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REGULAR ARTICLE

Variation in cadmium accumulation and speciation within the same population of the hyperaccumulator *Noccaea caerulescens* grown in a moderately contaminated soil

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Abstract

Background and aims Phytoextraction is an eco-friendly approach for remediation of heavy metal contaminated soil. The aim is to screen *Noccaea caerulescens* lines with higher cadmium (Cd) phytoextraction efficiency and investigate differences in Cd species and distribution in the leaves of high and low Cd accumulating lines.

Methods Biomass production and Cd bioaccumulation capacities of 29 *Noccaea caerulescens* lines, generated through single-seed-descent from a Cd hyperaccumulating calamine population, were assessed in a pot experiment with a moderately Cd contaminated soil (2.1 mg Cd kg^{-1}). Synchrotron-based techniques were employed to identify and characterize Cd speciation and distribution in *Noccaea caerulescens* leaves.

Results The largest biomass of *Noccaea caerulescens* reached 5.0 ± 3.3 g (D. W. pot^{-1}) after 6 months growth. The Cd concentrations in shoots varied from 85 to 203 mg kg^{-1}. The most efficient line removed 0.64 mg Cd pot^{-1} and lowered the total Cd in soil by 30%. Synchrotron-based X-ray absorption

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spectroscopy showed that the dominant Cd species was Cd-thiol complexes. Cadmium-carboxyl and Cd-phytate/phosphate were present in the leaves of high and low Cd accumulating lines, respectively. Micro X-ray fluorescence microscopy showed cadmium was concentrated in leaf veins.

Conclusions There are wide variations including both biomass production and Cd accumulation capacity among different lines within the same calamine ecotype of *Noccaea caerulescens*. Cadmium-thiol complexes play the most important role in Cd detoxification in leaves of *Noccaea caerulescens* grown in moderately Cd contaminated paddy soil. These findings provide a physiological basis for breeding high Cd accumulation varieties of *Noccaea caerulescens*.

Keywords *Noccaea caerulescens* · Hyperaccumulation · Phytoextraction · Cadmium speciation · X-ray fluorescence microscopy

Introduction

Human activities, such as mining, smelting, waste disposal, and industrial processing cause the redistribution of metals on the earth's surface. Cadmium (Cd) contamination is widespread and of significant environmental concern due to high bioaccumulation (Zhao et al. 2015; Tóth et al. 2016; Argüello et al. 2019; Wang et al. 2019). The mobility of Cd in soil-plant systems is relatively high, especially in aerobic and low pH conditions (Römkens et al. 2009; de Livera et al. 2011). Cadmium can accumulate in the human body through the food chain and can be retained in the human body for up to 10–30 years (Nordberg et al. 2014). Accordingly, even low levels of chronic Cd exposure can lead to diseases such as osteoporosis and renal dysfunction (Järup et al. 1998; Järup and Åkesson 2009). Cadmium is also associated with cancers of lung, prostate, urinary bladder, renal endometrial and breast (Nawrot et al. 2006; Huff et al. 2007; Järup and Åkesson 2009). Cadmium contamination in agricultural soil has posed a serious threat to food safety and human health. Hence, it is essential to remove Cd from contaminated agricultural soils.

In recent years, great efforts have been made in developing soil remediation techniques, including physical, chemical, and biological methods such as soil isolation, soil washing and phytoextraction (Gray et al. 2006; Bhargava et al. 2012; Bolan et al. 2014; Chaney and Baklanov 2017; Qayyum et al. 2020). Phytoextraction is an eco-friendly soil remediation technique, which uses metal-accumulating plants to remove contaminant metals from soil (Bhargava et al. 2012; Suman et al. 2018). Phytoextraction is realistically only feasible for moderately contaminated soils (Zhao et al. 2022), which are typical of Cd-contaminated agricultural soils in some areas in southern China that often lead to exceedance of the Cd limits in food crops (Zhao et al. 2015). To be efficient, this method requires both high biomass and metal hyperaccumulation (McGrath and Zhao 2003). Therefore, hyperaccumulators are good candidates for phytoextraction, due to their extraordinary ability to accumulate metals in aboveground biomass. Plants that contain over 100 mg kg^{-1} cadmium in their dry weight foliar tissue in their natural habitat are defined as Cd hyperaccumulators (Reeves et al. 2018). Nine Cd hyperaccumulators have been identified so far (Krämer 2010; Rosenfeld et al. 2018). *Noccaea caerulescens* (*N. caerulescens*, formerly *Thlaspi caerulescens*) is a well-known Zinc (Zn)/Cd/Nickel (Ni) hyperaccumulator (Baker et al. 1994; Reeves et al. 2001; Assunção et al. 2003; Kozhevnikova et al. 2020). *Noccaea caerulescens* is particularly abundant in parts of Europe (Krämer 2010), and can accumulate up to 0.1–0.4% Cd in the shoots, which makes it a potential candidate for effective phytoextraction of Cd from Cd-contaminated soil (Robinson et al. 1998; Reeves et al. 2001; Zhao et al. 2003).

Comparisons among different ecotypes of *N. caerulescens* showed that there are great variations in Cd accumulation abilities (Lombi et al. 2000; Lovy et al. 2013; Jacobs et al. 2018a, b; Kozhevnikova et al. 2020). For example, the Ganges accession (of the Cd hyperaccumulating calamine ecotype found in the Cevennes region in France) usually accumulate several times more Cd in shoots than accessions of other ecotypes, and is also highly Cd tolerant (Lombi et al. 2000). In a field experiment, Jacobs et al. (2018b) found that Cd uptake of Ganges reached 260 g ha^{-1} after 12-month growth, while the non-metallicolous accession Wilwerwiltz, from Luxemburg, accumulated 40 g ha^{-1}. Lombi et al. (2000) compared the bioaccumulation abilities of four *N. caerulescens* accessions in a pot experiment and found that the Cd accumulation increased with increasing soil Cd concentration, and the highest leaf Cd concentration reached 2,800 mg kg^{-1} in the Ganges accession, 5-fold higher than the Zn hyperaccumulating calamine accession Whitesike from the UK. In a hydroponic study, Kozhevnikova et al. (2020) compared the patterns of natural variation in Cd accumulation capacities among 28 accessions of *N. caerulescens*, and found the accessions from calamine populations showed significantly higher variations than the accessions from non-metallicolous or ultramafic populations. Although studies have shown that the Ganges accession is far superior in Cd accumulation to several other accessions (Lombi et al. 2000, 2002; Jacobs et al. 2018a), it is still unclear whether there is a difference in the Cd accumulation capacities between different individuals of the population in the Cevennes region from where the Ganges accession originated. Thus, a further investigation of the variations in Cd accumulation among different lines from this Cd hyperaccumulating Cevennes population is required.

