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Interaction of cadmium and zinc on accumulation and sub-cellular distribution in leaves of hyperaccumulator *Potentilla griffithii*

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ABSTRACT

Potentilla griffithii Hook is a newly found hyperaccumulator plant capable of high tolerance and accumulation of Zn and Cd. We investigated the interactive effects between Cd and Zn on accumulation and vacuolar sequestration in *P. griffithii*. Stimulatory effect of growth was noted at 0.2 mM Cd and 1.25 and 2.5 mM Zn tested. Accumulation of Zn and Cd in roots, petioles and leaves were increased significantly with addition of these metals individually. However, the Zn supplement decreased root Cd accumulation but increased the concentration of Cd in petioles and leaves. The results from sub-cellular distribution showed that up to 94% and 70% of the total Zn and Cd in the leaves were present in the protoplasts, and more than 90% Cd and Zn in the protoplasts were localized in the vacuoles. Nearly, 88% and 85% of total Cd and Zn were extracted in the cell sap of the leaves suggesting that most of the Cd and Zn in the leaves were available in soluble form. The present results indicate that Zn supplement significantly enhanced the petiole accumulation of Cd and further vacuolar sequestration plays an important role in tolerance, detoxification and hyperaccumulation of these metals in *P. griffithii*.

1. Introduction

Heavy metals, such as Cu, Zn, Mn, Co and Ni, are essential elements for plant growth and metabolism, although it is toxic in excess. Other heavy metals such as Cd are non-essential and toxic even at low concentration. Heavy metal uptake, translocation, and cellular sequestration are key factors of plant's hyperaccumulation and tolerance of heavy metals. Several hyperaccumulating plant species have been identified that tolerate in highly contaminated soils and accumulate metals to high concentrations. To date, more than 400 species of hyperaccumulators have been identified, of which only 18 species are Zn hyperaccumulators [1]. Among these hyperaccumulators, *Thlaspi caerulescens*, *Arabidopsis halleri*, and *Sedum alfredii* have also been identified as hyperaccumulators of both Cd and Zn [2]. These plants possess a range of potential detoxification and tolerance mechanisms both at the cellular and plant level. This includes complexation of metals by a range of ligands and compartmentation of metals into metabolically inactive cellular sites [3]. There is much evidence that vacuolar localization may be associated with metal detoxification in hyperaccumulators [4–7].

There is a need to study the influence of Zn on Cd toxicity since Cd has been described as an antimetabolite of Zn due to the observed Zn deficiency in Cd-treated systems [8]. Most of the experiments are dealing with the interaction between Cd and Zn in non-hyperaccumulator plants and the effects of Cd on Zn uptake and accumulation in plants are not consistent, which shows either synergistic [9–11] or antagonistic [8,12] pattern. These results are presumable due to difference in culture methods, species and conditions. Furthermore, such divergence may be also attributed to the genetic factors that govern the disposition, such as uptake, distribution, metabolism and excretion of a metal in different species or within species. The relationship between these two transition metals (Cd and Zn) in terms of their uptake, accumulation, and sub-cellular distribution in different parts of hyperaccumulators, *T. caerulescens* and *A. halleri* have been studied [13,14]. Assuncão et al. [15] observed a competition between Cd and Zn, in which the addition of Cd to the nutrient solution decreased shoot Zn uptake in *T. caerulescens* (Ganges). Further, the interactions of Cd and Fe were also reported in *T. caerulescens* (Ganges) [16] and *A. halleri* [17], in which the root uptake of Cd was up-regulated by low Fe status. Recently, Küpper and Kochian [18] investigated the involvement of Zn transporter genes in Cd and Zn uptake in shoots at various sub-cellular compartments using quantitative mRNA *in situ*

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Table 1
Individual and combination of different treatments of Cd or Zn (mM) used for this study. Note that all the treatment concentrations contain 0.5 μM L^{-1} of Zn as in medium.

