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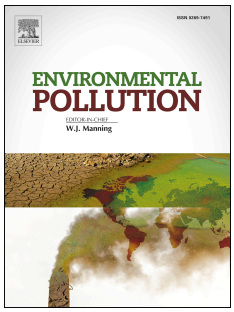

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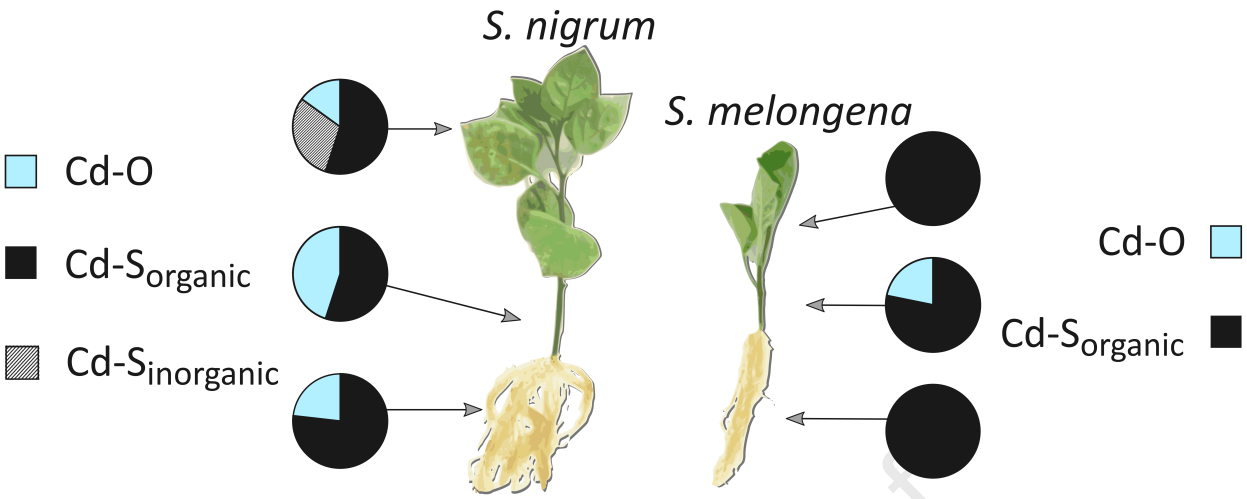

X-ray absorption spectroscopy evidence of sulfur-bound Cadmium in the Cd-hyperaccumulator *Solanum nigrum* and the non-accumulator *Solanum melongena*

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Abstract

It has been proposed that non-protein thiols and organic acids play a major role in cadmium phytoavailability and distribution in plants. In the Cd-hyperaccumulator *Solanum nigrum* and non-accumulator *Solanum melongena*, the role of these organic ligands in the accumulation and detoxification mechanisms of Cd are debated. In this study, we used X-ray absorption spectroscopy to investigate Cd speciation in these plants (roots, stem, leaves) and in the soils used for their culture to unravel the plants responses to Cd exposure. The results show that Cd in the 100 $mg.kg^{-1}$ Cd-doped clayey loam soil is sorbed onto iron oxyhydroxides. In both *S. nigrum* and *S. melongena*, Cd in roots and fresh leaves is mainly bound to thiol ligands, with a small contribution of inorganic S ligands in *S. nigrum* leaves. We interpret the Cd binding to sulfur ligands as detoxification mechanisms, possibly involving the sequestration of Cd complexed with glutathione or phytochelatins in the plant vacuoles. In the stems, results show an increase binding of Cd to -O ligands (>50% for *S. nigrum*). We suggest that Cd is partly complexed by organic acids for transportation in the sap.

Capsule

Cadmium speciation in Solanaceae was investigated using X-ray absorption spectroscopy. Results showed evidence of Cd detoxification through Cd binding with thiols and inorganic sulfur.

Keywords

Cadmium, Speciation, Toxicity, Solanaceae, X-ray absorption spectroscopy.

1. Introduction

Cadmium contamination in agricultural soils has raised worldwide concerns, as its release in the environment dramatically increased in the past decades due to various anthropogenic activities such as smelting, combustion of coal or inorganic phosphate fertilization (Cai et al., 2003; Cullen and Maldonado, 2013; Dharma-wardana, 2018; Zhou and Song, 2004). Cadmium is toxic toward almost all forms of life, even at low concentrations (Cullen and Maldonado, 2013; Das et al., 1997). With relatively high mobility in the soil/plant system, Cd easily enters the food chain and poses human health threats (Cullen and Maldonado, 2013; Das et al., 1997). Previous studies have demonstrated that the mobility and bioavailability of Cd in the soil/plant system – thus its toxicity in this system – depends ultimately on its chemical speciation (Cullen and Maldonado, 2013).

The development of direct, experimental characterization of trace elements speciation by X-ray absorption spectroscopy (XAS) has brought key information on trace metal behavior in the critical zone (Collin et al., 2014; Doelsch et al., 2006; Huguet et al., 2012; Isaure et al., 2015; Sarret, 2002; Sarret et al., 2013). However, applying this methodology to the determination of Cd speciation in biological samples remains challenging as in most plants, Cd concentration (e.g. 0.01-1 $mg.kg^{-1}$ dry weight) lies below the technical detection limit of most X-ray absorption synchrotron beamlines. That is why, to date, Cd speciation determination has mainly been carried out on Cd-hyperaccumulating and/or Cd-tolerant plant systems (Huguet et al., 2015, 2012; Isaure et al., 2015). These pioneering studies demonstrated a change in Cd speciation upon Cd uptake through the roots, and during Cd translocation and storage in the leaves, and the involvement of thiols, organic acids and phosphate ligands in response to Cd exposure (Huguet et al., 2012; Isaure et al., 2015, 2006). Recent progress in XAS spectroscopy triggered by the development of highly sensitive fluorescence detectors have opened the field of Cd speciation determination in

plants with very low Cd concentrations such as cacao (Vanderschueren et al., 2020) or rice (Wiggenhauser et al., 2020) and have identified new Cd ligands like histidine (Vanderschueren et al., 2020), proving that XAS techniques still have a lot to offer to better characterize Cd behavior in the soil/plant system.

Here, we investigated Cd speciation in plants from the solanum family, Cd-hyperaccumulator *Solanum nigrum* (*S. nigrum; black nightshade*) and non-tolerant (Sun et al., 2006) *Solanum melongena* (*S. melongena; eggplant*). Among the solanum family, several species are cultivated worldwide as food products – e.g. tomatoes, potatoes, bell peppers, eggplants, and have shown a tendency to Cd accumulation (e.g. eggplants, Sun et al., 2006; tomatoes, Hasan et al., 2019). Deciphering their responses to Cd exposure is of prime interest for food safety reasons. The mechanisms governing Cd distribution in these plants are still debated. Sun et al., (2006) study proposes that organic acids play a major role in solanum species behavior toward Cd (Li et al., 2020; Sun et al., 2006), as they found increasing carboxylic acid contents in *S. nigrum* and *S. melongena* leaves when exposed to high level of Cd. By chemical titration, they reported organic acid over Cd ratios of ~ 1000 (Sun et al., 2006) and suggested that Cd accumulation and detoxification in these plants is regulated by complexation with organic acids alone. The involvement of organic acids in the handling of Cd has been invoked in other Solanaceae, such as tomato (Bao et al., 2011b). However, other studies reported, by chemical extraction and titration, an increase of thiol compounds – cysteine, glutathione and phytochelatins – in solanum plants exposed to Cd (Chamseddine et al., 2008; Gao et al., 2010; Hasan et al., 2019, 2015), including *S. nigrum* (Gao et al., 2010). In this context, this study aims at deciphering the fate of Cd in the solanum plants at the molecular level by performing, for the first time, Cd speciation characterization in Solanaceae using XAS spectroscopy.

