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Compared to nitrate, ammonium enhances cadmium accumulation but does not change speciation in two hyperaccumulators

Running title: Ammonium enhances Cd accumulation in hyperaccumulators

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Highlights

Supply of NH_4^+ increased plant Cd uptake, translocation and accumulation.

Cd-S was dominant Cd species in *Carpobrotus rossii* and *Solanum nigrum* with Cd being transported as free ions in xylem.

Abstract

Nitrogen fertilization could improve the efficiency of Cd phytoextraction in contaminated soil and shorten the remediation time. However, limited information is available on the effect of N form on Cd phytoextraction and associated mechanisms in plants. This study examined the effect of N form on Cd accumulation, translocation and speciation in *Carpobrotus rossii* and *Solanum nigrum*. Plants were grown in nutrient solution with 5-15 μM Cd in the presence of 1000 μM NH_4^+ or NO_3^-. Plant growth and Cd uptake were measured, and Cd speciation analysed using synchrotron-based X-ray absorption spectroscopy. Shoot Cd accumulation was 30% greater with NH_4^+ than NO_3^- supply. *Carpobrotus rossii* accumulated three times more Cd than *S. nigrum.* However, Cd speciation in plants was not influenced by N form but varied with plant species and tissues. In *C. rossii*, up to 91% of Cd was bound to S-containing ligands in all tissues except the xylem sap where 87-95% were Cd-OH complexes. Furthermore, the proportion of Cd-S in shoots was substantially lower in *S. nigrum* (44-69%) than in *C. rossii* (60-91%). It is concluded that the application of NH_4^+ (instead of NO_3^-) increased shoot Cd accumulation by increasing uptake and translocation, rather than changing Cd speciation, and is potentially an effective approach for increasing Cd phytoextraction.

Key words: *Carpobrotus rossii*, Cd translocation, Cd speciation, halophytes, nitrogen form, phytoremediation, *Solanum nigrum*, synchrotron, XANES

Introduction

Although widespread in the environment, cadmium (Cd) is a non-essential element with no known physiological functions. It enters soil from a variety of sources including application of metal-containing sewage sludge, phosphate fertilizers, waste incinerators and other industrial wastes (Nicholson *et al.*, 1994). Cadmium presents a risk because it accumulates readily in plants to levels which are harmful in animal and human diets. It has been recognized that due to the efficient soil-to-plant transfer of Cd, dietary intake of food from crops grown on Cd-contaminated soils is a major route of Cd exposure to human health (WHO, 2010). Therefore, effective ways to remediate Cd-contaminated soils and mitigate Cd accumulation in crops are needed.

Among the various approaches for remediation, phytoextraction has attracted substantial attention because it is cost-efficient and environmentally friendly (Mahar *et al.*, 2016). Phytoextraction reduces metal concentrations in contaminated soils through the accumulation of these metals in the above-ground biomass of plants. For this purpose, hyperaccumulators are plants that can accumulate metals to levels at least 100 times that of most plant species (Baker & Brooks, 1989). Although many studies have emphasized the use of plants for remediation of Cd-contaminated soils, the main limitation of the phytoextraction approach is the long remediation time. To overcome this limitation, various strategies have been used to increase shoot biomass or increase the metal concentration in the harvestable portions of the plants.

Nitrogen (N) fertilization is an agronomic strategy to improve phytoextraction. As an essential nutrient, N fertilization increases shoot biomass of N-deficient plants, with this also potentially increasing Cd accumulation. However, the effect of N on Cd accumulation varies with the N source and the application rate (Mitchell *et al.*, 2000; Maier *et al.*, 2002; Wangstrand *et al.*, 2007). Nitrate (NO_3^-) and ammonium (NH_4^+) are the main sources of inorganic N taken up by plants, with N uptake comprising up to 80% of the total ion uptake. Therefore, the form of N supply plays an important role in the cation-anion balance, cellular pH and rhizospheric pH (Marschner, 2011). For NH_4^+, uptake results in an excess of cations over anions, with net extrusion of protons and acidification of the rhizosphere – this potentially increasing the availability of toxic metals such as Cd (Wu *et al.*, 1989). The opposite is true for NO_3^-, with uptake resulting in an excess of anions over cations. However, some studies have actually shown that supply of NO_3^- (rather than NH_4^+) increases uptake of Cd and other metals. For example, supply of NO_3^- enhanced Cd and Zn accumulation in *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) compared to NH_4^+, even though NH_4^+ lowered rhizoshphere pH (Monsant *et al.*, 2008; Xie *et al.*, 2009). Similarly, using nutrient solutions, supply of NO_3^- increased Cd uptake in tomato (*Solanum lycopersicum* Mill.), potato (*Solanum tuberosum* L.), hyperaccumulators *N. caerulescens* and *Sedum plumbizincicola* (Xie *et al.*, 2009; Luo *et al.*, 2012; Hu *et al.*, 2013; Jonsson & Asp, 2013). These findings indicate that, other than just influencing rhizosphere pH, N form also influences Cd uptake through additional mechanisms.

It is possible that the N form also influences Cd uptake and accumulation through changes in root morphology and plant growth (Rosen *et al.*, 1990; Bloom *et al.*, 2002) or through changes in the membrane potential of root cells (the driving force for cation uptake). For example, it has been reported that the uptake of NH_4^+ causes depolarization of cell membrane and thus reduces Cd uptake, whilst NO_3^- favors Cd uptake by hyperpolarising membrane potential (McClure *et al.*, 1990; Miller *et al.*, 2001; Zaccheo *et al.*, 2006). Furthermore, the N form may influence the expression of cation transporters, which may in turn alter Cd uptake and translocation given that Cd uptake is likely via the transport systems of other cations (Clemens *et al.*, 2002; Luo *et al.*, 2012).

The form of N supply may also alter the chemical speciation of Cd in plants, thereby influencing Cd translocation and accumulation. It is known that most transition metal ions in plants, including Cd, are bound by various ligands rather than being present as hydrated ions, and the chelation of Cd in plants determines its sequestration and mobility (Clemens, 2006). Moreover, uptake and assimilation of different N forms influence the production of amino acids and organic acids in plants, which in turn impacts the ligands for Cd complexation. Compared to NH_4^+, NO_3^- increases

the concentrations of organic acids but decreases amino acids (Roosta & Schjoerring, 2007; Marschner, 2011; White-Monsant & Tang, 2013). Furthermore, the form of N supplied influences the distribution of organic compounds in plant tissues, with NH_4^+ mainly being assimilated in roots whilst NO_3^- assimilated in both roots and shoots (Marschner, 2011). It has been reported that amino acids, peptides, proteins and organic acids are main ligands for Cd complexation, and Cd speciation in plants varies with plant species, plant tissues and environment conditions (Salt *et al.*, 1995, 1997; Vogel-Mikus *et al.*, 2010; Tian *et al.*, 2011). However, it remains unclear how the N form influences the speciation of Cd within plant tissues – this influencing Cd uptake, translocation and hence accumulation in plants.

The present study aimed to examine the effect of N form on Cd uptake and accumulation in two Australian native plant species, *Carpobrotus rossii* and *Solanum nigrum*, with both species having shown potential for Cd phytoextraction (Wei *et al.*, 2013; Zhang *et al.*, 2014). Specifically, this study aimed to determine whether the form of N-supply alters Cd accumulation and speciation in root and shoot tissues. It was hypothesized that the supply of nitrate, relative to ammonium, would increase Cd uptake and accumulation, and that the increased Cd accumulation would be associated with changes in Cd speciation of plant tissues. Synchrotron-based X-ray absorption spectroscopy (XAS) was used for *in situ* analyses of Cd speciation in plant tissues.

Materials and Methods

Plant growth

Carpobrotus rossii (Haw.) Schwantes (Aizoaceae) was collected from a rural landfill site (37°36′S, 143°35′E, Snake Valley, Shire of Pyrenees) in Victoria, Australia, while the seeds of *Solanum nigrum* L. were collected from plants grown on the La Trobe University farm (37°72′S, 145°4′E). Seeds of *S. nigrum* were germinated in a solution containing 600 µM $CaCl_2$ and 5 µM H_3BO_3 in a dark controlled-environment room for 5 d. For *C. rossii,* uniform cuttings (two nodes each cutting) were washed with tap water. The plant materials were transplanted to 5-L polyethylene pots filled with a basal nutrient solution aerated continuously. The basal nutrient solution had the following composition (µM): 500 NH_4NO_3; 200 $MgSO_4$; 10 KH_2PO_4; 600 K_2SO_4; 600 $CaCl_2$; 20 FeNaEDTA; 5 H_3BO_3; 1 $MnSO_4$; 0.2 $CuSO_4$; 0.03 Na_2MoO_4; 1 $ZnSO_4$. The root systems were well-developed after grown in these solutions for 15 d (*C. rossii*) and 30 d (*S. nigrum*). After this growth in basal solutions, three seedlings of *C. rossii* and five of *S. nigrum* were transferred to each of new pots containing the treatment solutions (below).