Zn	Cd		
	0	0.2	0.4
0	T0	T1	T2
1.25	T3	T5	T6
2.5	T4	T7	T8

hybridization (QISH). *Potentilla griffithii* Hook is a recently found Cd/Zn hyperaccumulator plant. This species has the ability to tolerate and accumulate high concentrations of both Zn [19] and Cd [20] from soil and nutrient solutions, suggesting that altered competitive interactions of both metals may occur in this plant. Hu et al. [21] demonstrated the tolerance and accumulation ability of Cd and Zn in *P. griffithii* by sub-cellular distribution in root and leaf tissues using TEM. In the present study, we addressed the interaction of Cd and Zn on accumulation and tolerance in terms of sub-cellular compartmentalization of these metals quantitatively by isolating protoplasts and vacuoles in the leaves of *P. griffithii*.

2. Materials and methods

2.1. Plant culture and metal treatments

Seeds of *P. griffithii* H. were germinated on agar medium which consisted of 6.5 g L^{-1} of agar, (in mg L^{-1}) NH_4NO_3 1650, KNO_3 1900, $CaCl_2{\cdot}2H_2O$ 440, $MgSO_4{\cdot}7H_2O$ 370, KH_2PO_4 170, $FeSO_4{\cdot}7H_2O$ 27.8, Na_2-EDTA·$2H_2O$ 37.3, KI 0.83, H_3BO_3 6.2, $MnSO_4{\cdot}H_2O$ 13.6, $ZnSO_4{\cdot}7H_2O$ 12.5, $Na_2MoO_4{\cdot}2H_2O$ 0.25, $CuSO_4{\cdot}5H_2O$ 0.025. One month after germination, seedlings were transferred to 1/2 strength Hoagland nutrient solution for 60 days in greenhouse (natural light; 28 °C/day and 18 °C/night; relative humidity 75%) for forth culture. The components of full strength solution includes: (in mmol L^{-1}) $Ca(NO_3)_2{\cdot}4H_2O$ 2.0, KH_2PO_4 0.10, $MgSO_4{\cdot}7H_2O$ 0.50, KCl 0.10, K_2SO_4 0.70, and (in μmol L^{-1}) H_3BO_3 10.00, $MnSO_4{\cdot}H_2O$ 0.50, $ZnSO_4{\cdot}7H_2O$ 0.50, $CuSO_4{\cdot}5H_2O$ 0.20, $(NH_4)_6Mo_7O_{24}$ 0.01, Fe-EDTA 100, and pH 5.8. The nutrient solution was renewed every seven days and aerated 1 h per day [21].

After 60 days plant culture, homogeneous seedlings were transferred into 0.5 L plastic vessels containing full strength Hoagland solution as described above. Eight concentrations of Cd ($CdCl_2$) and/or Zn ($ZnCl_2$) (mM) were used for the present study as depicted in Table 1. Each treatment was replicated in three containers, each containing three plants. The nutrient solution was aerated continuously and renewed every five days until the plants were harvested (30 days).

2.2. Extraction of cell sap and preparation of protoplasts

Sub-cellular distribution of Cd and Zn in *P. griffithii* leaves was investigated quantitatively by isolating protoplasts and vacuoles according to Shen et al. [6] and Ma et al. [22] with slight modifications. Protoplasts and vacuoles were purified by using different concentrations of Ficoll layering solutions, and normalized with acid phosphatase activity (EC 3.1.3.2), which is a marker enzyme for vacuoles [23]. Leaf samples (1 g) were collected, and washed with deionized water. After blotting, leaves were cut into two halves along with the midrib. One half (approximately 0.5 g) was ground with 800 μL 50 mM acetic acid–Tris buffer (pH 5.5) in a chilled mortar, and filtered through a very fine nylon mesh (230 mesh; 70 μm) for cell sap extraction [22]. Extracted cell sap was used for determination of Zn, Cd, and measurement of marker enzyme, acid phosphatase [23]. The remaining halves of the leaves were sliced into 1–2 mm pieces with sharp razor, and floated on protoplast medium containing 1.2% (w/v) Cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan), 0.3% (w/v) macerozyme R10, 0.1% (w/v) Pectolyase Y-23 (Kikkoman, Tokyo, Japan), 0.5 M mannitol, 20 mM MES (2-(N-morpholino)ethanesulfonic acid), 0.5 mM $CaCl_2$ and adjusted to pH 6.0 with 1 M KOH [6]. Approximately 0.5 g leaves were incubated in 15 mL medium in a Petri dish (9 cm in diameter) for 8 h in dark at the room temperature (26 °C) with gentle shaking (30 g) in a shaker. After incubation, the medium was filtered through a 40 μm filter and then centrifuged in a swinging-bucket rotor at 120 × *g* for 5 min. The supernatant was discarded, and the residue was resuspended in protoplast medium (without enzymes) containing 13% Ficoll. A discontinuous gradient was formed by successively layering solutions containing 8%, 5%, and 0% Ficoll in protoplast medium without enzymes. After centrifugation at 900 × *g* for 15 min, the interphase of the 5% and 8% layers was collected and the Ficoll was removed by washing with protoplast isolation medium without enzymes. Approximately 1 mL of purified protoplasts was used for measurement of acid phosphatase and determination of Cd and Zn. Due to low biomass and insufficient optimum sized leaves, we were unable to isolate the protoplasts and vacuoles at the highest treatment concentration T8. The viability of protoplasts was measured with the fluorescein diacetate (FDA) viability test according to Cosio et al. [13].