2. Materials and methods

2.1. Plant and soil material

500 seeds of *Solanum nigrum* – reference 'blackberry nightshade' – and *Solanum melongena* – reference 'black beauty eggplant' were purchased at a local gardening store. The soil used in this study is a clayey loam soil (LUFA Speyer standard 2.4, Table S1). The soil was freshly field collected in a meadow with apple trees, then sieved at 2 mm and analyzed before shipment. Upon reception at the CEREGE, the soil was incubated with 100 $mg.kg^{-1}$ Cd as dissolved cadmium nitrate tetrahydrate (98%, Sigma-Aldrich). Such elevated Cd concentration, albeit ~20 times higher than reported values for Cd agricultural soils from the industrialized Chenzhou region in China (Robson et al., 2014; Wang et al., 2019; Zhai et al., 2008) and ~200 times higher than the average Cd geochemical background in French soils (Baize, 1997), was chosen to ensure sufficient uptake of Cd by the plants prior to XAS analyses. We aimed at Cd content of >50 $\mu g.g^{-1}$ dry weight in the plant parts, which was previously reported in *S. nigrum* and *S. melongena* grown on a 100 $mg.kg^{-1}$ Cd-doped soil (Sun et al., 2006). The Cd-doped soil was brought to 70% of its maximum water holding capacity using deionized water, thoroughly mixed then stored for 4 weeks in a growth chamber with controlled humidity, at a temperature of 24°C. A control soil was also prepared, without Cd, but with the same amount of nitrate as dissolved $NaNO_3$, following the same procedure.

2.2. Plant cultures

Solanum nigrum and *S. melongena* seeds were placed on some clean KimTech™ wipes soaked with deionized water, in Plexiglas boxes, at 28°C for 14 days, for germination. Plastic gardening pots of 14 cm diameter were prepared with 2 cm of sand at the bottom

and ~500 g of incubated Cd-doped or control soil in each pot. Two-week-old seedlings of *S. nigrum* and *S. melongena* with shoots of about 2 cm were then transplanted into the pots (2 seedlings per pot). Six pots per plant type were prepared for the Cd-doped soil and for the control soil. Plants were cultivated in a controlled growth chamber with fixed temperature of 24°C, controlled humidity, and photoperiod (light: 6 am – 9 pm). Each pot was automatically watered individually with deionized water, twice a day (at 9 am and 5 pm), to maintain the soil at 70% of its maximum water holding capacity. No fertilizer was added. The plants where harvested after a growth period of 14 weeks. For each plant type and soil treatment, four replicates were used for total Cd content analyses and 3 replicates were prepared for XAS measurements, following two protocols detailed in the next two paragraphs.

2.3. Analysis of total Cd content in the plant parts (exposed and control samples)

After harvest, roots and aerial parts have been separated at the collar level and cleaned with ultrapure water (18.2 MΩ·cm). Roots received additional cleaning by gentle brushing and vortexing in water. Fresh leaves and stems were separated. Plant samples were dried in an oven set at 40°C during 48h. Roots, stems and fresh leaves were weighed before (fresh weight) and after drying (dry weight; Tables S6 and S7). Plant samples (roots, fresh leaves, stems) were ground for 2min at 20hz with a Retsch® Mixer Mill MM 400 to obtain a fine powder. 100 to 300 mg of each plant sample was then digested in a mixture of nitric acid, hydrochloric acid and peroxide using a microwave (MLS turboWAVE®). Total cadmium analyses were performed on the Q-ICPMS (quadrupole-inductively coupled plasma mass spectrometer, Perkin Elmer NexIon 300X).

2.4. Synchrotron X-ray absorption measurements

Plants and soils preparation for XAS measurements. After Cd incubation, and prior to the pot experiments, ~50 g of soil was frozen in a -80°C freezer to avoid changes in speciation. This initial soil will be referred as 'pre-culture soil t_0'. After 14 weeks of pot experiments, *S. nigrum* and *S. melongena* samples were carefully harvested and cleaned. Roots received additional cleaning by gentle brushing and vortexing in water. Special attention was paid to ensure that no soil residue would remain on any parts of the plants, especially on the roots of both *S. nigrum* and *S. melongena* and on the dead leaves of *S. melongena*, to prevent soil Cd contamination of the plant tissues. For XAS measurements, *S. nigrum* plants were cut and divided into: roots, top fresh leaves, bottom fresh leaves, and stem. *Solanum melongena* plants were cut and divided into: roots, fresh leaves, stem and dead leaves (detached from the plant). All plant parts were weighed then directly frozen in liquid nitrogen and stored in a -80°C freezer, to prevent any speciation change before the synchrotron measurements. All further preparation steps were performed frozen, using liquid nitrogen. Cryogenic grinding was performed on all plant and soil samples using zirconium oxide beads in 2 mL Eppendorf tubes with a Retsch® Mixer Mill MM 400. 7 mm diameter pellets were then prepared using a hand press with dies cooled in liquid nitrogen. Samples were transported to the Diamond Light Source and to Soleil Synchrotron on dry ice.

Cd reference compounds. A large database of Cd-containing reference compounds was analyzed for X-ray Absorption Near Edge Structure (XANES) and Extended X-ray Absorption Fine Structure (EXAFS) to allow the interpretation of XAS spectra obtained on the soil and plant natural samples (Huguet et al., 2015, 2012; Isaure et al., 2015; Vanderschueren et al., 2020). Some of these Cd reference materials were purchased chemicals, the others were prepared at the laboratory. Commercial Cd minerals included CdS, $Cd(OH)_2$, CdO, $CdCl_2$, $CdPO_4$, $CdCO_3$ and $CdSO_4$. A solution of aqueous Cd^{2+}

($Cd(H_2O)_6^{2+}$) reference was prepared by dissolving commercial $Cd(NO3)_2(H_2O)_4$ in Milli-Q water. A library of Cd-organic complexes, composed of several Cd bound to organic acids relevant to the solanum species (Sun et al., 2006) – Cd-acetate, Cd-malate, Cd-citrate, Cd-tartrate, Cd-phytate – as well as thiol Cd compounds – Cd-glutathione, Cd-cysteine – and amino acid Cd-histidine, were synthetized in the laboratory (Supplementary Material Part 1). To better constrain Cd speciation in the soil, we also analyzed a synthetic Cd-sorbed goethite (Hammer et al., 2006) and a naturally Cd-rich calcite collected at Dornach (NW Switzerland). The compounds CdS, $Cd(OH)_2$, CdO and $CdSO_4$ were prepared as pressed pellets diluted with PVP, while all other references were prepared as liquid solutions. Prior to XAS measurements, pellets or liquid references were frozen in liquid nitrogen. Spectra for all Cd-containing pellet references and Cd glutathione, Cd phosphate, Cd acetate and $Cd(H_2O)_6^{2+}$ solutions were recorded at Cd K-edge in transmission mode. Other liquid references were measured at Cd K-edge in fluorescence mode. The Cd K-edge EXAFS spectra of all Cd reference compounds and their Fourier transforms are presented in Figure S2. The Cd K-edge XANES spectra of all Cd reference compounds are presented in Figure S3.