The experiment consisted of eight treatments and was replicated three times. The eight treatments were two plant species (*C. rossii* and *S. nigrum*) × two forms of N (500 µM $(NH_4)_2SO_4$ and 1000 µM KNO_3) × two Cd concentrations (5 and 15 µM Cd). Solution pH was buffered with 2 mM MES [2-(N-morpholino)ethane-sulphonic acid] and 1.2 ml of 1 M KOH was used to adjust pH to ca. 6.0 which was maintained daily using 1 M KOH. Since sulfur (S) plays an important role in Cd binding, 500 µM K_2SO_4 was added to the KNO_3 treatments in order to ensure constant S concentrations in all treatments (hence, the K concentration was 1210 µM in the $(NH_4)_2SO_4$ treatments and 3210 µM in the KNO_3 treatments); the composition of other nutrients was the same as that of the basal nutrient solution. Solutions were renewed every 3 d. Plants were grown in a controlled-environment growth room with 14-h photoperiod, a light intensity of 400 µmol m^{-2} s^{-1}, 20 °C during the day and 18 °C during the night, and 50% relative humidity during the day, and 60% relative humidity during the night.

Xylem sap collection

After growth in the treatment solutions for 14 d, xylem sap was collected according to the methods of Monsant *et al.* (2011). Briefly, the stem was cut with fresh razor blades, with the root stumps immediately washed with deionized water and dried with paper tissue. Xylem sap was collected for 1 h using micropipette and transferred into 1.5-ml Eppendorf tubes. The xylem saps from the

various plants of each pot were pooled, with each treatment having three replicates. For *S. nigrum*, however, the volume of xylem sap collected was insufficient to permit analysis, and hence only samples from *C. rossii* seedlings are presented here.

Plant analysis

After collecting the xylem sap, the plants were harvested and fresh weights recorded. Roots were divided into two parts, with the first subsample immersed in ice-cold 20 mM $Ca(NO_3)_2$ for 15 min, washed with deionized water, frozen in liquid nitrogen, and stored at -80 °C. The second root subsample was immersed in ice-cold 20 mM Na_2-EDTA for 15 min to remove Cd adhering to the root surface and then washed with deionized water and weighed. The roots were examined using a root scanner at 600 dpi (Epson Perfection 4990 Scanner, model J131B, Epson Inc.) to determine morphological parameters (length and surface area) before being oven-dried (at 80 °C in paper bags) for analysis. After washing with deionized water, shoots were blot-dried and separated into stems, old leaves and young leaves. Like the roots, the shoots were subdivided into two subsamples. The first subsample was frozen in liquid nitrogen, and stored at -80 °C, with the second subsample oven-dried. For the plants grown in solutions contained with 5 μM Cd, a portion of the each tissue was also freeze-dried and stored at -80 °C for XAS analysis. The oven-dried samples were ground and digested using HNO_3 in a microwave digester (Multiwave 3000, Anton Paar). The concentrations of elements in digests and xylem sap (0.5 ml mixed with 2.5 ml of 5% HNO_3) were analyzed using an inductively coupled plasma optical emission spectrometry (ICP-OES) (Perkin Elmer Optima 8000, MA, USA).

Cadmium speciation by X-ray absorption spectroscopy (XAS)

The speciation of Cd in plant tissues was examined at the XAS beamline of the Australian Synchrotron (Victoria, Australia). The energy of each spectrum was calibrated by simultaneous measurement, in transmission, of a metallic Cd foil reference (K-edge at 27,711 eV). The spectra were collected in the fluorescence mode with a 100-element solid-state Ge detector. To minimize beam-induced artifacts and thermal disorder, samples were placed in a cryostat sample holder (maintained at ca. 12 K, liquid helium). The beam size was adjusted to ca. 2 × 0.5 mm.

To prepare plant tissues for analysis, approximately 1-2 g frozen or freeze-dried samples were ground under liquid nitrogen using an agate mortar and pestle. The homogenized tissues were placed into a sample holder with Kapton tape windows cooled with liquid nitrogen before being transferred directly to the cryostat for analysis. For the xylem sap, treated plants were transported to the Australian Synchrotron (ca. 45 min) before growth for an additional 24 h. The sap collected immediately prior to analysis. The xylem sap was mixed in 30 % glycerol (see below) and loaded into a sample holder sealed with Kapton tape.

A total of 14 Cd-containing standard compounds (eleven aqueous and three solids) were analyzed using Cd K-edge XANES spectroscopy to allow interpretation of the XANES spectra from the experimental tissue samples. The eleven aqueous standards consisted of (i) 1 mM $Cd(NO_3)_2$, (ii-x) 1 mM $Cd(NO_3)_2$ mixed with various ligands at a final concentration of 5 mM [phytate, histidine, citrate, malate, succinate, cysteine, glutathione (GSH), methionine (MT), and phytochelatin 2 (PC2)], and (xi) 1 mM $Cd(NO_3)_2$ mixed with 0.1 % polygalacturonic acid – this being the major charged component of the cell wall. Standards were prepared using a 10 mM $Cd(NO_3)_2$ stock solution together with 50 mM solutions of the various ligands, or 1% polygalacturonic acid stock solution. All aqueous standards (other than polygalacturonic acid) were mixed in 30 % glycerol to limit ice-crystal formation during cooling. The 1% of polygalacturonic acid stock solution, with 30.4 μmol mL^{-1} free carboxyl groups determined by titration with 0.02 M NaOH to neutrality, was prepared approximately 24 h before use and kept at 4°C until use (Kopittke *et al.*, 2011; Taylor & Walter, 2012). The citrate, GSH, malate, phytate, succinate, histidine and MT standards were adjusted to ca. pH 6 and cysteine to pH 7 and PC2 to pH 10 using

0.1 M NaOH, while pH was not adjusted for the polygalacturonic acid or the 1 mM aqueous $Cd(NO_3)_2$. Where constants were available, GEOCHEM-EZ was used to model the standard solutions, with the results indicating that >97% of Cd was complexed with citric acid, 85% with succinic acid, malic acid and histidine. The three solid standards, CdS, CdO and $CdCO_3$, were diluted to 100 mg Cd kg^{-1} using cellulose.

Multiple XANES scans were performed for each sample, with two scans per standard, and either two or three scans for each plant tissue sample. Beam-induced damage was assessed using a plant tissue sample, with two XANES scans conducted at the same sample location – the two scans then compared to see if they differed. For other samples, positions were changed after each scan to reduce the risk of beam damage and to obtain representative spectra.

The XANES spectrum for each sample was energy-normalized using the reference energy of the Cd foil, with replicate spectra for each sample merged using Athena (version 0.9.22) (Ravel & Newville, 2005). Principal component analysis (PCA) of the normalized sample spectra was employed to assess how many independent components contained in the samples, and target transformation (TT) was used to identify relevant standards for linear combination fitting (LCF) of the sample spectra. PCA and TT were undertaken using SixPack (Webb, 2005). Using PCA, the XANES spectra of the plant tissues were compared with those of the standards to evaluate the number of relevant components indicated by the indicator function (IND) reaching a minimum. The results of PCA indicate that the first four components accounted for 98.6% of the total variance of the XANES spectra, so only four components were needed to fit the data. Target transformation was then used to identify the standard spectra in the LCF analyses by selecting standards with a SPOIL value < 3. Nine of the 14 reference spectra met this criterion, these being Cd-citrate, Cd-malate, Cd-succinate, Cd-polygalaturonate, Cd-GSH, Cd-cysteine, Cd-PC2, $Cd(NO_3)_2$ and CdS (Table S1). The LCF was used to identify the relative proportions of standard spectra within the sample spectra as the XANES spectrum represents a combination of all Cd species present in the portion of sample transected by the beam, and the fitting energy range was –30 to +100 eV relative to the Cd K-edge.

Statistical analysis

The effect of N form and Cd concentration in solution on plant biomass, tissue Cd concentrations, shoot Cd content and Cd translocation was examined using a two-way analysis of variance for each species. Significant ($P \leq 0.05$) differences between means were identified using Tukey's HSD test using GenStat v. 11 (VSN international).

Results

Plant growth

All plants appeared healthy during the experimental period, with little visual difference between treatments. Overall, *C. rossii* had shoot biomass 5-fold higher than *S. nigrum* (Fig. 1). Despite this overall difference in biomass production between the two species, the form of N and the Cd concentration had no significant impact upon shoot biomass for either plant species. The N form and Cd level in solutions only influenced root biomass of *S. nigrum*, with biomass at 15 µM Cd 18% lower when supplied with NO_3^- than when supplied with NH_4^+. Moreover, increasing Cd concentration in solution from 5 to 15 µM decreased root biomass by 12% when supplied with NH_4^+ and by 29% when applied with NO_3^- ($P<0.05$). Furthermore, the root length and root surface area per plant were 16-40% greater when supplied with NH_4^+ than with NO_3^-, irrespective of the Cd concentration in nutrient solution (except the root surface area of *C. rossii* at 15 µM Cd) (Table S2).

