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ORIGINAL PAPER

Changes in phytochelatins and their biosynthetic intermediates in red spruce (*Picea rubens* Sarg.) cell suspension cultures under cadmium and zinc stress

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Abstract Cell suspension cultures of red spruce (*Picea rubens* Sarg.) were selected to study the effects of cadmium (Cd) and zinc (Zn) on phytochelatins (PCs) and related metabolites after 24 h exposure. The PC_2 and its precursor, γ-glutamylcysteine (γ-EC) increased two to four-fold with Cd concentrations ranging from 12.5 to 200 μM as compared to the control. However, Zn-treated cells showed a less than twofold increase in γ-EC and PC_2 levels as compared to the control even at the highest concentration of 800 μM. In addition, unidentified higher chain PCs were also found in both the Cd and Zn treated cells and they increased significantly with increasing concentrations of Cd and Zn. The cellular ratio of PC_2 : Cd or Zn content clearly indicated that Cd (with ratios ranging from 0.131 to 0.546) is a more effective inducer of PC_2 synthesis/accumulation than Zn (with ratios ranging from 0.032 to 0.102) in red spruce cells. A marginal decrease in glutathione (GSH) was observed in both Cd and Zn treated cells. However, the GSH precursor, cysteine, declined twofold with all Cd concentrations while the decrease with Zn was 1.5–2-fold only at the higher treatment concentrations of Zn as compared to control. In addition, changes in other free amino acids, polyamines, and inorganic ions were also studied. These results suggest that PCs and their biosynthetic intermediates play a significant role in red spruce cells protecting against Cd and Zn toxicity.

Keywords Amino acids · Cysteine · γ-Glutamylcysteine · Glutathione · Heavy metals · HPLC · Inorganic ions · Metal detoxification · Polyamines

Abbreviations

ACN	Acetonitrile
γ-EC	γ-glutamylcysteine
GSH	Glutathione
HPLC	High-performance liquid chromatography
mBBr	Monobromobimane
NAC	*N*-acetyl-L-cysteine
PCs	Phytochelatins
TFA	Trifluroacetic acid

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Introduction

Heavy metals are natural components of the biosphere, often with prolonged persistence in soils. The increased bioavailability of cadmium

(Cd) and zinc (Zn), both transition metals, renders them toxic to several agronomic crops (Sanità di Toppi and Gabbrielli 1999; Souza and Rauser 2003) and tree species (Kahle 1993) when they exceed a critical concentration. Plants respond to metal toxicity by initiating a wide range of cellular defense mechanisms. These include immobilization, exclusion and compartmentalization of metals and synthesis of phytochelatins [PCs, $(\gamma\text{-GluCys})_n\text{-Gly}$], stress proteins and ethylene (Sanità di Toppi and Gabbrielli 1999). Information available in literature mostly concerns chelating mechanisms. Potential metal binding ligands include amino acids and organic acids, and two classes of cysteine-rich peptides, the PCs and the metallothioneins (MTs). Mendoza-Cózatl et al. (2005) showed that the enhanced synthesis of PCs and their sulfur containing metabolic precursors, reduced form of glutathione (GSH), γ-glutamylcysteine (γ-EC), cysteine (Cys), and sulfide are mainly involved against Cd stress from yeast to plants. PCs are enzymatically synthesized using GSH as substrate. The number of γ-EC moiety varies (n = 2–11) depending upon the PC derivatives. The C-terminal end of the PCs that possess residues such as β-Ala, Ser, Glu or Gln instead of Gly, are collectively known as *iso*-PCs. The *iso*-PCs have been reported in some plants during metal exposure (Zenk 1996). Phytochelatin synthase (PCS) or γ-EC dipeptidyl transpeptidase (EC 2.3.2.15), a constitutive cytoplasmic enzyme is activated upon exposure to several heavy metals including Cd and Zn (Cobbett and Goldsbrough 2002). The regulation of PCS activity is the key factor in PC synthesis. Recently, Mendoza-Cózatl and Moreno-Sánchez (2006) proposed a kinetic modeling pathway for GSH and PC synthesis in plants. They demonstrated that in unstressed conditions, the rate limiting step in GSH synthesis is controlled by γ-glutamylcysteine synthetase (γ-ECS), while under Cd stress at least two enzymes (γ-ECS and PCS) play a major role to increase PC synthesis and/or alternatively diminishing the GSH-S-transferases partially to maintain the GSH demand.

Apart from metal chelation, GSH is involved in multiple metabolic roles such as intracellular redox state regulation, scavenger of reactive oxygen species, transport of GSH-conjugated amino acids, storage of sulfur and other functions related to the cell cycle, plant growth, pathogen-related responses, and cell death (Noctor et al. 1998). The level of GSH depends upon the availability of substrates, Cys and to some extent, glycine. Hence, it is noteworthy to observe the regulation of these amino acids in GSH biosynthesis and also in metal detoxification mechanisms.

Heavy metals are regarded as a potential risk for forest health but very little is known about the PC-mediated metal defense mechanisms in forest trees. Grill et al. (1988a) proposed that PCs function as a protective mechanism in trees. Very low levels of PCs were observed in roots of *Acer pseudoplatanus* on a Zn mine. The different clones of *Salix viminalis* were shown to vary in tolerance levels against a number of metals including Cd and Zn, even though PC_2 [$(\gamma\text{-EC})_2\text{-Gly}$] and PC_3 [$(\gamma\text{-EC})_3\text{-Gly}$] were not found in this species (Landberg and Greger 2004). Di Baccio et al. (2005) provided evidence of GSH synthesis and its redox status as the key factors of plant defense mechanisms in poplar exposed to Zn. The overexpression of γ-ECS gene in poplar allowed greater Cd accumulation and higher amounts of PC production under Cd toxicity (Koprivova et al. 2002). Bittsanszky et al. (2005) showed that elevated GSH levels and Zn accumulations in transgenic poplar overexpressing γ-ECS also indicated a marked stress tolerance upon Zn exposure.

According to Baker et al. (1990), Cd exists in the natural environment as a part of Pb/Zn mineralization rather than being independent. Hence, there is a need to study Cd as well as Zn in assessing the risk of Cd in natural environments (Chaney et al. 1995). In natural environments, various soil, and biological factors influence the bioavailability of metal by affecting uptake and transport by roots and that leads to variable plant growth responses. These factors may include site variations in soil pH, organic matter, cation exchange capacity, leaching rates, type of vegetation, mycorrhizal associations, and competition from other metals near rhizosphere. The response of plant cell cultures in vitro avoids this complexity and allows us to study the effects of a specific

metal. Even though studies are available for the PCs and their precursor metabolites in cell cultures of tomato (Scheller et al. 1987), *Silene vulgaris* (Leopold et al. 1999), marine green alga (Hirata et al. 2001), sunflower (Gallego et al. 2005), cucumber (Gzyl and Gwóźdź 2005), a marine diatom (Kawakami et al. 2006), and a perennial shrub (Israr et al. 2006), paucity of information is available for tree species under Cd and Zn toxicity. Therefore, the present investigation is mainly focused to evaluate the changes in non-protein thiols and free amino acids associated with Cd or Zn stress in red spruce (*Picea rubens* Sarg.) cell suspension cultures.