2.3. Preparation of vacuoles

The vacuoles were prepared following the method of Shen et al. [6]. One millilitre of purified protoplast suspension was mixed with 10 mL vacuole isolation medium containing 0.5 M mannitol, 1 mM EGTA (ethylene glycol tetraacetic acid), 0.5 mM CHAPS (3-[(3-cholamidoproyl) dimethylammonio]-1-propanesulfonate), and 20 mM MES (pH 8.0, adjusted with 2 M Tris solution), followed by gentle inversion by hand for 5 min, then centrifuged at 900 × *g* for 5 min. To remove the EGTA and CHAPS, the residue was resuspended with 0.5 M mannitol and 20 mM MES (pH 8.0). After centrifugation at 900 × *g* for 5 min, the residue was again resuspended in a solution containing 0.5 M mannitol, 20 mM MES (pH 8.0), and 10% Ficoll. A discontinuous gradient was formed by layering a solution containing 5% and 0% Ficoll in 0.5 M mannitol, 20 mM MES (pH 8.0). The fraction between the 0% and 5% layers was collected after centrifugation at 900 × *g* for 5 min and Ficoll was removed by washing with 0.5 M mannitol and 20 mM MES (pH 8.0). The purified vacuoles were used for measurement of acid phosphatase and determination of Cd and Zn.

2.4. Cd and Zn accumulation

After 30 days, the plants were harvested, and the root and shoot tissues were separated. The roots were transferred into 20 mM EDTA for 20 min to desorb metal ions bound to the roots. The roots and shoots were rinsed with deionized water and blotted dry. The samples were then oven-dried at 80 °C for 72 h and dry weights measured. The dried samples were digested with a mixture of concentrated HNO_3 and $HClO_4$ (4:1 ratio, v/v). The purified protoplasts and vacuoles were disrupted by dilution with water. The concentration of Cd and Zn in digests was measured by inductively coupled optical emission spectrometry (ICP-OES, Opitma 5300 DV, PerkinElmer, USA).

2.5. Statistical analysis

The plant biomass, Zn and Cd concentrations in plant tissues were analyzed using a one-way ANOVA followed by LSD test performed with the SPSS 11.0 for Windows Standard Version.

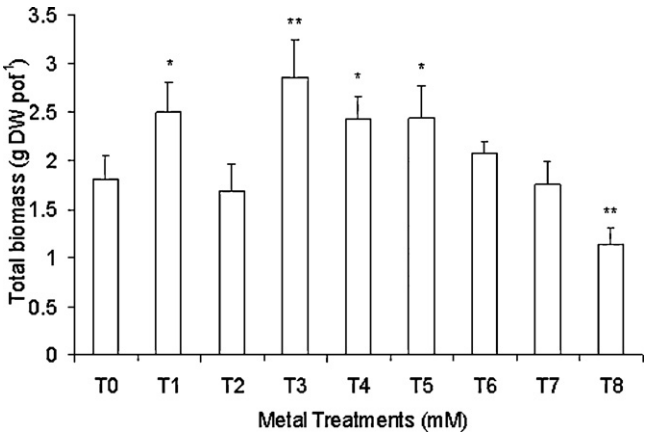

Fig. 1. Total biomass (g DW pot^{-1}) of *Potentilla griffithii* exposed to Cd and/or Zn in hydroponic culture for 30 days. Values are means ± SD of three replicates. Symbols with one ($P < 0.05$) and two asterisks ($P < 0.01$) indicate significant difference from control according to LSD test.