In recent years, synchrotron-based techniques such as X-ray absorption spectroscopy (XAS) and X-ray fluorescence microscopy (μ-XRF) are increasingly utilized in soil–plant systems to analyze metal speciation and distribution in biological samples (Kopittke et al. 2017). X-ray absorption spectroscopy includes two complementary techniques: the extended X-ray absorption of fine structure (EXAFS), which provides information on the local environment of investigated atoms in the sample, and X-ray absorption near-edge structure (XANES), which provides information concerning the chemical oxidation stage and coordination geometry of elements in complexes (Gardea-Torresdey et al. 2005). The EXAFS technique is an element-specific method that is particularly suited for analysing the in vivo ligand environment of Cd and Zn in plants (Küpper et al. 2004; Lu et al. 2014). XAS investigations have already been used for the determination of Cd ligand environments in the roots, stems, leaves and seeds of Cd-hyperaccumulating plants (Küpper et al. 2004; Ueno et al. 2005; Vogel-Mikuš et al. 2010; Koren et al. 2013; Isaure et al. 2015) both found Cd-organic acids were the dominant Cd species in *N. caerulescens*. Species of Cd bound to sulfur ligands (e.g. phytochelatins) were also detected in both leaves and stems in the Ganges accession with proportions of 20–35% (Küpper et al. 2004). A similar Cd ligand environment that Cd-O ligands prevailed over the Cd-S ligands in leaves, was also found in the related Cd hyperaccumulator *Noccaea praecox* and in *Arabidopsis halleri* (Koren et al. 2013; Isaure et al. 2015). In addition, distribution of Cd in leaves of *N. caerulescens* has also been studied, which indicated that most Cd was mainly concentrated at the edge of the leaves and in localized spots of higher concentration distributed throughout leaf surface (Cosio et al. 2005). It is worth noting that these studies on Cd speciation in hyperaccumulators were performed on plants that were grown in hydroponic conditions with very high levels of Cd (Küpper et al. 2004; Cosio et al. 2005; Ueno et al. 2005; Koren et al. 2013; Isaure et al. 2015).

In the present study, Cd bioaccumulation of 29 different *N. caerulescens* lines generated from individuals collected from the Cd-hyperaccumulating and Cd-hypertolerant population at the site of a former zinc smelter in Les Avinières, South Cevennes, France (Mahieu et al. 2011), was compared in a pot experiment with a moderately Cd-contaminated soil to evaluate the variations in biomass production and Cd accumulation capacity. To further elucidate the Cd detoxification mechanism in leaves of *N. caerulescens* grown in moderately Cd-contaminated soil, synchrotron radiation techniques, such as bulk XAS coupled with Linear combination fitting (LCF), were used to compare the differences in Cd speciation and distribution in leaves between two high and two low Cd accumulating *N. caerulescens* lines.

Materials and methods

Plant materials

Twenty-nine lines of *Noccaea caerulescens* were used. They are derived from 11 individual plants originating from an abandoned zinc smelter site at Les Avinières, France (43° 56’ 11.2’’ N, 3° 40’ 17.2’’ E)(Mahieu et al. 2011), also supposedly the origin of the Ganges accession (Jacobs et al. 2018a). Plants at this site belong to the Cd hyperaccumulating calamine ecotype (Kozhevnikova et al. 2020). Initially, from a set of around 25 seed samples, each sample collected from an individual plant at the site, five progeny plants were grown from each sample. These were numbered “AV-#-#” with the first # indicating the original sampled plant and the second # the plant grown from the seed sample. While several progenies displayed very poor seed set, progeny of 11 original individuals showed at least a few plants with high fecundity in the greenhouse. Most progenies display variation in plant morphology between plants, suggesting at least part of the sampled seed resulted from outbreeding. Plants with moderate to high seed production were harvested, and inbred for two generations of single seed descent, favouring plants with high fecundity and biomass. The seeds of the resulting 29 lines were used for this experiment. Two generations of inbreeding, and selection for fecundity, reduced the initially observed within-line variation, to yield phenotypically similar plants per line, while potentially maintaining the between-line variation.

Pot experiment

A Cd contaminated soil was collected from a paddy field (0–15 cm) in Xiangtan, Hunan Province, China. This paddy field has been suffering from Cd contamination due to rapid industrialization and agricultural intensification over the past four decades. Specifically, the Cd pollution is mainly caused by the discharge of pollutants from nearby chemical industry and steel plants and sewage irrigation in the Xiang River from the 1980s to the 2000s (Li et al. 2018). The soil contains 2.1 and 118 mg kg^{-1} of total Cd and Zn, respectively, and the soil pH is 6.4. The concentration of DTPA extractable Cd is 1.1 mg kg^{-1}. *Noccaea caerulescens* seeds were germinated in a petri dish filled with moistened vermiculite in the dark. After 10 days, three seedlings of each line were transferred to one pot filled with 1 kg air-dried soil. Each line was replicated in 3 pots. Plants were grown in a greenhouse with the following conditions: temperature 20–25 ^{o}C, natural sunlight supplemented with sodium vapour lamps to maintain light intensity > 300 µmol m^{-2} s^{-1} and a photoperiod of 12 h per day. Plants were watered every 3 days or as needed to maintain adequate soil moisture. The plants were cultured for six months and as they were not vernalized, remained in the rosette stage until harvesting. The plant shoots were harvested, washed with deionized water and blotted dry. Five leaves taken from the centre of rosette from 5 different plants of each line with similar size were immediately freeze-dried for synchrotron-based analysis. The remainder of the above-ground part was oven-dried at 65 ^{o}C for 3 days and weighed for biomass before elemental analysis.

Determination of Cd and Zn concentrations in plant samples

Dry plant samples were ground to fine powders and digested with 5 mL HNO_3/$HClO_4$ (85:15 v/v) in a heating block. Blanks and a certified reference material (GBW10015-spinach) were included in the digestion for quality control. The concentrations of Cd and Zn were determined using Flame Atomic Absorption Spectrometry (FAAS).

Cadmium speciation and elemental distribution by X-ray absorption spectroscopy (XAS) techniques

The Cd standard spectra used in this study are described in Yan et al. (2016), including 3 complexes of Cd bound to thiol groups (Cd-cysteine, Cd-glutathione and Cd-phytochelatin), 7 complexes of Cd bound to carboxyl groups (Cd-histidine, Cd-citrate, Cd-L-malate, Cd-methionine, Cd-glutamine, Cd-succinate, and Cd-polygalacturonic acid), and Cd-phytate. Freeze-dried plant leaves were ground into fine powders and mounted on sample holders for synchrotron analysis. Cadmium speciation in leaves was analysed by bulk X-ray absorption fine structure (bulk-XAFS) at the Cd K-edge (26,711 eV) at beamline 7 − 3, Stanford Synchrotron Radiation Laboratory (Menlo Park, CA, USA). A Cd metal foil was measured simultaneously in transmission mode as a reference. The energy calibration of each spectrum was conducted by setting the first inflection point of the X-ray absorption near edge structure (XANES) spectrum of the Cd metal foil to 26,711 eV. Each XAFS spectrum was collected from 300 eV below Cd K-edge to k values over 13 Å^{1} above the edge in fluorescence mode. Three scans were taken for each sample to maximize the signal-to-noise ratio.