Plant uptake and tissue Cd concentrations

Plant tissues differed significantly in Cd concentration (Fig. 2). Roots had the highest Cd concentrations in both plant species. In the shoots, *C. rossii* had highest Cd concentrations in young leaves, followed by stems and old leaves, resulting in a two-fold difference in Cd concentration between young and old leaves. In comparison, *S. nigrum* had similar Cd concentrations in the various above-ground tissues.

For both species, shoot Cd concentrations, especially leaf Cd concentrations were significantly higher when supplied with NH_4^+ than when supplied with NO_3^- (Fig. 2). For example, Cd concentrations in leaves of *C. rossii* were 50-60% higher when supplied with NH_4^+ than with NO_3^-, irrespective of the Cd concentration in nutrient solution (except the old leaves at 5 μM Cd). Similarly, leaf Cd concentrations of *S. nigrum* were 50-70% (5 μM Cd) and 30% (15 μM Cd) higher in the plants supplied with NH_4^+ than with NO_3^-. In addition, stem Cd concentrations of *S. nigrum* were 23% higher in NH_4^+-fed than NO_3^--fed plants at 5 μM Cd but did not differ between the N forms at 15 μM Cd. In addition, NH_4^+ treatment tended to have higher Cd concentrations in roots of both plant species although the N effect was not statistically significant for either of them.

Accordingly, shoot Cd amounts were higher in plants supplied with NH_4^+ than with NO_3^- although the effect of N form was greater for *S. nigrum* than *C. rossii* (Fig. 3a). Indeed, shoot Cd amounts of *C. rossii* were 20% higher in plants supplied with NH_4^+ than with NO_3^- at 15 μM Cd (P<0.05), while those of *S. nigrum* were 60% (5 μM Cd) and 23% higher (15 μM Cd) in plants supplied with NH_4^+ than with NO_3^-. Overall, for a given treatment, *C. rossii* accumulated 3 times more Cd in shoots than *S. nigrum.* Similar trends were observed for total amounts of Cd taken up by plants and Cd uptake per unit root mass of both plant species (Fig. S1).

For *C. rossii*, the translocation factor (i.e. the shoot-to-root concentration ratio) was not altered by the N form at 5 μM Cd, but at 15 μM Cd it was 29% higher when supplied with NH_4^+ (Fig. 3b). In comparison, the translocation factor of *S. nigrum* was 47% higher when supplied with NH_4^+ than with NO_3^- only at 5 μM Cd but not at 15 μM Cd. Overall, the translocation factors were approximately twice as high for *S. nigrum* than for *C. rossii*, and were 80% higher at 15 μM Cd than at 5 μM Cd.

Reference compounds for XAS

The Cd K-edge XANES spectra of various Cd standard compounds were first examined visually to identify the most likely forms of Cd within the plant tissues. Given that all standards examined were prepared using Cd^{2+}, the Cd K-edge XANES spectra from all standards exhibited a similar absorption edge, with white-line peaks at ca. 26,719 to 26,722 eV. However, the ligand to which the Cd was bound resulted in slight shifts in these white-line peaks, with Cd bound to S-containing ligands generally having white-line peaks at ca. 26,719 eV, whilst the white-line peaks for Cd bound to O- or N-containing ligands were at ca. 26,720 eV. Not only were slight shifts in the white-line peak observed, but differences in the shape of the spectra were also evident. For S-containing ligands, the spectra were comparatively flat and with distinctive features (Fukuda *et al.*, 2008) (Fig. 4). Furthermore, the spectra of CdO and $CdCO_3$ exhibit characteristic spectral features, especially at ca. 26,729 and 26,736 eV.

Although the various types of ligands influenced the spectra, the spectra obtained for $Cd(NO_3)_2$ and for Cd complexed by various carboxyl groups (e.g., Cd-citrate, Cd-malate, Cd-succinate, Cd-polygalaturonate) were all very similar in their appearance. Likewise, the spectra for many of the S-containing ligands (such as CdS, Cd-cysteine, Cd-GSH, and Cd-PC2) were similar. Given that some spectra were largely indistinguishable from each other, hereafter, the Cd present as either free Cd (i.e. the $Cd(NO_3)_2$ standard) or as Cd complexed by carboxyl-containing compounds (i.e. citrate, malate, pectin) are grouped and referred to as "Cd-OH". Similarly, Cd-cysteine, Cd-PC2, Cd-GSH and CdS were grouped and referred to as "Cd-S".

Cd speciation in plant samples using XAS

In all hydrated samples of plants grown at 15 μM Cd, the tissue Cd concentrations were sufficiently high to allow collection of XANES data with a good signal to noise ratio (Fig. 5). The spectra of plant samples from 5 μM Cd treatments were collected using the freeze-dried samples due to the low Cd concentrations. Thus the spectra of freeze-dried and frozen hydrated roots of *C. rossii* grown in 5 μM Cd were compared and were similar, indicating that Cd speciation was not altered by the freeze-drying treatment (Fig. S2). In addition, the spectra for comparable tissues of plants exposed to different Cd levels showed strong similarities, and the LCF revealed that Cd levels in solution did not alter Cd speciation in plants.

Firstly, giving consideration to *C. rossii*, the spectra for both root and shoot tissues were visually similar to that of Cd-S, whilst the spectra of xylem sap clearly differed (Fig. 5). Indeed, LCF predicted that the majority (60-91%) of Cd in all plant tissues (other than the xylem sap) was associated with S-containing ligands, while Cd-OH was the dominant form (87-95%) in xylem sap (with the remaining 5-13% of Cd in the xylem sap associated with S) (Table 1). Although Cd-S dominated in all tissues of *C. rossii* other than xylem sap, there was generally a higher proportion (ca. 90%) of S-bound Cd in young leaves than in old leaves and stems (ca. 60-80%). Furthermore, the spectra were visually similar for comparable tissues irrespective of the N form, indicating the N-form did not alter Cd speciation, although LCF subsequently predicted that the proportion of Cd-OH in root tissues, xylem sap, and stems of NO_3^--fed plants was ca. 10% higher than that in NH_4^+-fed plants (Table 1).

Overall, the proportion of Cd-OH was higher in the shoots of *S. nigrum* (31-56%) than that observed for the shoots of *C. rossii* (9-40%). As observed for *C. rossii*, the proportion of Cd associated with S-containing ligands in *S. nigrum* differed between tissues, with higher levels in young leaves (ca. 70%) than in old leaves and stems (44-55%). Finally, it was noted again for *S. nigrum* that the form of N supplied did not influence Cd speciation in any tissues.

Discussion

Ammonium enhances Cd uptake and translocation

The present study did not support our hypothesis that the supply of NO_3^-, compared to NH_4^+, would increase Cd uptake in these two plant species. Instead, NH_4^+ increased Cd uptake and translocation to the shoots. It is evident that supply with NH_4^+ increased both the shoot Cd concentration, especially Cd concentration in the leaves, and Cd accumulation in shoots, relative to that observed when supplied with NO_3^-. This increased Cd concentration when supplied with NH_4^+ was not due to a 'dilution' effect because plant growth and biomass production were similar for the two N forms. Furthermore, the difference in shoot Cd uptake between two N forms did not result from changes in solution pH or solution Cd speciation because buffered solution pH was constant and Cd speciation similar for both the NH_4^+ and NO_3^- treatments (Table S3). Considering the different compositions of the two N-treated solutions, the potentials (Ψ_0^o) of and the Cd^{2+} activity ($\{Cd^{2+}\}_0^o$) at the cell membrane surface were calculated according to Wang *et al*. (2011), and only differed slightly between the NH_4^+ and NO_3^- treatments (Table S4). Thus, it appears that the increased uptake and accumulation of Cd in solutions containing NH_4^+ was not the result of altered bioavailability of Cd or plant growth, but rather, due to other processes which influenced Cd accumulation, including Cd uptake, partitioning within the root and efficiency of translocation from root to shoot (Clemens *et al.*, 2002; Clemens, 2006).

