrations of glutathione were measured in shoots after Cd treatment (1 µM Cd) (Figure 6).

4 | DISCUSSION

Cadmium is an element that is not essential for the development of higher plants (Chmielowska-Bąk et al., 2013; EFSA, 2009). Therefore, plants evolved detoxification mechanisms (Clemens, 2006; Song et al., 2017; Viehweger, 2014) to avoid competitive displacement of divalent cations in living cells. Numerous studies showed that the accumulation of thiols allowed plants to survive (Cobbett & Goldsbrough, 2002;

TABLE 2 Zn and Cd concentrations of spinach and parsley cultivars

		1 µM Zn (Control) 0 µM Cd		10 µM Zn 0 µM Cd		1 µM Zn 1 µM Cd				1 µM Zn 1 µM Cd	
Zinc (mg kg^{-1} FW)		Shoot	Root	Shoot	Root	Shoot	Root	Cadmium (mg kg^{-1} FW)		Shoot	Root
Spinach	Camaro	6.28 ± 0.30^{D}	3.85 ± 0.50^{D}	23.13 ± 2.40^{A}	22.11 ± 1.34^{AB}	9.00 ± 0.73^{CD}	5.14 ± 0.10^{D}	**Spinach**	Camaro	10.95 ± 0.85^{B}	10.61^{B} ± 0.02
	Seychelles	5.97 ± 0.19^{D}	3.62 ± 0.19^{D}	19.37 ± 4.29^{AB}	15.56 ± 0.87^{BC}	7.42 ± 0.19^{D}	4.32 ± 0.31^{D}		Seychelles	9.47 ± 0.12^{B}	14.05 ± 0.79^{A}
Parsley	Gigante	4.75 ± 0.67^{d}	4.43 ± 0.35^{d}	17.63 ± 2.10^{b}	22.37 ± 1.45^{ab}	3.19 ± 0.17^{d}	2.94 ± 0.17^{d}	**Parsley**	Gigante	1.85 ± 0.15^{c}	11.88 ± 0.43^{b}
	Fidelio	8.41 ± 3.31^{cd}	4.90 ± 0.75^{d}	15.71 ± 4.25^{bc}	29.85 ± 1.20^{a}	2.93 ± 0.46^{d}	3.33 ± 0.26^{d}		Fidelio	1.84 ± 0.13^{c}	15.21 ± 0.91^{a}

Root and shoot concentrations of spinach and parsley cultivars after Zn and Cd treatment for 10 d in hydroponics; two cultivars of each plant species were grown for 24 d (spinach) or 45 d (parsley); means ($n = 4$) ± standard error; different letters indicate significant differences among spinach (capital letters) and parsley treatments (small letters); *toxic shoot zinc concentration (see Figure 1).