3. Results

3.1. Plant growth

P. griffithii plant grew healthy without showing any toxic symptoms during the experimental period in all treatments except T2 (0.4 mM Cd) and T8 (2.5 mM Zn with 0.4 mM Cd), in which plants had small leaves and stunted growth. The total dry biomass was measured at the end of the experiment and presented in Fig. 1. The dry biomass (2.43–2.86 g pot^{-1}) was significantly increased in T1, T3, T4 and T5 as compared to the control plants. However, the lowest biomass of 1.14 g pot^{-1} was observed at the highest treatment of 2.5 mM Zn with 0.4 mM Cd (T8). Further, high Cd treatment (0.4 mM) resulted in phytotoxicity symptoms, including reduced root and shoot biomass, while the addition of 1.25 mM Zn (T6) alleviated the phytotoxicity of Cd and slightly enhanced the growth. These results confirmed that *P. griffithii* has a high tolerance to Zn and Cd toxicity, and its growth can be stimulated by Zn and Cd at suitable levels.

3.2. Cd and Zn accumulation in P. griffithii

Zinc and Cd accumulation in the roots, petioles and leaves significantly ($P < 0.05$) increased with increasing concentrations of Zn and Cd. Zinc was primarily accumulated in the petioles (Fig. 2B), whereas Cd was mainly accumulated in the roots (Fig. 2A). The higher Cd accumulation was found in roots (6553 mg kg^{-1}) at individual 0.4 mM Cd (T2). Increasing Zn supplement (2.5 mM) to Cd treatments caused a significant reduction (~30%) in Cd accumulation in roots, whereas, a two fold increase in petioles and 36% increase in leaf Cd accumulation were recorded compared to the respective Cd treatments. The maximum concentration of Cd accumulation in petiole (4532 mg kg^{-1}) and leaves (1233 mg kg^{-1}) were observed in 2.5 mM Zn with 0.4 mM Cd treatments (T8) (Fig. 2A). Unlike Cd, the higher Zn accumulation was registered in roots (17295 mg kg^{-1}) at the combined treatments of 2.5 mM Zn with 0.2 mM Cd (T7) (Fig. 2B). The addition of Cd concentration to 2.5 mM Zn treatments showed a significant increase (63–65%) in root Zn accumulation (T7 and T8) except the addition of 0.2 mM Cd to 1.25 mM Zn (T5). On the contrary, Cd addition significantly reduced the petiole and leaf Zn accumulation as compared to the respective individual Zn treatments (Fig. 2B). The magnitude of decrease in Zn accumulation was higher in T5 in both petioles (57%) and leaves (113%) as compared to T7; whereas such decrease was higher in T6 for petioles (132%) and T8 in leaves (161%) as compared to each