The higher Cd accumulation in the shoots of NH_4^+-fed plants could be partially attributed to the higher Cd uptake by the root. It was evident that supply with NH_4^+ increased not only the total Cd uptake but also the Cd uptake per unit of root biomass in both plant species (Fig. S1). There are a number of possible reasons for this. Firstly, it is known that N form can alter root morphology. In the present study, although the N form did not affect root biomass of *C. rossii*, root length and surface area were greater when supplied with NH_4^+ than when supplied with NO_3^- (Table S2) – this being consistent with the findings of Liu *et al.* (2015). It has been reported previously that root development is altered depending upon the N form, with NH_4^+ accelerating cell division and subsequent root branching (Bloom *et al.*, 2002). Thus, the NH_4^+-fed plants in the present study

might have had a higher capacity to acquire Cd. Secondly, the regulation of Zn transporters involved in Cd uptake might potentially be enhanced in roots of NH_4^+-fed plants. The uptake of Cd is likely to occur through ZIP transporters (Zn/Fe-regulated transporter-like proteins) and it has been shown that increasing concentrations of tissue N can enhance the abundance of Zn-uptake transporters in the root (Erenoglu *et al.*, 2011). Considering the higher N concentration in the roots of NH_4^+-fed plants (Rosen *et al.*, 1990; Hassan *et al.*, 2005), the higher Cd and Zn uptake in NH_4^+-fed plants might be due to increased Zn transporters.

Other than this higher uptake by the roots, the higher Cd accumulation in the shoots of NH_4^+-fed plants could be partially attributed to an increased efficiency of Cd translocation to the shoots. This was evidenced by examination of translocation factors, with these being higher in NH_4^+- than NO_3^--fed plants. This is also consistent with the observation that the Cd concentration of the xylem sap in NH_4^+-fed plants was 20% higher than in NO_3^--fed plants (11.51 and 9.03 mg L^{-1} at 15 μM Cd, respectively). Xylem loading of Cd has been shown to be mediated by the P-type ATPase transporter *AtHMA4* in different plant species like *Arabidopsis thaliana, A. halleri, N. caerulescens and S. nigrum*, and overexpression of *AtHMA4* enhances Zn and Cd levels in leaves (Verret *et al.*, 2004; Verbruggen *et al.*, 2009; Xu *et al.*, 2012). The addition of NH_4^+, compared to NO_3^-, might also elevate the expression of Cd transporter *AtHMA4* and then increase Cd translocation, but this still needs further study.

Our finding that NH_4^+ increased Cd uptake and accumulation in both *C. rossii* and *S. nigrum* is consistent with that observed in lettuce (*Lactuca sativa* L.) (Florijn *et al.*, 1992), but differs from that reported for tomato (*solanum tuberosum* L.) and hyperaccumulators *S. plumbizincicola* and *N. caerulescens*, where NO_3^- increased Cd accumulation in shoots (Xie *et al.*, 2009; Luo *et al.*, 2012; Hu *et al.*, 2013). There are several possible reasons to explain the apparent discrepancies between these studies. First, plant species differ in their growth response to N form. In the present study, plant biomass was not affected by N form, consistent with the result of Liu *et al.* (2015) when *C. rossii* was grown in an acid soil. In other studies, however, NH_4^+ significantly decreased the growth of *Solanum lycopersicum*, *S. plumbizincicola* and *N. caerulescens* (Xie *et al.*, 2009; Luo *et al.*, 2012; Hu *et al.*, 2013), with this decreased growth potentially altering Cd uptake. Second, plant species differ in their mechanisms of Cd detoxification. For example, in *S. alfredii* (which belongs to same genus as *S. plumbizincicola*), and *N. caerulescens*, Cd is mainly coordinated with O-ligands (Kupper *et al.*, 2004; Tian *et al.*, 2011), whilst in *C. rossii* and *S. nigrum*, Cd was mainly associated with S-ligands. Thus, it may not be unexpected that the effect of N form differs between these plant species, given that NO_3^- nutrition enhances the concentration of organic acids (O-ligands) (which are important for Cd uptake and tolerance in *S. plumbizincicola* and *N. caerulescens*) whilst NH_4^+ nutrition increases amino acids and proteins (with Cd-S compounds important in *C. rossii* and *S. nigrum*). Third, plant species may differ in the mechanisms used to balance ion uptake when different N forms are applied. In the previous studies, NO_3^--fed plants accumulated more cations (including Cd) because of the antagonistic effects between NH_4^+ and Cd^{2+} (Xie *et al.*, 2009; Hu *et al.*, 2013), or the higher expression of *IRT1* in NO_3^--fed plants than NH_4^+-fed plants (Luo *et al.*, 2012). However, in the present study, significantly higher concentrations of Zn and Cd were found in NH_4^+-fed plants, with concentrations of other cations, including Fe, not affected by N form (Table S5).

Nitrogen form did not affect Cd speciation

Nitrogen form did not greatly affect Cd speciation in plant tissues, irrespective of treatment except the proportions of Cd-S in some tissues of *C. rossii* being slightly lower in NO_3^--fed plants (Table 1). These results indicate that the increased accumulation of Cd when supplied with NH_4^+ could not be ascribed to alterations in Cd speciation. This observation differs from the previously proposed (i.e. that differences in Cd concentrations and translocation in plants supplied with different N forms might result from differences in metabolites, such as organic acids and amino acids). For example, it is known that NO_3^- nutrition increases the concentration of organic acids in plants in

order to maintain the intercellular pH (Marschner, 2011). Our observation that the N form did not alter speciation is similar to those observed previously for Zn-hyperaccumulator *N. caerulescens* that N form did not alter Zn speciation in different tissues despite NO_3^- enhancing the production of organic acids (Monsant *et al.*, 2011; White-Monsant and Tang, 2013). Therefore, the formation of Cd-organic acid complexes in plants may not depend on the concentrations of organic acids, but rather, the efficient tonoplast transporters to facilitate Cd transport from the cytoplasm into vacuoles (Ueno *et al.*, 2005).

Plant species and tissues differ in their Cd speciation

Although Cd-S was the dominant Cd species in shoots of both plant species, the proportion of Cd-S in all the above-ground tissues was generally higher for of *C. rossii* (60-90%) than for *S. nigrum* (45-70%) (Table 1). Thus, O-containing ligands tended to be more important in *S. nigrum* than for *C. rossii* – this observation for *S. nigrum* being similar to that reported previously (Sun *et al.*, 2006). These differences are not unexpected, with Cd speciation reported to differ widely between species (Castillo-Michel *et al.*, 2009; Tian *et al.*, 2011; Huguet *et al.*, 2012; Lefevre *et al.*, 2014) and even between ecostypes of the same species (Ebbs *et al.*, 2009).

Not only did Cd speciation differ between plant species, but it also differed between tissues. Firstly, Cd was clearly bound to S-ligands in the roots of both plant species, with similar observation reported previously for other species, including corn (*Zea mays*), Indian mustard (*Brassica juncea* L.), and *A. thaliana* (Salt *et al.*, 1995; Isaure *et al.*, 2006; Castillo-Michel *et al.*, 2009). The high proportion of Cd-S in roots might explain the great Cd concentration in roots than shoots in both plant species, as Cd translocation was restricted by the complexation of Cd with strong ligands and sequestration in the vacuole of root cells. Secondly, the low proportion of Cd-S in the stems of both plant species is inconsistent with the finding of Kupper *et al*. (2004) who found a high concentration of Cd-S in stems of *N. caerulescens* and proposed that Cd was transported as PCs or MTs complexes. However, in the present study, analyses of the xylem sap of *C. rossii* showed that the free Cd^{2+} ion dominated in the xylem sap, although concentrations in other aerial tissues were low. This indicates that Cd was transported as either the free ion or complexed with carboxyl groups before being chelated by S-containing compounds, which may explain the low Cd-S proportion in stems. Moreover, the importance of free hydrated Cd^{2+} ions and/or Cd-O/N complexes for Cd translocation through xylem sap has also been found previously in other Cd-hyperaccumulating and non-hyperaccumulating species (Salt *et al.*, 1995; Ueno *et al.*, 2008; Hazama *et al.*, 2015). Thirdly, young leaves tended to have a higher proportion of Cd bound to S-containing ligands than did old leaves. This decrease in Cd-S with increasing leaf age has also been reported in *N. caerulescens* (Kupper *et al.*, 2004). The higher proportion of Cd-S in young than old leaves coincided with the accumulation of more Cd in epidermal cells of young leaves but in the mesophyll cells of old leaves, both of which would protect the highly sensitive metabolic processes in the young leaves from Cd^{2+} damage (Koren *et al*., 2013, Kupper *et al*., 1999).