Cd K-edge XANES and EXAFS Spectroscopy. Cd K-edge (E0=26711 eV) XANES and EXAFS experiments were performed during two synchrotron facility sessions carried out at the Diamond Light Source (Didcot, United Kingdom) and at the SOLEIL synchrotron facility (Saint-Aubin, France). At the Diamond Light Source, the experiments were performed on the B18 core XAS beamline (for more information on the beamline, see the supplementary materials, part 2), which is equipped with a Si (311) monochromator for measurements at high energy (>20 keV). The storage ring was operated at 3 GeV with a ring current of 300 mA. Samples were measured using a 35-element germanium Solid State Detector (SSD) in fluorescence detection mode. X-ray absorption spectra were scanned from 26523 eV to

197 27093 eV for the diluted samples and from 26523 eV to 27711 eV for the references in
198 quick EXAFS mode (~ 4 min per spectrum). For each sample, 100 to 180 spectra were
199 acquired, aligned, and merged. The energy calibration was performed using a Cd foil
200 sheet. The beam spot size was set to 1.5 x 1.5 mm. To avoid Cd speciation change during
201 measurements, the sample pellets and liquid references were measured using a liquid
202 nitrogen Oxford Optistat cryostat set at 93K. At the Soleil Synchrotron, the experiments
203 were performed on the SAMBA beamline (for more information on the beamline, see the
204 supplementary materials, part 2), which is equipped with a Si (220) monochromator for
205 measurements at high energy (>20 keV). The storage ring was operated at 2.75 GeV with
206 a ring current of 500 mA. Samples were measured using a 35-element germanium SSD in
207 fluorescence detection mode. X-ray absorption spectra were scanned from 26523 eV to
208 27093 eV for the diluted samples and from 26523 eV to 27711 eV for the references in
209 quick EXAFS mode (~ 3 min per spectrum). For each sample, 50 to 110 spectra were
210 acquired, aligned, and merged. The energy calibration was performed using a Cd foil
211 sheet. The beam spot size was set to 2 x 0.5 mm. All samples and references were
212 measured using a liquid nitrogen cryostat set at 80K.
213 For all samples, the extracted EXAFS spectra were k^2 weighted (with k: wave vector) to
214 enhance the high-k region. Data treatment was performed using the ATHENA (Ravel and
215 Newville, 2005), ARTEMIS (Ravel and Newville, 2005) and FASTOSH (Landrot, 2018)
216 softwares. Fourier transforms were performed of the k range from 2 to 9 $Å^{-1}$ to R space
217 using a Hanning apodization window. The structural and chemical parameters of the
218 references (Table S3) and of the soil samples (Table S10) were modelled by shell-by-shell
219 fitting using the ARTEMIS software. Shell-fitting analysis of the k^2-weighted EXAFS
220 spectra was performed in R-space. Theoretical EXAFS phase-shift and amplitude
221 functions for the paths were calculated using FEFF (Rehr et al., 2010). Principal
222 component analyses (PCA) were performed on the soil samples and on the plant samples

to determine the number of references needed to explain our datasets. PCA were the FASTOSH software (Landrot, 2018). Target transform were performed on every single Cd reference compound to assess whether they constitute good candidates to explain Cd speciation in our soil/plant system (Wasserman, 1997; Wasserman et al., 1999). The best linear combination fitting (LCF) of our samples EXAFS spectra were determined using the LCF fitting module of the FASTOSH software over the k range 2 to 9 $Å^{-1}$ with a fixed E_0 value of 26710.4 eV. Residual factor of LCF was calculated using the following equation:

$$R = \Sigma(k^2\chi(k)_{exp} - k^2\chi(k)_{fit})^2/\Sigma(k^2\chi(k)_{exp})^2$$

At each step of the fitting, an additional reference spectrum was added if the two following conditions were true: the R factor decreased by 20% or more and the additional reference had a contribution equal to or higher than 10% among Cd-species. For the same number of references, two different LCF fittings are considered undistinguishable if their R factor is comprised within 20%. The uncertainty of this LCF method was estimated at +/-15% (Doelsch et al., 2006; Le Bars et al., 2018). PCA, target transforms and LCF fittings are further discussed in the results part and in the supplementary material (Part 3).

3. Results and discussion

3.1. Plant Cd content and aspect

Solanum nigrum has been identified as a potential Cd-hyperaccumulator plant (Sun et al., 2006; Wei et al., 2005). The definition of hyperaccumulation depends on the considered metal. For Cd, several studies have proposed to define Cd hyperaccumulation ability as the accumulation of Cd in the stems or leaves of the plant exceeding the threshold of 100 $\mu g.g^{-1}$, dry weight (Baker et al., 2000; Huguet et al., 2015; Isaure et al., 2015; Lu et al., 2013; Sun et al., 2011, 2006; Uraguchi et al., 2006; Wei et al., 2005). In our experiments,

248 *S. nigrum* leaves show average values of 126.5 ± 12 µg.g^{-1} Cd in leaves (DW, Table 1).
249 60% of *S. nigrum* total Cd content is located in its shoots (6% in the stem, 54% in the
250 leaves, Table 1). In Sun et al., 2006, *S. melongena* has been described as 'less tolerant'
251 towards Cd than *S.*
252
253 *nigrum*. For *S. melongena* plants grown on a 100 mg.kg^{-1} Cd doped soil, they report Cd
254 concentrations of 64 µg.g^{-1} (DW) in leaves and 189 µg.g^{-1} (DW) in roots. Our results are
255 consistent with these values, with Cd concentrations of 59 µg.g^{-1} (DW) in leaves and 244
256 µg.g^{-1} (DW) in roots (Table 1). While *S. melongena* does not fit the definition of Cd-
257 hyperaccumulation, *S. melongena* does accumulate a significant amount of Cd when
258 exposed to this metal. Our results show that *S. nigrum* display enhanced capacity to
259 accumulate Cd in its aerial parts (60% total Cd in stem + leaves) compared to *S.*
260 *melongena* (41%, Table 1), with a slightly higher roots to aerial parts Cd translocation
261 factor (0.31 for *S. nigrum* and 0.24 for *S. melongena;* a Student t-test identifies the two
262 groups as distinct at a 93.7% confidence interval; Table S7).
263 *Solanum nigrum* plants grown on the Cd-doped soil show signs of leaf chlorosis in the
264 older, bottom leaves (Figure S4), as well as significantly (Student t-test, 95% C.I.) lower
265 roots, stem and leaves biomass compared to plants grown on the control soil (Figure 1,
266 Figure S5 and Table S6 and S7). *Solanum melongena* plants grown on 100 mg.kg^{-1} Cd-
267 doped soil have roots that display more friable texture than that of the control plants. No *S.*
268 *melongena* plants from the Cd doped experiment reached a stage where more than two
269 leaves were present at the same time, while *S. nigrum* plants reached a more mature
270 stage (up to 10 leaves). *Solanum melongena* plants exposed to Cd also show significantly
271 (Student t-test, 95% C.I.) lower roots biomass, and a tendency to lower stem and leaves
272 biomass compared to the control plants (Figure 1 and Table S6 and S7). For both *S.*
273 *nigrum* and *S. melongena*, all of the above constitute well-described consequences of

exposure to high levels of Cd (Das et al., 1997; Maqbool et al., 2019; Prasad, 1995; Rizwan et al., 2012).