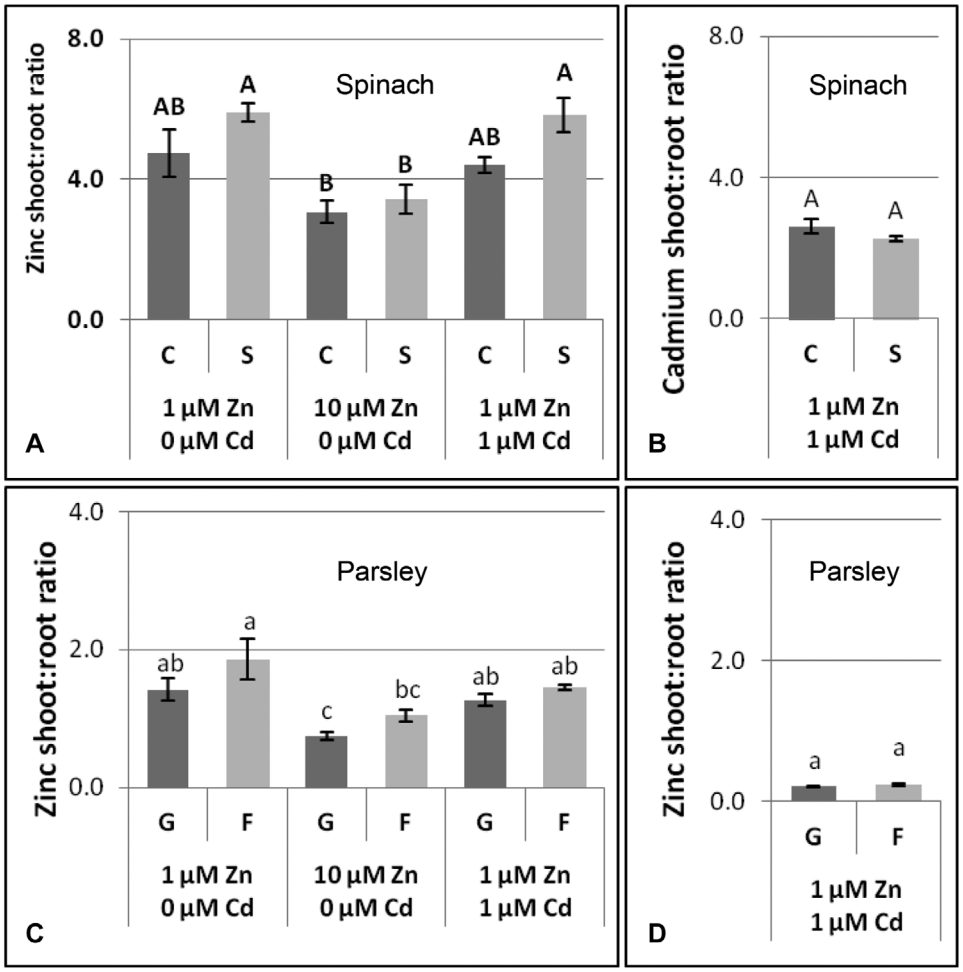

FIGURE 3 Shoot:root ratios of Zn and Cd. Zn and Cd shoot:root ratios of (A, B) spinach and (C, D) parsley after Zn and Cd treatment for 10 days in hydroponics; spinach cultivars Camaro (C) and Seychelles (S) were grown for 24 days and parsley cultivars Gigante (G) and Fidelio (F) were grown for 45 days; means ($n = 4$) ± standard error; FW, fresh weight; different letters indicate significant differences among treatments; different scaling of figures has to be considered

Rauser, 1990; Rea, 2012; Schmöger et al., 2000). After the application of 1 µM Cd, parsley cultivars accumulated thiols in roots (Figure 5) and hardly translocated Cd to the shoots (Figure 3D), which in turn maintained plant development (Table 1). These increases in thiol concentrations of parsley cultivars could not be explained in terms of glutathione accumulation (Figure 6). Also in spinach cultivars, thiol-containing substances significantly increased due to Cd application (Figure 4). However, spinach plants were more sensitive to the Cd treatment (1 µM Cd) and showed distinct Cd toxicity symptoms (Figure 1, Table 1), probably due to a higher translocation of Cd from root to shoot (Figure 3B) in contrast to parsley cultivars. A similar Cd concentration of the nutrient solution (1 µM Cd) affected a different degree of phytotoxicity; however, induction of thiol synthesis by Cd was clearly demonstrated for both plant species (Figures 4 and 5). The accumulation of thiols in spinach roots could therefore not be explained in terms of higher glutathione concentrations after Cd treatment (Figure 6).

A very sensitive detoxification mechanism of Cd is the synthesis of thiol-rich phytochelatins (Mendoza-Cózatl et al., 2005; Semane et al., 2007; Tennstedt et al., 2009). Cadmium is known as the most effective activator of the phytochelatin synthase (Grill et al., 1989; Nakazawa & Takenaga, 1998; Vatamaniuk et al., 2000). Phytochelatins accumulated within 2 h in maize roots after Cd application and phytochelatin concentrations increased with prolonged intervention period (Tukendorf & Rauser, 1990). Long-term Cd treatment (5–7 days) also enhanced transcription (Semane et al., 2007) and gene expression (Lee & Korban, 2002) of phytochelatin synthase 1. Thiol concentrations also accumulated in spinach and parsley cultivars after Cd application (Figures 4 and 5), indicating that phytochelatin synthesis was most probably induced by Cd treatment (1 µM Cd) in roots.