All experimental spectra were energy calibrated and merged prior to analysis. The extended X-ray absorption fine structure (EXAFS) analyses were obtained using the SixPack software package and Athena (version 0.9.26). Principal component analysis (PCA) of the EXAFS spectra with k values between 3 and 10 Å^{-1} was performed to estimate the likely number of Cd-bound species contained in the samples. The SPOIL values were calculated using target transformation (TT) function in SixPack. Linear combination fitting (LCF) was conducted to identify and characterize Cd speciation in each sample, in *k*-weight 1 with *k* values ranging from 3 to 10 Å^{-1}. The best fit with the lowest R-factor and reduced chi-square was chosen to represent Cd speciation and the proportion of each species.

Micro X-ray fluorescence (μ-XRF) and micro X-ray absorption near edge structure (μ-XANES) analysis were employed to detect the elemental distributions and Cd speciation at selected hot spots of intact freeze-dried leaves. Data were collected at beamline 20-ID-B, Advanced Photon Source (APS, Chicago, IL, USA). The step size was 8 μm and the dwell time was 1 s. The beam energy was set to 29,000 eV for μ-XRF analysis of Cd, Zn, Mn, and Cu distributions in the leaves. Micro XANES spectra were collected at hot spots, with energy ranging from 100 eV below to 200 eV above the Cd K-edge. Three to four scans were taken at each location to maximize the signal-to-noise ratio. Cadmium speciation in hot spots was identified as described above.

Results

Biomass production in different *N. caerulescens* lines

The 29 lines of the Avinières *N. caerulescens* population showed a 6.5-fold variation in shoot biomass (Fig. 1). The largest shoot biomass was observed in line AV-24-5, with a mean biomass of 5.0 ± 3.3 g per pot. By contrast, the smallest shoot biomass was produced by line AV-21-5, with a mean biomass of 0.8 ± 0.2 g per pot.

Cadmium and Zinc accumulation in different *N. caerulescens* lines

Cd and Zn concentrations in the shoot of the 29 tested *N. caerulescens* lines were analysed. A 2.4-fold difference was observed in shoot Cd concentrations among the lines, varying from 85 mg kg^{-1} in line

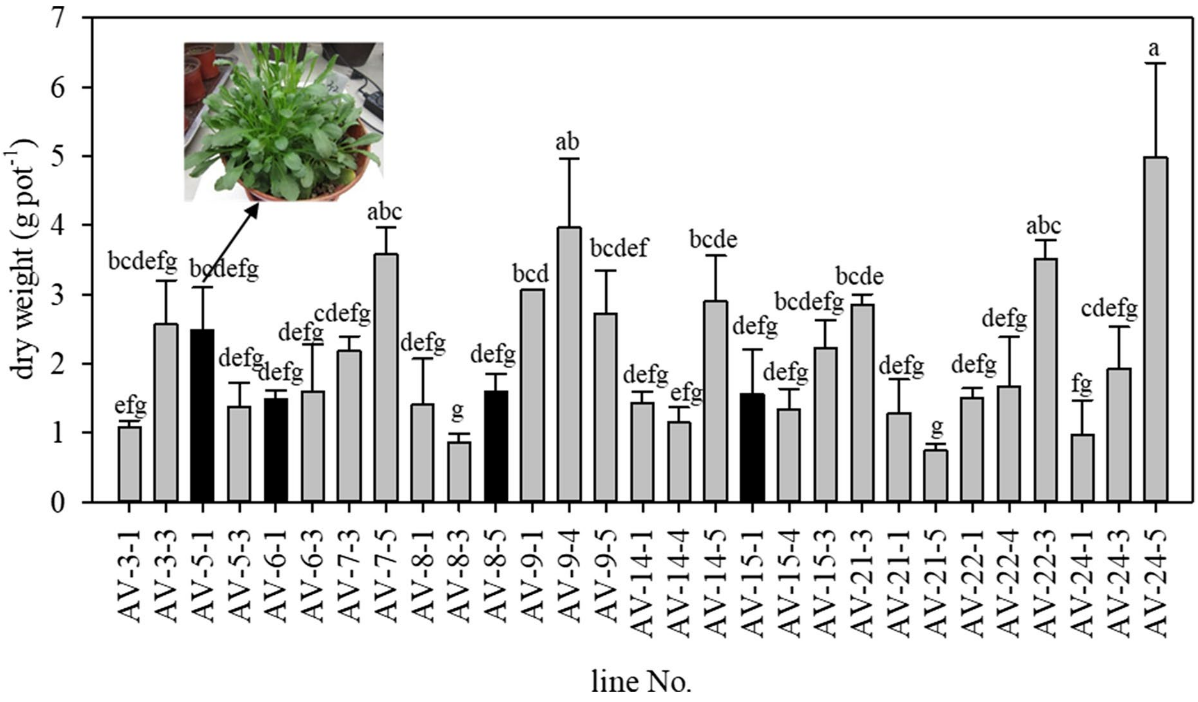
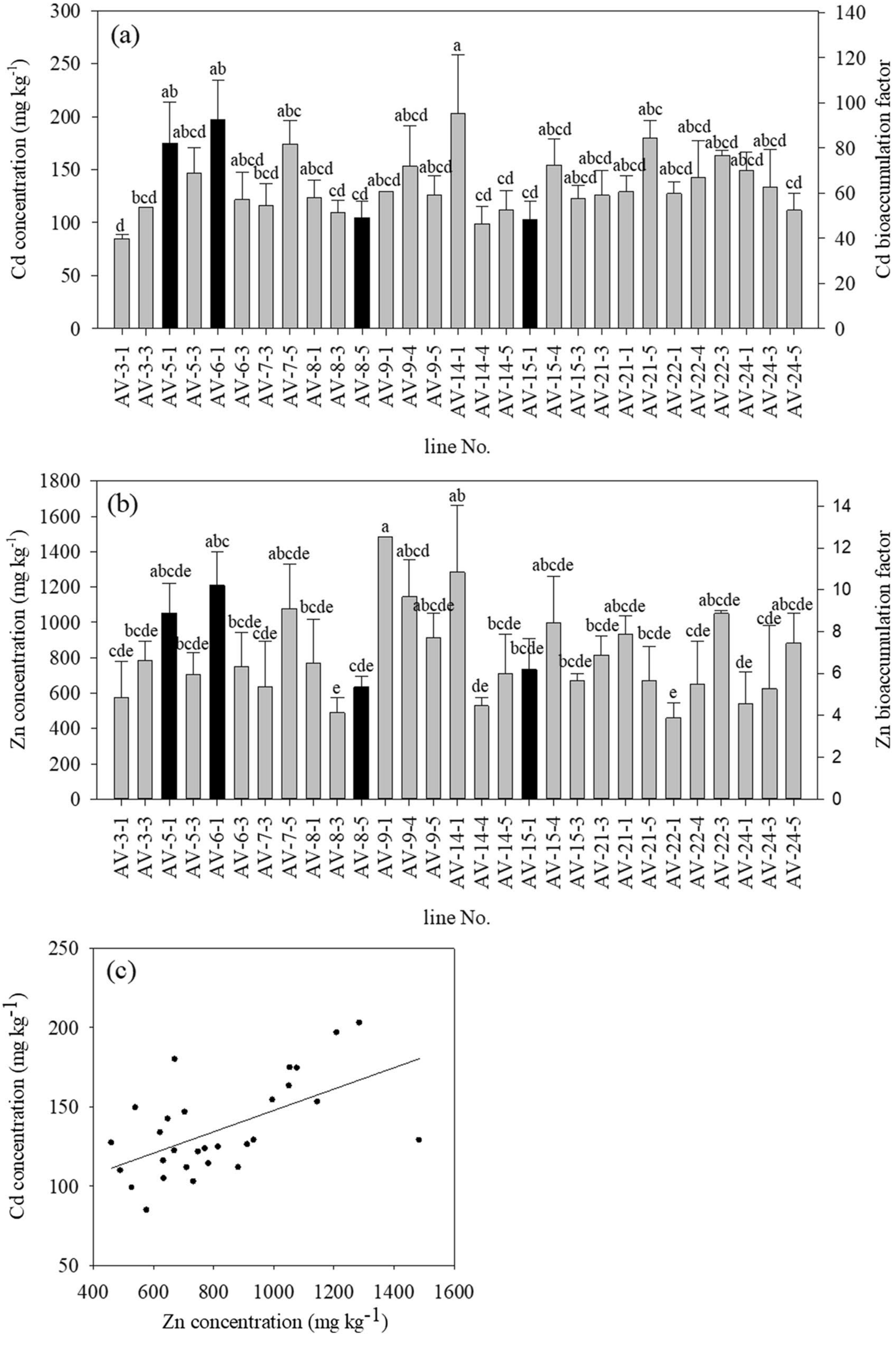