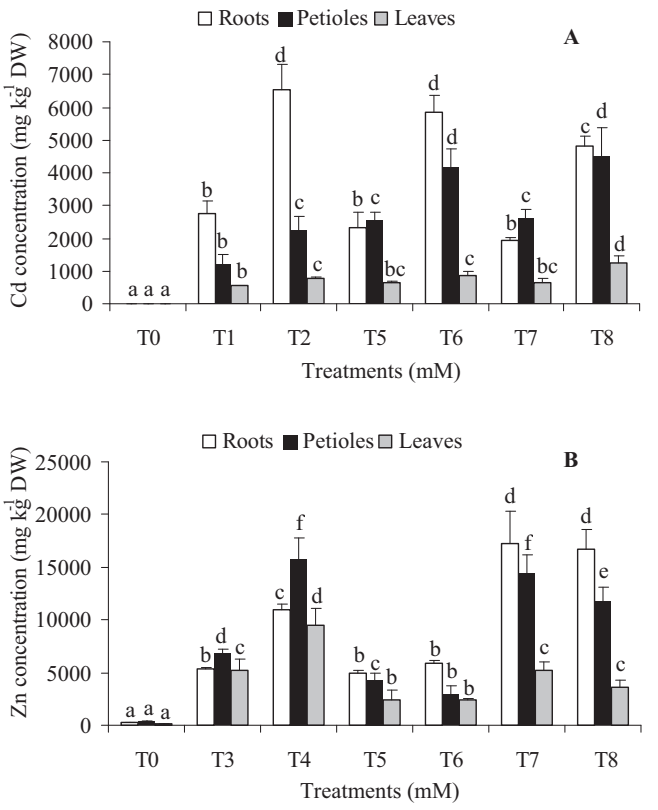

Fig. 2. Accumulation of Cd (A) and Zn (B) in roots, petioles and leaves of *P. griffithii* treated with Cd and/or Zn in hydroponic culture for 30 days. Values are means ± SD of three replicates. Different letters in each plant parts indicate significant difference from each other ($P < 0.05$) according to ANOVA followed by LSD test.

other. The higher Zn accumulation in petiole (15716 mg kg^{-1}) and leaves (9496 mg kg^{-1}) was found only in 2.5 mM Zn treatment (T4) (Fig. 2B).

3.3. Sub-cellular distribution of Cd and Zn in leaves of P. griffithii

Based on the FDA staining, the viability of protoplasts for Cd (ranged from 42% to 51%) and Zn (ranged from 45% to 49%) was almost similar. In addition, sizes of protoplasts were measured to assess the homogeneity between the different samples. The size of the protoplasts in control was 40 μm diameter and varied between 20 and 40 μm in Cd and Zn treated plants. Concentration of Cd and Zn in protoplast fraction was calculated by comparing the normalized Cd and Zn concentrations in the leaf and protoplasts. The results showed that 63% of the leaf Cd was localized in the protoplasts in 0.2 mM Cd treatment. Increasing the Cd concentration in the nutrient solution, increased the Cd localization in the protoplasts up to 70%. In the presence of 2.5 mM Zn (T7), the Cd localization in protoplasts also increased from 63% to 72% (Table 2). In addition, approximately 94% of the total leaf Zn was recovered in the protoplasts in individual Zn treatments (1.25 and 2.5 mM). However, in the presence of Cd, protoplasts localization of Zn was reduced from 94% (Zn alone) to 82% (2.5 mM Zn with 0.2 mM Cd) (Table 2).

Approximately 95% (T1) of the total Cd and 93% (T3) of the total Zn in the protoplasts was found in the vacuoles of *P. griffithii* leaves. Addition of Zn did not change the vacuolar Cd concentration (Table 3). Increasing the external concentration of Zn, increased the vacuolar concentration, but no significant change in percentage of Zn was recorded in vacuoles. The higher Cd addition (2.5 mM Zn

Table 2
Cd and Zn concentrations in fresh leaf tissue and leaf protoplasts from *P. griffithii* treated with Cd and/or Zn in hydroponic culture for 30 days. Cd and Zn concentrations are normalized to acid phosphatase activity (1 unit = 1 mg p-nitrophenol released in 1 h at 30 °C).

Treatments	Metal concentration (μmol unit^{-1})		% In protoplasts
	Leaves	Protoplasts	
Cd			
T1	0.98	0.62	63
T2	1.21	0.85	70
T5	1.14	0.79	69
T7	1.36	0.98	72
Zn			
T3	1.36	1.28	94
T4	1.42	1.33	94
T5	1.12	1.00	89
T7	1.04	0.85	82

Table 3
Cd and Zn concentrations in protoplast and vacuoles from leaves of *P. griffithii* treated with Cd and/or Zn in hydroponic culture for 30 days. Cd and Zn concentrations are normalized to acid phosphatase activity (1 unit = 1 mg p-nitrophenol released in 1 h at 30 °C).

Treatments	Metal concentration (μmol unit^{-1})		% In vacuole
	Protoplast	Vacuole	
Cd			
T1	0.38	0.36	95
T2	0.54	0.50	93
T5	0.46	0.43	93
T7	0.68	0.62	91
Zn			
T3	0.86	0.80	93
T4	0.98	0.94	96
T5	0.56	0.52	93
T7	0.42	0.43	102

with 0.2 mM Cd) increased the vacuolar sequestration of Zn (102%) in *P. griffithii* (Table 3).