Conclusions

This study used synchrotron-based XAS to investigate how the N form influences Cd speciation and subsequent Cd accumulation in two newly-defined Cd hyperaccumulators – this has not been examined previously. It has been demonstrated that the supply of NH_4^+, relative to NO_3^-, increased the shoot Cd accumulation by 30% in two native Australian plant species, *C. rossii* and *S. nigrum*, when grown in nutrient solution. Interestingly, this increase in Cd accumulation did not result from changes in Cd speciation within the plant tissues. Regardless, Cd speciation differed between the plant species and between tissues, with a higher proportion of Cd-S in the shoots of *C. rossii* than for *S. nigrum* and more Cd storing as Cd-S in roots and shoots but translocating as Cd-OH in xylem saps. In addition, *C. rossii* accumulated three times more Cd in shoots than *S. nigrum*. Thus, the application of NH_4^+-based fertilizers would potentially favor phytoextraction of Cd by *C. rossii* and *S. nigrum*, and *C. rossii* is a better candidate for phytoextraction in Cd-contaminated soils.

However, further work is required to confirm these findings, including experiments in soil- and field-based conditions.

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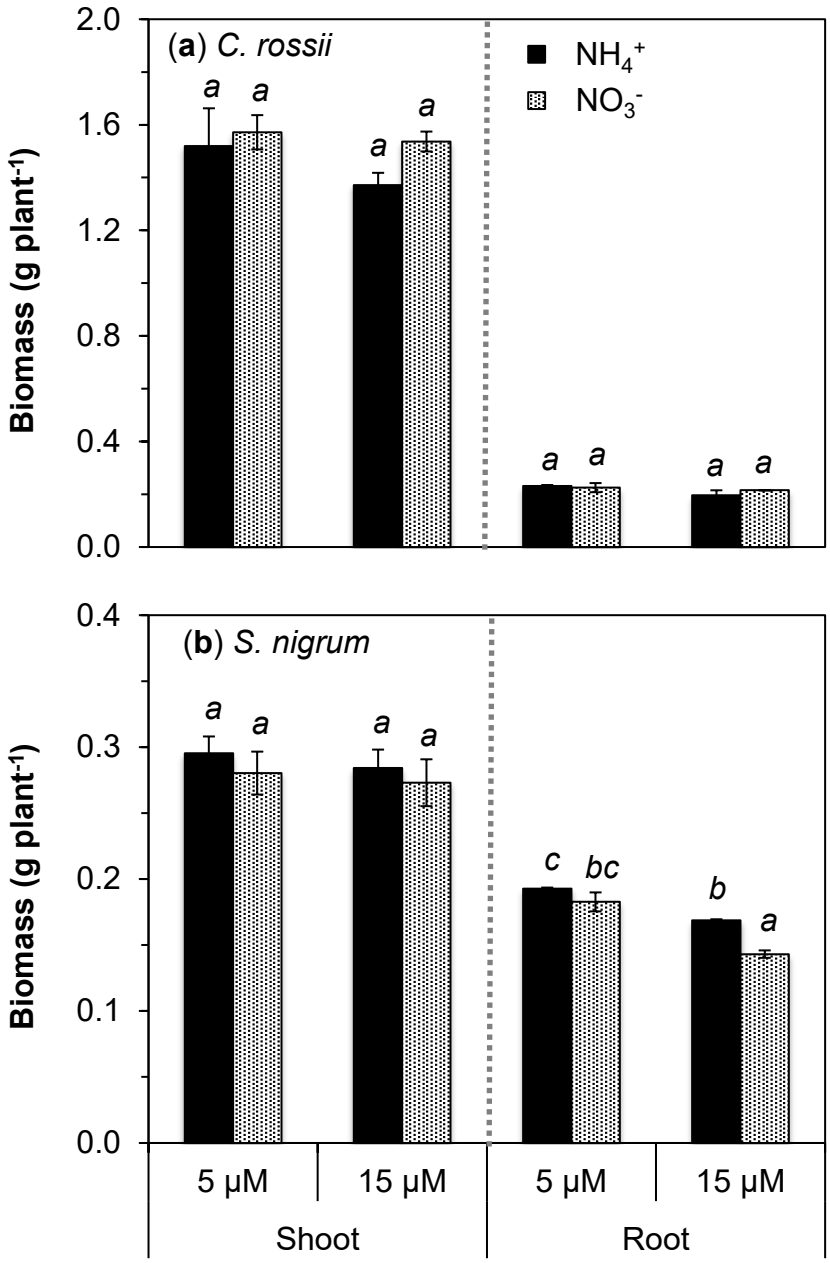

Figure 1. Effects of N forms on dry weights of *Carpobrotus rossii* (a) and *Solanum nigrum* (b) grown for 14 d in solutions containing Cd at a concentration of either 5 or 15 μM. Bars represent the standard errors (n = 3). For each panel, different letters above the bars indicate significant differences among treatments of each tissue of individual species (Tukey's test, $P<0.05$)

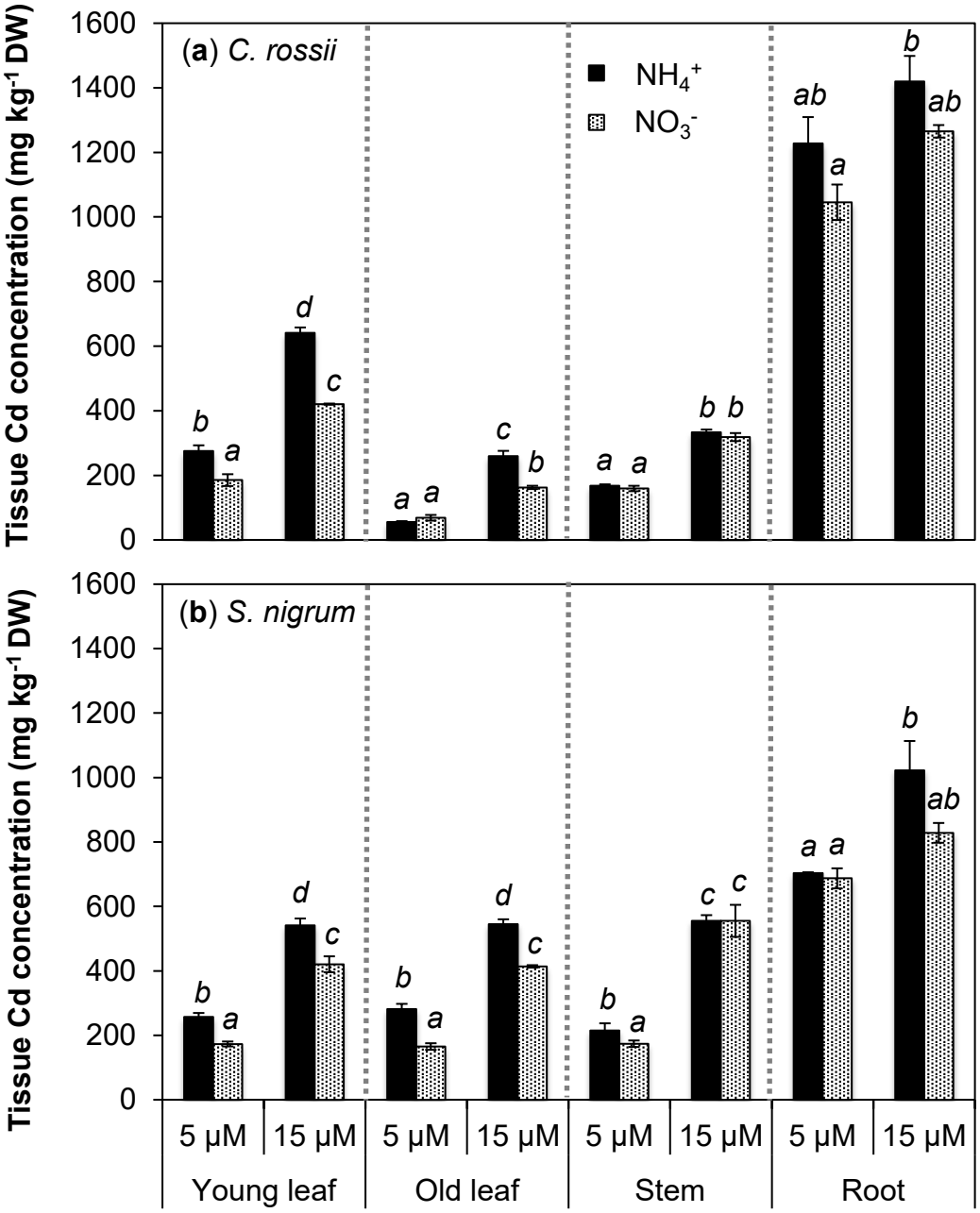

Figure 2. Effects of N form on Cd concentrations in the different tissues of *Carpobrotus rossii* (a) and *Solanum nigrum* (b) grown for 14 d in solutions containing Cd at a concentration of either 5 or 15 μM. Bars represent the standard errors (n = 3). For each panel, different letters above the bars indicate significant differences among treatments of each tissue of individual species (Tukey's test, $P<0.05$).