Fig. 1 Above ground biomass (dry weight) of *N. caerulescens* lines. Plants were cultivated in a greenhouse for six months. Data represent means ± SE of three pot replicates. The image shows a replicate of line AV-5-1. Four lines selected for synchrotron analysis are marked with black. Different letters indicate a significant difference (Duncan test, P < 0.05)


(a)
Cd concentration (mg kg^{-1})
Cd bioaccumulation factor
0
50
100
150
200
250
300
0
20
40
60
80
100
120
140
d
bcd
ab
abcd
ab
abcd
bcd
abc
abcd
cd
cd
abcd
abcd
abcd
a
cd
cd
cd
abcd
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AV-8-1
AV-8-3
AV-8-5
AV-9-1
AV-9-4
AV-9-5
AV-14-1
AV-14-4
AV-14-5
AV-15-1
AV-15-4
AV-15-3
AV-21-3
AV-21-1
AV-21-5
AV-22-1
AV-22-4
AV-22-3
AV-24-1
AV-24-3
AV-24-5
line No.
(b)
Zn concentration (mg kg^{-1})
Zn bioaccumulation factor
0
200
400
600
800
1000
1200
1400
1600
1800
0
2
4
6
8
10
12
14
cde
bcde
abcde
bcde
abc
bcde
cde
abcde
bcde
e
cde
a
abcd
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AV-9-4
AV-9-5
AV-14-1
AV-14-4
AV-14-5
AV-15-1
AV-15-4
AV-15-3
AV-21-3
AV-21-1
AV-21-5
AV-22-1
AV-22-4
AV-22-3
AV-24-1
AV-24-3
AV-24-5
line No.
(c)
Cd concentration (mg kg^{-1})
50
100
150
200
250
400
600
800
1000
1200
1400
1600
Zn concentration (mg kg^{-1})

◂**Fig. 2** Cadmium (**a**) and Zinc (**b**) concentrations and bioaccumulation factors in the shoots of *N. caerulescens* lines. (**c**) Relationship between shoot Zn and Cd concentrations. Plants were cultivated in a greenhouse for six months. Data represent means ± SE of three pot replicates. Four lines selected for synchrotron analysis are marked with black. Different letters indicate a significant difference (Duncan test, $P < 0.05$)

AV-3-1 to 203 mg kg^{-1} in line AV-14-1 (Fig. 2a). A total of 27 lines (93%) had Cd concentrations exceeding the Cd hyperaccumulation concentration criterion (> 100 mg kg^{-1}) (Krämer 2010), illustrating the Cd-hyperaccumulation property of the population. Furthermore, the Cd bioaccumulation factor (the ratio of shoot Cd concentration to soil Cd concentration) of the tested *N. caerulescens* lines varied from 40 to 95.

Shoot Zn concentration varied from 460 to 1483 mg kg^{-1}, with the Zn bioaccumulation factor ranging from 5.4 to 12.6 (Fig. 2b). There was a significant ($r = 0.58$, $P < 0.01$) correlation between shoot Zn and Cd concentrations among the 29 lines of *N. caerulescens* (Fig. 2c). In addition, a significant correlation ($r = 0.46$, $P < 0.01$) between shoot Zn concentration and shoot biomass was observed (Fig. 3A), whereas there was no significant correlation between shoot Cd concentration and shoot biomass ($r = 0.003$, $P > 0.05$) (Fig. 3B).

Cadmium and Zinc removal efficiency by *N. caerulescens*

The removal of Cd and Zn from soil by *N. caerulescens* lines was calculated as the products of shoot Cd or Zn concentration and shoot biomass (Fig. 4). The amount of Cd removed by the shoots varied from 0.09 to 0.64 mg pot^{-1}, accounting for 4.3–30.5% of the total Cd in the soil (Fig. 4a). The amount of Zn removal varied from 0.39 to 4.55 mg pot^{-1}, accounting for 0.4–3.8% of the total Zn in the soil (Fig. 4b).