Due to the chemical similarity of Cd and Zn (Kölelli et al., 2004; Küpper & Andresen, 2016), a similar induction of thiol synthesis is assumed in crops under Zn excess. Several studies showed that Zn exposure induced thiol accumulation in plants, specifically the accumulation of phytochelatins (Degola et al., 2014; Garg & Kaur, 2013; Ozdener & Aydin, 2010; Sofo et al., 2013; Tennstedt et al., 2009). The cell response to Zn stress was observed to be much lower than to Cd stress (Tennstedt et al., 2009). However, it was also assumed that phytochelatins were involved not only in detoxification of Zn but also contributed to

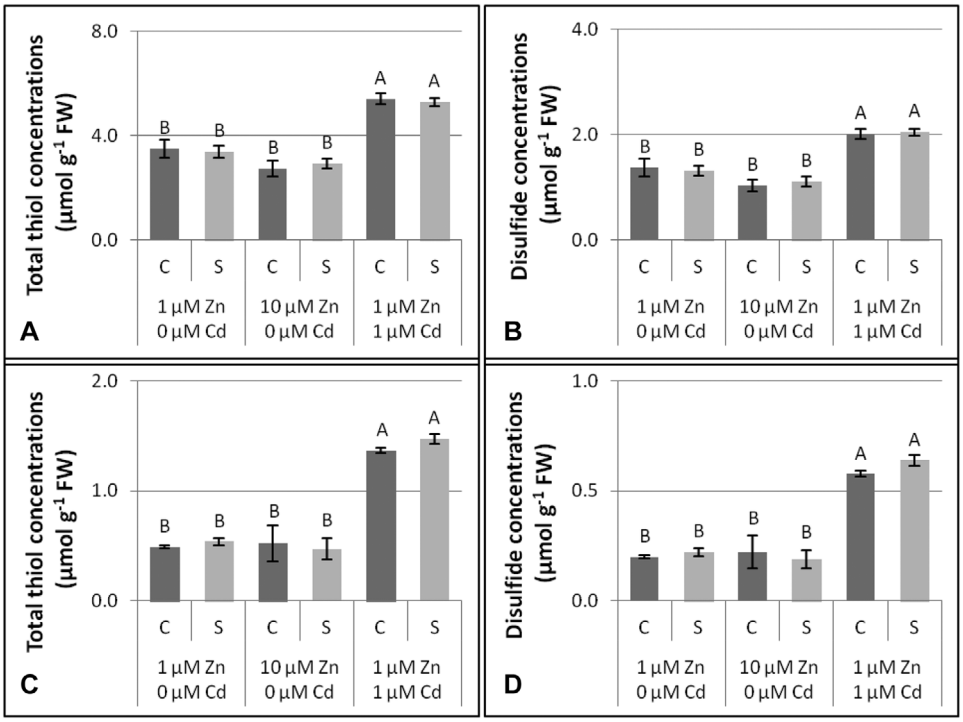

FIGURE 4 Total thiol and disulfide concentrations of spinach. Total thiol concentrations of spinach (A) shoots and (C) roots and disulfide concentrations of spinach (B) shoots and (D) roots after Zn and Cd treatment for 10 days in hydroponics; spinach cultivars Camaro (C) and Seychelles (S) were grown for 24 days; means ($n = 4$) ± standard error; FW, fresh weight; different letters indicate significant differences among treatments; different scaling of figures has to be considered

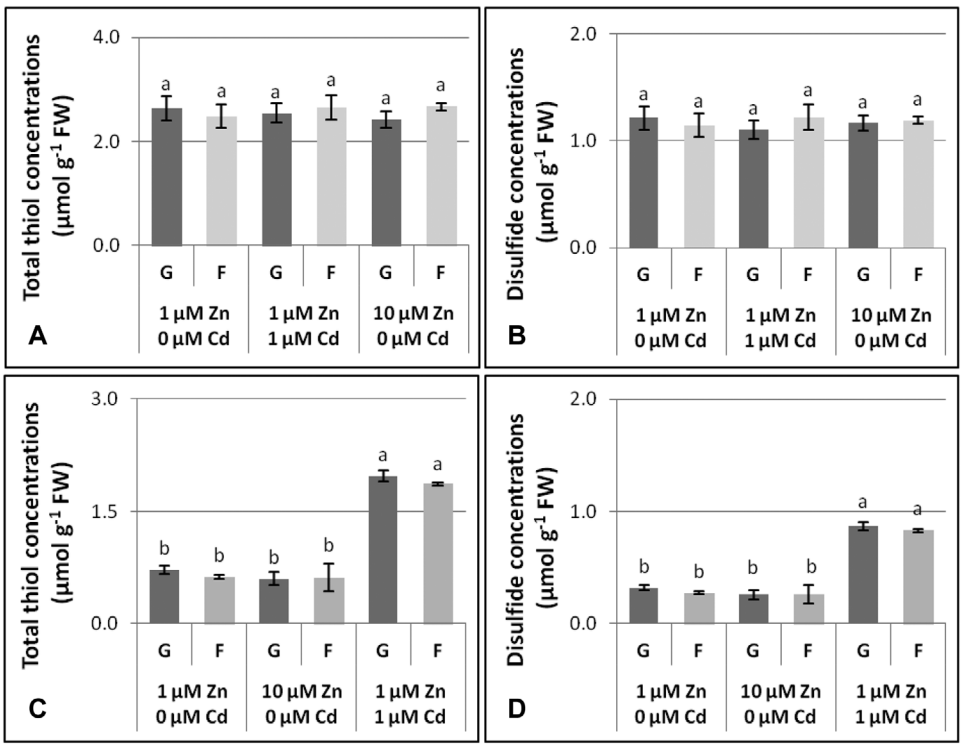

FIGURE 5 Total thiol and disulfide concentrations of parsley. Total thiol concentrations of parsley (A) shoots and (C) roots and disulfide concentrations of parsley (B) shoots and (D) roots after Zn and Cd treatment for 10 days in hydroponics; parsley cultivars Gigante (G) and Fidelio (F) were grown for 45 days; means ($n = 4$) ± standard error; FW, fresh weight; different letters indicate significant differences among treatments

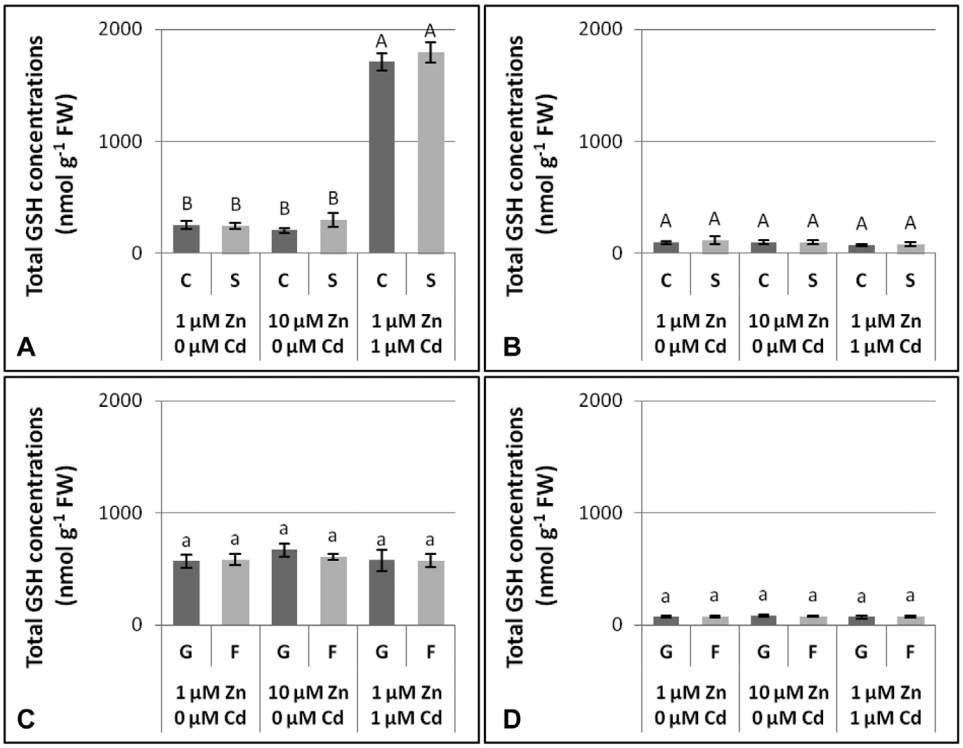

FIGURE 6 Total glutathione concentrations of spinach and parsley. Total glutathione concentrations of spinach (A) shoots, (B) spinach roots, parsley (C) shoots , and (D) parsley roots after Zn and Cd treatment for 10 days in hydroponics; spinach cultivars Camaro (C) and Seychelles (S) were grown for 24 days and parsley cultivars Gigante (G) and Fidelio (F) were grown for 45 days; means ($n = 4$) ± standard error; FW, fresh weight; different letters indicate significant differences among spinach (capital letters) and parsley treatments (small letters)

essential metal homeostasis in plants (Tennstedt et al., 2009; Clemens & Peršoh, 2009; Kühnlenz et al., 2016).

In the present study, accumulation of thiol-containing substances were neither observed for spinach nor for parsley cultivars at the high-Zn treatment (10 µM Zn) (Figures 4 and 5). When comparing the different studies, it has to be considered that the acquisition of Zn differed among plant species and that the intracellular free Zn concentration *in vivo* varied. Additionally in these studies, the induction of phytochelatin synthesis *in vivo* and activation of phytochelatin synthase *in vitro* were only observed at very high Zn levels (> 50 µM) accompanied with distinct growth reduction and severe toxicity symptoms (Tennstedt et al., 2009; Ozdener &Aydin et al., 2010; Sofo et al., 2013, Garg & Kaur, 2013; Degola et al., 2014) or for cells that were knocked out in a Zn homeostasis factor (Tennstedt et al., 2009) which was identified as important transporter for removal of excess Zn from cytosol (Boch et al., 2008).

An external concentration of 10 µM Zn used in the present study did not cause growth reduction of spinach and parsley cultivars (Table 1), but produced toxicity symptoms in spinach (Figure 1). Furthermore, an additional Zn supply did not lead to an increase of biomass production of spinach and parsley, respectively (Table 1). Therefore, plants that received 10 µM Zn were grown under elevated Zn conditions and accumulated Zn in root and shoot tissue (Table 2). It might be possible that Zn tissue concentrations in spinach were too high to allow controlled cell regulation of thiol groups under elevated Zn conditions (Table 2). However, thiol accumulation was also not detected even under comparable but non-toxic Zn tissue concentrations in parsley (Table 2). Although no obvious Zn toxicity symptoms were observed for parsley plants, an early accumulation would have been expected, if thiols were involved in Zn detoxification in vegetable crop plants. Such a premature accumulation of thiols, especially by an induction of phytochelatin synthesis was shown to prevent plants from metal stress (Tukendorf & Rauser, 1990; Mendoza-Cózatl et al., 2005). As glutathione concentrations did not differ from control plants (Figure 6), an involvement of glutathione in Zn detoxification and/or a limitation of the precursor for phytochelatin synthesis were also excluded for spinach and parsley cultivars under elevated Zn conditions (10 µM Zn).

The conserved N-terminal site of phytochelatin synthases seemed to be responsible for the catalyzed reaction, whereas the less conserved C-terminal site was shown to be important for stability and activation by metal ions (Ha et al., 1999; Filiz et al., 2019). Kühnlenz et al. (2016) identified a part of the AtPCS1 (phytochelatin synthase 1) protein which was required for the activation by Zn. In principle, activation of phytochelatin synthesis by Zn seemed to be possible *in vivo* (García-García et al., 2020). However, Filiz et al. (2019) showed a high variation in non-conserved parts of phytochelatins synthases proteins among plant species. It was also shown that metal binding per se was not responsible for catalytic activation of phytochelatin synthase (Vatamaniuk et al., 2000) and that exposure of 20 µM Zn did not induce transcription of phytochelatin synthase (Nguyen-Deroche et al., 2012). In

comparison to Cd, the transcription of phytochelatin-synthase genes was less sensitive to Zn excess (Fan et al., 2018). In roots of parsley cultivar Gigante d'Italia, an accumulation of thiols was measured long before applied Cd concentrations were toxic for plants (Figure 5). Phytochelatin synthesis might be sensitively activated in this case, whereas toxic Zn concentrations in spinach did not affect any thiol accumulation (Figure 4). Therefore, it is questionable that phytochelatins are involved in the homeostasis and specific detoxification of Zn because low dissociation constants of Zn-thiol complexes (Cheng et al., 2005; Krężel & Maret, 2016) would possibly impair the availability of the micronutrient Zn for growth processes. Possibly, not chelating thiols but other compounds such as metallochaperones could be involved in Zn detoxification (Khan et al., 2019, 2020).

5 | CONCLUSIONS

Spinach and parsley cultivars responded to increased Cd concentrations and reacted with defense mechanisms such as accumulation of thiols, which may be explained in terms of induction of phytochelatin synthesis. Under the conditions studied, excess Zn did not affect thiol concentration or glutathione concentrations. Excessive Zn was therefore not detoxified by chelating thiols in spinach and parsley cultivars as it was shown for Cd.

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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.

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