Cell sap was extracted from leaves and the results showed that the soluble forms of Cd increased with increasing supply of Cd in medium. The cell sap concentration of Zn decreased from 8.3 to 4.8 mM in the presence of 0.2 mM Cd with 1.25 mM Zn treatments (T5), whereas no significant change was observed in 2.5 mM Zn with 0.2 mM Cd concentration (T7). Irrespective to treatments, about 78–88% of total leaf Cd and 71–85% of total leaf Zn was present in the cell sap (Table 4). These results indicated that most of the Cd and Zn were available in soluble forms in the leaves.

Table 4
Cd and Zn concentrations in the leaves and cell sap of *P. griffithii* treated with Cd and/or Zn in hydroponic culture for 30 days. Values are means ± SD of three replicates.

Treatments	Leaf concentration (mmol kg^{-1} fresh wt)	Cell sap concentration (mM)	% In cell sap
Cd			
T1	1.25 ± 0.38	0.98 ± 0.15	86.40 ± 4.08
T2	1.54 ± 0.24	1.36 ± 0.24	88.16 ± 13.60
T5	1.30 ± 0.64	0.82 ± 0.14	81.19 ± 7.38
T7	1.58 ± 0.58	1.02 ± 0.38	78.06 ± 6.41
Zn			
T3	10.12 ± 1.62	8.3 ± 1.94	80.50 ± 5.09
T4	13.42 ± 1.04	9.6 ± 1.16	71.37 ± 3.12
T5	6.56 ± 1.62	4.8 ± 1.24	73.02 ± 0.90
T7	8.98 ± 1.30	7.6 ± 0.96	84.79 ± 1.60

4. Discussion

Studies on the interaction between Cd and Zn were mainly focused on conventional plant species and paucity of information is available for hyperaccumulators. In the present study, we compared *P. griffithii* plants grown in Zn and/or Cd treatments in terms of their ability to tolerate and accumulate these heavy metals. The plant biomass was enhanced by moderate addition of Cd (0.2 mM) and Zn (1.25 and 2.5 mM) individually as well as in combination (1.25 mM Zn with 0.2 mM Cd) in nutrient solution (Fig. 1). It is interesting to note that the stimulatory effect on plant biomass can also be induced by non-essential element Cd. Similar increase in biomass was observed at 160 mg L^{-1} Zn for 21 days [19] and 20 mg L^{-1} Cd exposed for 60 days [21] in the same plant. However, the plant growth was sustained similar to control when the concentration of Zn increased to 240 mg L^{-1} and a decrease in plant growth was evident at the highest concentration of 320 mg L^{-1} Zn (1.96 fold higher than the present study) for 21 days [19]. Irrespective of whether they are beneficial or toxic, stimulatory effect to some extent was also observed in other hyperaccumulators, such as *Arabis paniculata* [24], *S. alfredii* [25] and *Viola baoshanensis* [26], but inhibitory at higher concentrations (hormetic response). It is generally believed that since Cd has no known biological function, its entry into the plant takes place via transport processes that are normally functioning for Zn [27]. Whereas, Liu et al. [28] reported that Cd may play a physiological role in the Cd hyperaccumulation of *T. caerulescens*.

No phytotoxic symptoms were observed in petioles and leaves for both individual Cd and Zn treatments and in combination except in T2 and T8. The order of Cd accumulation in *P. griffithii* was: roots > petioles > leaves (T1 and T2); whereas in Zn addition treatments to 0.2 mM Cd (T5 and T7) showed as petioles > roots > leaves. Similar Zn accumulation pattern (petioles > roots > leaves) was evident at 2.5 mM Zn for 30 days in the present study as in Hu et al. [21] for 60 days of exposure. Unlike Cd, Zn plays a dual role either as a micronutrient or toxic element and their faster accumulation ability in shoots leads to increased Zn accumulation in the aerial plant parts, whereas the complete exclusion mechanism is not possible for Zn in plants [29].