Table 1: Cd average concentrations, dry weights, and total Cd contents measured in *S. nigrum* and *S. melongena* grown on 100 mg.kg^{-1} Cd doped clayey loam soil.

			Cd (µg.g^{-1})		DW (mg)		Cd (µg)	Cd (%)
plant type	**soil Cd concentration**	**Plant Part**	**measured on dry sample (± 1 s.d.)**	**n**	**Dry weight (± 1 s.d.)**	**n**	**Average total Cd content**	**Average total Cd content**
S. melongena	100 mg.kg^{-1}	Roots	243.8 ± 9	4	39.8 ± 10	4	9.7	59
		Stem	58.5 ± 10	4	28.9 ± 4	4	1.7	10
		Fresh leaves	58.8 ± 10	4	87.1 ± 38	4	5.2	31
S. nigrum	100 mg.kg^{-1}	Roots	376.8 ± 87	4	33.6 ± 17	4	12.5	40
		Stem	43.8 ± 6	4	41.4 ± 26	4	1.8	6
		Fresh leaves	126.5 ± 12	4	130.8 ± 40	4	16.7	54

3.2. No change in soil Cd speciation upon plant culture

Cadmium speciation in the 100 mg.kg^{-1} Cd doped soil before and after culture of both *Solanum nigrum* and *S. melongena* was assessed by EXAFS spectroscopy. Principal component analysis (PCA) performed on EXAFS spectra of six soil samples identified up to two components (Supplementary material Part 3), meaning one to two Cd reference compounds are needed to reconstruct soil samples EXAFS spectra through linear combination fitting (LCF). XAS spectra of pre- and post-culture soils for both *S. nigrum* and *S. melongena* are visually almost identical (Figure S6). They are also very similar to the spectrum of the Cd-sorbed artificial goethite we measured (Figure S6). Sorption of Cd onto oxides and hydroxides in soils is a known mechanism that has been documented in several studies, for example Cd sorption on manganese oxyhydroxides (Chen et al., 2019;

Kubier et al., 2019; Lin et al., 2016; Wasylenki et al., 2014) or iron oxyhydroxides, including goethite (Barrow et al., 1989; Bruemmer et al., 1988; Gerth et al., 1993; Gerth and Bruemmer, 1983). However, due to the low amount of Cd in the goethite reference (~50 $mg.kg^{-1}$ Cd), the Cd-sorbed goethite EXAFS spectrum presents significant noise in the high k region. For these reasons, we chose to use the XANES part of our soil samples spectrum (from -30 eV to +100 eV) to perform linear combination fittings. Figure 2 shows our soil samples XANES spectra and their best fit, along with Cd-sorbed goethite, Cd-phytate and Cd-organic acids reference spectra. In the pre-culture, 100 $mg.kg^{-1}$ Cd-doped soil, the spectrum was best reconstructed with 100% Cd-sorbed goethite (Figure 3; Supplementary Table S9). Post-culture *S. nigrum* soil XANES LCF fit gives the same result: 100 % Cd sorbed onto goethite. For *S. melongena* post-culture soil, our fitting method suggests a contribution of Cd bound to organic acids, with the best LCF fits giving 85% Cd sorbed to goethite and 15% Cd bound to acetate. Our LCF method also gave acceptable results for citrate, malate and tartrate as oxygen ligand candidates. The secretion of organic acids in roots exudates is a well-documented phenomenon (Adeleke et al., 2017; Bao et al., 2011b; Han et al., 2006; Loganathan et al., 2012). In various plant families, including solanum plants, the root exudation of organic acids enhances the bioavailability of nutrient metals such as zinc or iron in soils (Bao et al., 2011a; Samardjieva et al., 2015). Cadmium is a non-essential cation with similar properties as zinc. The release of organic acids by *S. melongena* roots in the soil might consequently cause partial Cd desorption from oxyhydroxides and enhance Cd phytoavailability. Changes in soil Cd speciation from Cd mineral species to Cd bound to organic acids after plant culture has been previously identified by Huguet et al (Huguet et al., 2015). Overall, considering our LCF method gives an uncertainty of +/-15% on the percentage of Cd species, our results for pre- and post-culture soils, regardless of the plant type, are quite similar. This is consistent with the structural and chemical parameters we obtained by

performing a first shell fit of our soil samples Cd k-edge EXAFS spectra using the ARTEMIS software (Ravel and Newville, 2005). All three samples show the same Cd coordination to O atoms (N ~ 5.6) with Cd-O bond length of 2.26 Å (see Supplementary Table S10), which is also very close to the structural parameters modelled for the Cd sorbed goethite reference (backscatterer in the first coordination sphere: O; N = 5.7, Cd-O R = 2.25 Å). We interpret this overall lack of differences in Cd speciation in soil before and after culture as the expression of a mass balance effect. The total Cd content in *S. melongena* and *S. nigrum* is ~30 µg (Table 1). Considering two plants per pot, the plants have taken up 60 µg of Cd per pot, which represents 0.12% of the initial Cd content. Despite the roots of both plants having colonized the whole pot, the bulk 500 g, 100 $mg.kg^{-1}$ Cd-doped soil is not significantly affected by the plants.

3.3. Cd speciation in *S. nigrum* and *S. melongena*

Cd k-edge EXAFS spectra of plant parts and corresponding Fourier transforms are presented in figure 4. Linear combination fitting (LCF) of *S. nigrum* and *S. melongena* plant parts EXAFS spectra (from k=2 to k=9) were performed using the EXAFS spectra of all of the Cd reference compounds measured in this study. The best linear combination fittings of samples EXAFS spectra and associated Fourier transforms are also plotted in Figure 4 (for LCF numerical result, see Table S11). In our plant samples, the fitting of EXAFS spectra allowed us to identify the first neighbor of Cd as oxygen or sulfur. As detailed in the following paragraphs, potential -O ligands included organic acids, histidine, phytate or $Cd(H_2O)_6^{2+}$. Potential -S ligands included binding to mineral sulfur (CdS) and organic sulfur as glutathione or cysteine. Obtained proportion of Cd-O and Cd-S species for best EXAFS LCF fits are displayed in Figure 5. Cd k-edge XANES spectra of the plant samples are presented in Figure S7. Linear combination fitting of *S. nigrum* and *S. melongena* plant

parts XANES spectra (from 26680 to 26810 eV) were performed using the XANES spectra of all of the Cd reference compounds measured in this study (displayed in Figure S3). The best linear combination fittings of samples XANES spectra are also plotted in Figure S7 (for LCF numerical result, see Table S11). Obtained proportion of Cd-O and Cd-S species for best XANES LCF fits are displayed in Figure S8, alongside best EXAFS LCF fits for comparison. As demonstrated by Figure S8 and LCF results in Table S11, both EXAFS and XANES analyses give the same results. For this reason, we chose to use the EXAFS data analyses for the plant samples.