Materials and methods

Chemicals

All chemicals used in the analyzes were of the highest purity available. Trifluroacetic acid (TFA), methanesulfonic acid (MSA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), *N*-acetyl-L-cysteine (NAC), GSH, γ-EC, α-methyl-DL-phenylalanine, zinc sulfate were purchased from Sigma, St. Louis, MO, USA; cadmium chloride was obtained from Fisher Scientific Company, Fair Lawn, NJ, USA; diethylenetriamine-pentaacetic acid (DTPA) was obtained from Fluka, Milwaukee, WI, USA; acetic acid and perchloric acid ($HClO_4$) were obtained from J.T.Baker, Phillipsburg, NJ, USA; monobromobimane (mBBr), and phytochelatin [PC_2, $(\gamma\text{-Glu-Cys})_2$-Gly] (custom ordered) were obtained from Anaspec, San Jose, CA, USA. The HPLC-grade solvents, acetonitrile (ACN) (Burdick and Jackson, Muskegon, MI, USA), 1-propanol and methanol (EMD Chemicals Inc., Gibbstown, NJ, USA), Photrex toluene and acetone (J.T.Baker) used in the present study were filtered with 0.45 μm nylon filter before use. Water was purified by a Milli-Q system (Millipore).

Cell suspension culture

Embryogenic cell suspension cultures of *P. rubens* Sarg. (cell line RS 61.1, 03–92) were grown in modified ½ strength Litvay's medium (Litvay et al. 1981) as described in Minocha et al. (1996). The following were the modifications: 3.42 mM glutamine (Gln), 9.05 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.44 μM N^6-benzyl adenine (BA), 1.0 g l^{-1} casein hydrolysate, and 2% (w/v) sucrose. Also, Sequestrine (40 mg l^{-1}) containing 7% (w/v) iron chelate (Plant Products, Brampton, ON, Canada) was replaced with 25 mM Fe-EDTA. The ½ strength Litvay's medium components included 150 μM Zn, an essential micronutrient required for adequate growth of conifer cell suspension cultures (Litvay et al. 1985; Teasdale et al. 1986). Before autoclaving, the pH of the medium was adjusted to 5.7. The cells were routinely subcultured by transferring 15 ml of cell suspension to 45 ml of fresh medium in 250 ml Erlenmeyer flasks at a regular interval of 7 days. All cultures were incubated in the dark on a gyratory shaker at 120 rpm and 25 ± 2°C.

Metal exposure

Seven-day-old cell suspensions from 2 to 4 flasks (each containing 60 ml of suspension) were pooled and mixed with an equal volume of fresh medium. These diluted cell suspensions were stirred constantly at a low speed for obtaining a uniform distribution of cells. Cultures were initiated by aseptically adding an aliquot of 10 ml diluted cell suspension culture in to 50 ml experimental flasks containing 10 ml of fresh medium to provide a final cell density comparable with routine subcultures on Day 0. On Day 3, different concentrations of Cd ($CdCl_2.2½\ H_2O$; 0, 12.5, 25, 50, 100, and 200 μM) or Zn ($ZnSO_4.7H_2O$; 0, 50, 100, 200, 400, and 800 μM) were added to the cell suspension cultures that already contained 150 μM Zn. All the experiments were performed 3–4 times with each treatment replicated three times unless specified otherwise.

Cell collection

The cells were harvested after 24 h incubation by vacuum filtration using Miracloth (Calbiochem–Novabiochem Corporation, La Jolla, CA, USA). The residue was washed twice with an equal volume of deionized water. The total fresh weight

was determined and the cells were subdivided into fractions for analyzes of thiol compounds, free amino acids, polyamines and exchangeable inorganic ions. The collected cell fractions were placed in appropriate solutions described below and stored at –20°C until further analyzes.

Derivatization of thiol compounds

Cell fractions (approximately 100 mg) were placed in 1 ml of extraction buffer (6.3 mM DTPA containing 0.1% (v/v) TFA). The samples were frozen (–20°C) and thawed three times prior to analysis to release cellular contents according to the procedure for the extraction of polyamines as described in Minocha et al. (1994). The supernatant was collected by centrifuging at 13,000× *g* for 10 min and used for subsequent analysis. The derivatization of thiol compounds using mBBr was carried out based on the methods of Rijstenbil and Wijnholds (1996) and Sneller et al. (2000). Briefly, 615 μl of 200 mM HEPPS buffer containing 6.3 mM DTPA, pH 8.2 was mixed with 25 μl of 20 mM TCEP, used as a disulfur reductant. To this mixture, 250 μl mix of standards or sample extract was added. This reaction mix was pre-incubated at 45°C for 10 min. Then 10 μl of 0.5 mM NAC was added as an internal standard (50 pmol/injection). The derivatization was carried out in the dark for 30 min at 45°C after adding 10 μl of 50 mM mBBr (a fluorescent molecule that binds specifically to thiol groups). The reaction was terminated by adding 100 μl of 1 M MSA. The derivatized samples were filtered with 0.45 μm nylon syringe filter (Pall-Gelman Labs., Ann Arbor, MI, USA). The blank reaction mix included extraction buffer in place of sample to identify peaks arising from reagents. GSH, γ-EC and custom ordered PC_2 were used as standards.

Dansylation of free amino acids and polyamines

Approximately 100 mg of fresh cell fractions were placed in 1 ml of 5% (v/v) ice-cold $HClO_4$. The samples were frozen (-20°C) and thawed three times prior to analyzes (Minocha et al. 1994). The supernatant was collected by centrifuging at 13,000× *g* for 10 min and subjected to simultaneous dansylation of amino acids and polyamines following the procedure described in Minocha and Long (2004) with the following modification. The reaction was terminated using 50 μl of L-asparagine (20 mg ml^{-1} in water) instead of L-alanine. The amino acid standards kit (Fluka) and ornithine, γ-aminobutyric acid (GABA), polyamine standards (Sigma) were used. Heptanediamine was used as an internal standard for polyamines. One hundred μl of sample extracts or standards mix (21 amino acids + ornithine + GABA and three common polyamines) in 5% $HClO_4$ was used in the dansylation procedure.

