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Effect of combined arsenic and lead exposure on their uptake and translocation in Indian mustard \star



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ABSTRACT

Phytoremediation makes use of hyperaccumulating plants to remove potentially toxic elements (PTEs) from soil selectively. Most researches examining hyperaccumulators focused on how they act on a single PTE contaminant, However, there is more than one kind of PTEs in most contaminated soils. Phytoremediation approaches could be less effective in environments containing multiple PTEs contaminants. Here we examine arsenic (As) and lead (Pb) accumulation in Indian Mustard (Brassica juncea) from solutions with one or both pollutants. Indian mustard accumulates As or Pb when exposed in the single liquid exposure of As or Pb, and the highest concentrations of As and Pb in Indian Mustard reach 1,786 mg/kg and 47,200 mg/kg, respectively. But the absorption efficiencies of As and Pb decrease (by >90% for As, and ~10–30% for Pb) when both As and Pb are present. The translocation of As and Pb from the root to leaf is also impeded by 36%-88% for As and 55-85% for Pb when treated with both PTEs. In As and Pb co-treatment, significant negative correlations between As (V) and P and between Pb and other elements (including K, Mg and Ca) were found in Indian mustard. X-ray absorption near edge (XANES) spectroscopy and subcellular extraction experiments indicate that much of the accumulated Pb bound within lead phosphate particles, and often located within the cell wall. Pb could decrease the percentage of water-soluble As and increase protein combined As in subcellular levels within Indian mustard. Based on these data, we suggest that the competition between Pb and monovalent and divalent nutrients (e.g., Ca(II), Mg(II) and K(I)), and the formation of lead phosphates within cell walls play critical roles in decreasing As and Pb co-uptake efficiencies for Indian mustard.

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1. Introduction

Mining, smelting, industrial use and disposal have resulted in widespread potentially toxic elements (PTEs) contamination of soil, water, and atmospheric conditions (Milačič et al., 2017; Palansooriya et al., 2020). Arsenic (As) and lead (Pb) are two of the most prevalent PTEs contaminants with a myriad of adverse health effects, including neurological disorders, heart diseases, and

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various cancers associated with both chronic and acute exposures (Duruibe et al., 2007; Ekholm et al., 2009; Järup, 2003). Arsenic and Pb often exist together in the soil as contamination sources often have both elements present. For example, mining and processing of sulfide minerals in lead-zinc mining areas can directly pollute the soil, causing As and Pb content in the soil to exceed the geobaseline or regulatory levels by several orders of magnitude (Nriagu, 1996; Rodríguez et al., 2009; Zhou et al., 2007). Arsenic and Pb are also both relatively volatile elements that can be released from smokestack emissions during smelting into the surrounding soils. Soil As and Pb concentrations approached 1,500 mg/kg and 50 mg/kg, respectively, near an ore smelter (Lynch et al., 1980). The historical usage of lead arsenate pesticides on croplands also



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contributes to As and Pb contamination in agricultural soils, reaching 1,000 and 400 mg/kg as reported, respectively (Salido et al., 2003).

Globally, more than 5 million sites, covering 20 million ha of soil lands, are contaminated by multiple PTEs, and most of them urgently need to be remediated (He et al., 2015; Wuana and Okieimen, 2011). Phytoremediation is a green, cheap, and potentially efficient means of remediating inorganic pollutants (Chaney, 1997; Liu et al., 2018; Salt et al., 1998; Tangahu et al., 2011). It is essential to study phytoremediation in more representative environments, such as those containing both As and Pb (Macfarlane and Burchett, 2002), since As and Pb have distinct physical and chemical properties in soil resulting from their contrasts in speciation, solubility, and bioavailability (Sun et al., 2011). Meanwhile, the combination of As and Pb can also inhibit plant growth (Sun et al., 2011) and cause additional stress to plants (Li and Wong, 2012), both of which can prevent effective remediation. Therefore, soils containing multiple contaminants are much more complicated, challenging, and expensive to be remediated than that of single pollutants (Khalid et al., 2017; Ye et al., 2017). The difficulties in addressing multiple contaminants cause most phytoremediation efforts to focus on the selective removal of a single pollutant. The limited data available from such studies suggest that remediation efficiencies decrease in some areas with complex contaminant mixtures (Kamnev and Lelie, 2000).

Indian mustard (*Brassica juncea*) is an ideal plant to study phytoremediation mechanisms since it could accumulate As and Pb (Bassegio et al., 2020; Bennett et al., 2003; Blaylock et al., 1997; Pickering et al., 2000). These recent studies suggest that As and Pb levels in Indian Mustard can reach over 500 mg/kg and 10,000 mg/ kg in similar experiments containing either As or Pb. As far as we know, limited researches was on its application on complex As and Pb pollution. Pb uptake efficiency of Indian mustard was reported to be improved by adding EDTA into Pb and As complex contaminated soil, but no characters about the As uptake efficiency were studied (Salido et al., 2003). The reduction of phytoremediation efficiencies on As and Pb contaminated soils need to be evaluated, and related mechanisms need to be grasped before large-scale applications.