A change of Cd speciation upon plant uptake in the roots. Our results show that the main first neighbor of Cd in both *S. nigrum* and *S. melongena* roots is sulfur (77% and 100% respectively, Figures 4 and 5). In *S. nigrum* roots, the remaining Cd is bound to oxygen. All the acceptable linear combination fittings involve the Cd-glutathione model compound, a thiol molecule, as the S-bearing ligand (Supplementary material Part 1). For the oxygen counterpart in *S. nigrum*, while the best residue (R factor) was obtained with Cd-tartrate, the LCF method could not distinguish between $Cd(H_2O)_6^{2+}$, Cd-histidine, or any of the Cd-organic acids analyzed (malate, tartrate, citrate, and acetate).

These results first demonstrate a major change of Cd speciation between the soil (Cd sorbed to goethite) and the roots (Cd mainly bound to organic -S) for both solanum species. From our results alone, it is unclear how the Cd sorbed to goethite in the soil becomes available to the plants. Several studies suggest that plants can participate in the release and dissolution of Cd sorbed onto oxyhydroxides, as root exudation of organic acids – particularly tartrate and acetate – is involved in heavy metal uptake by roots (Bao et al., 2011b; Fan et al., 1997; Sun et al., 2006). The Cd in *S. melongena* and *S. nigrum* roots is then mainly complexed with thiol ligands (100% for *S. melongena* and 77% for *S. nigrum*). In *S. melongena*, most of the Cd is located in the roots (Table 1), that show Cd

concentration of ~5 times that of the stem and fresh leaves (Table 1) and a roots to aerial parts translocation factor of ~0.24. In non-accumulator plants, Cd binding to thiols and vacuolar sequestration of these complexes has been previously described as a detoxification mechanism (Cobbett, 2000; Marentes and Rauser, 2007; Mendoza-Cózatl et al., 2008; Pál et al., 2018; Uraguchi and Fujiwara, 2012; Wawrzyński et al., 2006), with possible storage of these complexes in the roots (Cao et al., 2018; Nocito et al., 2011; Rauser, 2003; Salt et al., 2002, 1995; Ueno et al., 2010; Wiggenhauser et al., 2020). The binding of Cd to non-chelating thiols such as glutathione and cysteine or to chelating thiols such as phytochelatins in the roots could immobilize Cd and prevent its translocation to the aerial parts of the plant (Cao et al., 2018; Nocito et al., 2011; Salt et al., 2002). In rice, a recent XAS study by Wiggenhauser and collaborators (Wiggenhauser et al., 2020) demonstrated that Cd in roots is 100% bound to S ligands, bringing new, direct evidence of the role of Cd binding to S in Cd sequestration in roots and echoing our findings. We therefore conclude that Cd bound to organic -S ligands in *S. melongena* roots constitutes a storage form that limits Cd translocation to aerial parts and participates in its detoxification. In the Cd-hyperaccumulator *S. nigrum*, most of the Cd is bound to S ligands (77%) and the Cd translocation factor from roots to aerial parts is 0.31. Like *S. melongena*, we suggest that Cd in roots is mainly immobilized and stored as Cd-thiol complexes. Such mechanism has also been suggested in the Cd-hyperaccumulator *Brassica juncea L.* for which Cd coordination with S was demonstrated using XAS spectroscopy (Salt et al., 1995). For the remaining 23% of Cd bound to oxygen in *S. nigrum* roots, we considered two scenarios: (i) this Cd-O fraction is also a storage form or (ii) it represents a transient fraction that could be translocated to the aerial parts. (i) Storage of Cd as Cd-O has been previously reported in Cd-hyperaccumulator plants. In the Cd-hyperaccumulator plant *Thlaspi praecox,* a Brassicaceae that can accumulate very high amount of Cd in its shoot (>7500 $\mu g.g^{-1}$, DW, Lombi et al., 2000; Vogel-Mikuš et al., 2006), Vogel-Mikuš and her collaborators performed

399 XAS measurements and reported that, of the 858 µg.g^{-1} (DW) Cd in *T. praecox* roots, 79%
400 was bound to -O ligands (as Cd-O-C), and the remaining 21% was bound to -S ligands (as
401 thiols, Vogel-Mikuš et al., 2010). They suggest that Cd is mainly bound to cell-wall -O
402 ligand components or stored in the vacuoles, complexed with organic acids. Organic acid
403 vacuolar sequestration of cadmium, albeit limited in the roots compared to the shoots, is a
404 well-described detoxification mechanism in Cd hyperaccumulator plants (Han et al., 2006;
405 Huguet et al., 2012; Isaure et al., 2015; Küpper et al., 2004; Lombi et al., 2000; Maqbool et
406 al., 2019; Sun et al., 2011; Vogel-Mikuš et al., 2010). We cannot rule out that such
407 mechanism takes place in *S. nigrum* roots and account for part of the Cd-O we reported.
408 (ii) However, given that Cd speciation in *S. nigrum* stem shows an increase in Cd binding
409 to O ligands (55%, Figure 5) for an amount of ~ 3.3% of the plant total Cd (Table 1), we
410 consider that the Cd bound to O in the roots (~9.2% of the plant total Cd) could be a Cd
411 transient form for roots to aerial parts translocation. This Cd transport form hypothesis will
412 be further discussed in the next paragraph on Cd speciation in the stems.
413
414 **Cadmium transport forms in *S. nigrum* and *S. melongena* stems.** The linear
415 combination fittings of stem EXAFS spectra show contrasting results for *S. nigrum* and *S.*
416 *melongena*, as the main first neighbor of Cd for *S. nigrum* is O (55% O, 45 % S, Figure 5)
417 while it is S for *S. melongena* (78% S, 22% O, Figure 5). For *S. nigrum*, the best LCF
418 fittings involve non-protein thiol glutathione as the S-bearing ligand. For the O ligand in *S.*
419 *nigrum*, the best fit is obtained with acetate, but other organic acids malate, citrate, and
420 tartrate, as well as $Cd(H_2O)_6^{2+}$, histidine, give acceptable results according to our LCF
421 method. As for *S. melongena*, all the best LCF give glutathione as the S bearing ligand,
422 and tartrate as the best O ligand. Other organic acids, $Cd(H_2O)_6^{2+}$, histidine and phosphate
423 also give acceptable results.