Cadmium speciation in *N. caerulescens* leaves

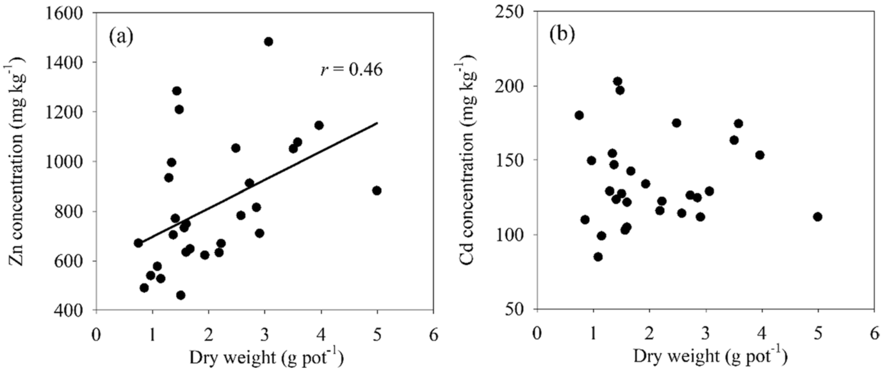
To investigate the Cd speciation in *N. caerulescens* leaves, two high Cd accumulating lines (AV-5-1 and AV-6-1) and two low Cd accumulating lines (AV-8-5 and AV-15-1) were selected for synchrotron analysis. Leaves from the centre of rosette were sampled for Cd speciation analysis using EXAFS. Total Cd concentration in the leaf samples varied from 103 to 197 mg kg^{-1}. The EXAFS spectra of the reference standards are shown in Fig. S1. All Cd standards were divided into three ligand groups: thiol, phytate/phosphate, and carboxyl, as the spectral differences between the compounds within each group were small. Principal component analysis (PCA) of the EXAFS spectra from the leaf samples showed that the cumulative variance reached a minimum at the second component, with the fitting of two standards accounting for 98% of the variance (Table S1). Based on this information, two groups of standards were used in linear combination fitting (LCF) of the EXAFS spectra of leaf samples. Linear combination fitting is influenced by k weight. A higher k weight will amplify the data at higher k ranges (over 7). Metals (e.g., Fe, Mn) have a larger impact on the EXAFS spectrum at higher k space (7–12 Å^{-1}), while light elements are more impacted by scattering (i.e., shoulder features and oscillations) at low k values (3–5 Å^{-1}). In the present study, the most important nearest neighbouring scattering atoms for Cd are light elements, such as oxygen, phosphorus, or sulphur. For this reason, a k-weight of 1 was set in the present study. The best fits for the Cd EXAFS spectra in the leaf samples are shown in Fig. 5. In all four lines of *N. caerulescens*, the majority of Cd (61–69%) in the leaves was complexed with thiol compounds. The remainder was complexed with carboxy ligands in the two high Cd accumulating lines (AV-5-1 and AV-6-1), but with phytate/phosphate in the two low Cd accumulating lines (AV-8-5 and AV-15-1).

Elemental distribution and μ-XANES analyses

Micro-X-ray fluorescence maps of Cd, Cu, Mn, Zn, and Fe distribution in leaf sections of two high Cd accumulating lines (AV-5-1 and AV-6-1) and a low Cd accumulating line (AV-15-1) were obtained (Fig. 6a-c). All three lines showed generally similar distribution patterns of the five metals analysed. Cadmium was mainly distributed in the veins (Fig. 6a-c). There were also hot spots near the vein where Cd was locally concentrated. Cadmium was also concentrated at the edge of the leaf in the low Cd accumulating line AV-15-1 (Fig. 6c). The distribution patterns of Cu and Mn were similar to that of Cd, whereas Zn showed a diffused distribution throughout the leaves.

The μ-XANES spectra at the Cd K-edge were collected at Cd hot spots in the μ-XRF maps of the plant leaves. The best fits obtained from LCF and

Fig. 3 Relationship between shoot biomass and Zn (**A**), Cd (**B**) concentrations of *N. caerulescens* lines



the proportions of Cd speciation in each hot spot are shown in Fig. 6d; Table 1, respectively. In hot spots of the two high Cd lines, the majority (54–70%) of the Cd was bound to thiol groups, with the remainder (30–46%) being bound to carboxyl groups. By contrast, in hot spots of the low Cd content line AV-15-1, nearly half of the Cd was bound to phytate/phosphate, while the other half was bound to thiol groups. These results are consistent with Cd speciation in the bulk samples of whole leaves (Fig. 5).

Discussion

Noccaea caerulescens is a well-known hyperaccumulator of Zn, Ni and Cd (Reeves et al. 2001; Assunção et al. 2003; Krämer 2010; Kozhevnikova et al. 2020). It is typically found as small, scattered populations on non-metalliferous soils (Besnard et al. 2009) or as large populations on abandoned mine spoils containing high concentrations or Zn and/or Cd. Few populations grow on ultramafic soils (Gonneau et al. 2017). There is considerable variation among different populations of this species in their metal accumulation ability, especially for Cd (Lombi et al. 2001; Gonneau et al. 2014; Sterckeman et al. 2017). Currently three different edaphic ecotypes are distinguished, formed by non-metallicolous, calamine and ultramafic populations (Koshevnikova et al., 2020). The Ganges accession from southern France has a particularly high Cd accumulation (Lombi et al. 2001; Sterckeman et al. 2017). The exact origin of this accession is obscure ("collected in the vicinity of St. Laurent le Minier"), and may well be from the large population present at the Les Avinières former smelter site. Variation within populations has not been investigated extensively. In the present study, we found that 29 lines of the Avinières population of *N. caerulescens* varied substantially in biomass production and accumulation of Cd and Zn in the shoots (Figs. 1 and 2). These results suggest that there is substantial genetic variability among *N. caerulescens* lines of the same population. These differences can be useful in improving biomass production in *N. caerulescens*. Plant biomass production is a complex trait depending on numerous genes and environmental conditions (Demura and Ye 2010). Under the growth conditions in the present study, the largest shoot biomass produced by line AV-24-5 was weighed at 1.67 g dry matter (DM) per plant (5.0 g per pot with 3 plants), which could theoretically yield up to 1.5 t DM ha^{-1} with a sowing density of 90 plants m^{-2} in field conditions (McGrath et al. 2000; Simmons et al. 2015). Although the biomass produced by *N. caerulescens* lines in the present study may not be enough to fully decontaminate Cd-contaminated soils within a reasonable time, *N. caerulescens* has the potential to produce up to 10 t dry matter ha^{-1} with appropriate genotypes and sowing density (McGrath et al. 2000; Zhao et al. 2003; Koopmans et al. 2008; Sterckeman et al. 2019). Some fields trials have also demonstrated that hyperaccumulators with optimized agronomic strategies have great potential to be applied in remediation of metal contaminated soil. For example, Deng et al. (2016) optimized the phytoextraction efficiency of Zn/Cd hyperaccumulator *Sedum plumbizincicola* in metal contaminated soil by two agronomic strategies of intercropping with maize and plant densities through two long-term (8 years) field experiments. Results indicated that *S. plumbizincicola* at an appropriate planting density and intercropped with maize can achieve high remediation

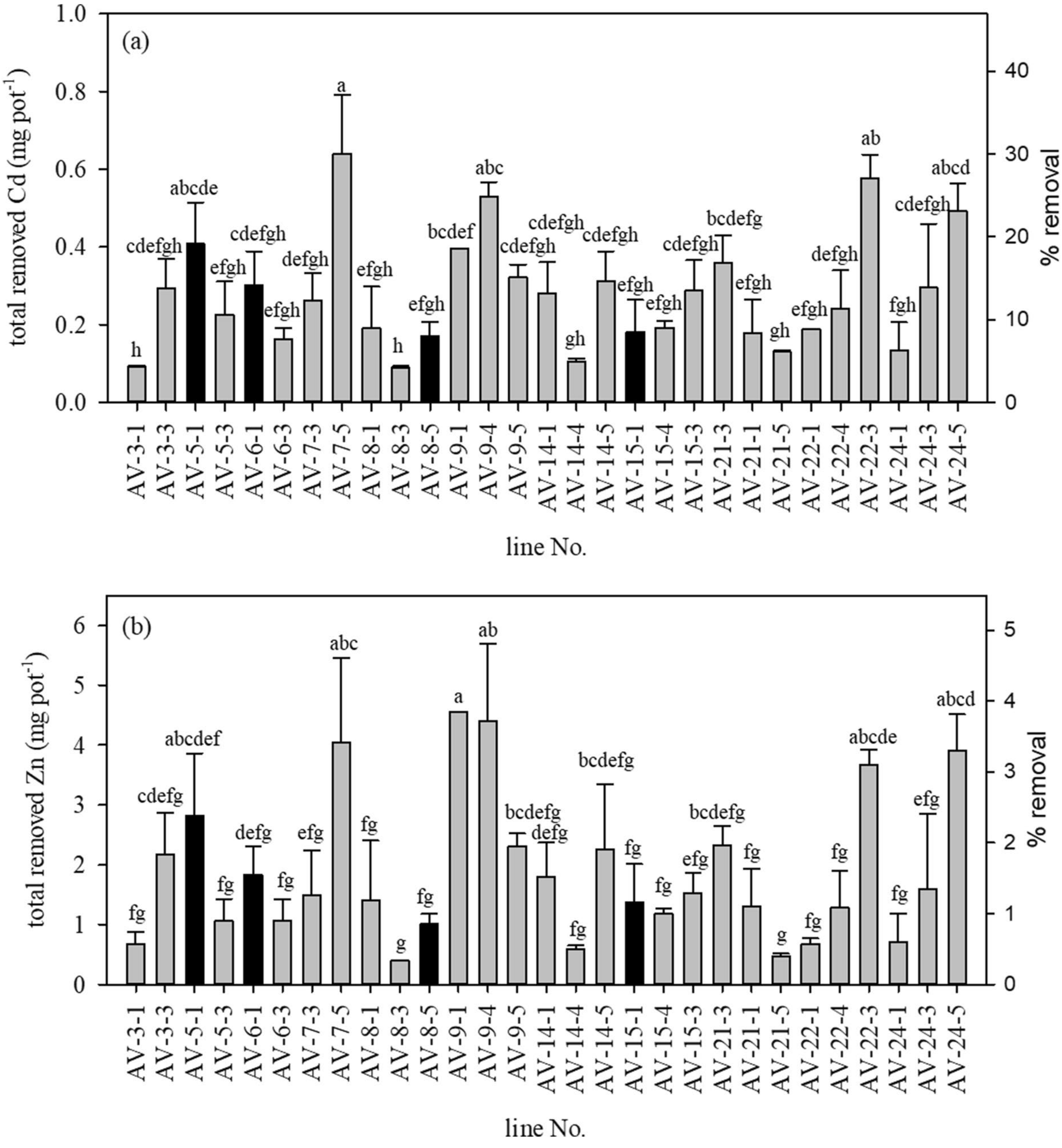


Fig. 4 Total extracted (**a**) Cd and (**b**) Zn from a pot filled with 1 kg of soil by *N. caerulescens* lines. The soil has 2.1 mg kg^{-1} Cd and 118 mg kg^{-1} Zn. Data represent means ± SE of three pot replicates. Four lines selected for synchrotron analysis are marked with black. Different letters indicate a significant difference (Duncan test, $P < 0.05$)

efficiency to contaminated soil without affecting the cereal crop productivity. In a large scale field experiment on a moderately contaminated soil in southern China, *S. plumbizincicola* produced 2–6 t ha^{-1} dry biomass per season and reduced soil Cd from 0.6 to 0.3 mg kg^{-1} within 2–3 years (Hu et al. 2019; Simmons et al. 2015) conducted a field trial to investigate the potential of *N. caerulescens* to phytoextract localized Cd/Zn contamination in Thailand and suggested that optimizing the use of fungicidal sprays, acidic

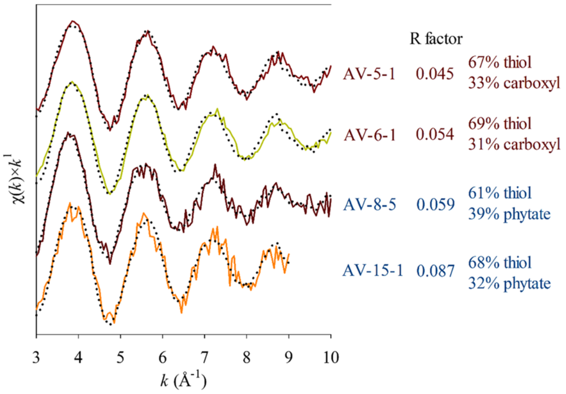


Fig. 5 Linear combination fitting (LCF) results of bulk-EXAFS spectra of *N. caerulescens* leaves. Solid lines represent k1-weighting spectra and dotted lines represent the best fits

soil pH, planting density and increasing the effective cropping period will increase rates of Cd and Zn removal enough to facilitate practical Cd phytoextraction from rice paddy soils.

Cadmium and Zn concentration in the shoots of the 29 *N. caerulescens* lines ranged from 85 mg kg^{-1} to 203 mg kg^{-1} and 460 to 1483 mg kg^{-1}, respectively, reflecting the natural variability of Cd and Zn accumulation among *N. caerulescens* lines. The significant correlation between shoot Cd and Zn concentrations (Fig. 2c) suggests a common pathway of uptake and transport shared by the two metals. The variation in Cd and Zn accumulation could be caused by variation in the expression of metal transporter genes. A previous study showed that NcZNT1 is a plasma membrane transporter with a high affinity for Zn and a low affinity for Cd and is possibly involved in Zn and Cd uptake in *N. caerulescens* (Pence et al. 2000), and in transgenic *Arabidopsis thaliana* expressing *NcZNT1* (Lin et al. 2016), albeit a later study suggested that NcZNT1 is involved in Zn and not Cd uptake and translocation in *N. caerulescens* (Milner et al. 2012). Another transporter, NcNRAMP1, is involved in the influx of Cd across the endodermal plasma membrane and may play a role in Cd flux into the stele and root-to-shoot Cd translocation (Milner et al. 2014). Contribution of NcNRAMP1 to the increased Cd hyperaccumulation in the high Cd-accumulating Ganges accession appears to be due to the higher expression level than that in the low Cd-accumulating Prayon accession (Milner et al. 2014). The iron transporter NcIRT1 may also contribute to Cd uptake. Previous studies have shown that Fe deficiency enhanced Cd uptake and the expression of *NcITR1* in the Ganges accession (Lombi et al. 2002), and NcIRT1 from Ganges also showed Cd transport activity in yeast assay (Halimaa et al. 2019). NcHMA3 is a tonoplast-localized transporter responsible for Cd sequestration in leaf vacuoles, which showed sevenfold higher expression in the high Cd-accumulating Ganges accession than in the low Cd-accumulating Prayon accession (Ueno et al. 2011). The gene copy number and expression of *NcHMA4* among *N. caerulescens* populations were also reported to be associated with Cd tolerance and accumulation (Craciun et al. 2012). Whether the expression levels of *NcZNT1*, *NcNRAMP1*, *NcIRT1*, *NcHMA3* and *NcHMA4* vary among the different lines of *N. caerulescens* used in the present study remains to be investigated.

Interestingly, there is no trade-off between shoot Cd and Zn concentrations and shoot biomass. In fact, there was a significant positive correlation between shoot Zn concentration and shoot biomass (Fig. 3 A), suggesting that *N. caerulescens* lines producing larger biomass may also have a higher ability to accumulate Zn. Our results are consistent with the study of Sterckeman et al. (2017), who also found no trade-off between biomass production and metal concentrations in *N. caerulescens*. Gonneau et al. (2014) reported no trade-off in the calamine and non-metalliferous populations of *N. caerulescens*, but substantial trade-off in the serpentine populations, which are very Cd sensitive (Koshevnikova et al., 2020). These findings suggest that metal accumulation and plant biomass are genetically independent, and that selection and breeding for large biomass would not lead to decreased concentrations of Zn and Cd, at least for the calamine populations such as those used in the present study. In a study spanning three generations of *N. caerulescens*, Sterckeman et al. (2019) showed that pure-line selection could improve Cd and Zn accumulation capacities, but the high biomass traits could not be fixed readily due to the complex genetic basis of biomass production and the interactions between genotype and environmental conditions.

The plant metal bioaccumulation factor is also an important factor affecting the efficiency of phytoextraction. Under the experimental conditions of the present study, the efficiency of Cd phytoextraction was much greater than that of Zn phytoextraction.

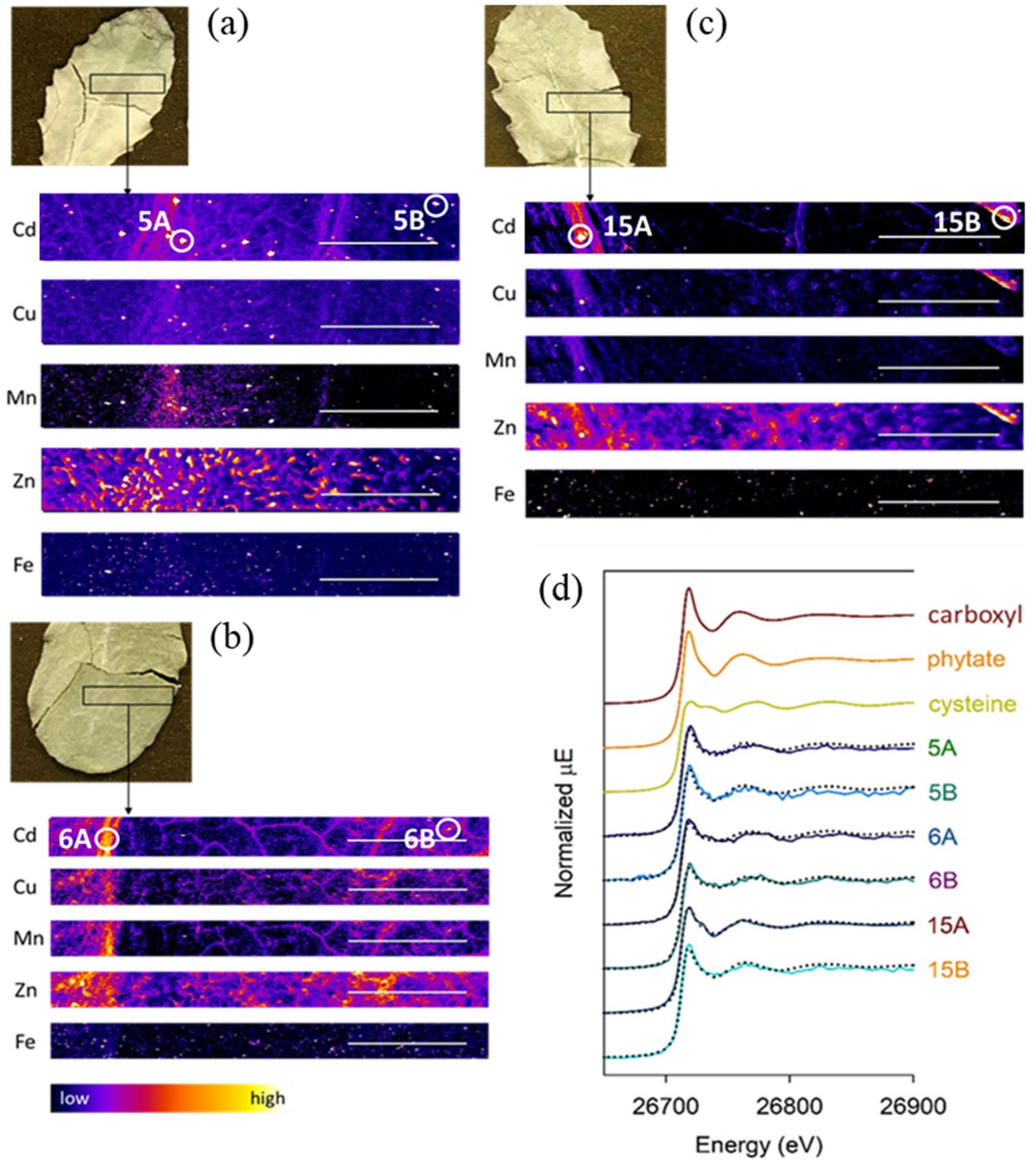


Fig. 6 Elemental distribution of Cd, Cu, Mn and Zn in leaves of *N. caerulescens* line AV-5-1 (**a**), AV-6-1 (**b**), and AV-15-1 (**c**) using µ-XRF mapping. (**d**) µ-XANES spectra at Cd-K-edge of Cd standards (in solid lines) and different spots taken in 5 A, 5B, 6 A, 6B, 15 A and 15B, and LCF results (in dotted lines). Bars (White line) = 1 mm

One reason is that the soil used in the present study was not contaminated by Zn. These results suggest that *N. caerulescens* has great phytoremediation potential for Cd-contaminated soil. On average, of all *N. caerulescens* lines tested, the percentage of the total Cd removed from soil by the shoots was 13.2%.

Table 1 The proportion of Cd species at hotspots from the XANES spectra from µ-XRF maps

Hotspots	carboxyl	phytate	thiol	R-factor
5 A	46%	-	54%	0.0064
5B	42%	-	58%	0.0071
6 A	30%	-	70%	0.0053
6B	32%	-	68%	0.0064
15 A	-	45%	55%	0.0029
15B	-	52%	48%	0.0039

Hotspots name represent the hotspots from Fig. 6

The best performing line of *N. caerulescens* removed 30.5% of the total soil Cd. Therefore, phytoextraction of Cd from moderately contaminated soils is feasible, which is in agreement with previous model calculations (Zhao et al. 2003). In the present study, the Cd bioaccumulation factor of the tested *N. caerulescens* lines varied from 40 to 95, which is consistent with previous studies (Yanai et al. 2006; Maxted et al. 2007; Lovy et al. 2013). The higher the bioaccumulation factor, the more efficient the phytoextraction of Cd by *N. caerulescens*. The large variation (7-fold) among the 29 lines of *N. caerulescens* in the Cd phytoextraction efficiency suggests considerable scope for genotype improvement by line selection and subsequent interline cross progeny selection. Although the Cd bioaccumulation factor is also soil pH and soil Cd dependent, this parameter could be used to select the populations or the individuals most suitable for Cd extraction.

In addition to the variation in Cd accumulation, differences in Cd speciation between high- and low-Cd accumulating lines of *N. caerulescens* were also observed through synchrotron-based EXAFS (Fig. 5). Approximately two thirds of the leaf Cd were complexed with thiol compounds in all lines of *N. caerulescens* investigated (Fig. 4). However, the remaining one third of Cd was found to be complexed with carboxyl groups, likely to be organic acids, in the high-Cd accumulating lines, but with phytate/phosphate in the low-Cd accumulating lines. This difference was confirmed by both bulk EXAFS and µ-XANES. The results suggest that the complexation of Cd with organic acids may become more important with increasing Cd accumulation in the leaves of *N. caerulescens.* Previously, Vogel-Mikuš et al. (2010) found that Cd was coordinated to phytate in the embryonic tissues of *Noccaea praecox*, while Monsant et al. (2011) reported Zn-phytate complexes in *N. caerulescens*. To our knowledge, it is the first time that Cd-phytate/phosphate was detected in the leaves of relatively low-Cd accumulating lines of *N. caerulescens* plants. These results indicate that differences in the detoxification mechanism are likely to exist between high- and low-Cd accumulating lines within the Avinières population of *N. caerulescens*. While *N. caerulescens* in greenhouse conditions is largely autogamous, especially in natural, high-density, calamine populations in the field, there can be high incurrence of cross-fertilisation, which will support maintenance of high levels of heterozygosity and genetic variation in the populations (Mousset et al. 2016), explaining the variation found in the Avinières population.

The dominance of Cd-thiol complexes in the present study is also surprising, as previous reports using either XAS or ^{113}Cd NMR showed Cd-O (Cd-organic acids) to be the dominant species in the leaves of *N. caerulescens* plants (Küpper et al. 2004; Ueno et al. 2005). Huguet et al.(2012), using EXAFS spectroscopy analysis, showed that Cd was predominantly bound to COOH/OH groups of organic acids in the leaves of *Arabidopsis halleri*, while Cd bound to thiol groups was found as a secondary species (less than 25%). Similar results were observed in Cd hyperaccumulator *N. praecox*, where the Cd-O ligands prevailed over the Cd-S ligands in bulk leaf tissues of *N. praecox* treated with different Cd salts and concentrations (Koren et al. 2013). Isaure et al. (2015) also found that the proportion of Cd-O ligands increased in the Cd-hyperaccumulating *A. halleri* and Cd tolerant progenies from the cross between *A. halleri* and the non-tolerant and non-hyperaccumulating relative *A. lyrata*, suggesting the binding of Cd with O ligands was associated with Cd tolerance. Küpper et al. (2004) found that only about a third of the Cd in the young and mature leaves of *N. caerulescens* was complexed with thiol ligands. However, both studies grew *N. caerulescens* (Ganges) in hydroponic culture supplied with high levels of Cd (50–100 µM) and, as a result, much higher concentrations of Cd were accumulated in the leaves (approximately 20–100 times higher than those obtained in our study). Moreover, the plants were suffering from Cd toxicity in the study of Küpper et al. (2004). However, in the non-hyperaccumulator *Brassica juncea*, Salt et al. (1997) showed that Cd was mainly associated with S ligands (60%) in the seedling after 36 h of 1 μM Cd exposure. It is reasonable to argue that thiol compounds provide stronger but more costly ligands for Cd complexation that would be suitable at relatively low levels of Cd accumulation, as observed in our study and the study by Salt et al. (1997). In contrast, organic acids provide weaker and low cost ligands for Cd complexation at very high levels of Cd accumulation. Thus, Cd speciation observed in our study represents *N. caerulescens* plants growing on slightly contaminated soils.

With respect to the distribution of Cd in leaves, Cosio et al. (2005) showed that Cd was concentrated at the edge of *N. caerulescens* leaves, and there were hot spots on the leaf surface. Cadmium imaging using laser ablation inductively coupled plasma mass spectrometry also showed increased Cd concentration on the leaf edges of *N. caerulescens* (Callahan et al. 2016). These results are consistent with the findings of the present study. The elemental distribution pattern was similar in the three leaves from different lines (Fig. 6), which showed that Cd, Cu, and Mn were co-localized at the veins. This similarity indicates that likely the same accumulation pathway is shared by these three elements. Though Zn and Cd may also share a similar uptake and transport mechanism, the distribution of Zn was more diffuse than Cd (Fig. 6). This was possibly due to the significantly higher Zn concentration in *N. caerulescens* leaves. At lower Zn concentrations, Zn also localizes mainly to the veins (van der Zee et al. 2021).

Conclusions

Significant differences existed in Cd accumulation and biomass production among different hyperaccumulator *N. caerulescens* lines of the Les Avinières population. Selection for higher Cd accumulation and biomass production lines of Cd hyperaccumulating calamine populations could provide more efficient *N. caerulescens* for soil Cd phytoextraction. The EXAFS analysis identified that Cd-thiol and Cd-carboxyl complexes exist in leaves of high Cd accumulating lines while Cd-thiol and Cd-phytate/phosphate complexes are present in leaves of low Cd accumulating lines. In addition, cadmium-thiol complexes were the most dominant species in the *N. caerulescens* leaves (61–69%) when plants were grown on a moderately Cd-contaminated soil. These new results show that the mechanisms of Cd storage and detoxification in *N. caerulescens* differ from what was previously found.

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