The study aims to examine the effects of combined As and Pb exposure on their respective uptake efficiencies on potential multiple-phytoremediation plants, and explain the mechanism of the absorption efficiency decline from the aspects of the changes of elements competitive uptakes, As and Pb localization characteristics at the subcellular level, and element species. Greenhouse experiments are highly useful because they allow researchers to control conditions and optimize phytoremediation methods carefully. X-ray absorption near-edge structure (XANES) spectroscopy is important *in situ* technique to study the element chemical species in plants (Bovenkamp et al., 2013; Liu and Luo, 2019; Luo et al., 2016). We compared the absorption and transport characteristics of As and Pb by Indian mustard under separate or simultaneous exposure to As and Pb with variable levels based on greenhouse experiment. Following uptake, selective extractions and XANES are used to study the localization characters in subcellular level and speciation of As and Pb changes within plant tissues. This study is among the first to examine the effects of concurrent As and Pb exposure on their uptake, transportation, and detoxification in Indian mustard.

2. Material and methods

2.1. Greenhouse Pb-As gradient exposure experiment

Indian mustard seeds were surface sterilized with 2.6% (w/v)

NaClO for 30 min, rinsed with deionized water, transferred into a Petri dish filled with deionized water (Pickering et al., 2000). Then seeds were vernalized in darkness within a 4 °C fridge for 5 days, then moved outside for germination (natural light, 25 °C). Seedlings with similar size were selected and cultivated in 250 mL mason jars filled with Hoagland's nutrient solutions for two weeks. Indian mustard seedlings were then washed and transferred into jars filled with modified Hoagland's nutrient solutions (KH₂PO₃ was removed from the formula to reduce the formation of lead phosphate precipitation in the solution) amended with variable levels of Na₂HAsO₄ and/or Pb(NO₃)₂. Treatments containing only As or Pb are referred to as "As-only" and "Pb-only"; while treatments containing both As and Pb are called "As + Pb" treatments. Four levels of As and Pb concentrations were used, corresponding to 0, 100, 200, and 400 μ mol/L As or Pb, equivalent to up to about 30 mg/L As and 82 mg/L Pb. All growth experiments were done in triplicate.

Plants were exposed to these variable As and Pb-containing solutions for three days (natural light, 25 °C), and then collected for analysis. Plants were washed, soaked in 2 mmol/L Na₂H₂EDTA (ethylene diamine tetraacetic acid, disodium salt) solutions for 15 min to remove As and Pb adsorbed on the root surface, rinsed with distilled water for more than five times, air-dried, and then divided into roots, stems and leaves, respectively, freeze-dried, flash-frozen in liquid nitrogen gas, and grounded into homogeneous powders with a clean mortar and pestle.

2.2. Sample digestion and analysis of the total contents of elements

0.50 g sample powders for each sample were weighted and then pre-digested in Teflon beakers with 5 mL concentrated HNO₃ (68%) and 2 mL H_2O_2 (30%) for 10 h. Then beakers were heated on a hot plate for 2 h (50 °C) and then set to 80 °C to evaporate the liquid until almost dry. Then 5 mL concentrated HNO₃ (68%) and 2 mL H_2O_2 (30%) were added into the beakers again, cover the sealing cap and digest for 300 min (150 °C), deionized water was added until the liquid was weighted to 50.00 g and then diluted five times before analyzing. An inductively coupled plasma mass spectrometry (ICP-MS) (Elmer NexION 300, Perkin) was used to measure the concentrations of Pb and As, and an inductively coupled plasmaoptical emission spectrometry (ICP-OES) (Optima 8300) was processed to measure the concentrations of other elements using established procedures, including K, Ca, Mg, Na, Mn, S, and P. (See quality controls in supporting information Part S1). All results obtained are reported as dry weight (DW).