S. melongena stem and fresh leaves display very similar cadmium concentrations (~58 µg.g^{-1} DW, Table 1), much lower than that of the roots (~250 µg.g^{-1} DW). Compared to *S. nigrum*, for which fresh leaves Cd concentration is three times higher than in stem (~127 and ~44 µg.g^{-1} DW respectively, Table 1), our results point towards a more limited translocation of Cd in *S. melongena* stem. Previous studies demonstrated – using XAS spectroscopy(Salt et al., 2002, 1995) – or suggested (using chemical titration, Mendoza-Cózatl et al., 2008) that Cd can be transported in the xylem sap bound to oxygen bearing components. Our EXAFS measurements show that the binding of Cd to O ligands in *S. melongena* is restricted to the stem, which we interpret as Cd transportation form in the saps. In *S. nigrum*, Cd concentration and contents in the plant parts (Table 1) indicate a more intense translocation of Cd from the roots to the leaves, where Cd is stored. As mentioned earlier, the amount of Cd bound to oxygen ligands in the stem (55% of stem Cd; ~3.3% of the plant total Cd) is close to that of the roots (~9.2% total Cd). This value is also similar to the one in *S. nigrum* fresh top leaves (15% Cd-O in the leaves; 8.1% of the plant total Cd, Figure 5). We interpret these results as an argument in favor of Cd-O being the translocation form for Cd in *S. nigrum*.

Like *S.* melongena, we propose that the prevalence of O ligands in the stem of *S. nigrum* is to be attributed to the transport form of Cd in the xylem sap. Our results suggest that Cd is transported bound to O ligands, unlike what was observed in *Arabidopsis halleri* and *A. lyrata* (Isaure et al., 2015) or Cd-hyperaccumulator *Noccaea caerulescens* (Küpper et al., 2004), where Cd is associated with glutathione. The possible transportation of Cd as $Cd(H_2O)_6^{2+}$ in the xylem sap of *A. halleri* has been proposed by Ueno et al., 2008 (Ueno et al., 2008). In *Brassica napus*, Mendoza-Cózatl et al. (2008) suggested that Cd in xylem sap could also be complexed by oxygen ligands. As for the specific O ligands candidates, histidine has been reported for zinc transport in sap (Küpper et al., 2004), and complexation of Zn by organic acids has been invoked in *A. halleri* xylem sap (Cornu et al.,

2015). Given that Cd is considered Zn sister element, and that Sun et al 2006 (Sun et al., 2006) identified increased amount of organic acids in *S. nigrum* when exposed to Cd, we hypothesize that Cd could be transported bound to organic acids in *S. nigrum* xylem sap, though further investigation is needed to rule out the involvement of histidine or $Cd(H_2O)_6^{2+}$. As for the phloem, Mendoza-Cózatl (2011, 2008) suggested transport of Cd by non-protein thiol compounds, namely glutathione, and possibly phytochelatins, in *Brassica napus*. This could explain the ligand mixture we measured in *S. nigrum* stem, with Cd being complexed with O ligands in the xylem sap and with S ligands in the phloem sap (Figure 5).

Storage of S-bound Cd in *S. nigrum* and *S. melongena* leaves. Our results show that the main first neighbor of Cd in both *S. nigrum* and *S. melongena* fresh leaves is sulfur (Figure 5). In *S. melongena* fresh leaves, 100% of Cd is bound to organic sulfur ligands (glutathione, Figures 4 and 5). In *S. nigrum*, Cd speciation depends on the leaf maturity. Younger, top leaves EXAFS spectrum is best reproduced with three components: Cd bound to organic sulfur (best fit with glutathione, 58.4%), Cd bound to inorganic sulfur (best fit with CdS, 27.1%) and Cd bound to oxygen ligands (best fit: 14.5% -O). While the best LCF fit involves Cd bound to tartrate, the method gives acceptable results for Cd-histidine, $Cd(H_2O)_6^{2+}$ and the other organic acids measured. In *S. nigrum*, older, bottom leaves, we found that 100% of Cd is bound to sulfur ligands: 74.4% to organic sulfur ligands (Cd-glutathione) and 25.6% to inorganic sulfur (CdS). For both *S. nigrum* and *S. melongena*, our results do not validate Sun et al., 2006 hypothesis that, for plants grown in the same conditions, Cd could be entirely complexed by organic acids in their leaves (Sun et al., 2006), as they reported high amounts of tartrate and acetate in leaves of *S. melongena* and of malate in leaves of *S. nigrum*.

Cd in *S. melongena* leaves.

Previous EXAFS studies performed on leaves from Cd-hyperaccumulators *T. praecox* (Vogel-Mikuš et al., 2010) and *Arabidospsis halleri* (Huguet et al., 2012; Isaure et al., 2015) or Cd-tolerant *Arabidopsis halleri* x *A. lyrata* progenies (Isaure et al., 2015) plants demonstrated that Cd is mainly bound to -O ligands, and that S ligands are secondary species. They link complexation of Cd to oxygen ligands to the plant Cd tolerance and its Cd accumulation capacity (Isaure et al., 2015). Interestingly, in non-accumulator *Arabidospis lyrata* and Cd non-tolerant *Arabidopsis halleri* x *A. lyrata* progenies from Isaure et al. study (Isaure et al., 2015), Cd is coordinated by S atoms only or with a small contribution of O ligands, which is very similar to what we observed in *S. melongena* (100% Cd bound to thiols). As mentioned earlier, Cd chelation by thiol ligands and vacuolar sequestration of these complexes is a known detoxification mechanism in various plants and fungi (Cobbett, 2000; Marentes and Rauser, 2007; Mendoza-Cózatl et al., 2008; Pál et al., 2018; Uraguchi and Fujiwara, 2012; Wawrzyński et al., 2006). Among the non-protein thiol ligands, glutathione is of particular interest. As an antioxidant compound, it plays a major role in the defense against oxidative stress in plants (Cobbett, 2000; Mendoza-Cózatl et al., 2008; Ulrich and Jakob, 2019). Glutathione is also the precursor and building block of phytochelatins (Noctor et al., 2011; Ulrich and Jakob, 2019), which have been identified as Cd chelating agents involved in Cd detoxification mechanisms (Cobbett, 2000; Szalai et al., 2009). Our results point toward a major role of these detoxification mechanisms in *S. melongena*. Genetic and cellular studies show that the vacuolar sequestration of Cd complexed by non-protein thiols are active processes that consume energy to maintain vacuolar to cytoplasmic Cd gradient (Cobbett, 2000; Ulrich and Jakob, 2019). Interestingly, in the dead leaves of *S. melongena*, the amount of Cd bound to thiol ligands dramatically decreases compared to fresh leaves, with values of

40% total Cd bound to S ligands (Figures 4 and 5), the remaining Cd being bound to -O ligands (accepted fits with Cd-malate and Cd-tartrate). This is in very good agreement with the existence of an active Cd detoxification mechanism taking place in *S. melongena* fresh tissues.

Cd in *S. nigrum* leaves.