The process of translocation from roots to shoots involves several steps: (i) symplastic absorption in roots; (ii) root sequestration; (iii) xylem loading from roots to shoots, and (iv) the storage in leaf epidermal and mesophyll cells. Several transporters (ZNT1, ZNT5, HMA4, FRD3, and MTP1, etc.) have been suggested to regulate these processes in plants. The efficient root to shoot translocation system is one of the main criteria for hyperaccumulators used for phytoextraction purposes and the xylem loading may play an important role in Cd hyperaccumulation. In Cd/Zn hyperaccumulator, *T. caerulescens* showed that high-Zn-grown plants maintained significantly lower root Cd concentrations compared with roots from plants grown at lower Zn concentrations [14]. The authors also suggested that the stimulated shoot Cd accumulation is associated with enhanced xylem transport of Cd from the root to shoot. Cd uptake in *T. caerulescens* was probably mediated by specific Cd transporters [30] and/or by a high affinity Zn transporter, ZNT1 [31]. In *A. halleri*, co-accumulation of Cd and Zn occurred in aerial parts and the uptake of these metals to a certain degree by the same transporters or the uptake mechanism is controlled by common regulators if the metal transporters are different [32]. In our study, both 1.25 and 2.5 mM Zn supplement to Cd treated plants showed a significant reduction in root and an elevation in petiole Cd accumulation as compared to the respective individual Cd treatments (Fig. 2). These results may suggest the synergistic behavior between Cd and Zn and the optimum level of Zn addition involve the process of Cd translocation from solution to petiole accumulation in *P. griffithii*. Similar dual response pattern was also observed in

Cd/Zn hyperaccumulator *S. alfredii* [33] when exposed to combination of Cd–Zn treatments. The authors described that the Zn supply at levels $\leq$500 μmol L^{-1} increased the Cd concentration in *S. alfredii*, whereas high Zn supply decreased root Cd but did not affect leaf Cd concentrations in *S. alfredii*. Also, Li et al. [34] reported that a strong positive interaction between these metals at 500 μM Zn +100 μM Cd treatment and enhanced both the uptake and translocation of Cd in hyperaccumulator ecotype of *S. alfredii* under hydroponic conditions which is also reported in *T. caerulescens* [14]. Further, it is well known that the chemical similarity of Cd and Zn results in significant levels of Cd-affinity for binding sites designed for Zn, so that the Zn transporters ZNT1, ZNT5 and MTP1 should transport or at least bind Cd as well [18]. Recently, Lu et al. [35] reported that the influx of Cd decreased in the roots of *S. alfredii* (HE) upon higher Ca supply probably due to the usage of same transport systems both by Ca and Cd but with different affinities. Hart et al. [36] also reported that decrease in Cd uptake by roots with increasing Zn concentration in non-hyperaccumulators such as duram and bread wheat. On the other hand, either 0.2 or 0.4 mM Cd addition showed a significant increase (~65%) in the root Zn accumulation at T7 and T8 (Fig. 2A and B) and such synergistic pattern was also observed in wheat [11]. Further, no significant changes in the root Zn accumulation pattern were evident in both the Cd addition to 1.25 mM Zn (T5 and T6). Although a significant decrease in Zn was observed in both petioles and leaves, the magnitude of decrease was higher (116% and 161%) in leaves especially for 0.4 mM Cd addition (T6 and T8). These results indicate that root Zn uptake was not affected by the Cd but some how it interferes in the translocation of Zn in hyperaccumulator *P. griffithii.* Recently, Küpper and Kochian [18] found that Cd addition strongly decreased the Zn translocation into shoots of Ganges ecotype of Cd/Zn hyperaccumulator *T. caerulescens.* Also, the elevated Cd induced Zn deficiency in the mesophyll involved the downregulation of ZNT1 expression in *T. caerulescens*. This may be one of the possible reasons for reduced petiole and leaf Zn concentrations at elevated Cd as observed in our study. Further, Assuncão et al. [15] also suggested that such competitive interactions between these transition metals might occur in different phases of hyperaccumulation processes including from root uptake to their final sub-cellular compartmentalization. This reciprocal competitive or synergistic interaction between Zn and Cd in *P. griffithii* strongly suggests that these two cations enter in to the plant via a common transport system. As Cd utilizes the other transporters including the Zn transporter for their uptake and translocation, the addition of Zn to Cd-treated plants facilitate the Cd translocation whereas the addition of Cd to Zn-treated plants reduced the Zn translocation as a result of competitive interaction between these metals. In addition, the increased affinity of Cd into xylem loading may also contribute for the increased Cd content in petioles of *P. griffithii*. Although different patterns of Cd–Zn interactions occurred in uptake and translocation process, it is evident that *P. griffithii* possess co-tolerant ability for both Cd and Zn (up to 2.5 mM Zn with 0.2 mM Cd) without any toxicity symptoms and it could be used as a suitable hyperaccumulator for co-contaminated sites with Cd and Zn. Once these metals enter into the plant cells, the efficient internal detoxification may be achieved through sub-cellular compartmentation especially in vacuoles of leaves.

Although several qualitative methods are available for sub-cellular elemental distribution using advanced analytical techniques [2,5,37], the quantitative method to isolate the protoplasts and vacuoles from the leaves is sensitive for even at low metal concentrations in leaves [22] which was deployed in the present study. Vacuolar sequestration in leaves could play an important role in hyperaccumulation of heavy metals [7]. Ma et al. [22] found more than 90% of the total Cd in the mesophyll tissues was present in the protoplasts and all Cd in the protoplasts was localized in the vacuoles of hyperaccumulator *T. caerulescens*. Further, Küpper et al. [2] reported that *A. halleri* mesophyll cells accumulated substantial amounts of Cd and Zn, most likely in their vacuoles, particularly when the external supply of Cd and Zn was high. The present results showed that the 70% of the total Cd and 94% of the total Zn in the leaves was present in the protoplasts. The Zn addition favoured the Cd localization in the protoplasts but decreased the Zn localization in protoplasts treated with Cd. Furthermore, we found that 93% (T2 and T5) of total Cd and almost 96% (T4) of the total Zn in the protoplasts was present in the vacuoles. These results suggest that vacuolar compartmentation is an important mechanism for Zn and Cd detoxification and hyperaccumulation of leaves in *P. griffithii*. This may be due to the chemical similarities such as electron configuration, valence state as well as affinity to S, N and O donor ligands of both Cd and Zn. The CAX (CAX2 and CAX4), HMA (HMA3) and CDF (MTP1) transporters are responsible for Cd and Zn transport in to vacuoles [7] as these transporters occurred in most plant species at the tonoplast. The expression of MTP1 is switched off once the vacuoles were full with Zn sequestration [18].

Cd and Zn may be complexed with O-donor ligands such as citrate and present in a soluble form, or more likely as metal oxides in solid forms because of extraordinary concentrations [2]. A large proportion of the Zn and Cd accumulated in the shoots of *T. caerulescens* [22,38] and barley plants [39] were present in the soluble form. Our study, 78–88% Cd and 71–85% of Zn in the leaves of *P. griffithii* was available in the soluble form. There is a possibility of binding of metals with other ligands during the preparation of cell sap extraction. Although this may contribute for the higher percentage of metal as compared to the total metal content in leaves but such artifact are only minimal. The high proportion of soluble Zn and Cd in the shoots of *P. griffithii* rules out apoplast precipitation as an important detoxification mechanism, but suggests that the excess Zn and Cd are complexed with soluble organic compounds and stored in the vacuoles. Evidence from SEM–EDX analysis also shows that Zn and Cd were accumulated predominantly in the vacuoles of epidermal cells of *P. griffithii* leaves [21].

5. Conclusions

In conclusion, Zn supplement significantly reduced the Cd concentration in roots and further enhances the Cd translocation in shoots, especially petioles. This indicates strong competition between Cd and Zn uptake in root level and efficiently translocated these metals into the shoots via a common transport system. Quantitative study of sub-cellular localization of *P. griffithii* showed Cd and Zn were mainly stored in leaves as soluble form and more than 90% of Cd and Zn in leaves were localized in vacuoles. Irrespective of either synergistic or antagonistic pattern in uptake and translocation mechanisms between Cd and Zn in different plant parts, vacuolar compartmentation plays a major role in tolerance, detoxification and hyperaccumulation of these metals in *P. griffithii*. Studies on the involvement of other N and S containing metabolites and molecular aspects are needed to explore the entire mechanism involved for the uptake, translocation and tolerance for this plant deserve further investigation.

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