2.3. Subcellular selective fraction extraction experiment

A subcellular selective extractions method was used to separate water-soluble, membrane and cell wall-bound, hydrophobic proteins-bound, solid phases like carbonates and less soluble organically-bound elements and residues in specific plant tissues (Jedynak and Kowalska, 2011; Jedynak et al., 2010; Połeć-Pawlak et al., 2005). The extraction method was sequential to remove the most labile forms of elements first, with stronger extraction conditions step by step to remove increasingly recalcitrant forms of As and Pb as follows: Step 1: 50.0 mg plant powders were added into 1.5 mL conical centrifuge tubes. Then 1 mL 10 mmol Tris-HCl (tris(hydroxymethyl)aminomethane -HCl, adjusted to pH 7.4) was added into the tubes to extract water-soluble complexes that are frequently isolated within the cytosol and/or vacuole. Each extraction step was processed within an ultrasonic tank (100% ultrasonic frequency, 22 °C) for 1 h. Then tubes were kept in an ice bath for 10 min, and then centrifuge twice with a centrifuge (15,000 rpm, centrifuge for 15 min). The liquid in the tube was transferred out, filtered (0.45 µm filters), and analyzed using ICP-MS and ICP-OES. The residues were kept in the centrifuge tubes for further extraction procedure of the next step. Step 2: 1 mL 2% DriselaseTM (D9515, Sigma) in 10 mmol Tris-HCl (pH 7.4) was added into the tubes with the residues from step 1 to quantify membrane and cell wall-bound species released during enzymatic digestion of cell walls. Copy the rest of the procedures in step 1 and move to step 3. Step 3: 1 mL 1% sodium dodecyl sulfate (SDS) in 10 mmol Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.4) was added into the tubes with the residues from step 2 to disrupt biological membranes and extract species binding with hydrophobic proteins. Copy the rest of the procedures in step 3 as well and move to step 4. Step 4: 1 mL 10 mmol CH₃COONH₄ (pH 4.6) was added into the tubes with the residues from step 3 to extract some solid phases like carbonates and less soluble organically-bound species. Step 4 is the last extraction step, so the residues from this step were digested and analyzed with ICP-MS and ICP-OES following the method introduced in 2.2. All results here were reported as dry weight (DW).

2.4. XANES spectrometry analysis and data processing

Pb L_{III}-edge XANES spectroscopy was performed at beamline 14W1 at Shanghai Synchrotron Radiation Facility (SSRF). This beamline was configured with a double channel Si (111) monochromator. The spot size of the focused beam was set to 300 μ m × 300 μ m with Kirkpatrick-Baez mirrors. The sample X-ray fluorescence signal was monitored with a 32-element Ge detector oriented 45 degrees off the sample and orthogonal to the incident radiation. The spectra energy range was 12,980–13,240 eV, with the dwell time of 1s and edge step of 0.5 eV, near the instrument resolution (about 1 eV) and smaller than the theoretical width of structural features (several eV) based on the core-hole lifetime of Pb. This is adequate for the accurate collection of XANES data. Pb L_{III}-edge (13,035 eV) energy was calibrated with Pb foil before analysis.

Indian mustard (treated with 400 μ mol/L Pb and 400 μ mol/L As + Pb, respectively) roots were washed with 2 mmol/L Na₂H₂EDTA and deionized water, freeze-dried, and fixed on the Kapton tape before analysis. Samples were folded three times before fixed on the sample holder to increase the Pb detection mass. To get a good XANES signal to noise ratio, XRF signals were monitored to target the spot with relative higher Pb concentrations on the main root. Two parallel runs were performed for each sample at room temperature.

Spectral processing and analysis were performed using the software package Athena (Ravel and Newville, 2005), including averaging replicate spectra, curve smoothing, and normalization. The identity of Pb in plant tissue was determined by comparison with reference spectra of potential Pb target complexes and mineral solids, and was quantified using linear combination fitting (LCF) of those references as previously described (Ostergren et al., 1999; Sun et al., 2018). The fitting presented here includes the following species: Pb₅(PO₄)₃Cl, cellulose-Pb, Pb(CH₃COO)₄. Other phases, including (C₁₇H₃₅COO)₂Pb, Pb(NO₃)₂, Pb₃(PO₄)₂, PbCO₃, $Pb(OH)_2$ were also considered, but these compounds were not required in the fitting. Cellulose-Pb was synthesized by adding 1.0 mmol/L Pb(NO₃)₂ solution (pH 6) into cellulose (Sigma Aldrich) for 72 h, rinsed in deionized water, and freeze-dried for storage. Other references were purchased (Sigma). The quality of those fits was estimated using the residual (the fraction of the spectrum not fit with a combination of reference spectra) and the R-factor for the final fits.

3. Results and discussions

3.1. Arsenic and lead uptake by Indian Mustard

To properly assess the accumulation of As and Pb under complex treatment, it is essential to be able to compare uptake of elements in experiments containing only As or Pb. In experiments containing only As in media, arsenic concentrations in plants increased proportionally to increases in aqueous concentration (Fig. 1-a). The highest As concentration in plant reaches 1,786 mg/kg (DW), around 60 times higher than that in the media (highest only-As treatment level, 30 mg/L). Lead followed similar patterns, its concentrations increased to 47,200 mg/kg, 600 times as that of in the media (~82 mg/L Pb). Both As and Pb uptake decreased considerably when both were present in the media (Fig. 1). The presence of Pb decreases the plant As concentrations sharply (more than 90% in each treatment), while plant Pb concentrations decreased <40% due to As addition.