Chromatographic conditions

The liquid chromatographic system consisted of PerkinElmer (Wellesley, MA, USA) Series 200 pump and autosampler fitted with a 200 μl loop (10 μl injection volume for thiol compounds and 20 μl for free amino acids and polyamines), a Phenomenex Synergi Hydro-RP C_{18} column (4 μm particle size, 100 × 4.6 mm i.d.) heated to 40°C, a Phenomenex Security Guard C_{18} cartridge guard column and a fluorescence detector (Series 200 PerkinElmer). Separate analytical columns were used for amino acids and thiol compound analyzes. The details of the gradient profile used for the separation of amino acids and polyamines are described in Minocha and Long (2004). The excitation and emission wavelengths were set at 340 and 510 nm, respectively.

The thiol compounds were separated by using solvents A (ACN) and B (water) both containing 0.1% TFA. The gradient profile is described in Table 1. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. The total run time was 33.8 min including column cleaning and stabilization before the next injection. The data were integrated using TotalChrom HPLC software package (PerkinElmer, Version 6.2.1).

Exchangeable inorganic ions

The 3× frozen and thawed cell extracts were centrifuged at 13,000× *g* for 10 min. The supernatant

Table 1 Solvent gradient profile for separation of thiol compounds using monobromobimane (*mBBr*) derivatization

Step	Time (min)	Flow (ml min^{-1})	ACN + 0.1% TFA (%)	Water + 0.1% TFA (%)	Curve[a]	Cumulative time (min)
1	0.5	1.0	10.0	90.0	0	0.5
2	11.2	1.0	19.5	80.5	1	11.7
3	13.6	1.0	29.0	71.0	1	25.3
4	4.0	2.5	100.0	0.0	0	29.3
5	4.0	2.5	10.0	90.0	0	33.3
6	0.5	1.0	10.0	90.0	0	33.8

[a] 1 = linear and 0 = step

of 5% $HClO_4$ extract was diluted 50× with deionized water and subjected to exchangeable inorganic ion (fraction of total ions within cells, that is, soluble in dilute acid, Minocha et al. 1994) analyzes including Cd and Zn by simultaneous axial inductively coupled plasma emission spectrophotometer (Varian, Palo Alto, CA, USA) Vista CCD and Vista Pro (Version 4.0) software.

Statistical analyzes

The data for each treatment were analyzed as a series of one-way analysis of variance (ANOVA) to determine statistically significant differences between the control and metal treated cells. If the *F* values were significant, Tukey's test was performed to determine differences between the treatment concentrations. Pearson correlation coefficients were used to assess the interactions between various parameters studied. All analyzes were done using SYSTAT Version 10.2 for Windows (SYSTAT, Richmond, CA, USA) with a probability level of 0.05 unless specified otherwise.

Results

Metal accumulation

Cellular Cd content proportionately increased at higher treatment concentrations ranging from 50 to 200 μM Cd; the increase at 100 and 200 μM was statistically significant. The control as well as the lower treatment concentrations did not show detectable Cd accumulation (Fig. 1A). A significant progressive increase (ranging between 0.545 and 2.294 μmol g^{-1} FW) was found in cellular Zn levels with increasing treatment concentrations of Zn (Fig. 1B). The magnitude of increase varied from one to sixfold in Zn treatments with respect to control. It should be pointed out that the culture medium also contained 150 μM Zn as an essential element for growth. The cell growth as measured by fresh cell mass was insignificantly inhibited with increasing treatment concentrations of both Cd and Zn (data not shown).

HPLC profile of thiol compounds

The present study involved a pre-column derivatization of thiols using mBBr. The linear range of detection for different thiol compounds was from 5 to 100 pmol. The r^2 value for standard curves was 0.99 for all thiol compounds tested (Table 2). The representative chromatographic profiles of 24 h Cd (25 μM) exposed red spruce cell suspension cultures along with suitable blank and known standards profile are depicted in Figs. 2A–C. The known monothiols (GSH and γ-EC) along with internal standard (NAC) and PC_2 in the present study were eluted within 13.5 min. The identification of these compounds was based on: (1) similar retention times with the standards and (2) spiking of peaks. The unidentified long chain PCs were also found in Cd and Zn exposed red spruce cells but the amounts were not quantified due to the absence of appropriate standards. Changes in these unidentified polythiol compounds were compared using relative fluorescence values given as areas. The total run time is significantly shorter than most other published methods for non-protein thiol analyzes using HPLC.

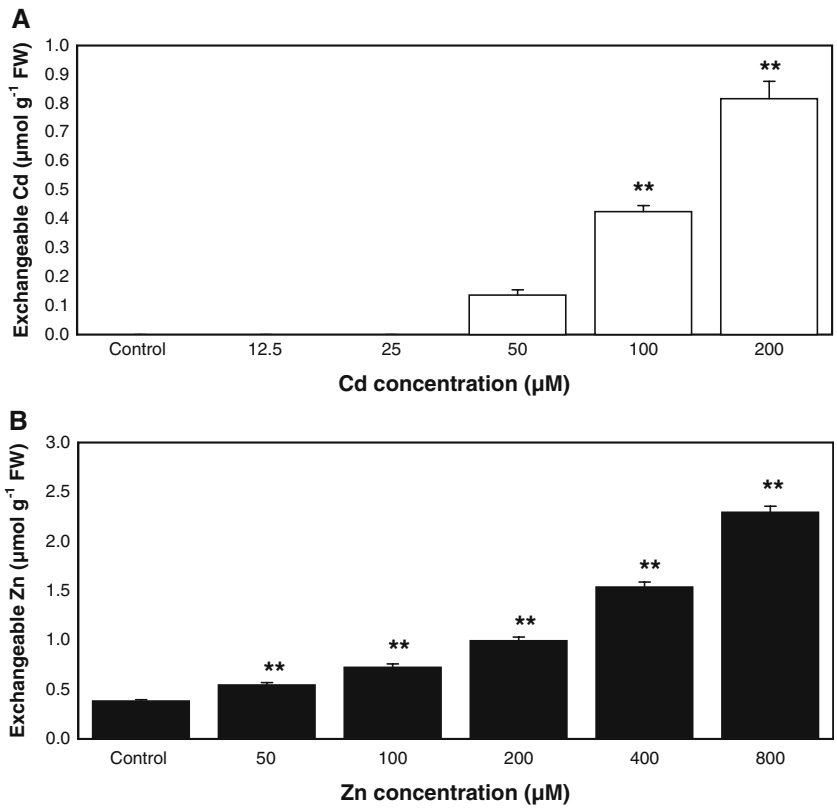

Fig. 1 Cadmium (**A**) and zinc (**B**) accumulation in 3-day-old red spruce cell suspension cultures exposed to varying concentrations of Cd (□) and Zn (■) for 24 h. All treatments had 150 μM Zn as part of Litvay's growth medium. Values represent the mean ± SE [n = 9–12 except for 400 and 800 μM Zn (n = 6)]. Note that the vertical scaling is not similar between Cd and Zn. **Statistically significant ($P < 0.05$) compared to control

Table 2 Linear ranges and r^2 values for quantification of thiol compounds (n = 6)

Component name	Retention time (min)	Linear curve points	Linear range tested (pmol)	r^2		Slope (×10^2)		Intercept (×10^2)	
				Mean	SE	Mean	SE	Mean	SE
GSH	6.34	7	5–100	0.998	0.001	106.47	9.35	2.33	1.88
γ-EC	6.79	7	5–100	0.996	0.002	82.20	1.75	–0.05	1.35
PC_2	13.43	7	5–100	0.999	0.0005	127.99	2.09	–0.25	0.91

Cellular thiol levels

The levels of GSH showed no significant decrease in Cd treated cells (Fig. 3A). On the contrary, γ-EC (Fig. 3B) and PC_2 (Fig. 3C) levels significantly and steadily increased between 25 and 200 μM Cd. Nearly a fourfold increase in both γ-EC and PC_2 was seen at 100 and 200 μM Cd with respect to the control.