As for *S. melongena*, Cd complexation by thiol ligands plays an important role in *S. nigrum*, with values of ~75% in the bottom, more mature leaves. Our results suggest that the remaining Cd in those leaves is bound to inorganic sulfur (Figures 4 and 5). Previous studies reported that Cd may be stored in plants, including *Solanum lycopersicum*, in a stable high molecular complex that incorporate phytochelatins and sulfides (Collin et al., 2014; Pickering et al., 1999; Reese et al., 1992). In fungi, CdS crystallite core coated with phytochelatins were isolated and identified by titration and optical spectroscopy (Bae and Mehra, 1998; Dameron and Winge, 1990). The possibility of a polynuclear Cd cluster with inorganic, bridging sulfides was also suggested by sulfur K-edge EXAFS performed on synthetic Cd-phytochelatin (Pickering et al., 1999). Bae and Mehra proposed that the stability of Cd-phytochelatin complexes is enhanced by the addition of sulfides (Bae and Mehra, 1998). Cadmium detoxification through binding to mineral sulfur has also been observed by Cd K-edge EXAFS spectroscopy, in the bacterium *Stenotrophomonas maltophilia* (Pages et al., 2008). We also conclude that the combination of inorganic CdS and Cd bound to thiols compound observed in *S. nigrum* mature leaves constitutes a Cd detoxification mechanism and could explain the enhanced Cd accumulation capacity of *S. nigrum* compared to *S. melongena*. In *S. nigrum* younger, top leaves, our results suggest that the same mechanism is involved, as 58% of Cd is bound to thiols and 27% to mineral S. In addition, ~15% of Cd is bound to -O ligands. As the more mature leaves do not show Cd complexation with oxygen ligands, this reinforces our hypothesis that Cd bound to O

528 ligands is a transient form in younger leaves, that could be associated with the
529 translocation and transport of Cd from the roots to the leaves, where Cd would eventually
530 be stored bound to S ligands.
531
532
533
534 **3.4. Environmental implications**
535 Since Cd phytoavailability is tightly linked to its chemical speciation (Cullen and
536 Maldonado, 2013), it is crucial to get direct, experimental access to this parameter. In this
537 context, the developing use of XAS spectroscopy to determine Cd ligands keeps on
538 bringing new information on this metal fate in plants (Huguet et al., 2015; Isaure et al.,
539 2015; Wiggenhauser et al., 2020). We reported here, to our knowledge, the first direct
540 determination of Cd speciation in Solanaceae. We demonstrated that sulfur plays a major
541 role in the detoxification of Cd in these plants, and that oxygen ligands only take part as
542 transport forms for Cd translocation. Our findings do not agree with Sun et al. titration-
543 based hypothesis of a full Cd complexation with organic acids in *S. nigrum* and *S.*
544 *melongena* (Sun et al., 2006), which proves that XAS spectroscopy has a lot to offer to
545 understand Cd distribution in plants. For the first time, a combination of Cd binding to
546 thiolates and to inorganic sulfur was determined in plants, confirming Salt and
547 collaborators intuition in 2002 (Salt et al., 2002). Cao et al. showed that sulfur fertilizer
548 application in rice reduces Cd uptake and translocation to the grains (Cao et al., 2018). In
549 *S. melongena*, a similar result was obtained for chromium: high sulfur supply decreases
550 Cr(VI) toxicity (Singh et al., 2017). As we showed that S is a major agent of Cd
551 detoxification in *S. nigrum* and *S. melongena*, it would be of interest to investigate whether
552 high sulfur supply to these plants may also reduce Cd toxicity.
553

554

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Figures

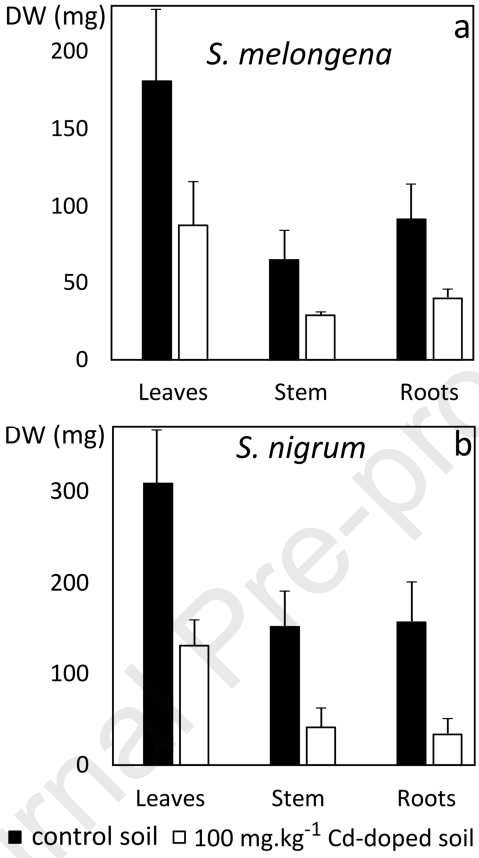

Fig. 1. Dry weight (DW) in milligrams of plant parts from *S. melongena* (a) and *S. nigrum* (b) grown on 100 mg.kg^{-1} Cd-doped soil and control soil. Error bars are 1 s.d.

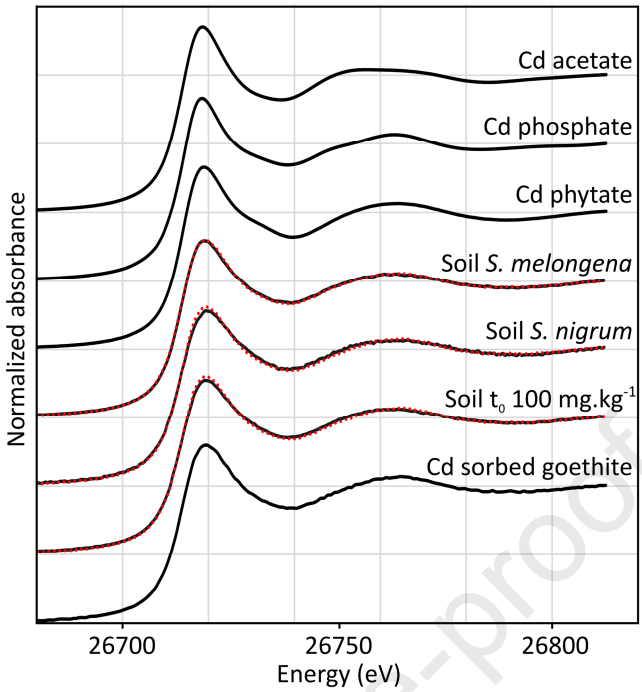

Fig. 2. Cd K-edge XANES spectra of 100 mg.kg^{-1} Cd-doped pre-culture soil (Soil t_0) and post-culture soils (Soil *S. melongena* and Soil *S. nigrum*; solid dark grey lines) and their respective reconstructed best fits (LCF; dotted red lines). LCF parameters are given in Supplementary Table S9. Cd K-edge XANES spectra of some relevant Cd reference compounds for comparison (Cd sorbed to goethite, Cd-phytate, Cd-phosphate and Cd-acetate).