It is important to note that bioavailability of As and Pb in culture medium could affect phytoaccumulation. The bioavailability differences between single and complex treatment need to be evaluated before other mechanisms were considered. Firstly, precipitation in solutions could affect the bioavailability of ions in hydroponic experiments. We evaluated the solution composition and saturation indices (SI) in hydroponic nutrient solutions with Visual MINTEQ 3.1 using the default database for mineral and aqueous complexes (See supporting information Part S2). The SI of Pb₃(AsO₄)₂ were -1.361 (undersaturated), 0.140 (undersaturated), 1.636 (slight saturated) at As + Pb co-treatment level of 100, 200, and 400 μ mol/L, respectively. For 400 μ mol/L As + Pb co-treatment level, the solution was slightly supersaturated with lead arsenate, which is relatively insoluble, but much more soluble than lead phosphate analogs. This solution is also slightly saturated with anglesite (PbSO₄, SI = 0.3) because of the high concentration of sulfate counterions. The presence of As and Pb on element uptake affects As incorporation much more, despite potential precipitation



Fig. 1. The concentrations (DW) of As and Pb in Indian mustard plants after cultured in nutrition solutions with variable levels of As-only, Pb-only and As + Pb treatments. (a) Comparation of As concentrations in Indian mustard after treated in the media with variable As and As + Pb levels (0 μ mol/L, 100 μ mol/L, 200 μ mol/L and 400 μ mol/L). (b) Comparation of Pb concentrations in Indian mustard after treated in the media with variable Pb and As + Pb levels (0 μ mol/L, 100 μ mol/L, 200 μ mol/L and 400 μ mol/L).

affecting Pb levels more. For example, arsenic concentrations could decrease by 30% or so at most, whereas Pb concentrations could conceivably decrease by up to 50%. Given that As incorporation is the most affected, we conclude based on these measurements that As and Pb uptake is not highly affected by lead arsenate precipitation in these experiments, and that the difference in their plant concentrations results from differences in their absorption. Thus, these data suggest that the potential effects of lead arsenate precipitation in the media alone do not explain differences in plant uptake, this effect is smaller than interactive uptake effects. Meanwhile, complexing with ligands on dissolved organic matters like small-molecule organic acids, amino acids, etc., secreted by plants may also affect the bioavailability of ions in solutions (Niyogi and Wood, 2004). But the amount of free ionic added in this study is extremely higher, it might be much higher than those organic bound ones that may affect the plant uptakes in the single element and co-treatment. Therefore, contribution of organic ligands to the reduction of co-uptake is also limited.

The distribution and concentration of As and Pb in roots do appear to be affected by their combined presence (Figure S3 and Table 1); however, they affect As and Pb in ways that are not readily explained by precipitation in the rhizosphere or roots. Arsenic concentrations in roots differ most, increasing from 440 mg/kg (in the absence of Pb) to 833 mg/kg and 1,214 mg/kg as Pb concentration increased. Meanwhile, Pb concentrations in the roots actually decreased somewhat due to As exposure. If lead arsenate precipitation did explain this increase in As concentrations within the root or on the root surface, about 700–800 mg/kg As could be attributed to mineral precipitation, corresponding to increases of 2,000–3,000 mg/kg Pb. Lead phosphate precipitation in the roots would further enhance Pb incorporation (Zeng et al., 2017), while not affecting As substantially. The presence of elevated As and Pb concentrations in the rhizosphere or root could also enhance their precipitation (Chappell et al., 1995). If lead arsenate precipitation occurred in the rhizosphere, it could decrease soluble levels and the plant's uptake of As and Pb. Conversely, if this mineral phase was to precipitate within the root tissues, then mineral formation could prevent translocation from the roots into above-ground biomass (Anawar et al., 2018). Although Pb was added to phosphate-free media for exposure treatment, phosphate is abundant in the plant comparing to which in the media, and also could precipitate with Pb. If this occurred in the roots, then, like lead arsenates, lead phosphate mineral precipitations could possibly inhibit Pb translocation into the stems and leaves.

The translocation of As and Pb upwards into stem and leaf also appear to be affected by the co-treatment of As and Pb (Table 1). The Pb transport efficiency aboveground appeared to decrease 70-90% due to As addition. Arsenic concentrations in the stem and leaf are very low, suggesting that most As is bound firmly within the root tissue particularly in the presence of Pb. The effective uptake and translocation of elements into aboveground biomass is key to effective phytoremediation. Bioconcentration factors (BCF, element concentration ratio of root to soil) and translocation factors (TF, element concentration ratio of shoot to root) are used to measure the phytoremediation efficiency (Yoon et al., 2006). Since the planting medium in this study is nutrient solutions, so we calculated BCF with the element concentration ratio of root to concentrations in the nutrient solution. A greater BCF value indicates better hyperaccumulate ability. The TF value determines the transport efficiency of PTEs from roots to branches. A TF value of less than 1 indicates that element transfer is ineffective, which indicates that these types of plants accumulate more elements in roots and rhizomes than in branches or leaves (Yoon et al., 2006). Phytoremediation is effective with high BCF and high TF. This is due to the efficient element transportation system. Moreover, fastgrowing rate, higher aerial biomass yield and other factors could also be the key for phytoextraction efficiencies (Liu et al., 2018). Root sequestration, which was significant for As, decreased the transport efficiencies of As into aboveground biomass. Table 1 calculates BCF and TF factors for Pb and As uptake by Indian mustard in each treatment.