A statistically insignificant reduction in GSH levels was observed only at the highest treatment concentration of 800 μM Zn (Fig. 3D). On the contrary, the GSH precursor, γ-EC significantly increased at 400 and 800 μM Zn (Fig. 3E). There was also a significant dose-dependent increase in PC_2 levels for all treatment concentrations except 50 μM Zn (Fig. 3F). The magnitude of increase was highest (69% for γ-EC and 82% for PC_2) in 800 μM Zn with respect to control. Unlike Cd, Zn treated cells showed a less than twofold increase in γ-EC and PC_2 levels even at the highest treatment concentration of 800 μM.

In addition to PC_2, unidentified long chain polythiols were also found in both Cd (Figs. 4A–D) and Zn (Figs. 4E–H) treated cells. Such polythiols significantly increased (three to

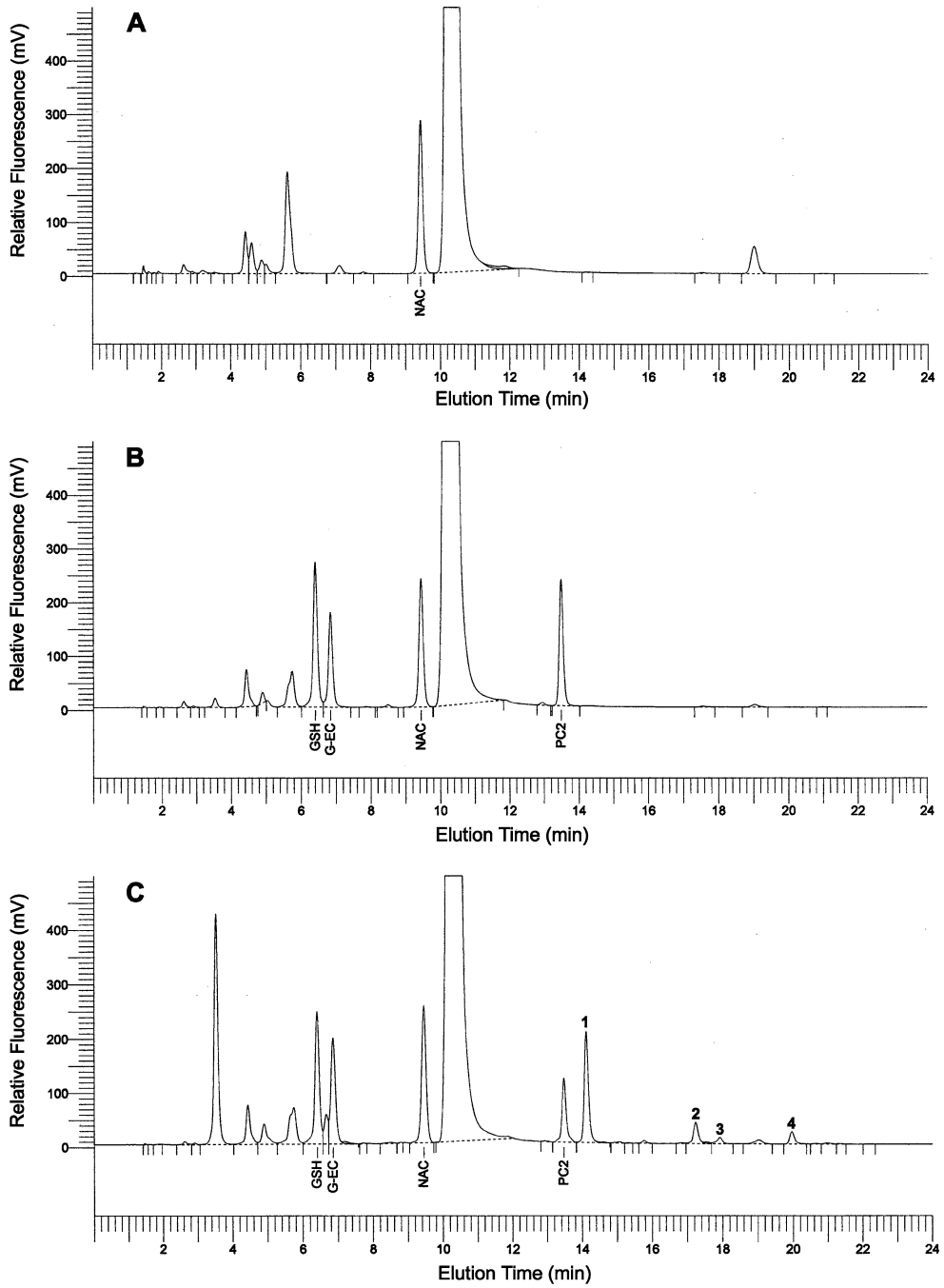

Fig. 2 Representative HPLC elution profile for different thiol compounds: blank run (Extraction buffer: 6.3 mM DTPA containing 0.1% TFA) (**A**), standard mix (**B**), and 25 μM Cd-treated red spruce cell suspension cultures for 24 h (**C**) along with 0.5 mM *N*-acetyl-L-cysteine (*NAC*) as an internal standard even in the blank run. *Peaks* 1–4 indicate long chain unidentified polythiols (PC_n). The gradient profile and chromatographic conditions are described in the text