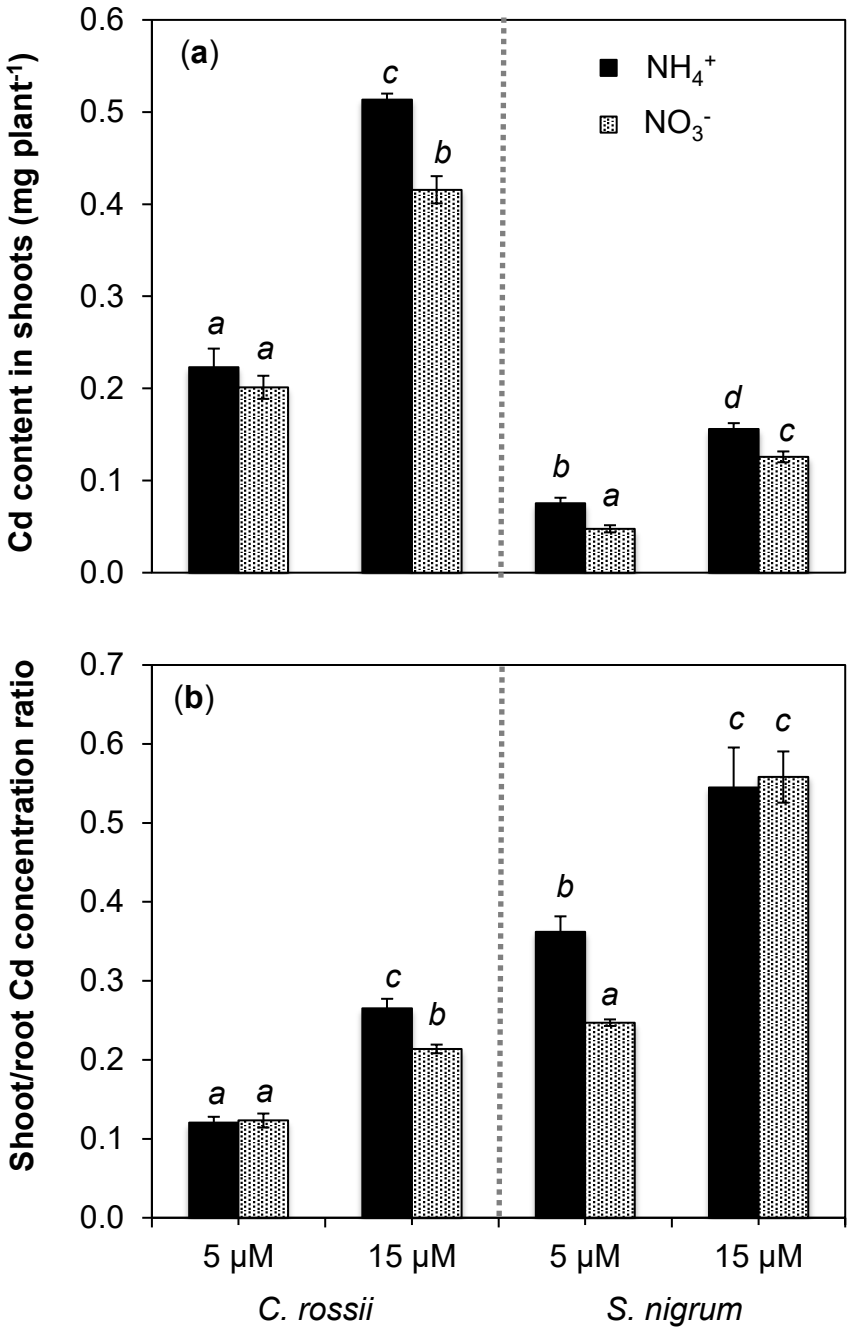

Figure 3. Effects of N form on Cd shoot content (a) and shoot-to-root Cd concentration ratio (translocation factor) (b) of *Carpobrotus rossii* and *Solanum nigrum* grown for 14 d in solutions containing Cd at a concentration of either 5 or 15 µM. Bars represent the standard errors (n = 3). For each panel, different letters above the bars indicate significant differences among treatments of individual species (Tukey's test, $P<0.05$).

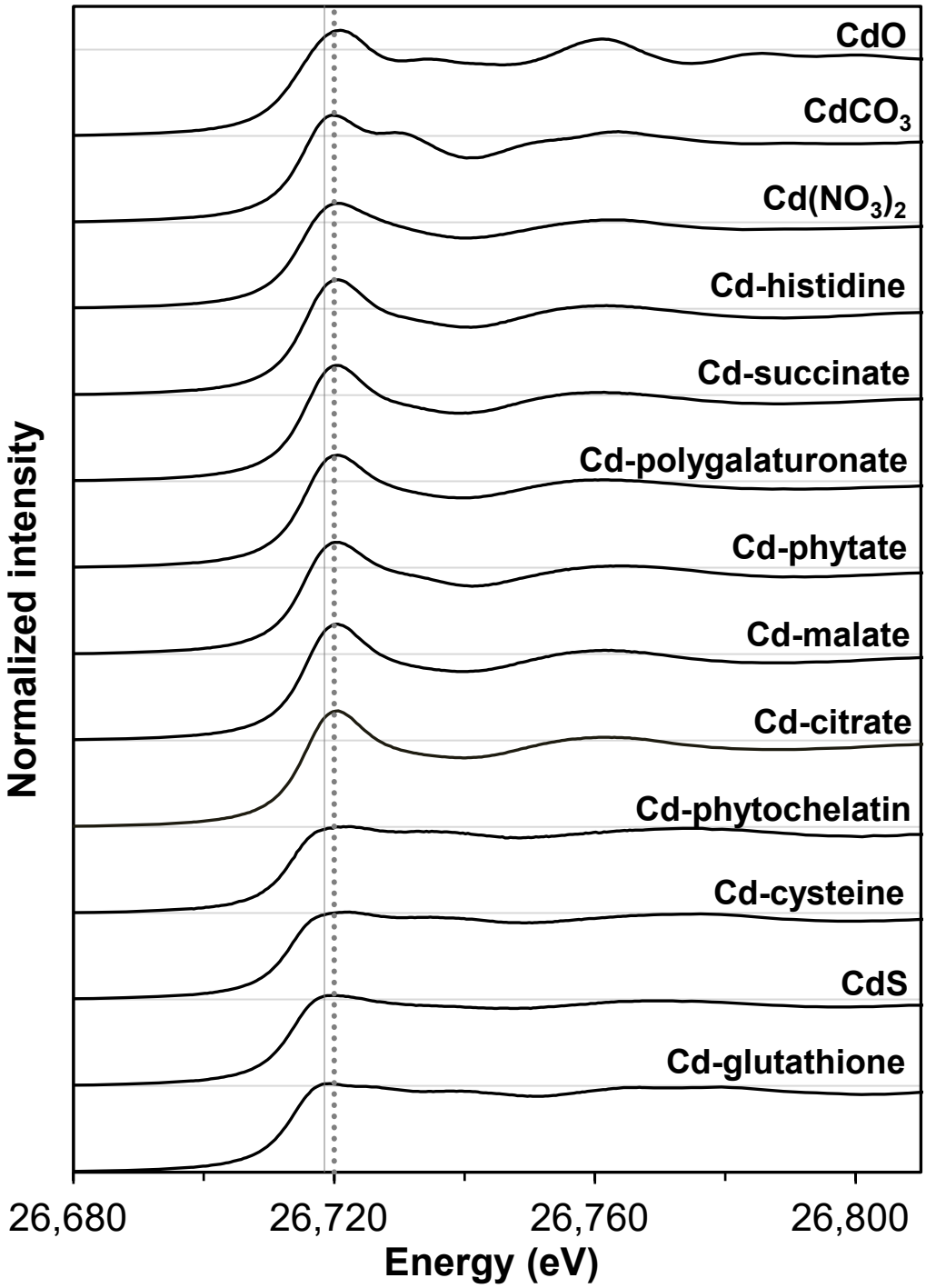

Figure 4. Normalized K-edge XANES spectra of the Cd standards. The horizontal grey lines represent a value of 1 for each of the normalized spectra, while the vertical grey lines represent white-line peaks of Cd-S (solid line) and Cd-OH (dotted line) standards.