In general, the BCF and TF decrease as element concentrations increase in solution. In experiments containing only As, its BCF decreased modestly, from 8.5 ± 0.2 to 4.5 ± 0.2 as As treatment level increased, while its TF decreased from 0.93 ± 0.07 to 0.47 ± 0.01 . In experiments containing only Pb, the effect was reversed. The BCF of Pb increased from 78 ± 3.7 to 118 ± 3.8 as Pb exposure increased, while its TF increased from 0.27 ± 0.01 to 0.33 ± 0.02 . The differences in the absorption characteristics of individual As and Pb may be due to the different detoxification capabilities and mechanisms of Indian mustard. It was reported that the majority of the As in Indian Mustard root was an As(III)-tris-thiolate complex (Pickering

Table 1

Bioconcentration factor (BCF) and translocation factor	r (TF) of As and Pb in Indian mustard under trea	atment with only As (or only Pb) and both As + Pb.
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Treatment	Treatment Level	Root	Stem	Leaf	BCF ^a	TF ^b	
	(µmol/L)	As (mg/kg, DW*)					
Control	0	0.7 ± 0.19	0.59 ± 0.06	0.52 ± 0.03	-	-	
As	100	441 ± 20	238 ± 19	172 ± 12	8.5 ± 0.2	0.93 ± 0.07	
	200	834 ± 15	287 ± 16	197 ± 15	6.6 ± 0.2	0.58 ± 0.05	
	400	$1,215 \pm 57$	342 ± 4.4	230 ± 18	4.5 ± 0.2	0.47 ± 0.01	
As + Pb	100	61 ± 4.4	4.4 ± 2.2	3.8 ± 1.0	0.69 ± 0.07	0.14 ± 0.04	
	200	46 ± 6.4	6.3 ± 1.2	4.9 ± 1.3	0.23 ± 0.05	0.24 ± 0.03	
	400	32 ± 3.2	5.1 ± 1.7	4.2 ± 1.8	0.08 ± 0.02	0.29 ± 0.08	
	Pb (mg/kg, DW)						
Control	0	3.0 ± 1.2	0.98 ± 0.29	1.2 ± 0.27	-	-	
Pb	100	6,132 ± 281	1,637 ± 117	34 ± 14	78 ± 3.7	0.27 ± 0.01	
	200	$14,380 \pm 702$	$4,201 \pm 219$	109 ± 17	93 ± 4.6	0.30 ± 0.00	
	400	35,592 ± 977	11,383 ± 695	226 ± 10	118 ± 3.8	0.33 ± 0.02	
As + Pb	100	4,158 ± 158	487 ± 17	19 ± 4.8	47 ± 1.7	0.12 ± 0.00	
	200	$15,502 \pm 470$	671 ± 27	105 ± 10	81 ± 2.5	0.05 ± 0.00	
	400	$30,737 \pm 1360$	$1,286 \pm 65$	120 ± 17	80 ± 3.4	0.05 ± 0.00	

Notes.

(*): DW: All the concentrations here are reported as dry weight.

(a): BCF: bioconcentration factors, normally calculated with element concentration ratio of root to solution.

(b): TF: translocation factors, which is element concentration ratio of shoot to root.

(±): The number after the symbol " \pm " represents "the average data in the group" \pm "the standard deviation of the group", n = 3.

et al., 2000), the limitation of the formation of the As-thiolate because of the limited S addition might cause the decreasing of the As uptake in Indian Mustard. On the contrary, mineral precipitates formed within the plant, potentially affected the uptake, translocation, bioavailability, and toxicity of the PTEs (Kodera et al., 2008; Tian et al., 2011), particularly for Pb (Blaylock et al., 1997). As the concentration of Pb increases, so does the chemical precipitation. The species of Pb and its subcellular localizations are further discussed in the following parts.

The BCF and TF of As and Pb both decreased significantly when both As and Pb were present. In experiments containing both As and Pb, BCF of As decreased by 91%–95% and As TF decreased by 36%–88% relative to As-only experiments. The effect of As on Pb accumulation and translocation was less obvious. The presence of As reduced the BCF and TF of Pb by 13–40% and 55–85%, respectively. Measured tissue concentrations do not provide direct evidence for the underlying basis for this decreased efficiency of uptake and translocation. It could result from changes in the concentration or speciation of As and/or Pb. However, it could also relate to the changes in biochemistry or physiology of the plant caused by exposure to one or both elements. We discuss evidence for correlations among variable elements that could explain As and Pb uptake in our experimental systems below.