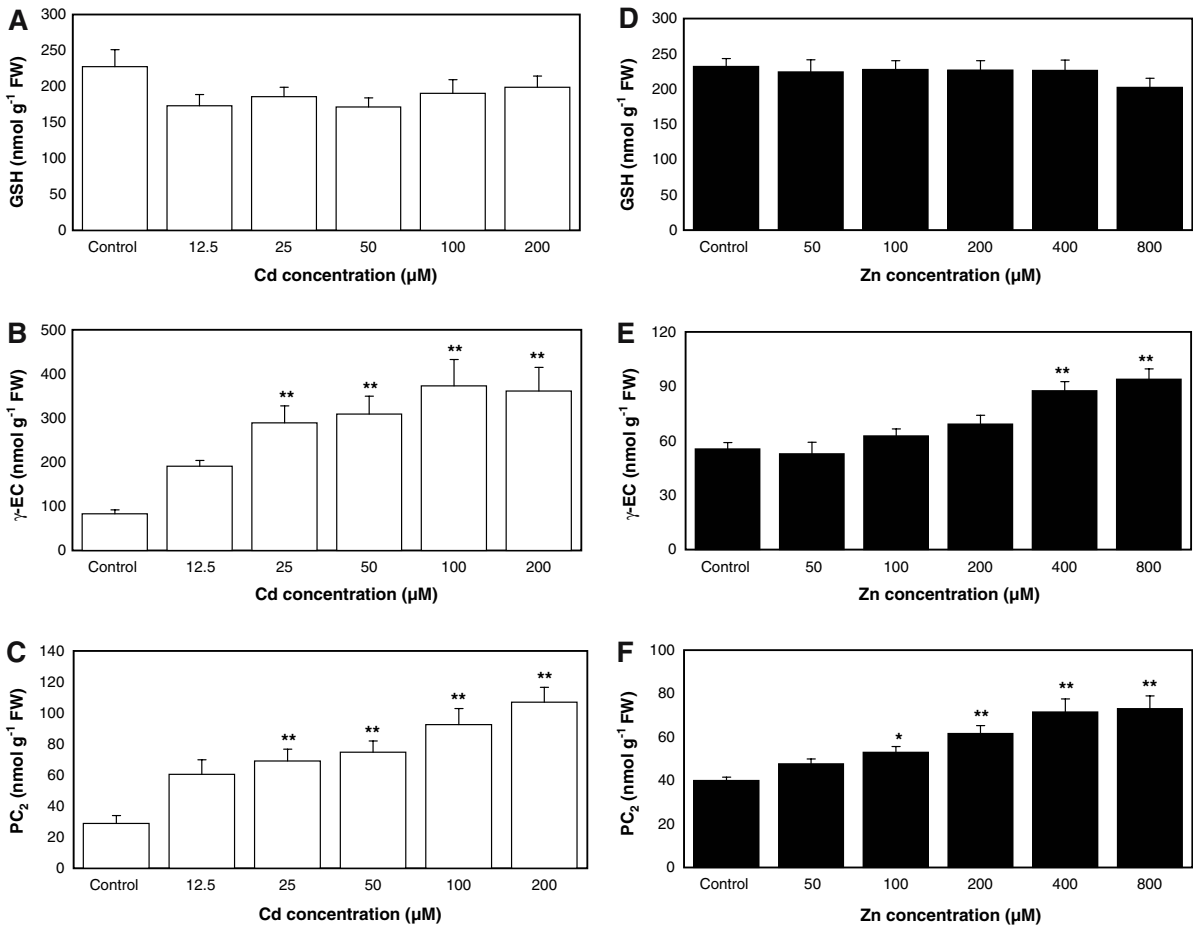

Fig. 3 Mean thiol concentrations in 3-day-old red spruce cell suspension cultures exposed to different concentrations of Cd (□) and Zn (■) for 24 h (**A** & **D**, GSH; **B** & **E**, γ-EC; **C** & **F**, PC_2). All treatments had 150 μM Zn as part of Litvay's growth medium. Thiols were tagged with mBBr and analyzed by HPLC. Values are given as mean ± SE (n = 9–12). Note that the vertical scaling is not similar between Cd and Zn for γ-EC and PC_2. **Statistically significant ($P < 0.05$); *statistically significant ($P < 0.10$) compared to control

ninefold for Cd and two to threefold for Zn) with increasing treatment concentrations of Cd and Zn.

Cellular free amino acids

An increase was observed in Gln and arginine (Arg) levels upon Cd exposure and was significant only at the higher concentrations of 100 and 200 μM for Gln (Fig. 5A) and 200 μM for Arg (Fig. 5B). The sulfur-containing amino acids, Cys + Cystine (our HPLC system could not separate Cys from cystine) significantly declined twofold from control level (Fig. 5C).

A significant increase in Gln levels was observed only at the higher concentrations of 400 and 800 μM Zn (Fig. 5D). The Arg levels did not show any specific trend in Zn treated cells (Fig. 5E), while Cys + Cystine showed a significant decline at 400 and 800 μM Zn (Fig. 5F). The remaining 19 amino acids tested did not exhibit any significant changes in either Cd or Zn treated cells (data not shown).

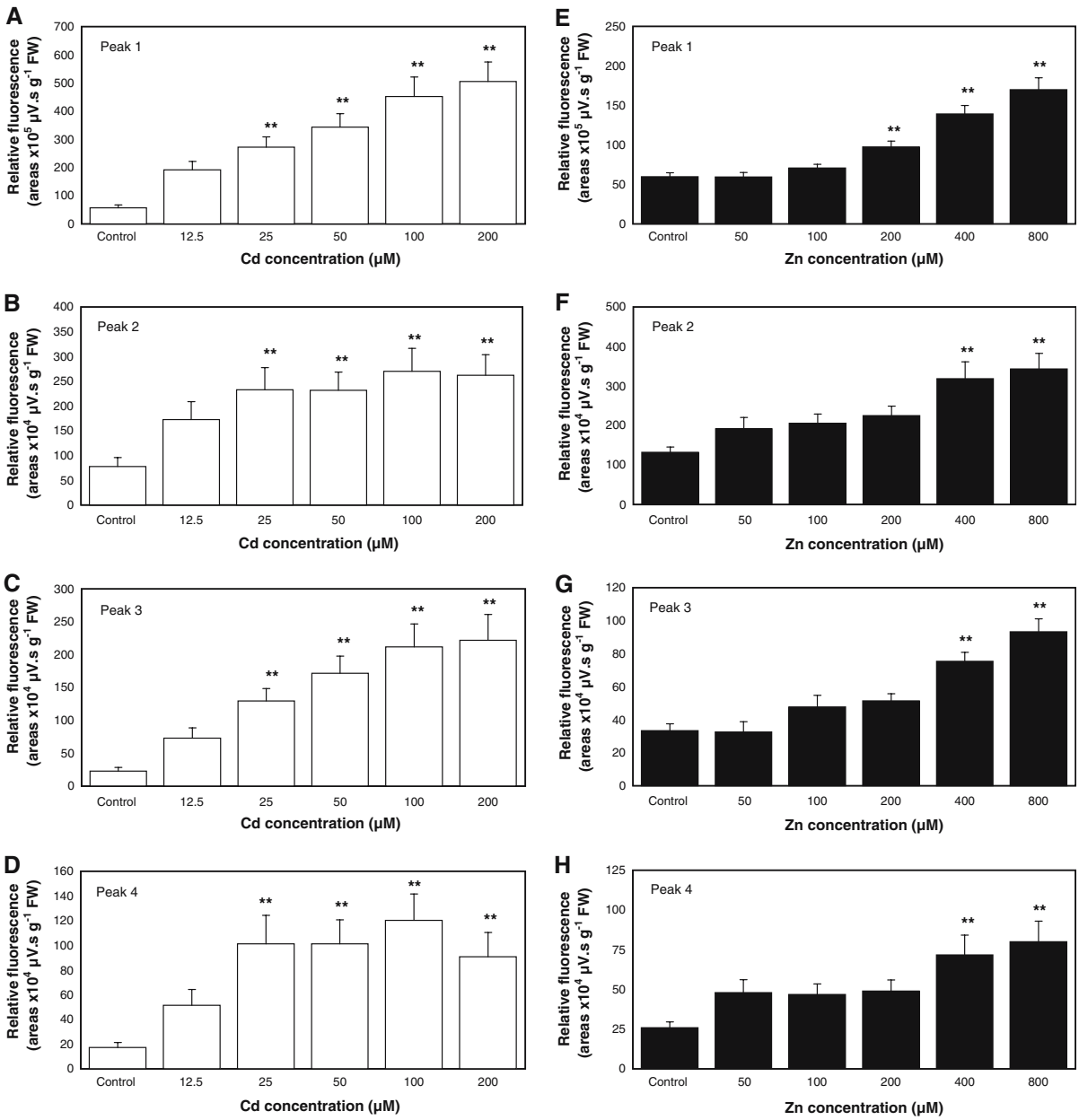

Fig. 4 Long chain polythiol contents in 3-day-old red spruce cell suspension cultures exposed to different concentrations of Cd (□) and Zn (■) for 24 h (unidentified peaks 1–4 in Fig. 2C). All treatments had 150 μM Zn as part of Litvay's growth medium. Retention times of peak 1 (**A** & **E**), 2 (**B** & **F**), 3 (**C** & **G**), and 4 (**D** & **H**) were 14.12, 17.23, 17.92, and 19.96 min, respectively. Data represents the mean ± SE (n = 9–12). Note that the vertical scaling is not similar between Cd and Zn. **Statistically significant ($P < 0.05$) compared to control

Cellular free polyamines and exchangeable inorganic ions

The changes in polyamines and ions (Ca, Mg, P, Mn, and Fe) were not statistically significant with one exception where K decreased significantly with 800 μM Zn (Fig. 6B). However, there was a trend toward increase in putrescine (baseline levels in control cell lines were about 600 nmol g^{-1} FW) and a decrease in spermidine (data

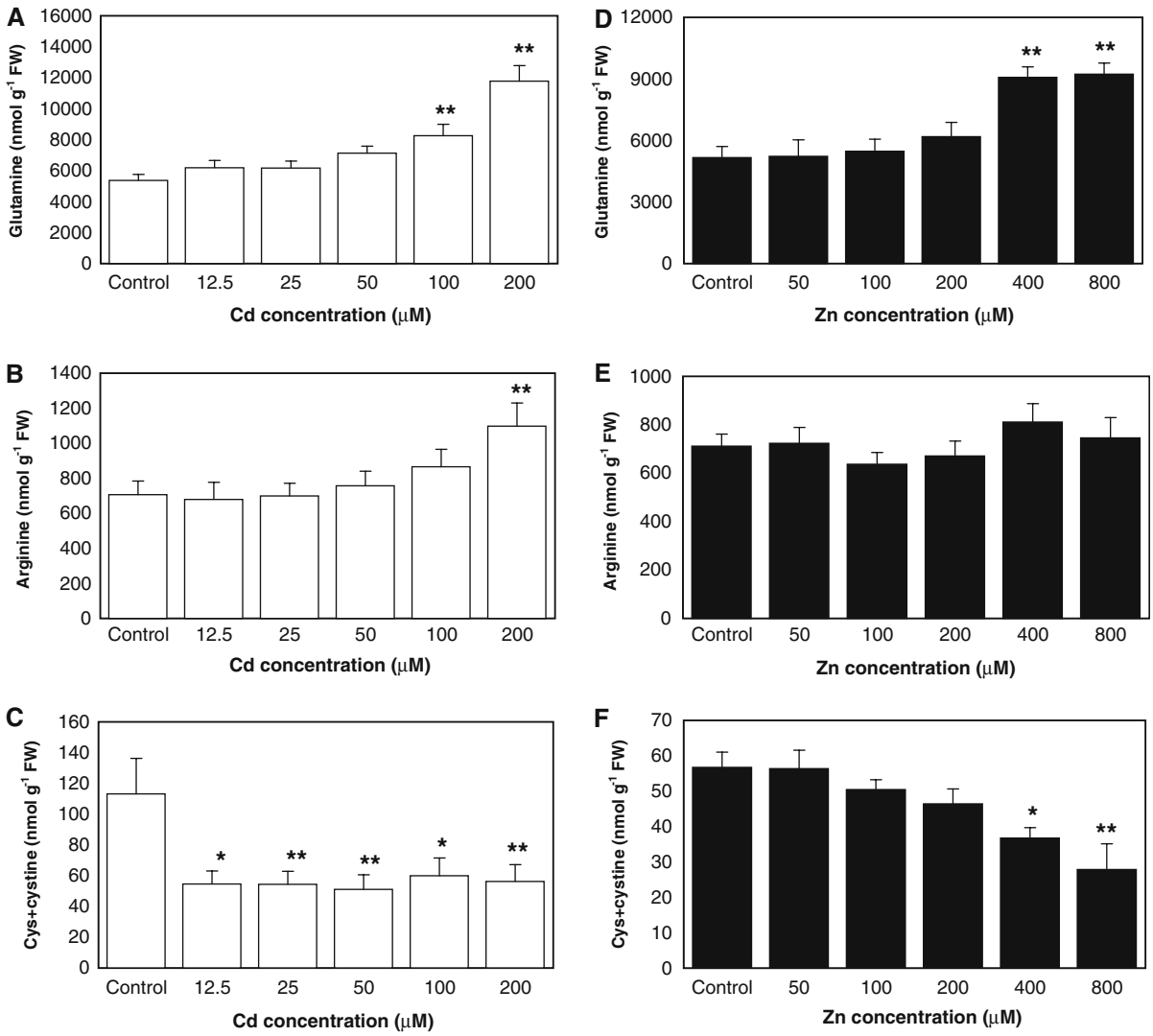

Fig. 5 Cellular free amino acids content in 3-day-old red spruce cell suspension cultures exposed to varying concentrations of Cd (□) (**A**–**C**) and Zn (■) (**D**–**F**) for 24 h. All treatments had 150 μM Zn as part of Litvay's growth medium. The free amino acids in the cell extracts were derivatized with dansyl chloride and quantified by HPLC. Each *bar* represent the mean ± SE [n = 9–12 except for 400 and 800 μM Zn (n = 6)]. The vertical scaling is not similar between Cd and Zn for different amino acids is noted. **Statistically significant ($P < 0.05$); *statistically significant ($P < 0.10$) compared to control

not shown) as well as K in Cd exposed cells (Fig. 6A).

Discussion

Phytochelatins

The synthesis of PCs and their sulfur containing metabolic precursors, GSH, γ-EC, Cys and sulfide are common detoxification responses to Cd stress in yeast, algae and higher plants (Mendoza-Cózatl et al. 2005). The type and amount of PCs synthesized in response to Cd depends on the concentration of Cd. The higher PC_2 levels (109–270%) in Cd-exposed red spruce cells compared to control suggested that tolerance to higher Cd toxicity levels is correlated with an elevated PC synthesis. Similar results have been reported in *Betula pendula* (Gussarsson 1994), *Datura innoxia*

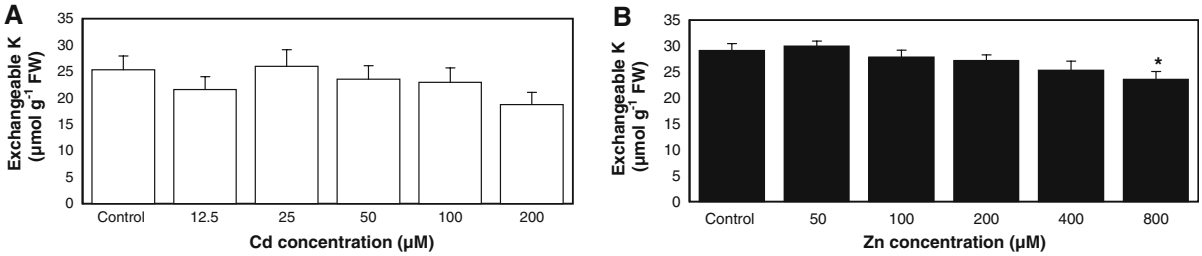

Fig. 6 Exchangeable K contents in 3-day-old red spruce cell suspension cultures exposed to varying concentrations of Cd (□) (**A**) and Zn (■) (**B**) for 24 h. All treatments had 150 μM Zn as part of Litvay's growth medium. Values are the mean ± SE [n = 9–12 except for 400 and 800 μM Zn (n = 6)]. *Statistically significant ($P < 0.10$) compared to control

(Delhaize et al. 1989), *Zea mays* (Tukendorf and Rauser 1990), *Pisum sativum* (Lima et al. 2006), and *Thlaspi caerulescens* (Wójcik et al. 2006) exposed to Cd. The presence of considerable amount of PCs in the control red spruce cells is consistent with the results of Keltjens and van Beusichem (1998), Maier et al. (2003) and Le Faucheur et al. (2005). It has been hypothesized that besides their functions in detoxification, PCs also play an important role in intracellular metal homeostasis in *Rauvolfia serpentina* cells (Grill et al. 1988b). However, in the present study, the culture medium contained 150 μM Zn, which may account for the minimal induction of PCs in control cells. Earlier reports also confirmed that 150 μM Zn in Litvay's medium is required for proliferative growth of conifer cell suspensions and maintaining them for prolonged periods (Litvay et al. 1985; Teasdale et al. 1986). Several other plant cell culture media also contain Zn and elicited PCs production in *R. serpentina* cells as reported in Grill et al. (1988b).

Depending on the metal species, Hg, Cd, As, Ag, and Fe have been reported as strong inducers of PCs while Pb and Zn are weak inducers and Cu and Ni are moderate inducers (Zenk 1996). In our study, the ratios of PC_2 to the cellular metal contents (both on nmol g^{-1} FW basis) clearly indicated that Cd (with ratios ranging from 0.131 to 0.546) is a more potent inducer of PC synthesis than Zn (with ratios ranging from 0.032 to 0.102) in red spruce cell suspension cultures. This observation corroborates reports on maize and radish exposed to different concentrations of Cd or Zn (Souza and Rauser 2003). These authors suggested that radish and maize exposed to Zn contained concentrations of monothiols and polythiols in both the roots and shoots that were too low to account for excess Zn sequestration. The root cultures of *Rubia tinctorum* exposed to either 100 μM Cd or 1,000 μM Zn showed similar PC_2 levels (7.0 nmol g^{-1} FW) although the culture medium contained 30 μM excess Zn (Maitani et al. 1996). In our case, Zn treated cells showed a less than twofold increase in γ-EC and PC_2 levels as compared to control even at the highest treatment concentration of 800 μM Zn whereas two to fourfold increase was observed in both the γ-EC and PC_2 levels in Cd (50–200 μM) treated cells (Figs. 3B & E, C & F). These results strongly suggested that Cd is a strong inducer than Zn even though excess Zn (150 μM) was present in the medium that accounted for only one-fifth of the exposed dose of Zn concentration. The longer chain species form more stable complexes with metals even though they represent only a small proportion of total PCs (Wójcik and Tukiendorf 2004). In our study, longer chain PCs were only a small proportion of total PCs but the changes in these were significant in Cd (Figs. 4A–D) and Zn (Figs. 4E–H) treated cells compared to controls. Unlike Cd, Zn chelated by lower molecular weight ligands, probably organic acids (Wójcik et al. 2006) is also a feature of tolerance mechanisms. Salt et al. (1999) showed that different ligands such as histidine, citrate, and oxalate in hyperaccumulator, *T. caerulescens* may facilitate metal loading into xylem, translocation to shoot, and presumably metal storage in cell vacuoles. They demonstrated that 70% of the

intracellular root Zn was found to be coordinated with histidine and it acts to transport Zn within cells, while organic acids act to coordinate Zn during long distance transport and storage in shoots. Data obtained for tomato and *S. vulgaris* cell cultures exposed to Pb and Zn ions showed that heavy metals are bound to lower molecular weight ligands, but not to PCs (Piechalak et al. 2002). Hence, PC formation plays only a partial role in Zn resistance while other mechanisms may be activated at higher degrees of tolerance.

In addition to detoxification mechanism, foliar PC levels also served as an indicator of heavy metal stress in forest trees, *P. rubens* (Gawel et al. 1996), *Betula papyrifera*, *Abies balsamea*, *Populus tremuloides* (Gawel et al. 2001), *Rhamnus frangula*, *Acer platanoides*, and *Betula populifolia* (Gawel and Hemond 2004) grown on metal contaminated environments. However, these authors did not report any soil metal levels, to establish which metal was actually responsible for the changes in PC levels. The major limitation in field conditions is that PC production in plants cannot be related to a specific metal as other metals may also be involved in such process (Rauser 1995).

Glutathione and cysteine

The monothiols, Cys, and GSH are actively involved in PC synthesis, and also metal sequestration in plants. As an antioxidant and PC precursor, GSH and its metabolism play an important role in plant responses and adaptation to various natural stress conditions. Nagalakshmi and Prasad (2001) reported a progressive decrease in the level of GSH in Cu stressed *Scenedesmus bijugatus* and suggested that it alters the equilibrium between the synthesis and utilization of GSH either for antioxidant role or PC production. PC synthesis induced by metals is accompanied by a rapid depletion of GSH in plant cell suspensions and intact plants (Foyer and Rennenberg 2000). The use of BSO (buthionine sulfoximine), an inhibitor of GSH biosynthesis, provides strong evidence that GSH pathway is necessary for PC synthesis and metal detoxification in *B. pendula* (Gussarsson et al. 1996) and other plant species (Schat et al. 2002; Wójcik and Tukiendorf 2004).

Several authors have reported a significant decrease in GSH levels in seedlings and cell cultures of different plants treated with varying Cd and Zn concentrations (Scheller et al. 1987; Tukendorf and Rauser 1990; Di Baccio et al. 2005; Le Faucheur et al. 2005; Wójcik et al. 2005; Lima et al. 2006). In agreement with the results reported in *B. pendula* (Koricheva et al. 1997), *Dunaliella tertiolecta* (Hirata et al. 2001), *Arabidopsis thaliana* (Wójcik and Tukiendorf 2004), and *Triticum aestivum* (Sun et al. 2005), the GSH contents did not change significantly in the present study after 24 h of Cd or Zn exposure in red spruce cell suspension cultures indicating that GSH was continuously synthesized to meet the requirement for PC synthesis and other stress related processes. However, there was a decreasing trend in GSH with all concentrations of Cd and only at 800 μM Zn as compared to control in these cultures. On the other hand, an increase in GSH level was evident in Norway spruce (*Picea abies* L. Karst.) cell suspension cultures under $CdSO_4$ exposure (Schröder et al. 2003) may be time dependent and/or species specific response. Howden et al. (1995) reported that intracellular GSH acts as a 'first line defense' against Cd toxicity in *A. thaliana*. One of the possible reasons for the decrease in GSH in red spruce cells may be that initially GSH acts as a 'first line of defense' against Cd toxicity by complexing the internal metal ions before the induction of PC synthesis becomes effective. GSH is also actively involved in secondary defensive mechanisms such as antioxidants, by scavenging free radicals in Cd induced oxidative stress in *P. abies* (Schröder et al. 2003) and other plants such as *T. aestivum* L. (Ranieri et al. 2005) and *Helianthus annus* L. (Gallego et al. 2005).

Cysteine, the precursor molecule for the synthesis of GSH, acts as a putative ligand for metals in several cultured plant cells. Along with Cys, Gly availability may also contribute to modulate GSH synthesis (Noctor et al. 1998). In this study, Gly did not vary significantly from the control in both Cd and Zn treated cells (data not shown). The Cys + Cystine content significantly and uniformly declined (50%) in all Cd treatment

concentrations possibly to maintain the increased GSH demand for PCs production. This decrease in Cys + Cystine was directly correlated with a decline in GSH ($r^2 = 0.591$, $P < 0.05$, $n = 70$). There was a weak correlation between Cys + Cystine and γ-EC ($r^2 = -0.324$, $P < 0.05$, $n = 70$) and no correlation between Cys + Cystine and PC_2 production. Therefore, it can be assumed that GSH in these cells was being consumed not only for PC_2 production but also for other functions such as antioxidant role under Cd exposure. A weak correlation between GSH and PC_2 ($r^2 = 0.311$, $P < 0.05$, $n = 70$) also confirms different roles of GSH under Cd stress. Lima et al. (2006) also demonstrated that decreased Cys and GSH levels in *P. sativum* are necessary for thiol production at the higher concentration of 120 μM Cd exposure. The Cd tolerant strain of *Chlamydomonas acidophila* KT-1 exhibited a decreased Cys levels after 4 days of 60 μM Cd, which lead to a poor GSH pool (Nishikawa et al. 2006).

A significant decrease of 35 and 51% was evident in Cys + Cystine only at 400 and 800 μM Zn as a result of increased γ-EC (Cys + Cystine versus γ-EC: $r^2 = -0.728$, $P < 0.05$, $n = 57$) and PC_2 levels (Cys + Cystine versus PC_2: $r^2 = -0.657$, $P < 0.05$, $n = 57$) in Zn treated red spruce cells. The lack of correlation between Cys + Cystine and GSH indicates Cys + Cystine may be needed for syntheses of metabolites other than GSH under Zn stress. The GSH pool was maintained constantly in Zn treated red spruce cells up to 400 μM. In poplar cuttings exposed between 1 and 10 mM Zn, the Cys contents were not significantly different from control (Di Baccio et al. 2005).

Free amino acids and polyamines

Very limited data are available on the effect of Cd on amino acid composition in plants (Costa and Spitz 1997; Leskó and Simon-Sarkadi 2002; Wu et al. 2004). Some amino acids (such as proline, Arg, GABA, and glutamic acid) are directly or indirectly involved in the regulation of plant responses to various environmental stress conditions (Bauer et al. 2004; Galili and Höfgen 2002). In this study, except for Gln, Arg, and Cys + Cystine, there was no significant variation observed for amino acids or polyamines profiles in red spruce cells exposed to different Cd and Zn treatment concentrations. The significant increase in Gln content in red spruce cells is consistent with the results of Chaffei et al. (2004) in Cd treated tomato leaves. The putrescine precursor, Arg significantly increased (55%) in red spruce cells after 24 h of 200 μM Cd exposure. Similarly, Leskó and Simon-Sarkadi (2002) suggested that the putrescine and the precursor Arg accumulated at higher Cd concentrations in both the roots and shoots of wheat seedlings.

Conclusions

The present study clearly shows changes in PCs and their biosynthetic intermediates such as GSH, γ-EC and Cys + Cystine in red spruce cell suspension cultures exposed to varying concentrations of Cd and Zn. An increase in both γ-EC and PC_2 with increasing Cd and Zn concentrations indicates a role for such non-protein thiols involved in Cd and Zn detoxification mechanisms. Further, GSH may serve as a 'first line of defense' to cope with Cd stress and not in Zn stress in red spruce cells. Due to different physiological roles of GSH in plants, it did not show a unique response for metal stress, which leads to the debate for GSH to serve as a suitable biomarker for metal toxicity even under laboratory conditions. Unlike GSH, considerable attention has been paid to PCs as a potential biomarker for metal toxicity because of their unique regulation by metal contents. Based on the cellular level study, PCs serve as an 'early warning signal' for Cd stress and to a lesser extent for Zn stress in red spruce cells only under controlled laboratory conditions. However, the mechanism deserves further investigation for individual and combination of metals under chronic exposures. Also, additional studies at higher hierarchical levels (organism and ecosystem) under field conditions are required to strengthen the value of PCs as a biomarker to assess the forest health.

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