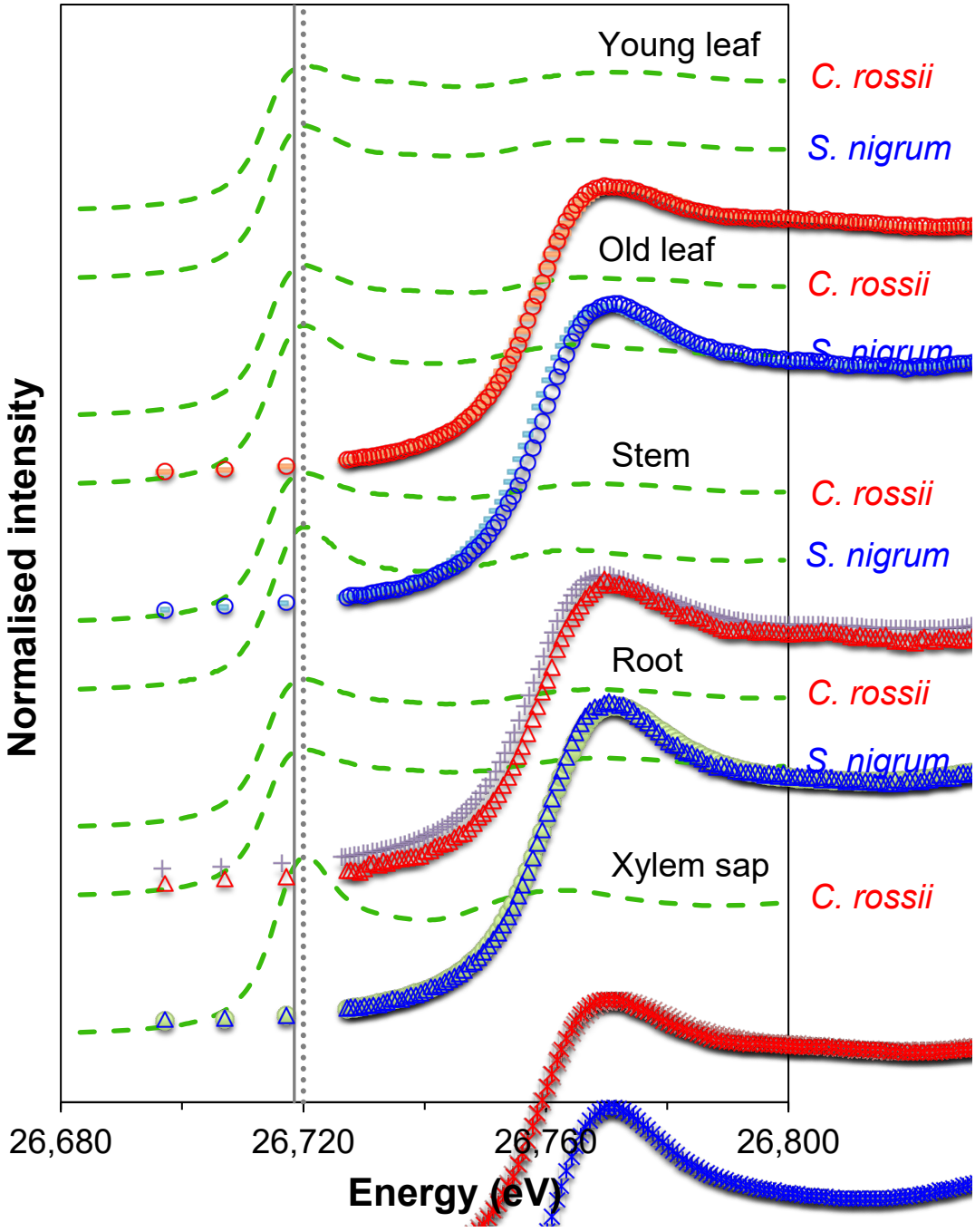

Figure 5. Normalized Cd K-edge XANES spectra for various tissues of two Cd hyperaccumulator plants (*Carpobrotus rossii* and *Solanum nigrum*) grown in nutrient solutions containing $(NH_4)_2SO_4$ and 15 μM Cd. Dashed green lines show the best fits of reference spectra obtained using linear combination fitting (LCF) and the vertical grey lines represent white-line peaks of Cd-S (solid line) and Cd-OH (dotted line) standards.

Table 1. The predicted speciation of Cd in various tissues of plants of *Carpobrotus rossii* and *Solanum nigrum* grown for 14 d in nutrient solutions containing 15 µM Cd as calculated using linear combination fitting (LCF) of the K-edge XANES spectra.

Treatment	*C. rossii*			*S. nigrum*		
	Cd-S (%)	Cd-OH (%)	R-factor	Cd-S (%)	Cd-OH (%)	R-factor
Young leaf						
$(NH_4)_2SO_4$	89 (0.8)	11 (1.2)	0.00012	68 (0.6)	32 (0.9)	0.00006
KNO_3	91 (0.8)	9 (1.7)	0.00011	69 (1.0)	31 (0.6)	0.00006
Old leaf						
$(NH_4)_2SO_4$	75 (0.8)	25 (1.4)	0.00010	55 (1.0)	45 (1.0)	0.00019
KNO_3	75 (1.1)	25 (1.3)	0.00020	44 (1.0)	56 (1.0)	0.00018
Stem						
$(NH_4)_2SO_4$	79 (0.8)	21 (2.2)	0.00013	45 (0.8)	55 (0.8)	0.00012
KNO_3	60 (0.8)	40 (1.6)	0.00010	46 (0.8)	54 (0.8)	0.00010
Root						
$(NH_4)_2SO_4$	80 (1.0)	20 (1.5)	0.00017	83 (0.6)	17 (1.1)	0.00006
KNO_3	68 (0.6)	32 (1.6)	0.00008	85 (1.0)	15 (1.0)	0.00005
Xylem sap						
$(NH_4)_2SO_4$	13 (0.9)	87 (1.9)	0.00014			
KNO_3	5 (0.7)	95 (1.8)	0.00010			

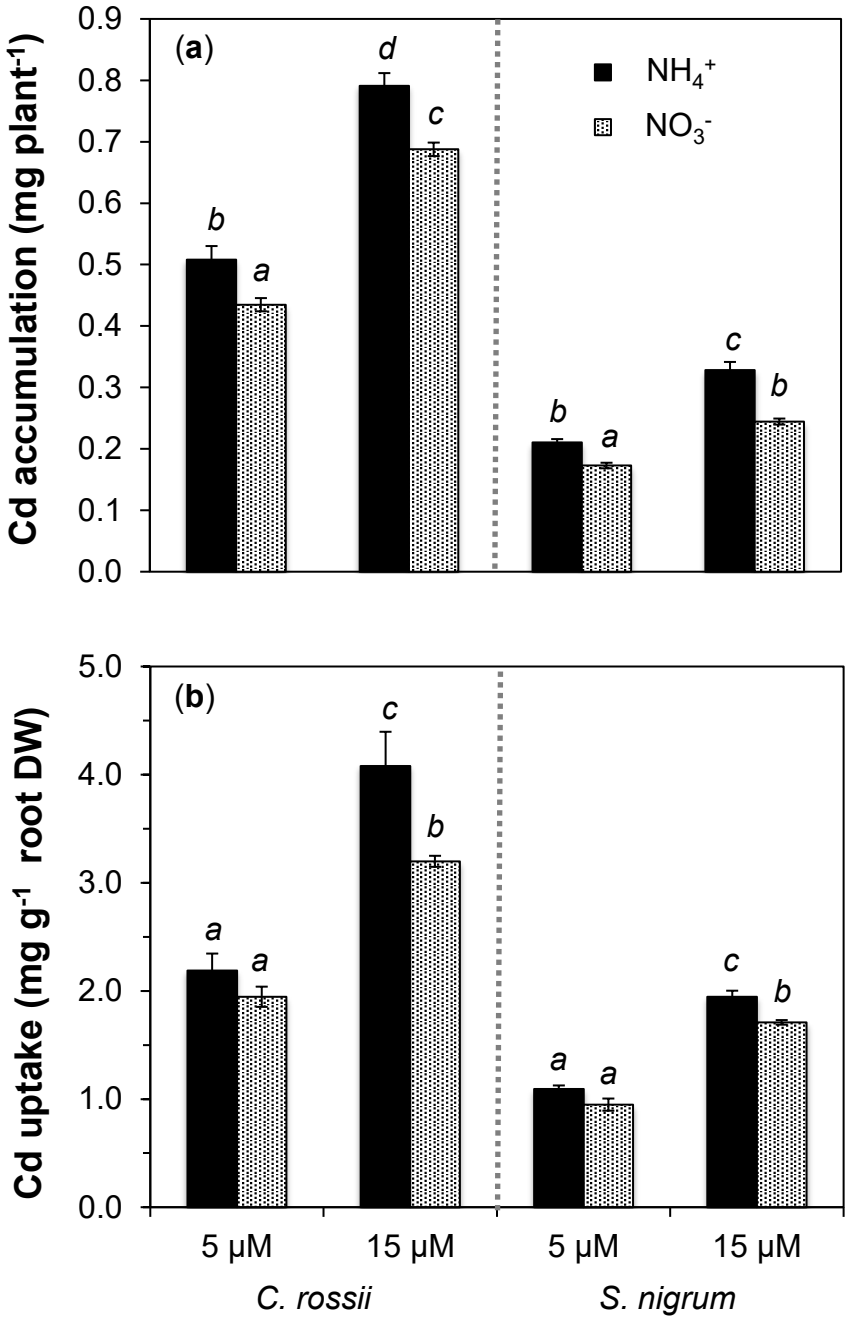

Figure S1. Effects of N form on Cd total content (a) and Cd uptake per root dry weight (b) of *Carpobrotus rossii* and *Solanum nigrum* grown for 14 d in solutions containing Cd at a concentration of either 5 or 15 μM. Bars represent the standard errors (n = 3). For each panel, different letters above the bars indicate significant differences among treatments of each tissue of individual species (Tukey's test, $P<0.05$).

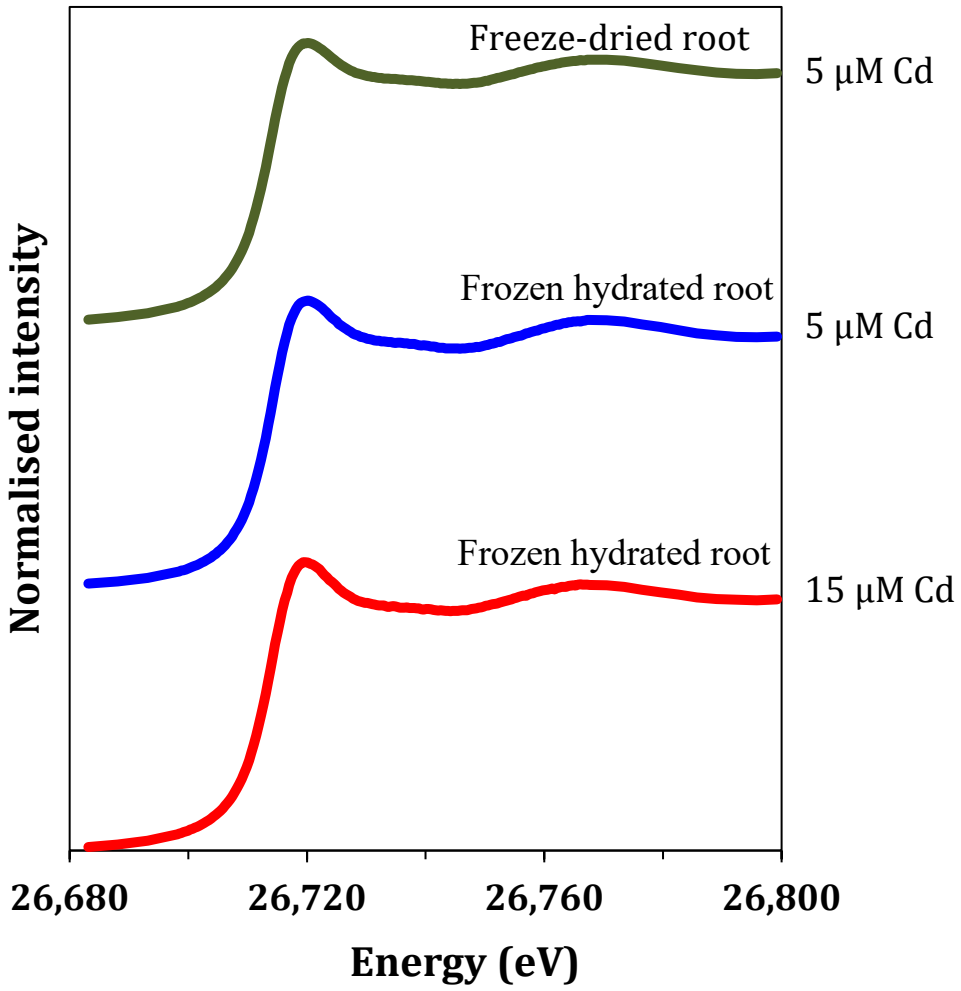

Figure S2. Normalized Cd K-edge XANES spectra for freeze-dried roots and frozen hydrated roots of NH_4^+-fed *Carpobrotus rossii*.

Table S1. Target transformation SPOIL values of selected reference spectra obtained by principle component analysis (PCA). Reference spectra are classified as excellent (SPOIL < 1.5), good (1.5-3.0), acceptable (3.0-4.5), poor (4.5-6.0), or unacceptable (> 6.0). The standard compounds with SPOIL values < 3 were included in the subsequent linear combination fitting (LCF) analyses.

	References	SPOIL VALUES
1	Cd-citrate	1.913
2	Cd-glutathione	1.977
3	Cd-malate	1.999
4	Cd-phytochelatin	2.085
5	Cd-succinate	2.113
6	$Cd(NO_3)_2$	2.402
7	Cd-cysteine	2.388
8	Cd-polygalaturonate	2.815
9	CdS	2.951
10	Cd-metallothinein	4.026
11	Cd-phytate	4.425
12	Cd-histidine	7.827
13	CdO	10.184
14	$CdCO_3$	15.046

Table S2. Root length and surface area per plant of *Carpobrotus rossii* and *Solanum nigrum* grown for 14 d in solutions containing either 5 or 15 µM Cd. Data are means ± standard errors (n = 3). The means followed by a same letter do not differ significantly within a column (Tukey's test, $P<0.05$).

Treatments	*C. rossii*		*S. nigrum*	
	Root length (m plant^{-1})	Root surface (cm^2 plant^{-1})	Root length (m plant^{-1})	Root surface (cm^2 plant^{-1})
NH_4^+ + 5 µM Cd	72.7 ± 3.5 c	633 ± 26 b	46.8 ± 2.7 c	617 ± 18 c
NO_3^- + 5 µM Cd	59.3 ± 2.6 ab	501 ± 25 a	38 ± 1.7 b	525 ± 14 b
NH_4^+ + 15 µM Cd	61.3 ± 1.5 b	488 ± 17 a	32.7 ± 1.5 b	525 ± 16 b
NO_3^- + 15 µM Cd	52.7 ± 1.5 a	429 ± 19 a	23.3 ± 2.0 a	417 ± 9 a

Table S3. Speciation of Cd in the nutrient solution of different treatments.

Treatments	Cd^{2+}	$CdCl^{+}$	$CdSO_4$ (aq)	$CdHPO_4$ (aq)	$CdEDTA^{2-}$
NH_4^+ + 5 µM Cd	83.31	7.11	6.80	0.17	2.49
NO_3^- + 5 µM Cd	83.62	6.95	6.49	0.16	2.49
NH_4^+ + 15 µM Cd	83.84	7.27	6.83	0.17	1.78
NO_3^- + 15 µM Cd	84.15	7.10	6.52	0.16	1.78

Values were estimated according to the Visual MINTEQ software and expressed as percentages of the total Cd added to the nutrient solution.

Table S4. The calculated Cd^{2+} activities in the bulk treatment solution ($\{Cd^{2+}\}_b$), the cell membrane surface potentials ($\Psi_0{}^o$), and Cd^{2+} activities at the cell membrane surface ($\{Cd^{2+}\}_0{}^o$) in the different treatments.

Treatments	$\{Cd^{2+}\}_b$ (µM)	$\Psi_0{}^o$ (mV)	$\{Cd^{2+}\}_0{}^o$ (µM)
NH_4^+ + 5 µM Cd	3.0	-40.7	71
NO_3^- + 5 µM Cd	2.9	-40.3	67
NH_4^+ + 15 µM Cd	9.0	-37.9	172
NO_3^- + 15 µM Cd	8.8	-37.1	159

Table S5. Concentrations of Ca, Mg, Zn and Fe in shoots of *Carpobrotus rossii* and *Solanum nigrum* grown for 14 d in solutions containing Cd at a concentration of either 5 or 15 µM. Data are means ± standard errors (n = 3).

Plant species	Cd (µM)	N treatment	Ca (g kg^{-1})	Mg (g kg^{-1})	Zn (mg kg^{-1})	Fe (mg kg^{-1})
C. rossii	5	NH_4^+	35.2 ± 2.1	7.6 ± 0.3	150 ± 28	194 ± 22
		NO_3^-	36.1 ± 2.4	6.9 ± 0.3	153 ± 13	226 ± 36
	15	NH_4^+	46.2 ± 2.7	7.3 ± 0.2	210 ± 15	116 ± 2
		NO_3^-	39.3 ± 3.6	5.7 ± 0.3	156 ± 4	120 ± 7
S. nigrum	5	NH_4^+	18.0 ± 1.0	5.9 ± 0.2	108 ± 13	41 ± 10
		NO_3^-	20.0 ± 0.6	6.4 ± 0.1	90 ± 7	27 ± 17
	15	NH_4^+	18.9 ± 0.9	7.0 ± 0.3	94 ± 5	106 ± 1
		NO_3^-	21.9 ± 2.9	7.0 ± 0.5	79 ± 25	117 ± 24