3.2. Element co-uptake and competition characteristics

To properly assess how to facilitate best the uptake of As and Pb during phytoremediation, it is essential to consider how other elements are competing with As and Pb through plant uptake. We can gain considerable insight into the mechanisms for the uptake and exposure of elements and ions by examining the uptake and translocation of other elements and ions. Transport and ion channels are important ways of absorbing metal ions in plants. As reported, As and Pb enter plant cells through transport systems involved in micronutrient uptake. Many of these elements have well-studied pathways and are structural or ionic analogs of As and Pb; these other elements are often taken up and transported in parallel processes.

Arsenic uptake by plant can be affected by several elements (e.g., Fe, P, Si, and S) depending on soil conditions (Shin et al., 2010). Phosphate (PO_4^{3-}) transporters are involved in the uptake and transport of arsenate (AsO_4^{3-}) by *Arabidopsis thaliana* (pht1; 1–3) in oxidized soils (Catarecha et al., 2007). The structural similarity of silicate and arsenite, both of which are tetrahedral/trigonal planar uncharged oxyacids (H_4SiO_4 and H_3AsO_3) allows As to be transported into plants through Si transporters in more reduced soils containing As(III) (Zhao et al., 2010). Here in this study As(V) in plant were correlated to several other elements in Pb-free experiments, that is, when the only variable in the experiment involved changes in As activity (Table 2). In general, arsenic concentrations were negatively correlated with the concentrations of Ca, Mg and

Mn in plants (Table 2), while positively correlated to Na levels, which might be attributed by the additives were Na₃AsO₄. Although previous studies of As hyperaccumulators suggest that As is absorbed by *Arabidopsis thaliana* and other plants through silica or phosphate transporters, arsenic uptake has also been linked to Ca and other divalent ions (Cong and Ma, 2002). The correlations between As and Mn was weaker in treatments containing both As and Pb, although the more limited range of As concentrations may make any trends difficult to identify. The negative correlation between As and P was significant when treated within both As and Pb, which suggest that the competition between As(V) and soluble PO_4^{3-} was more obvious, might also explain why As concentrations decreased when exposed in Pb.

However, the negative correlations between Pb and K, Ca, Mg, and P levels were found when As was also present compared with Pb-only treatment (Table 2). This indicates that Pb also affects competitive absorptions of some of the divalent and monovalent cations, such as K, Ca and Mg, and P but through distinct interactions as opposed to that of As. This result is expected for divalent and monovalent cation and the complexation of thiols because Pb(II) is also a divalent cation that can complex with thiols (Jacquart et al., 2017; Mandavian et al., 2016). However, we suggest that the reason for the lack of correlation between Pb and S (Table 2) is that most of the Pb in plants is stored mainly in phases that contain little or no S, such as some hard dissolved Pb precipitation materials. So unlike arsenic, the lack of S in the experiment may seldomly decrease the uptake of Pb in Indian Mustard. This result is probably due to the extremely high Pb concentration in these tissues of the plant (Fig. 1). Lead, on the other hand, usually is found as free or complexed Pb cations that enter plant cells through cationic transporters and channels designed for Ca(II), Mg(II), K(I), and Fe(II) (Antosiewicz, 2005; Brunet et al., 2008; Sharma and Dubey, 2005; Zaier et al., 2010). Of these pathways, the Ca ion channels appear to be the most important for Pb to enter plant roots (Bertrand et al., 2008; Wang et al., 2007).

3.3. Characteristics of element subcellular localization and species under single or complex exposure of As and Pb

Selective extractions can help to identify plant components that contain As and/or Pb, and how those associations change in response to changes in solution composition (Luo and Rimmer, 1995). Most As extracted from plant tissues were water-soluble and cell-wall fractions in Indian mustard regardless of treatment (Fig. 2-a). The abundance of water-soluble As likely impacts its uptake and redistribution within the plant. Although water-soluble As is translocated efficiently, it is bioavailable and likely toxic to plants. Therefore, this likely causes the As to inhibit accumulate in the plant during growth. In As-only experiments, the water-soluble fraction of As gradually increases from the root to the leaf, while the cell wall binding fraction and protein fraction of As gradually

Table 2

Correlation coefficients between As/Pb an	l other elements in leaf, stei	m, and root under the only- an	d co-treatment of As and Pb.
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Treatment	Element		Correlation coefficients					
		K	Ca	Mg	Mn	Na	Р	S
As $(n = 36)$	As	-0.41*	-0.69**	-0.46**	- 0.61 **	0.74**	-0.39*	0.01
As + Pb $(n = 27)$		-0.44*	-0.87**	-0.70**	-0.39*	0.66**	- 0.54 **	-0.22
Pb (n = 36)	Pb	-0.14	-0.49**	-0.23	-0.34*	0.66 **	0.12	-0.07
As + Pb (n = 27)		- 0.50 **	-0.70**	- 0.55 **	-0.28	0.31	-0.45*	-0.19

Notes: Pearson correlation analysis test (two-tail) was processed by SPSS, n is the number of the samples in each test group.

(-)/(+): Negative or positive correlation between As/Pb and other elements.

(**): The correlation is significant at the p < 0.05 level.

(*): The correlation is significant at the p < 0.1 level.



Fig. 2. Subcellular localizations of As (a) and Pb (b) in leaf, stem, and root with As-only, Pb-only treatment and co-treatment of As + Pb (all for 400 $\mu mol/L$).

decreases toward the leaf. This result is consistent with soluble complexes being most effective when transported upward (Pickering et al., 2000), while detoxification by complexation with glutathione (GSH) and proteins is most effective where their overall concentrations are highest (Bleeker et al., 2010; Raab et al., 2005). In As + Pb experiments, the protein-bound As fraction increased (relative to As-only experiments) in stems and roots at the expense of both the water-soluble As fraction and the cell-wall bound As fractions. At the same time, overall, arsenic concentrations decreased sharply. This effect suggests that Pb is not only affecting As uptake but also enhancing the formation of less-bioavailable As-thiol complexes.

Lead was also partitioned into water-soluble and cell-wall fractions; however, a large residual fraction also remained, indicating that Pb had formed much more recalcitrant phases than that of As (Fig. 2-b). This recalcitrance likely reflects the formation of insoluble mineral forms of Pb, though it does not provide

information about the locus of that precipitation within the plant. The largest residual Pb fraction was present in the roots and stems, but some were also found in the leaves. There is no systematic difference between Pb partitioning with As-only and As + Pb experiments, except possibly in the leaves where the residual fraction increases from about 5% to 15% when As is present. The formation of Pb precipitates in the roots and stems likely explains the plant's high overall uptake capacity and the weak upward translocation into aboveground biomass. The presence of a large (>25%) residual Pb fraction in root and stem tissues suggests that these insoluble Pb phases continue to form as more soluble forms of Pb are transported upward. However, their formation is incomplete, and watersoluble Pb continues to be the most abundant Pb fraction in the leaves. Extractions of organic-acid and protein-bound Pb were fairly consistent across all tissues and treatments, suggesting that they may be present and affect Pb solubility and mobility (Senden et al., 2006; Wei et al., 2007), however, these complexes were not concentrated enough to impact the bulk of Pb speciation. The formation of both cell-wall bound and protein-bound Pb are effective in decreasing Pb solubility and mobility in plants (Gupta et al., 2010; Kopittke et al., 2008; Lombi and Susini, 2009; Phang et al., 2011). This result is possible given that the Pb concentrations are so high (approaching percent levels in tissues). Most of Pb is insoluble both internally and externally to plants, under normal conditions, so Pb is transported in or through the plant when complexed by organic acids or thiols (Gupta et al., 2010; Lombi and Susini, 2009; Tian et al., 2011). Alternatively, Pb chelates of small, soluble organic acids, proteins, and thiols may be extracted in the water-soluble step. The first extraction step capture most of the Pb complex existing in intercellular substance and vacuole, containing alkaloids, protein enzymes, inorganic salts and sugars (Połeć-Pawlak et al., 2005; Tan et al., 2019). It was also reported that inorganic sulfide (if present), and phosphate may also act to sequester heavy metal ion activity in the vacuole by forming insoluble sulfides and phosphates (Wang et al., 1991). Therefore, the substances separated by this extraction method are more representative of the content of the substances present in different parts of the plant cells, and of course it is also limited by the solubility of the substances under the corresponding experimental conditions during the extraction experiment. This result is quite plausible given the strong complexation of Pb to a variety of ligands and the prevalence of relatively insoluble Pb in the soluble and mobile fraction.

XANES spectroscopy suggests that the dominant Pb species is different in Pb-only and As + Pb experiments (Table 3). In Pb-only experiments, XANES suggests that Pb mainly exists as lead phosphate precipitates (98%) in the root of Pb-only treatments, but that the formation of these phosphates is suppressed significantly with As + Pb treatments, which have almost equal fractions of Pb phosphate, Pb-cellulose, and Pb acetate complexes. This may be

Table 3

Pb species and fractions in the root of Indian mustard exposure in single Pb and coexposure of As and Pb based on Pb L_{III} -edge XANES analysis and linear combination fitting.

Components	Pb Species Fraction \pm STD			
	Pb-only treatment	Pb + As treatment		
Pb ₅ (PO ₄) ₃ Cl Cellulose-Pb Pb(CH ₃ COO) ₂ R-factor γ ²	$\begin{array}{l} 0.98 \pm 0.06 \\ 0.03 \pm 0.00 \\ 0.00 \pm 0.06 \\ 0.000178 \\ 0.00131 \end{array}$	$\begin{array}{c} 0.32 \pm 0.23 \\ 0.45 \pm 0.24 \\ 0.18 \pm 0.23 \\ 0.001945 \\ 0.01356 \end{array}$		
Reduced χ^2	0.0000365	0.000377		

Note: Single Pb and co-treatment of Pb and As + Pb were both 100 μ mol/L.

because after the addition of As, it strongly competes with phosphate for ion competition channels, which makes it difficult for the limited P in the medium to be absorbed by plants, so the phosphate precipitation of Pb formed in this case is very limited. Our fitting results suggest that pyromorphite, Pb₅(PO₄)₃(OH, Cl) is the mineralogical form of phosphate present (see linear combination fitting results in Figure S6). However, in XANES analysis, some other minerals composed of Pb and phosphate may also have spectral data similar to Pb₅(PO₄)₃(OH, Cl), such as Pb₃(PO₄)₂. Therefore, Pb₅(PO₄)₃(OH, Cl), Pb₃(PO₄)₂ and other Pb phosphates could all be possible forms of Pb precipitation in Indian mustard root cells. The exceedingly low solubilities of lead phosphates are consistent with the sequestration of Pb within the roots, and the limited translocation of Pb to stems and leaves. Similar phosphate precipitates have been documented in Arabidopsis thaliana roots (Klassen et al., 2000). Both Pb-substituted hydroxyapatite and pyromorphite (Pb₅(PO₄)₃Cl) structures were found in plant roots (Gardea-Torresdey et al., 2005). Pb is usually retained in vascular bundles and cell walls as Pb₃(PO₄)₂ and Pb₅(PO₄)₃Cl (Kodera et al., 2008; Tian et al., 2011). Therefore, lead phosphate precipitation in the Indian mustard root is likely to be an effective detoxification strategy to keep the plant function while taking up large quantities of Pb. Cell wall components, for example, pectin, can also enhance metal tolerance (Krzeslowska et al., 2016) and affect translocation efficiency (Kopittke et al., 2008; Phang et al., 2011). Under the complex Pb–As exposure, the Pb entering the Indian mustard roots was greatly reduced compared to the Pb-only exposure (Fig. S3). It can be seen from the results of XANES that at this time, the percentages of cellulose-Pb (45%) and small-molecule organic acid-Pb (18%), which originally occupied the main proportion of the complex form, have dropped sharply, and the more difficult dissolve Pb (percentage of phosphates) has increased. Therefore, Indian mustard may be able to greatly stimulate the Pb accumulation capacity and detoxification mechanism of Indian mustard through the formation of Pb compounds (such as phosphate) with lower solubility; however, under the complex As + Pb exposure conditions, less soluble organic, cellulose binding state and phosphate are sufficient for enrichment and detoxification.

There is some disagreement between estimates of Pb speciation obtained by XANES and those inferred from extractions because of the limitation of the speciation analysis methods. Although the residual fraction was significant in most samples, the fraction of Pb bound in the residual fraction of the roots was much lower (about one half) than the fraction of phosphate determined by XANES (98%). XANES also suggests a large decrease in insoluble phosphate formation in As + Pb treatments relative to As-only treatments (Table 3), which were only weakly shown by changes in the residual fraction (Fig. 2). These discrepancies could reflect the limited selectivity of extractions. For example, water-soluble and cell-wall bound fractions could also contain Pb from the partial extraction of smaller, impure, and more soluble Pb phosphates. At the same time, for in-situ XANES analysis, it is possible that the spectral characteristics of XANES more reflect the information of the root surface, while the subcellular extraction experiment is aimed at the analysis of all cells in the entire tissue, which may also bring differences in results. This also indicates that during a single exposure of Pb, when excessive Pb enters Indian mustard roots, compared with the inner structure of the root, the outer layer structure like border cells may have a stronger ability to barrier Pb as a deposit seizure (Huskey et al., 2018). Alternatively, we may be overestimating phosphate mineral fractions because multiple potential components have similar spectra, or we lack a specific mineral standard needed for fitting. Although more data is needed to establish which of these explanations is most significant, they both likely influence the results.

4. Conclusions

The phytoremediation process is ideally effective in an environment affected by a variety of contaminants. In the present study, we designed a set of greenhouse experiments, using hyperaccumulator plant Indian mustard as the research object. We revealed that compared with single PTEs treatment, both As and Pb uptake in Indian mustard were reduced to various degrees under combined As and Pb treatment. This could seriously affect the phytoremediation abilities in a complex contaminated field. As and Pb uptake are found competitive, which may be caused by the change of competition uptake between Pb and monovalent or divalent cations like Ca, Mg, and K which are important nutrients in soil as well. The decrepitation of Pb phosphates, As and Pb species and bio-availability changes in cell walls and vacuoles play critical roles in affecting the plant uptake efficiency. This study may be applied to access the remediation efficiency reduction and provide valuable information needed to improve phytoremediation in complex soil solutions.

Credit author statement

Wenlei Yang: Performing the experiments, Data collection, Writing-Original draft preparation. Liqiang Luo: Conceptualization, Methodology, Supervision. Benjamin C. Bostick: Reviewing and Editing. Elizabeth Wiita: Reviewing and Editing. Youfa Cheng: Provision of greenhouse study materials and other resources. Yating Shen*: Project administration, Writing- Reviewing and Editing.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116549.

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