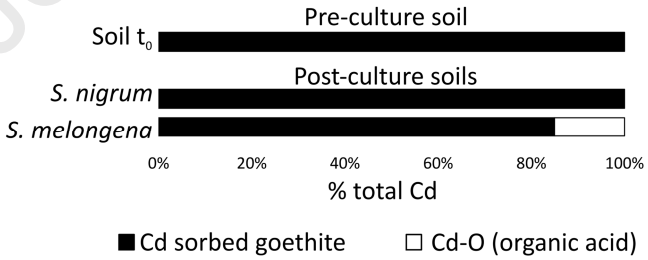

Fig. 3. Proportion of Cd species (in % mole fraction) in pre- and post-culture soils treated with 100 mg.kg^{-1} Cd, obtained by XANES linear combination fitting. The percentages were normalized to 100%. The uncertainty of each compound proportion was estimated to ±15 %. LCF parameters are given in Supplementary Table S9.

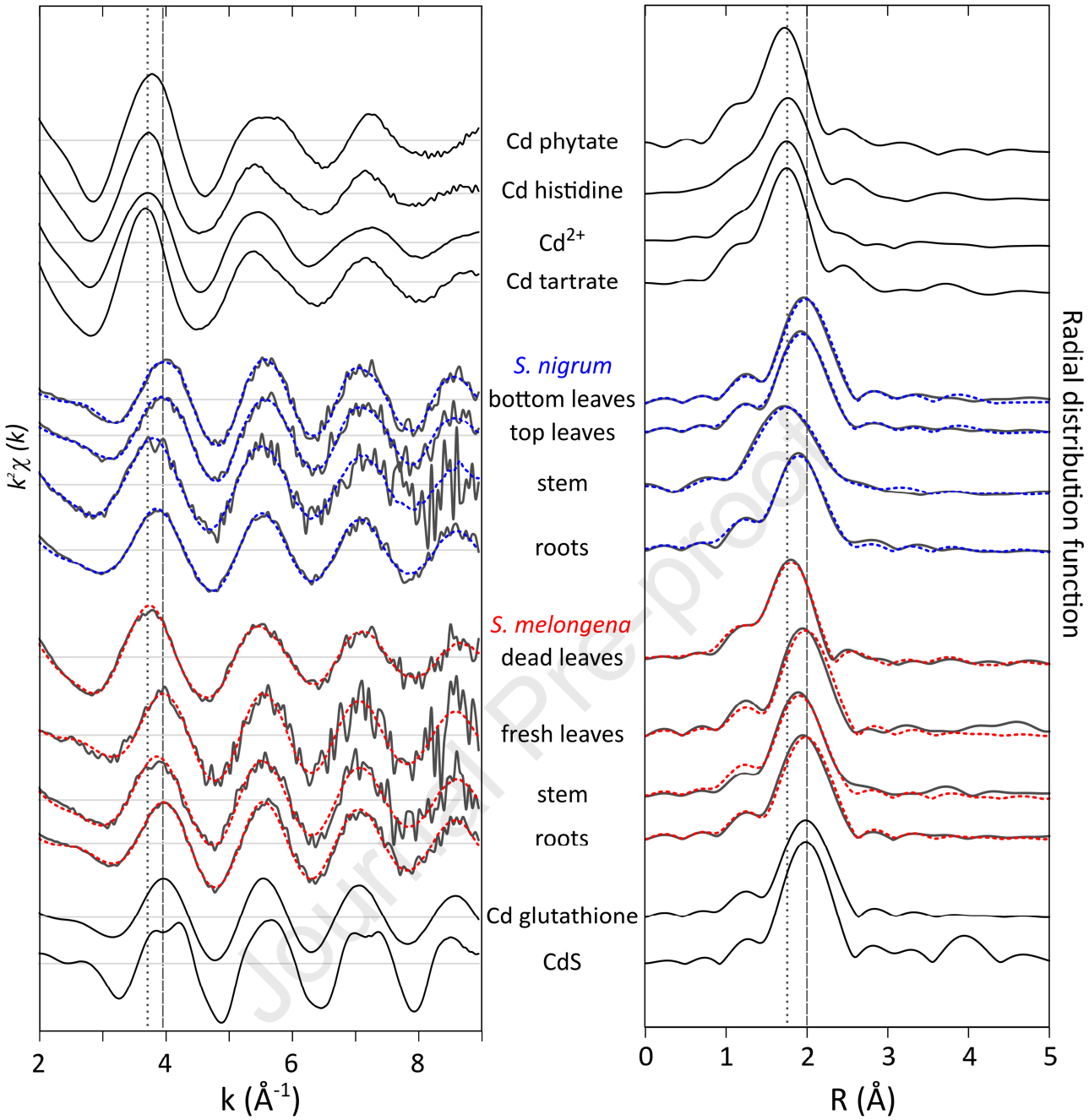

Fig. 4. Cd K-edge EXAFS spectra (left) and Fourier transforms (FT, radial distribution function – uncorrected for phase shift functions; right) giving the radial distances of the nearest neighbors to Cd for plant parts of *S. nigrum* and *S. melongena* (dark grey solid lines) after 14 weeks of Cd exposure on a 100 mg.kg^{-1} Cd-doped soil. Red dotted lines: reconstructed best fits (LCF) for *S. melongena* EXAFS spectra and associated FT. Blue dotted lines: reconstructed best fits (LCF) for *S. nigrum* EXAFS spectra and associated FT. LCF parameters are given in Supplementary Table S11. Black solid lines: EXAFS spectra and FT of some relevant Cd reference compounds for comparison. Vertical dotted lines: distance R or wave-number k associated with Cd bound to O atoms in its first shell. Vertical dashed lines: distance R or wave-number k associated with Cd bound to S atoms in its first shell.

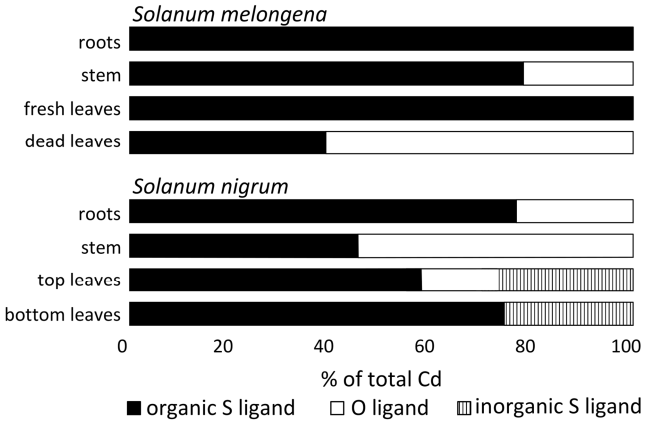

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Fig. 5. Proportion of Cd species (in % mole fraction) in *S. melongena* and *S. nigrum* plant parts (plants grown on a 100 $mg.kg^{-1}$ Cd-doped soil) obtained by EXAFS linear combination fitting. The percentages were normalized to 100%. The uncertainty of each compound proportion was estimated to ±15 %. LCF parameters are given in Supplementary Table S11.

Highlights

- X-ray absorption Cd speciation in *Solanum nigrum and S. melongena* was reported.
- Cd in the pre- and post-culture clayey loam soils was bound to iron oxyhydroxides.
- In *S. melongena* roots and fresh leaves, Cd was fully bound to thiol ligands.
- In *S. nigrum* leaves, Cd detoxification involved binding to thiols and inorganic S.
- In both plants, Cd transportation involved partial complexation with organic acids.

Declaration of interests

x The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: