

How do phosphate and methionine affect distribution and chemical forms of cadmium in valerian (*Valeriana officinalis* L.)?

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Abstract

Controlling the uptake and translocation of heavy metals in medicinal plants is crucial due to its strong impact on the safety and quality of derived medicinal plant products. This study investigated the effects of phosphate and methionine on the distribution and chemical forms of Cd in valerian (*Valeriana officinalis* L.) roots and shoots. Seedlings were grown for two weeks in a Cd-free nutrient solution containing either 400 or 1200 μM phosphate and then exposed for 10 days to 10 μM Cd either in combination with 400 μM or no methionine in the nutrient solution. “Pectate- and protein-bound Cd” and “insoluble Cd phosphates” were the dominant Cd forms in sequential extractions of both, roots and shoots. Phosphate increased the fraction of “insoluble Cd phosphates” in the roots, but not in the shoots. Methionine strongly increased the soluble Cd fractions in roots and shoots. While it also increased the “pectate- and protein-bound Cd fraction” in the shoots, it decreased this fraction and the share of apoplastic Cd in the roots. The results indicate that methionine induced root-to-shoot Cd translocation by mobilizing Cd bound to root cell walls. This could help limit Cd accumulation in valerian roots grown for medicinal use.

1. Introduction

Accumulation of potentially toxic heavy metals in plants is a serious public health issue due to their adverse effects on consumers. Due to its high toxicity, wide distribution in the environment, comparatively easy uptake by plants and long term accumulation in the human body with a half-life of more than 10 years, cadmium (Cd) is of particular concern in this regard, as it is easily accumulated in the edible parts of many food plants at levels that are toxic for humans without causing visible toxicity to the plants (Zhou et al. 2016; Ismael et al. 2019). The uptake of Cd by plants usually occurs through transfer from the soil solution into the roots, from where it is translocated into the shoot. Apoplastic and symplastic pathways are involved in uptake and subsequent translocation (Redjala et al. 2009; Khodamoradi et al. 2015; Dalir and Khoshgoftarmanesh 2014). Their contributions vary in importance with species, physiological factors, Cd concentration and presence of other ions and ligands (Rabêlo et al. 2017; Zhao et al. 2002). While the first step in root uptake unavoidably is transfer into the apoplast of the root cortex, symplastic transport is generally considered to play a key role in metal transfer from the root cortex into the root stele, which is a necessary step required for solutes to be translocated into the shoots. Transport within the symplast allows by-passing the Casparian strips of the endodermis, which restrain radial water movement in the roots along the apoplastic pathway into the xylem (Barberon et al. 2016). The apoplast, which consists of the extracellular space of cell walls and inter-cellular voids, is not just a space through which solutes are moving passively, driven by diffusion. Due to the presence of negatively charged pectins in the cell walls, it also has a high capacity to bind cations (Meychik and Yermakov 2001; Sattelmacher 2001), making the root apoplast a major compartment for the inactivation of toxic metals (Guigues et al. 2014). Hence, the partitioning between apoplastic and symplastic Cd in plant roots is an important factor determining Cd loading into the xylem and thereby its translocation from roots to shoots.

The fate of heavy metals in plants is closely related to the chemical forms in which they are present in their tissues (Yan et al. 2020). Cd ions bind to inorganic (e.g. chloride) and organic ligands (e.g. organic acids such as citrate), which affect their mobility and reactivity depending on the water-solubility and stability of the complexes formed (Qiu et al. 2011; Uddin et al. 2020). In particular, complexes with water-soluble organic ligands can greatly increase Cd mobility in plants, and many studies have demonstrated the important role which complexes with organic ligands, especially chelates with low-molecular-weight organic acids, can play in the accumulation, allocation and detoxification of Cd in plants (Fernández et al. 2014; Mousavi et al. 2021b; Zhang et al. 2020). Special attention in studies on ligand-assisted plant Cd uptake has been devoted to Cd complexation with amino acids, as the latter are often readily available as organic ligands in the rhizosphere solution and can serve in the same time as immediate sources for the nitrogen nutrition of plants (Khodamoradi et al. 2015; Callahan et al. 2006). Because of the latter effect, amino acids also can affect Cd uptake indirectly by their fertilizer effects on plant growth (Wang et al. 2017; Nigam and Srivastava 2005). Cadmium ions have been shown to form complexes with almost all natural amino acids (Sóvágó and Várnagy 2013). An amino acid with higher affinity than other amino acids for binding Cd is methionine (Met) due to its S-methyl side chain (Sóvágó and Várnagy 2013). The coordination of Cd with the thioether sulfur atoms in addition to the carboxylate and amino groups of methionine results in the formation of particularly stable complexes with Cd, as reflected in the high stability constant of Cd-Met complexes (Sóvágó and Várnagy 2013). In addition, there are other effects that make methionine application an attractive soil amendment to ameliorate Cd stress. Methionine is a substrate for the synthesis of the antioxidant glutathione, which plays an important role in Cd detoxification, and as a sulfur (S) source it can improve the Cd stress tolerance of plants in other ways (Sohn et al. 2014; Cui et al. 2020). Various studies have shown that S supply can mitigate deleterious impacts of Cd on plant growth and reduce Cd accumulation in plants, including rice (Wu et al. 2020), pakchoi (Li et al. 2020) and tartary buckwheat (Lu et al. 2019). Furthermore, methionine is involved in the biosynthesis of phytohormones controlling plant root development, the target organ in valerian production (Ravanel et al. 1998; Chapman and Estelle 2009).

Among the inorganic ligands that can have a strong effect on the mobility and phytoavailability of Cd in soil, phosphate (PO_4) is of particular importance. It can impact Cd phytoavailability through various mechanisms. Many studies reported an immobilizing effect of phosphate compounds on soil Cd, which is attributed to (i) the formation of insoluble phases, (ii) adsorption of Cd-phosphate complexes and (iii) formation of surface complexes (Ruangcharus et al. 2020). On the other hand, because of antagonistic interactions between Cd and its sister element zinc (Zn), high phosphate concentrations may also have an enhancing effect on plant Cd uptake due to reduced Zn absorption (Tkalec et al. 2014; Ova et al. 2015).

Different binding forms do not only govern the retention of Cd in the soil, but are also related to the accumulation, distribution and toxicity of Cd in plants (Yang et al. 2021). Sequential extraction of Cd fractions with extractant solutions of increasing extraction efficiency is a method often used to characterize chemical binding forms of Cd in soils and plants. For plants, the sequential extraction

scheme described by Yang et al. (1995) has often been applied to explain differences among plants in their ability to accumulate and tolerate Cd and in their responses to experimental treatments proposed to influence these abilities. For instance Wang et al. (2017) found that differences in the Cd-translocating ability of two species of cabbage plants were closely related to Cd extractable with 1M NaCl ("pectate/protein integrated Cd fraction"). Another study found that a larger enhancement effect of citric acid on Cd accumulation and translocation in tall fescue as compared to Kentucky bluegrass was associated with differences in the promotion of water-soluble Cd-organic acid complexes (Wang et al. 2017).

Valerian (*Valeriana officinalis* L.) is an economically important and widely used medicinal herb, the roots of which have been traditionally utilized as a relaxing and sleep-promoting agent (Bent et al. 2006; Mousavi et al. 2021a). Pharmaceutical preparations made of valerian root have a positive role in treating heart disorders, hypertension, sweating and other disorders (Garges et al. 1998). Excessive heavy metal uptake by medicinal herbs may not only impair the efficacy and quality of derived products, but even pose serious health risks to consumers (Luo et al. 2020). Improving our knowledge on Cd accumulation and allocation in valerian plants could help avoid these risks.

In a previous study, we found a distinct difference in Cd accumulation (per plant) by valerian in the presence and absence of methionine in nutrient solution (Mousavi et al. 2021b). Associated with a strong shift from apoplastic to symplastic Cd accumulation in the roots, methionine application decreased Cd retention in the roots, while enhancing Cd accumulation in the shoots. Phosphate increased Cd accumulation in root symplasts and shoots in combination with methionine, but not in its absence. On the other hand, phosphate increased apoplastic Cd accumulation in the absence but not in the presence of methionine. While these effects suggested that methionine increased Cd mobility in the plants due to formation of Cd-methionine complexes, it remained unclear apart from this mobilization effect how the treatments affected Cd binding forms in the experimental plants. Thus, here we performed an experiment with valerian in hydroponics addressing the following two questions: 1) How does methionine application affect the chemical forms of Cd in roots and shoots of Cd-exposed valerian plants? 2) How do these methionine effects depend on phosphate supply to the plants?

2. Material And Methods

2.1. Plant cultivation

Valerian seeds (*Valeriana officinalis* L.) were surface sterilized for 30 min with 1% hydrogen peroxide, rinsed, placed for 5 days on wet filter paper at 25 °C in the dark, and then transplanted for germination into a sand culture moistened with deionized water. Two weeks after transplanting, the seedlings were transferred to opaque barrels containing nutrient solutions with a phosphate concentration of either 400 or 1200 µM. The nutrient solutions were produced using greenhouse tap water with a pH of 6.9 and an EC of 0.74 dS m⁻¹. They were aerated continuously in the growth containers by air bubbling from a capillary tube, and they were replenished with fresh solutions twice a week. The pH was maintained between 5.8

and 6.0 by adding 0.1 M HCl or KOH as needed. The chemical composition of the tap water and of the nutrient solutions is shown in Table 1. Plants were grown under glasshouse conditions at ambient temperature (day/night temperature of ~ 20–40 °C) and at least 12 h natural light per day.

Table 1
The chemical composition of tap water used for preparation of nutrient solutions and the final composition of prepared nutrient solution.

Nutrients	Tap water	Nutrient solution	
Ca	0.90	2.76	mM
Mg	0.25	1.25	
K	0.02	3.22	
NH ₄	—	0.35	
Na	4.00	4.04	
NO ₃	0.09	10.07	
SO ₄	1.20	1.51,1.21	
HCO ₃	1.92	0.70	
PO ₄	—	0.40,1.20	
Cl	1.92	1.92	μM
Fe	—	30.00	
Mn	—	7.00	
Cu	—	1.00	
Zn	—	5.00	
B	19.4	35.00	
Mo	—	0.10	
pH	6.93	5.80	
EC	0.74	1.60	

2.2. Experimental treatments

The experiment was set up as a completely randomized 2x2 factorial design with each treatment replicated three times as follows: After 2 weeks of growth in Cd-free nutrient solution, 48 uniformly sized seedlings were chosen and groups of 4 plants per replicate were transplanted into 5-l beakers. Here, for the next 10 days, the plants were exposed to 10 μM Cd in the form of (NO₃)₂ added to the aerated nutrient

solutions with either 400 μM or no methionine (Met) and with either 400 or 1200 μM phosphate. Otherwise, all conditions remained the same as before. The solutions were renewed every two days and to minimize positional non-uniformity effects, the positions of the containers were randomly changed every day. Before the treatment solutions were applied, all containers were washed with 5% sodium hypochlorite to prevent rapid degradation of Met.

2.3. Sampling and Cd analysis of wet-digested samples

After 10 days exposure to the treatment solutions, when they were 43 days old, the plants were harvested. Roots and shoots were separated by cutting the stems at the upper surface level of the plant-supporting plates. Shoot and root length were measured using a graded ruler. Two out of the 4 seedlings per replicate were used for the assessment of chemical Cd forms by means of sequential extraction, as described below. These samples were frozen in liquid nitrogen until analysis as described below. The other two plants per replicate were used for the assessment of symplastic and apoplastic root Cd accumulation and Cd translocation into the shoots. The roots and shoots of these plants were washed with pure water, blotted dry with tissue paper and weighed. The shoots were then oven dried at 65°C for 72 h until constant weight and ground into a fine powder for elemental analysis after wet digestion. As described by Mousavi et al. (2021), the root samples were divided into two aliquots: one was directly oven-dried and ground like the shoot samples, while the other was first washed with an EDTA solution to desorb apoplastic Cd, as suggested by Zhou et al. (2007) before oven-drying and grinding. Briefly, about one gram of fresh root material was filled into a 100-mL centrifuge tube containing 50 mL of 10 mM EDTA (pH 6.0), which had been precooled in a refrigerator. The tube was stirred at 100 rpm on a rotation bed at 4°C for 1 h. The Cd that remained after the extraction with the EDTA-washing solution (EDTA-washed roots) was considered as symplastic Cd and the difference in Cd content between EDTA-washed and unwashed roots as apoplastic Cd. Dried and ground tissue samples were wet-digested using a mixture of concentrated HNO_3 and HCl (1:3 v/v). The Cd concentration of all root and shoot digests were determined by means of atomic absorption spectroscopy (AAS). The translocation of Cd from root to shoot was determined as the ratio between corresponding Cd concentrations in the shoots and roots.

2.4 Sequential extraction

To determine fractions of different chemical Cd forms in root and shoot tissue, the following extraction solutions were applied sequentially, following the stepwise extraction procedure described by (Yang et al. 1995):

- 1) 80% ethanol to extract water-soluble Cd complexes with chloride, aminophenols etc. (F_E , “water-soluble inorganic Cd fraction”);
- 2) deionized water ($\text{d-H}_2\text{O}$) to extract water-soluble Cd complexes with organic acids and $\text{Cd}(\text{H}_2\text{PO}_4)_2$ (F_W , “water-soluble organic Cd fraction”);
- 3) 1M sodium chloride (NaCl) to extract Cd bound to pectates and proteins (F_{NaCl} , “pectate- and protein-associated Cd fraction”);

4) 2% acetic acid (HAc) to extract insoluble CdHPO_4 , $\text{Cd}_3(\text{PO}_4)_2$ and other Cd phosphate complexes of low solubility (F_{HAc} , “Cd phosphate fraction”);

5) 0.6 M hydrochloric acid (HCl) to extract Cd oxalate (F_{HCl} , “Cd oxalate fraction”).

The Cd remaining in the plant material after the five extraction steps with these solutions is denoted as the “residual Cd fraction” (F_R).

Briefly, the extraction proceeded as follows: Frozen root and shoot tissue samples (1.5 g each) were homogenized and suspended in 37.5 ml of the first extraction solution. After incubation for 18 h at 30°C the suspensions were centrifuged for 10 min at 2500 rpm. After separating the supernatant, the residual pellets in the centrifuge tubes were resuspended in 37.5 ml of the same extraction solution for 2 h. After centrifugation the supernatant was separated again. This procedure was repeated 2 more times. After each extraction step the whole procedure was repeated with the remaining residue with the extraction solution of the subsequent step. The pooled supernatant solutions of each sequential extraction step (150 ml) and the final residue were evaporated at 70 °C to constant weight, digested with concentrated HNO_3 and HCl (1:3 v/v), and analyzed for Cd and Zn by atomic absorption spectroscopy (AAS).

3. Statistical Analysis

Statistical analysis was performed using SAS software (SAS Inc., Cary, NC; version 9.0). ANOVA was used to determine treatment effects. Differences between treatment means were considered significant if the error probability was $p < 0.05$ (confidence level > 95%) in Duncan’s multiple range tests.

4. Results

4.1. Plant biomass production and height

In presence of Met, root fresh weight, root length and shoot length, but not shoot fresh weight were larger at the low than at the high phosphate level (Table 2). In absence of Met, there was no significant phosphate effect on any of these parameters (Table 2). The largest root fresh weight (13.47 g) and root-to-shoot ratio (1.39) were obtained in the 400 μM phosphate treatment in combination with Met in the nutrient solution. Met increased root fresh weight and shoot length at the low phosphate level, but had no effect on these growth parameters at the high phosphate level. Moreover, there was no significant Met effect on shoot fresh weight and root length at both phosphate levels. At the low level of phosphate application, Met caused a 22% increase in root-to-shoot ratio, while it had no significant effect on this ratio at the high phosphate level. In summary, the combination of Met application with the low phosphate level was the only treatment that led to significant growth effects compared to other treatments, although not for shoot biomass. While Met application increased root fresh weight and shoot length at the low phosphate level, the only influence of the phosphate level on growth was that these growth-stimulating Met effects disappeared at the high phosphate level.

Table 2

Morphological traits in 43-day-old valerian seedlings treated with phosphate and methionine (Met) in nutrient solution over a 10-day experimental period.

Treatments	Phosphate (μM)			
	400		1200	
	-Met	+Met	-Met	+Met
Root fresh weight (g)	11.24 ± 0.55^b	13.47 ± 0.39^a	11.85 ± 0.44^{ab}	11.12 ± 0.85^b
Shoot fresh weight (g)	9.86 ± 0.21^a	9.69 ± 0.26^a	9.48 ± 0.47^a	9.87 ± 0.33^a
Root length (cm)	35.33 ± 6.39^{ab}	40.00 ± 2.89^a	30.00 ± 1.15^{ab}	25.33 ± 0.88^b
Shoot length (cm)	19.67 ± 2.03^b	31.00 ± 2.08^a	18.67 ± 1.20^b	21.83 ± 3.35^b
Root to shoot ratio	1.14 ± 0.04^b	1.39 ± 0.02^a	1.26 ± 0.03^{ab}	1.13 ± 0.12^b

4.2. Cadmium accumulation in roots and shoots

All root Cd concentrations were higher than shoot Cd concentrations (Fig. 1). The mean Cd concentrations ranged between 27 and 36 $\mu\text{g g}^{-1}$ FW in the roots and between 12 and 19 $\mu\text{g g}^{-1}$ FW in the shoots. Increasing the phosphate treatment level from 400 to 1200 μM had no effect on root and shoot Cd accumulation in the absence of Met, but increased root Cd in combination with Met, mirroring the negative effect of elevated phosphate on root growth stimulation by Met. Met application increased shoot Cd concentrations at both phosphate levels, but had no significant effect on root Cd concentrations.

The root-to-shoot translocation factor, defined as the ratio between shoot and root Cd concentration, reached a maximum value of 0.7 in the low phosphate treatment combined with Met, where it was 60% higher than in average (0.44) in the other treatments.

4.3. Ratio between symplastic and apoplastic root Cd concentration

Overall, apoplastic Cd represented 44–76% of total root Cd concentrations, with a mean value of 62%. Correspondingly, symplastic Cd represented in average 38% (23–56%) of total root Cd (Fig. 2). The phosphate concentration of the nutrient solution had no significant effect on the partitioning between symplastic and apoplastic root Cd, while methionine had a strong effect at both phosphate levels. In the presence of Met, the ratio between symplastic and apoplastic root Cd concentrations increased by a factor of 2.0 in the treatment with 400 μM phosphate and by a factor of 1.6 in the treatment with 1200 μM phosphate. Met decreased the mean apoplastic Cd concentration from 70–44% and from 77–56% at 400 μM and 1200 μM phosphate, respectively.

4.4. Chemical forms of root Cd

There were obvious differences between the treatments in the chemical fractionation of root Cd (Figs. 3 and 5A). In general, the two largest Cd fractions were the “pectate- and protein-associated fraction” (F_{NaCl}) and the “phosphate fraction” (F_{HAC}). Together they represented between 59% and 78% of total root Cd across all four treatments. On the other hand, the two smallest fractions always were the “oxalate fraction” (F_{HCl}) and the “residual fraction” (F_{R}). In general, under low phosphate conditions, the Cd concentrations of the forms were in the order of $F_{\text{NaCl}} > F_{\text{HAC}} > F_{\text{E}} > F_{\text{W}} > F_{\text{HCl}} \approx F_{\text{R}}$, while the order changed to $F_{\text{NaCl}} > F_{\text{HAC}} \approx F_{\text{E}} > F_{\text{W}} > F_{\text{HCl}} > F_{\text{R}}$ when Met was also supplied. Under high phosphate application, the order was $F_{\text{NaCl}} > F_{\text{HAC}} > F_{\text{W}} > F_{\text{E}} > F_{\text{HCl}} \approx F_{\text{R}}$ and it became $F_{\text{HAC}} > F_{\text{NaCl}} \approx F_{\text{W}} > F_{\text{E}} > F_{\text{HCl}} > F_{\text{R}}$ when phosphate was combined with Met in the nutrient solution (Fig. 5A). Figure 3 shows that increased phosphate application led to a strong increase in the “phosphate fraction” (F_{HAC}), and at least in presence of Met, it also increased the “water-soluble organic fraction” (F_{W} , which also includes soluble Cd phosphate forms), whereas it had no influence on the “water-soluble inorganic fraction” (F_{E}) and tended to decrease the “residual fraction”. The effects of the phosphate treatment level on the other two fractions depended on the application of Met: a decreasing effect in the absence of Met, but no effect in the presence of Met on the “pectate- and protein-associated fraction” (F_{NaCl}) and an enhancing effect in the presence of Met, but a non-significant decreasing effect in the absence of Met on the “oxalate fraction” (F_{HCl}).

The Met treatment showed two clear effects that were independent of the phosphate level: a strong increase in the “water-soluble inorganic fraction” (F_{W}) and a strong decrease in the “pectate- and protein-associated fraction” (F_{NaCl}). Met also led to a strong increase in the “oxalate fraction” (F_{HCl}) at the high phosphate level, but showed only a tendency to increase this fraction at the low phosphate level. There was also a tendency to increase the “water-soluble organic fraction” (F_{W}) and a tendency to decrease the “residual fraction” (F_{R}), but no Met effect at all on the “phosphate fraction” (F_{HAC}).

Looking at groups of different fractions, Met led to a strong increase in the proportion of the “soluble fractions” ($F_{\text{E}} + F_{\text{W}}$) relative to the total of the other four fractions, whereas the phosphate level had no visible effect on this proportion (Fig. 5A). The main effect of phosphate application was to increase the share of the respective fraction with “Cd phosphate” forms within each of these groups, i.e. the share of F_{W} in the group of soluble Cd forms and the share of F_{HAC} in the group of Cd forms with low solubility.

4.5. Chemical forms of shoot Cd

As in the roots, the “pectate- and protein-associated fraction” (F_{NaCl}) and the “phosphate fraction” (F_{HAC}) always were the two largest Cd fractions also in the shoots, representing 48%-79% of total shoot Cd, while the “oxalate fraction” (F_{HCl}) and the “residual fraction” (F_{R}) again were the two smallest fractions (Fig. 4). However, there was less variation in magnitude among the various fractions. The fractions F_{NaCl} , F_{HAC}

and F_E , which all three were clearly larger in each treatment than each of the other three fractions in the roots, had only about half their size by concentration in the shoots as in the roots, while the “residual fraction” (F_R) and the “water-soluble organic fraction” (F_W) were of similar magnitude in both plant parts, and the “oxalate fraction” was rather larger in the shoots than in the roots.

The treatment effects on shoot Cd fractionation showed many similarities with those on Cd fractionation in the roots, but also notable differences (Fig. 4). The only significant phosphate effect was a decrease in the “water-soluble inorganic fraction” (F_E) with elevating the phosphate level in the presence of Met. Met increased the “water-soluble organic fraction” (F_W) at both phosphate levels, the “inorganic water-soluble fraction” (F_E) at the low phosphate level and the “pectate- and protein-associated fraction” (F_{NaCl}) at the high phosphate level. Being opposite in direction to the decreasing Met effect on the “pectate- and protein-associated fraction” (F_{NaCl}) in the roots, the positive Met effect on this Cd fraction in the shoots represents the most striking difference in the Met effects on Cd fractionation between the two plant parts, while the lacking phosphate effects on the “phosphate fraction” (F_{HAc}) and the “water-soluble organic fraction” (F_W) of shoot Cd are the most obvious differences to the phosphate effects on root Cd fractionation.

Comparing groups of fractions, the Met had an enhancing effect on the share of the “soluble” fractions ($F_E + F_W$) also in the shoots, which was similar in magnitude as in the roots at the low phosphate level, but less strong than in the roots at the high phosphate level (Fig. 5B). In contrast to the respective treatment effects on root Cd forms, the phosphate level had little or no effect on the shares of the fractions with “Cd phosphate” forms within each of the two solubility groups, i.e. on the ratio between F_W and F_E in the group of “soluble” fractions and the ratio between F_{HAc} and $F_{NaCl} + F_{HCl} + F_R$ within the group of the “insoluble” fractions. Also, in contrast to the treatment effects on root Cd forms, Met decreased the proportion of the “phosphate fraction” (F_{HAc}) within the insoluble shoot Cd fractions, as a result of the opposite Met effect on the “pectate- and protein-associated fraction” (F_{NaCl}) of shoot Cd.

4.6. Zinc accumulation in roots and shoots and Zn/Cd ratio

As shown in Fig. 6, root concentrations were much lower at the high than at the low phosphate level in absence of Met. In presence of Met the difference was not significant. Shoot Zn concentrations showed no phosphate effect but were significantly increased by Met application at the high phosphate level. Met also tended to increase root Zn concentrations and shoot Zn at the low phosphate level. But none of these trends was strong enough to reach statistical significance.

As a result of the various treatment effects on root and shoot Cd and Zn concentrations, the ratio between overall plant Zn and Cd concentration was much lower at the higher than at the lower phosphate level and tended to be higher with than without Met application (Fig. 6). The maximum Zn/Cd ratio of approximately 1.6, which was achieved with Met application in the treatment with 400 μ M phosphate,

was more than 76% higher than minimum Zn/Cd ratio, which was obtained in the high-phosphate treatment without Met (0.91).

5. Discussion

5.1. Plant growth

Although the phosphate level differed by a factor of 3 between the two phosphate treatments, there was no enhancement of plant growth with the increased phosphate level. Also, Met application enhanced only root but not shoot biomass production, and this effects was limited to plants exposed to the low phosphate level. Thus, it appears that plant growth was not limited by P or N supply, and also co-limitation by insufficient P and N supply can be excluded. In that case, we should have expected enhanced growth in the treatment with Met application at the high and not at the low phosphate level. The very small extent of variation among treatments in growth parameters rather indicates that growth was inhibited by Cd stress, in line with our previous study (Mousavi et al. 2021b). In other studies, significant growth inhibition has been reported in plants grown at Cd concentrations of 5–10 μM under hydroponic conditions (Benáková et al. 2017; Huang et al. 2015). The fact that our experimental plants showed no symptoms of Cd toxicity may be due to the short time of Cd exposure, after which the plants were harvested immediately, leaving little time for the development of noticeable toxicity symptoms. The fact that the application of Met enhanced root growth in combination with a low phosphate level may lend some further support to the hypothesis that growth was limited by Cd stress and that this stress was reduced in the Met treatment by the formation of Cd-methionine complexes. However, it is not clear why Met application then did not have this effect at the high phosphate level.

5.2. Cd accumulation and allocation

As far as the two studies are comparable, the results of the study here are in good overall agreement with those of Mousavi et al. (2021). The main findings of both studies, as far as they are comparable, are that Met application led to a strong shift from apoplastic to symplastic Cd accumulation in the roots and increased the accumulation of Cd in the shoots. Irrespective of the effect on root Cd accumulation, Met increased the root-to-shoot Cd translocation factor at low phosphate but not at high phosphate levels. Increasing the phosphate concentration of the nutrient solution increased Cd accumulation in the roots in some treatments, but it did not increase the shoot-to-root Cd translocation factor in any treatment. These results are in agreement with the expectation that methionine enhanced Cd transfer from apoplast into the symplast of the root cortex by formation of mobile Cd-methionine complexes, leading to the desorption of cell-wall bound Cd, thereby to enhanced cellular Cd uptake in the root cells and thus ultimately also to increased root-to-shoot translocation. The antagonistic effect of increased phosphate supply on root Cd accumulation and thereby on the translocation factor indicates that phosphate strengthened Cd binding, counteracting the mobilization effect of methionine. This phosphate effect appears to have been stronger in the apoplast than in the symplast, as the data show a tendency to a

higher ratio between apoplastic and symplastic Cd with increased phosphate level, although this tendency did not reach statistical significance (Fig. 2).

5.3. Relationships between accumulation and chemical forms of Cd

The results of the sequential extractions lend further support to the view that the effects of the Met and phosphate treatments resulted from direct chemical interactions of methionine and phosphate with Cd in the roots, especially in the apoplast, leading to Cd mobilization in the case of methionine, while binding to phosphate promoted retention. This view implies that the observed increase in soluble Cd in the roots was primarily due to the formation of Cd-methionine complexes. This increase mainly occurred in the ethanol-extractable fraction (i.e. the “water-soluble inorganic Cd fraction”), suggesting that this fraction to a large part actually consisted of water-soluble organic Cd compounds. In fact, already Yang et al. (1995) mentioned that apart from inorganic Cd complexes, such as Cd chloride complexes, this fraction also includes organic complexes such as Cd complexes with amino phenols.

The NaCl-extractable fraction was the only fraction of root Cd that showed a strong decrease with Met application, indicating that the mobilized Cd came from this fraction. Cadmium in this fraction is assumed to be bound to pectates and proteins. Plant cell walls predominantly consist of cellulose, hemicellulose and other carbohydrates that provide a range of metal-binding functional groups, such as amino, hydroxyl, carboxyl and aldehyde groups (Wójcik et al. 2005). Also, it was found in previous studies using the same sequential extraction scheme as the study here that the fraction of NaCl-extractable Cd, i.e. the fraction assumed to be associated with proteins and pectates, showed positive correlations with the proportions of Cd bound to cell walls (Qiu et al. 2011; Xu and Wang 2013). The decrease in this Cd fraction thus is in full agreement with the reduced share in the roots of apoplastic Cd in the Met treatments and the postulated mechanism of Cd mobilization in the roots by desorption of apoplastic Cd through formation of soluble complexes with methionine.

Interestingly, the Met treatment had no effect on HAc-extractable Cd, the other large root Cd fraction insoluble in ethanol and water. This fraction, called the “Cd phosphate fraction”, is assumed to be made up of Cd phosphate complexes of low solubility according to Yang et al. (1995). In fact, this was the only root Cd fraction that showed a strong increase when the phosphate level was increased, suggesting that Cd binding to phosphate groups indeed prevailed in this fraction. Complexes and precipitates with phosphate are important forms of Cd binding in cell walls (Jiang et al. 2007). But apparently this fraction was not the Cd that was mobilized by complex formation with methionine.

The other Cd fraction which showed a consistent increase with increasing level of phosphate application was the water-extractable Cd fraction. Although referred to as “water-soluble organic Cd fraction”, already Yang et al. (1995) mentioned that this fraction also includes water-soluble Cd complexes with phosphoric acid besides Cd complexes with low-molecular-weight organic compounds, in particular organic acids.

Finally, it is noteworthy that in combination with methionine also the HCl-extractable root Cd fraction increased with the level of supplied phosphate. This interactive effect may be understood as the result of a two-step mechanism: first mobilization by methionine, then again immobilization in a different form. This is plausible if we assume for example that the mobilized Cd became internalized in root cells before re-immobilization, which would have occurred then by a different mechanism at a different location. The HCl-extractable Cd fraction is considered to represent Cd oxalate precipitates, in accordance with its designation “Cd oxalate fraction” by Yang et al. (1995). Vacuolar sequestration of Cd in form of Cd oxalate has been reported as an important process in the inactivation and thus detoxification of Cd in plants (Krotz et al. 1989; Pongrac et al. 2018; Kopittke et al. 2014). The question that arises from the finding that phosphate increased the “Cd oxalate fraction” is that of the mechanism. Either this fraction would include also some form of insoluble Cd phosphate not extractable with HAc in the previous step of the extraction procedure, or that the effect of increased phosphate supply in combination with methionine was by enhancing strong Cd-binding in some indirect way, e.g. by enhancing the production of oxalates in root cortex cells.

Comparing the treatment effects on Cd fractionation in shoots and roots, there were many similarities but also some striking differences. The major similarity was that the Met treatment increased ethanol- and water-soluble Cd in both plant parts, showing that the mobilization effect translated from the roots into root-to-shoot translocation. This does not mean that Cd was actually mobilized in the shoots. In fact, the finding that there was no net decrease in the “insoluble” shoot Cd fractions, indicating that the increase in ethanol- and water-soluble shoot Cd in the Met treatments was rather due to increased import of Cd from the roots. Also, such an increase in root-to-shoot translocation does not necessarily imply that Met-mobilized Cd was actually transported into the shoots in form of Cd-methionine complexes, although this is a possibility. Various authors found evidence indicating that Cd complexes with certain amino acids can be transferred across root cell membranes in undissociated form (Gramlich et al. 2013). In any case, the fact that the Met treatments had the effect to increase the “fraction of pectate- and protein-associated Cd” in the shoots in contrast to the roots, where they led to a strong decrease in this Cd fraction, means that the treatment resulted in a major addition of soluble Cd available for chemisorption to the binding sites of the NaCl-extractable Cd fraction in the shoots, not prevented by the presence of major amounts of methionine.

The other major difference in the treatment effects on the chemical forms of Cd between roots and shoots was that the phosphate level had no influence on the “insoluble” Cd fractions in the shoots, not even on the “Cd phosphate fraction”, which was almost doubled in the roots by increasing the concentration of phosphate in the nutrient solution from 400 to 1200 μM . This lack of effect indicates that, if there was any increase in shoot phosphate with the higher level of phosphate supply, then it was not available to any substantial extent for interaction with increased amounts of Cd translocated into the shoots, as observed in the combined treatment with Met. This finding also provides further support to the notion that the phosphate effects on root Cd fractionation were primarily due to Cd reactions with phosphate in the apoplast.

5.4. Zn accumulation and translocation

Apart from interactions with ligands such as organic acids and phosphate, competition for binding sites with its “sister” element Zn is another important factor influencing Cd uptake by and translocation in plants

(Chesworth 1991); (Dong et al. 2019); (Loneragan and Webb 1993). Antagonism due to such competition was postulated to explain the opposite effects of increased phosphate application on root Cd and Zn, i.e. a decrease in root Zn vs. an increase in root Cd, in the study of Mousavi et al. (2021). With the application of methionine, the antagonistic effect disappeared, as there was sufficient methionine to mobilize both elements, given the strong affinity also of Zn to form complexes with this amino acid (Sóvágó and Várnagy 2013). The results of our study here confirm these findings. The most striking treatment effect on Zn uptake in our study here was the decrease in root Zn with elevated phosphate application in presence of Met, which was opposite to the effect of this treatment on root Cd and thus led to a strong decrease in the Cd:Zn ratio. Negative effects of phosphate on root uptake of Zn have been reported many times in different plant species (Watts-Williams et al. 2014; Yousaf et al. 2019; Bukvić et al. 2003). One mechanism to which they are often attributed is precipitation of Zn phosphates (McGowen et al. 2001). However, no precipitation was observed in the nutrient solutions of our study. Also, a “dilution effect” that could be related to increased biomass production can be excluded, as the higher phosphate level did not lead to larger biomass. While we refer to Mousavi et al. (2021) for further discussion, the relevant point in the context of the present study is that, similar as for Cd, the observed phosphate effects on Zn uptake remained restricted to the roots, whereas Met-induced mobilization propagated into the shoots, suggesting that also the phosphate effect on Zn retention was largely limited to a change in Zn binding in the root apoplast without much effect on Zn mobility and thus Zn transfer into the root symplast.

6. Conclusions

Overall, this study demonstrates that methionine has the ability to mobilize Cd in the roots of valerian and thereby to enhance its translocation into the shoots. Increased phosphate supply had little to no effect on shoot Cd and only a minor effect in presence of methionine on root Cd, indicating that it primarily affected Cd binding in the root apoplast through direct interaction with Cd. The main binding forms of Cd in roots and shoots were NaCl-extractable Cd (“Cd fraction associated with pectates and proteins”) and HAc-extractable Cd (“Cd phosphate fraction”). Interestingly, these two fractions in particular (but also the other fractions) were affected very differently by phosphate and methionine application, and the effects also differed between roots and shoots: The NaCl-extractable Cd fraction was strongly reduced in the roots and increased in the shoots by methionine, while phosphate had no effect on this fraction in the shoots and in presence of methionine also in the roots but decreased it in absence of methionine. Conversely, HAc-extractable Cd was affected to a significant extent only in the roots and only by phosphate application. As methionine application also increased the water-soluble Cd fractions in roots and shoots, it can be concluded that the increase in NaCl-extractable Cd in shoots was due to the re-immobilization of translocated Cd mobilized by methionine in the roots.

The results show that the applied sequential extraction scheme provides a valuable method to characterize Cd binding and dynamics in response to environmental and other factors in plants. Caution should be applied, however, to the chemical interpretation of the fractions obtained. Their meaning is just operational and their tentative designation should not be mistaken as chemical identification without validation by proper chemical analysis.

The fact that methionine application reduced Cd accumulation in the roots by increasing root-to shoot translocation in combination with low phosphate supply shows that it can help in limiting Cd accumulation in valerian roots grown for medicinal use. This is a promising result given that Cd pollution of soils used for the cultivation of valerian is posing risks for the health of consumers, given that the roots are the commonly used parts of these plants in medicinal applications.

Statement and Declaration

- Funding

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- Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethical Responsibilities of Authors

All of the guidelines regarding to ethical responsibilities have been respected and authors respect third parties rights such as copyright and/or moral rights. This manuscript is original and has not been submitted to a preprint server before submitting it to Environmental Science and Pollution Research.

Authorship Principles

- Authorship Clarified

All authors whose names appear on the submission made substantial contributions to the conception of the work and approved the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

- Author Contributions

Masoud Rahimi: Carried out the experiment.

Neda Dalir: Design of the experiment, analysis of the data, writing of first draft.

Rainer Schulin: Contributed to the analysis of the data, reviewing and editing of the manuscript.

All authors discussed the results and contributed to the final manuscript.

Declarations

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- Author Contributions

Masoud Rahimi: Carried out the experiment.

Neda Dalir: Design of the experiment, analysis of the data, writing of first draft.

Rainer Schulin: Contributed to the analysis of the data, reviewing and editing of the manuscript.

All authors discussed the results and contributed to the final manuscript.

Compliance with Ethical Standards

- Potential conflicts of 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.

- Human and/or Animals Participants

This research does not involve human or animal participant.

- Informed consent

All authors agree with the content and that all gave explicit consent to submit and publish and that they obtained consent from the responsible authorities at the institute where the work has been carried out, before the work is submitted.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Figures

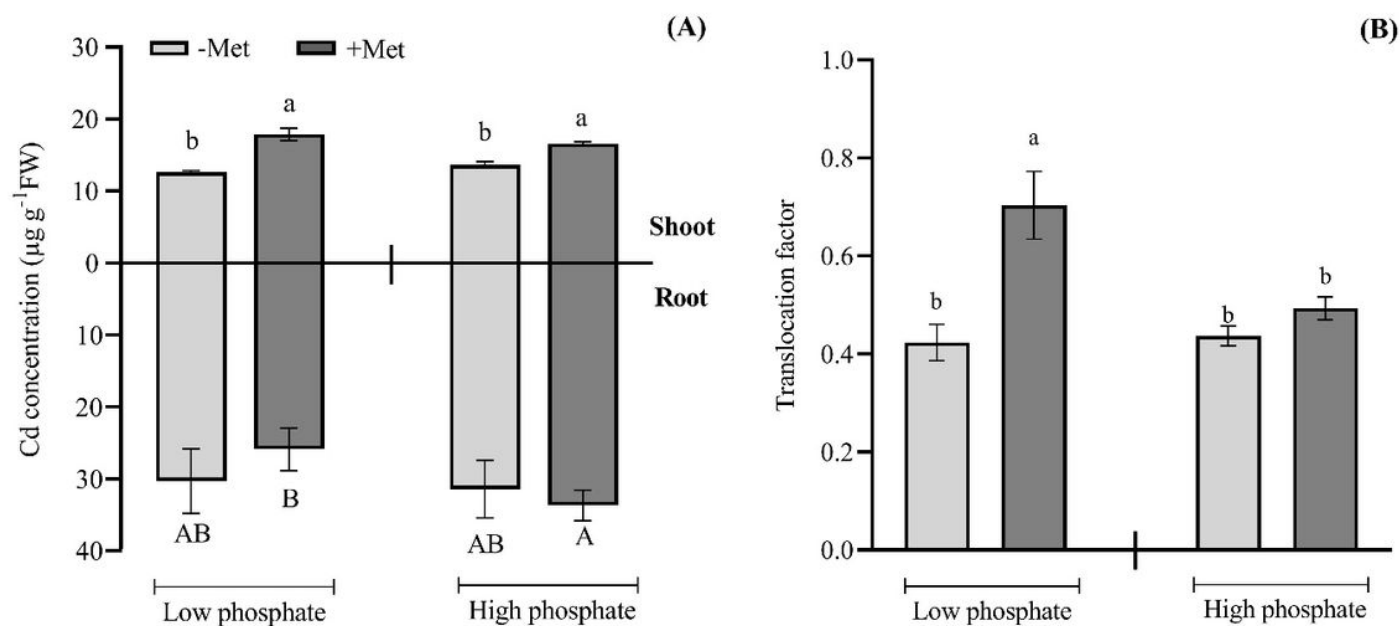


Figure 1

Root and shoot Cd concentration (A) and translocation factor of Cd from roots to shoots (B) of 43-day-old valerian plants treated with phosphate and methionine (Met) in nutrient solution over a 10-day experimental period. Columns represent mean values ($n = 3$) and bars represent standard errors. Means with the same letter are not significantly different from each other, using Duncan's multiple test ($P < 0.05$).

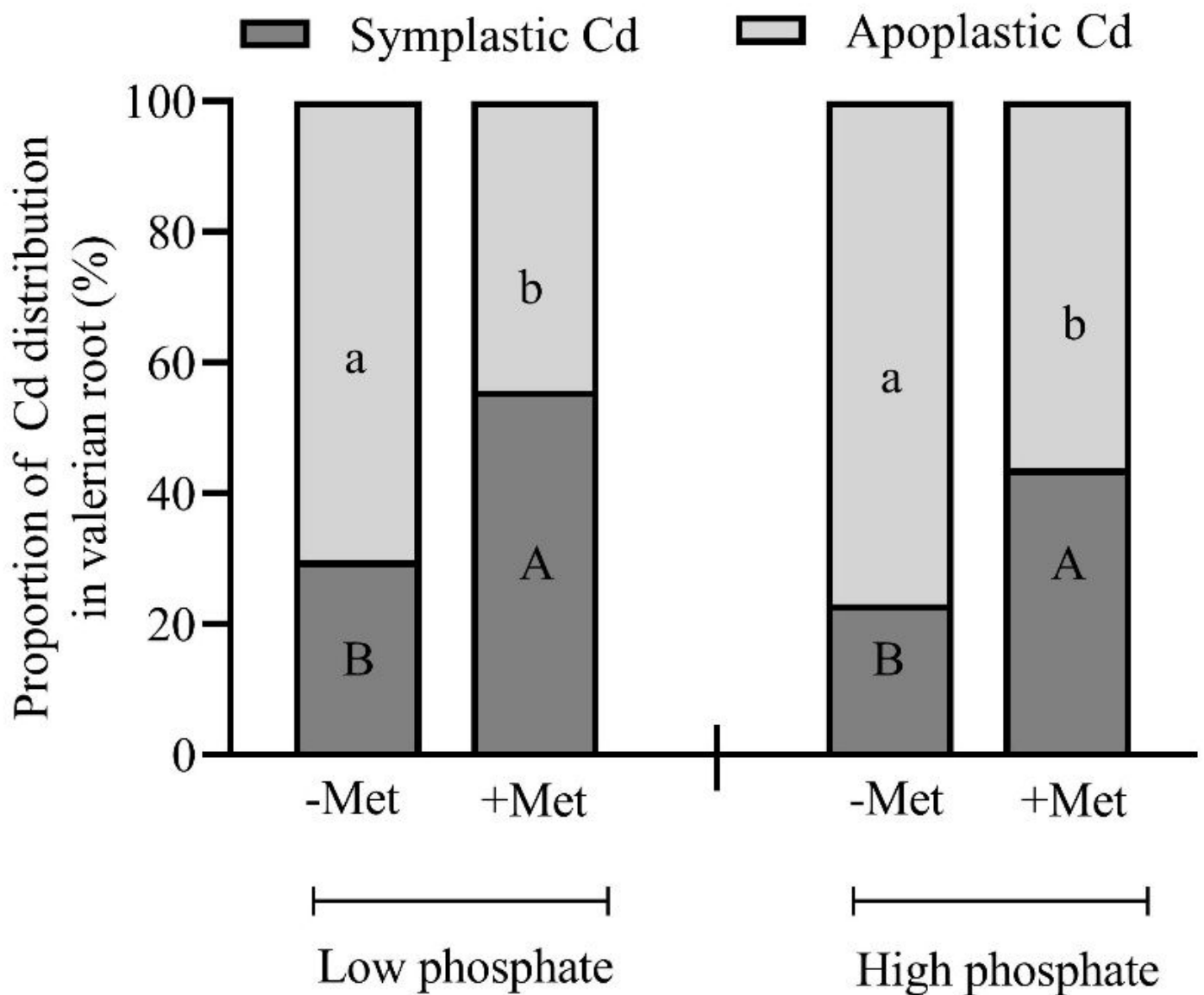


Figure 2

Contribution of symplastic and apoplastic Cd in roots of 43-day-old valerian plants treated with phosphate and methionine (Met) in nutrient solution over a 10-day experimental period. Columns represent mean values ($n = 3$) and bars represent standard errors. Means with the same letter are not significantly different from each other, using Duncan's multiple test ($P < 0.05$).

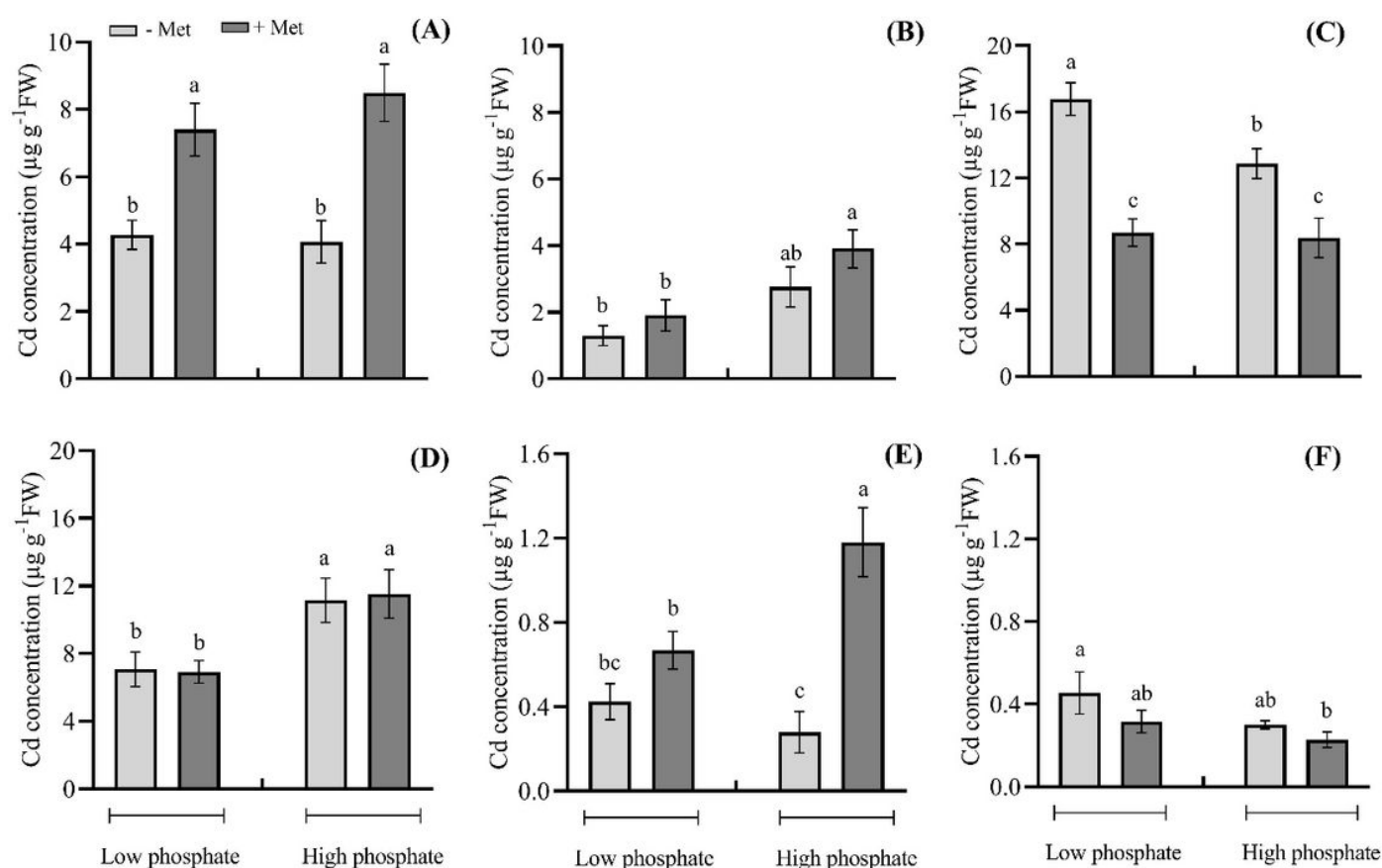


Figure 3

Concentration of different chemical forms of Cd in the roots of 43-day-old valerian plants treated with phosphate and methionine (Met) in nutrient solution over a 10-day experimental period. A: inorganic Cd including nitrate, chloride, and aminophenol cadmium (F_E), B: water-soluble Cd with organic acids and Cd(H₂PO₄)₂ (F_W), C: pectate- and protein-integrated Cd (F_{NaCl}), D: insoluble CdHPO₄, Cd₃(PO₄)₂, and other Cd phosphate complexes (F_{HAC}), E: cadmium oxalate (F_{HCl}), F: residual (F_R). Columns represent mean values (n = 3) and bars represent standard errors. Means with the same letter are not significantly different from each other, using Duncan's multiple test (*P* < 0.05).

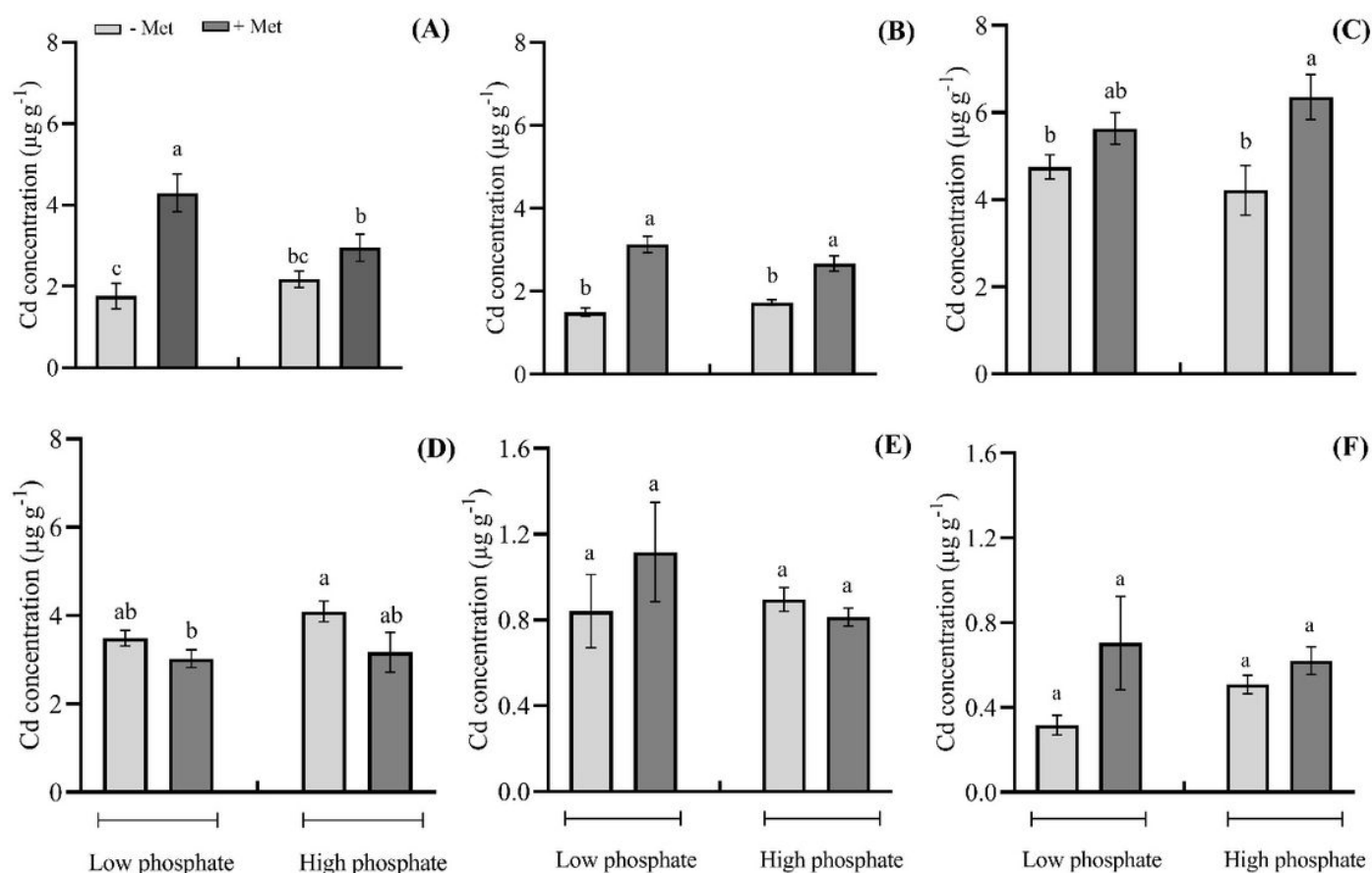


Figure 4

Concentration of different chemical forms of Cd in the shoots of 43-day-old valerian plants treated with phosphate and methionine (Met) in nutrient solution over a 10-day experimental period. A: inorganic Cd including nitrate, chloride, and aminophenol cadmium (F_E), B: water-soluble Cd with organic acids and $Cd(H_2PO_4)_2$ (F_W), C: pectate- and protein-integrated Cd (F_{NaCl}), D: insoluble $CdHPO_4$, $Cd_3(PO_4)_2$, and other Cd phosphate complexes (F_{HAC}), E: cadmium oxalate (F_{HCl}), F: residual (F_R). Columns represent mean values ($n = 3$) and bars represent standard errors. Means with the same letter are not significantly different from each other, using Duncan's multiple test ($P < 0.05$).

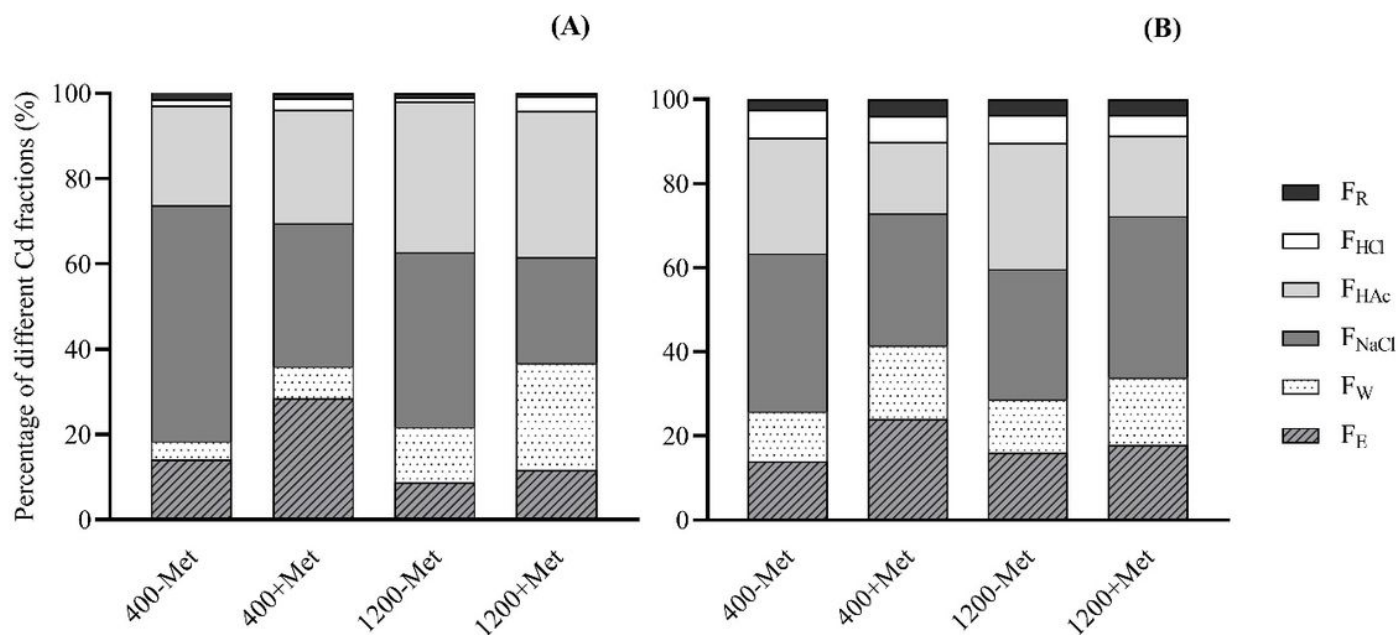


Figure 5

The proportion of different chemical forms of Cd in the roots (A) and shoots (B) of 43-day-old valerian plants treated with 400 and 1200 μM phosphate and methionine (Met) in nutrient solution over a 10-day experimental period. F_E : inorganic Cd including nitrate, chloride, and aminophenol cadmium, F_W : water-soluble Cd with organic acids and $\text{Cd}(\text{H}_2\text{PO}_4)_2$, F_{NaCl} : pectate- and protein-integrated Cd, F_{HAc} : insoluble CdHPO_4 , $\text{Cd}_3(\text{PO}_4)_2$, and other Cd phosphate complexes, F_{HCl} : cadmium oxalate, F_R : residual. Columns represent mean values ($n = 3$) and bars represent standard errors. Means with the same letter are not significantly different from each other, using Duncan's multiple test ($P < 0.05$).

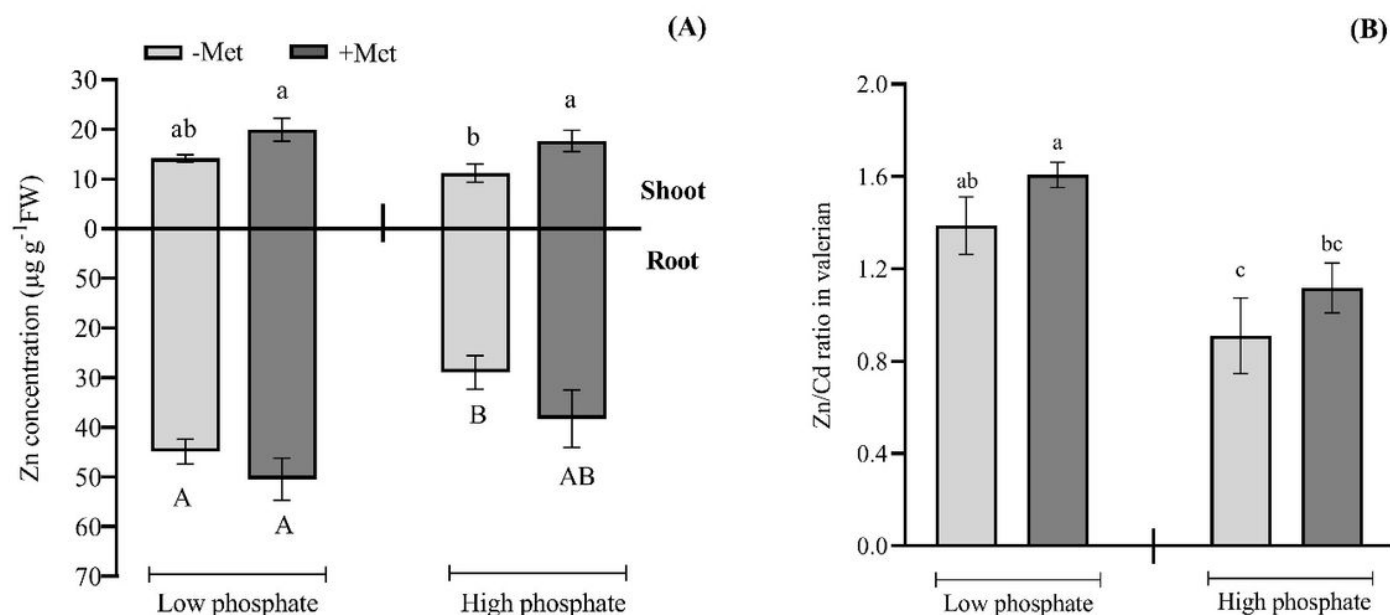


Figure 6

Root and shoot Zn concentration (A) and Zn/ Cd ratio (B) of 43-day-old valerian plants treated with phosphate and methionine (Met) in nutrient solution over a 10-day experimental period. Columns represent mean values ($n = 3$) and bars represent standard errors. Means with the same letter are not significantly different from each other, using Duncan's multiple test ($P < 0.05$).