Effect of fulvic acids on lead-induced oxidative stress to metal sensitive *Vicia faba* L. plant

Muhammad Shahid · Camille Dumat · Jérôme Silvestre · Eric Pinelli

Abstract Lead (Pb) is a ubiquitous environmental pollutant capable to induce various morphological, physiological, and biochemical functions in plants. Only few publications focus on the influence of Pb speciation both on its phytoavailability and phytotoxicity. Therefore, Pb toxicity (in terms of lipid peroxidation, hydrogen peroxide induction, and photosynthetic pigments contents) was studied in *Vicia faba* plants in relation with Pb uptake and speciation. *V. faba* seedlings were exposed to Pb supplied as $Pb(NO_3)_2$ or complexed by two fulvic acids (FAs), i.e. Suwannee River fulvic acid (SRFA) and Elliott Soil fulvic acid (ESFA), for 1, 12, and 24 h under controlled hydroponic conditions. For both FAs, Pb uptake and translocation by *Vicia faba* increased at low level (5 mg l^{-1}), whereas decreased at high level of application (25 mg l^{-1}). Despite the increased Pb uptake with FAs at low concentrations, there was no influence on the Pb toxicity to the plants. However, at high concentrations, FAs reduced Pb toxicity by reducing its uptake. These results highlighted the role of the dilution factor for FAs reactivity in relation with structure; SRFA was more effective than ESFA in reducing Pb uptake and alleviating Pb toxicity to *V. faba* due to comparatively strong binding affinity for the heavy metal.

Keywords Speciation · Pigment contents · Metal toxicity · Reactive oxygen species

Abbreviations

Chl-a	Chlorophyll a
Chl-b	Chlorophyll b
EDTA	Ethylenediaminetetraacetic acid
ESFA	Elliott Soil fulvic acid
FAs	Fulvic acids
HSs	Humic substances
PCs	Phytochelatins
ROS	Reactive oxygen species
V. faba	*Vicia faba*
SRFA	Suwannee River fulvic acid
TBARS	Thiobarbituric acid reactive substances
WHAM VI	Windermere humic aqueous model VI

Introduction

Lead is widely used in many industrial processes and can reach in soil (Uzu et al. 2010; Shahid et al. 2011a). This metal is known to interfere with morphological, physiological, and biochemical functioning of exposed plants and can induce a broad range of noxious effects (Islam et al. 2008; Shahid et al. 2011b) such as, decrease in seed germination, root elongation, and plant biomass and inhibition of

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chlorophyll biosynthesis (Shahid et al. 2011c). Inside the cell, Pb can affect respiration and enzyme reactions (Shahid et al. 2011c). The presence of Pb in plants can also induce the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which can disrupt the redox status of cells and cause lipid peroxidation (Shahid et al. 2012). According to Shahid et al. (2011c), Pb-induced toxicity to plant is closely related to its form in the soil solution.

Humic substances (HSs) influence the speciation, the bioavailability, and the acute toxicity of metals in soils and natural waters (Dumat and Staunton 1999; Staunton et al. 2002; Dumat et al. 2006). These substances are a complex mixture of partially "decomposed" and otherwise transformed organic materials and constitute a major sorbent phase for contaminants in soils (Essington 2004). The properties of HSs are due to their physico–chemical characteristics such as molecular weight, molecular size, and substructures, and their functionality depends on the nature and source of organic matter and on their functional groups. The carboxylic and phenolic hydroxyl groups of HSs are the main binding sites for metals (Zeng et al. 2002). Most of the data on HSs refer to average properties and structure of a large assembly of components with diverse structure and molecular weight.

Over the last decades, more than 200 short-term bioassays utilizing plants, microorganisms, or insects have been developed and used to evaluate the environmental risk (Debenest et al. 2008 and 2009; Marcato-Romain et al. 2009a). Plant assays are highly sensitive, easy to be used in experiment, inexpensive, and good predictors of carcinogenicity (Ennever et al. 1988).

In the present study, the effect of Pb speciation on its uptake by *Vicia faba* and toxicity to the plant was assessed by measuring different stress biomarker parameters, such as lipid peroxidation, ROS, and pigment contents. These parameters were selected due to their high sensitivity and common use to evaluate plant toxicity to environmental pollutants, such as heavy metal (Mishra et al. 2006; Cenkci et al. 2010; Singh et al. 2010). Lead was supplied as salt or complexed with FAs.

Materials and methods

Plant materials and growth conditions

The *V. faba* (common name, broad beans; cultivar, Primabel; type, aguadulce; family, Fabaceae) seedlings were cultured according to El Hajjouji et al. (2007) and Marcato-Romain et al. (2009a). The *V. faba* seeds were germinated under optimal germination conditions, i.e., under the darkness at 22°C and 100% of moisture. When the primary roots were about 2–3-cm long, the seedlings were transplanted and cultured in modified Hoagland solution (Sigma) with the following macro elements: 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM KH_2PO_4, and 1.5 mM $MgSO_4$; the micro elements were: 9.11 μM $MnSO_4$, 1.53 μM $ZnSO_4$, 0.235 μM $CuSO_4$, 24.05 μM H_3BO_3, 0.1 μM Na_2MoO_4, and 268 μM Fe/ethylenediaminetetraacetic acid (EDTA). Nutrient solution was renewed on alternate days to keep the nutrient composition and pH constant. Plants were grown under controlled conditions in a phytotron with 16-h photoperiod at 70% relative humidity and day/night temperatures of 24/22°C. Light was supplied by 600 W Osram Nav-T super high pressure sodium lamp providing a minimum photosynthetical photon flux density of 500 μmol m^{-2} s^{-1} at the top of the plant (Pourrut et al. 2008).

Treatments

After preculturing of 15 days (fifth to sixth foliar stage), the plants were exposed to 5 μM Pb in the presence or absence of two FAs, i.e., Suwannee River fulvic acid (SRFA) and Elliott Soil fulvic acid (ESFA), from the International Humic Substances Society (IHSS, Colorado School of Mines, Golden, CO, USA), used as model of natural dissolved organic matter. The chemical properties of these FAs are given in Table 1. These substances were applied at two levels, i.e., 5 and 25 mg l^{-1}. The HSs were also applied alone as control at the highest level (25 mg l^{-1}). The concentration of KH_2PO_4 in the nutrient solution was reduced, also in the control, to 0.2 mM in order to prevent phosphate precipitation (Shahid et al. 2011c). All Pb treatments were applied for 1, 12, and 24 h. Since organic ligands have no effects on the studied parameters of *V. faba* up to 24 h (Shahid 2010), FAs alone was only applied for 24 h.

Calculation of Pb speciation in nutrient solution

The speciation of Pb in nutrient solution (free Pb^{2+} ions and complexed by FAs, Table 2) was calculated using Windermere

Table 1 Properties of the two standard FAs from International Humic Substance Society

Parameters	SRFA	ESFA
Cat. No	2S101F	2S102F
C (% *w*/*w*)	52.34	40.12
H (% *w*/*w*)	4.36	4.28
O (% *w*/*w*)	42.98	42.61
N (% *w*/*w*)	0.67	3.75
S (% *w*/*w*)	0.46	0.89
P (% *w*/*w*)	0.004	0.12
Carboxyl (mEq/g C)	11.17	13.24
Phenolic (mEq/g C)	2.84	2.27

Log K1 values are 3.76 and 3.67, and log K2 values are 9.84 and 9.53 for SRFA and ESFA, respectively

Humic Aqueous Model VI (WHAM VI) (Tipping et al. 1998) considering the applied level of FAs and the experimental conditions. WHAM VI is a speciation model designed to model chemical equilibria in oxic waters but can also be applied to soil solution where HSs are the main complexants of metals. According to this model, humic compounds are rigid spheres of homogenous size, which carry metal–humic binding sites positioned on the surface. These sites can have different binding strength and can be bidentate and tridentate binding sites. The model considers two types of acid groups for metal binding, which are represented by COOH and OH. The intrinsic WHAM VI Pb binding constants (log K1 3.2 and log K2 9.4) of COOH and OH were replaced with those of SRFA and ESFA obtained from IHSS site (Table 1).

Lead content analysis

Before all experiments, Pb content was analyzed in the nutrient solution so as to determine the possible precipitation of Pb. All solutions (Table 2) were prepared from solutions of Pb [50 μM Pb as $Pb(NO_3)_2$, Sigma] and FAs (100 mg l^{-1} SRFA and ESFA) in milli-Q water. The pH values of all solutions were adjusted to 5±0.1 using distilled HNO_3 (15 M, suprapur 99.9%, Sigma). Inductively coupled plasma–atomic emission spectrometry (ICP–AES, Jobin Yvon) with an IRIS Intrepid II XDL/ Thermo Electron Corporation was used to determine Pb contents after filtration (0.22 μm). Both the pH and total Pb concentration remained constant in all treatments after 24 h (Table 2).

Lead cell uptake by *V. faba* seedlings was determined according to Pourrut et al. (2008). After harvest, the seedlings were separated into root stem and leaves. Lead bound to the rhizoderm was removed by HCl as described by Uzu et al. (2009). Plants tissues were dried at 80°C for 48 h and digested by a 1:1 mixture of 65% HNO_3 (Sigma) and 30% H_2O_2 (Sigma) at 80°C over 6 h using DigiPrep Jr (SCP Sciences). After dilution with milli-Q water, the metal contents were analyzed using ICP-AES. Virginia tobacco leaves (CTA-VTL-2, polish certified reference material; ICHTJ) were used as a reference material for verifying the accuracy of the analytical procedure. Certified values for Pb in tobacco leaves were 22.1±1.2 mg Pb kg^{-1} dry weights. Measured values of the three replicates were 22.1±0.9, 22.3±1.0, and 22.0±0.7 mg Pb kg^{-1} dry weight.

Determination of lipid peroxidation

In order to evaluate the Pb-induced oxidative damages on cell membrane, thiobarbituric acid reactive substances (TBARS, Sigma) were determined as reported by Hodges et al. (1999). Plant samples were homogenized in hydro-alcoholic solution (80/20: *v*/*v*, Sigma) under liquid nitrogen at 4°C under darkness followed by incubation at 95°C with thiobarbituric acid. After centrifugation (3,000*g* for 10 min), the absorbance of the supernatant was recorded at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The amount of TBARS was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}) and the equations reported by Hodges et al. (1999).

Evaluation of H_2O_2 contents

The H_2O_2 content was measured according to Islam et al. (2008). Each sample (500 mg) was homogenized with 5 mL 0.1% (*w*/*v*) trichloroacetic acid (Sigma) under liquid nitrogen followed by centrifugation at 12,000*g* for 20 min. The mixture assay contained 0.5 mL of the supernatant added to 0.5 mL of 10-mM potassium phosphate buffer (pH 7.0, Sigma) and 1 mL of 1 M KI (Sigma). Absorbance was determined at 390 nm and the content of H_2O_2 was calculated using a standard curve under the same conditions.

Pigment content assay

The frozen leaves samples (600 mg) of *V. faba* were ground with liquid nitrogen. Pigments were extracted by incubating leaves in 10-mL acetone 80% (*v*/*v*, Sigma) for 24 h at 4°C under darkness. After centrifugation at 1,000*g* for 10 min,

Table 2 Experimental design and speciation of Pb in nutrient solution calculated by the WHAM VI

Treatments	Composition	Pb-chelated (%)	Pb-free (%)	Measured Pb (μM)
Control	Hoagland solution (HS)	–	–	0
ESFA-25	HS + 25 mg l^{-1} ESFA	–	–	–
SRFA-25	HS + 25 mg l^{-1} SRFA	–	–	–
Pb	HS + 5 μM Pb	0	84	4.99±0.04
Pb-ESFA-5	HS + 5 μM Pb + 5 mg l^{-1} ESFA	16	70	5.01±0.03
Pb-ESFA-25	HS + 5 μM Pb + 25 mg l^{-1} ESFA	36	57	5.01±0.02
Pb-SRFA-5	HS + 5 μM Pb + 5 mg l^{-1} SRFA	12	74	4.98±0.03
Pb-SRFA-5	HS + 5 μM Pb + 5 mg l^{-1} SRFA	12	74	4.98±0.03

Measured Pb (last column) indicates the Pb concentration (in micromolars) determined by ICP-AES in the nutrient solution after 24 h without plants

absorbance of the supernatant was recorded at 663, 645, and 480 nm. Concentrations of chlorophyll a (Chl-a), chlorophyll b (Chl-b), and carotenoids were calculated according to extinction coefficients and equations reported by Lichtenthaler (1987).

Statistical analysis

The variables were tested for differences between treatments using an analysis of variance (one-way) followed by LSD Fisher's test. This statistical analysis was performed using the software Statistica, edition'98 (StatSoft Inc., Tulsa, OK, USA). In each bioassay, the mean values are average of 12 replicates and one separate plant was used for each replicate. These 12 replicates were run in two separate experiments (six replicates in each experiment). An asterisk or a solid dot indicates significant differences at $P<0.05$ as measured by LSD Fisher's test.

Results and discussions

Effect of FAs on Pb uptake by *V. faba*

Table 3 presents Pb contents in *V. faba* roots, stem, and leaves in function of time. *V. faba* roots were exposed to Pb in the presence or absence of FAs. In roots, without FAs, there was a rapid uptake and accumulation of 27±8, 59±8, and 95±9 mg g^{-1} of Pb after 1, 12, and 24 h, respectively. In contrast to Pb uptake, its translocation towards shoot tissues was very slow and only 3.6±1.1 and 3.2±0.5 mg g^{-1} of Pb were transferred, respectively, into stem and leaves after 24 h. This limited translocation of Pb from roots to shoot tissues has been already reported (Shahid et al. 2011b). Indeed, in plant roots, Pb precipitates as insoluble salts or is immobilized by molecule such as sugar, pectins, celluloses, and hemicelluloses (Shahid et al. 2011b). This metal sequestration in roots is not common to all heavy metals but its intensity is very specific to Pb. Several studies reported that more than 90% of absorbed Pb is stored in the plant roots (Uzu et al. 2009; Shahid et al. 2011b). In our case, 93% of absorbed Pb was sequestered in *V. faba* roots.

Table 3 Effect of SRFA and ESFA on Pb concentration (in micrograms per gram dry weight) in *V. faba* roots, stem, and leaves

Treatments	Time (h)	Roots Mean ± SD	Stem Mean ± SD	Leaves Mean ± SD
Pb	1	27±8	0.14±0.01	0.03±0.01
Pb-SRFA-5		31±4	0.14±0.05	0.03±0.01
Pb-SRFA-25		27±2	0.11±0.02	0.02±0.01
Pb-ESFA-5		29±8	0.11±0.04	0.04±0.03
Pb-ESFA-25		28±2	0.14±0.03	0.03±0.01
Pb	12	59±8	2.9±0.7	2.2±0.4
Pb-SRFA-5		60±13	3.1±0.4	2.3±0.3
Pb-SRFA-25		47±12	2.4±0.6	2±0.3
Pb-ESFA-5		69±5	3.5±0.5	2.7±0.5
Pb-ESFA-25		50±6	2.5±0.3	2±0.5
Pb	24	95±9	3.6±1.1	3.2±0.5
Pb-SRFA-5		106±13	4.5±0.8	3.6±0.6
Pb-SRFA-25		62±19*	2.6±0.6*	2.4±0.2*
Pb-ESFA-5		110±15	5.1±0.3	3.9±1.1
Pb-ESFA-25		74±8*	2.8±0.4	2.6±0.3

Values are means of 12 replicates

*$P<0.05$, significant

Application of both FAs at low levels (Pb-SRFA-5 and Pb-ESFA-5) increased Pb uptake and Pb translocation to shoot tissues. The Pb accumulation in roots, stem, and leaves increased, respectively, by 16%, 42%, and 22% for Pb-ESFA-5 and by 12, 25% and 13% for Pb-SRFA-5 after 24 h. In contrast, at higher levels of FAs (Pb-SRFA-25 and Pb-ESFA-25), a significant ($P<0.05$) decrease in Pb uptake and translocation was observed except for Pb-ESFA-25 (Table 3). The decrease in Pb accumulation by Pb-ESFA-25 in roots, stem, and leaves was, respectively, 22%, 22%, and 19% after 24 h. In the case of Pb-SRFA-25, this decrease was 35%, 28%, and 25%, respectively, in roots, stem, and leaves after 24 h.

Several previous studies described the interaction between Pb and HSs and their results are contradictory. Some studies observed a significant increase in Pb uptake under HSs influence (Halim et al. 2003; Karaca et al. 2006; Salati et al. 2010), whereas others observed a decrease (Worms et al. 2010). In our experiment, the contrasting effects of FAs towards Pb uptake, at low and high concentrations could be explained by FA reactivity due to its structure (Dumat et al. 2000; Staunton et al. 2002; Kleber and Johnson 2010). When added at high concentration, FA adopts a ball structure, which can strongly complex Pb and reduce its plant uptake. Under diluted conditions, FA adopts a more hydrophilic fibrous structure that forms soluble and mobile complexes with Pb, which can enter the plants more easily due to small size and this increases Pb plant uptake (Evangelou et al. 2004; El-Ghamry et al. 2009).

The other possible explanation of the reduced Pb uptake at high level of FAs could be the competition between Pb and other nutrient ions. For example, Nardi et al. (2002) reported that FAs increased plant growth by positively influencing the uptake of nutrients. This increased uptake of nutrients at high level of FAs might have competed with Pb uptake. This can explain the Pb reduced uptake during the first 24 h.

The kinetic of Pb uptake by *V. faba* roots and translocation to aerial parts is shown in Fig. 1. The rate of Pb uptake

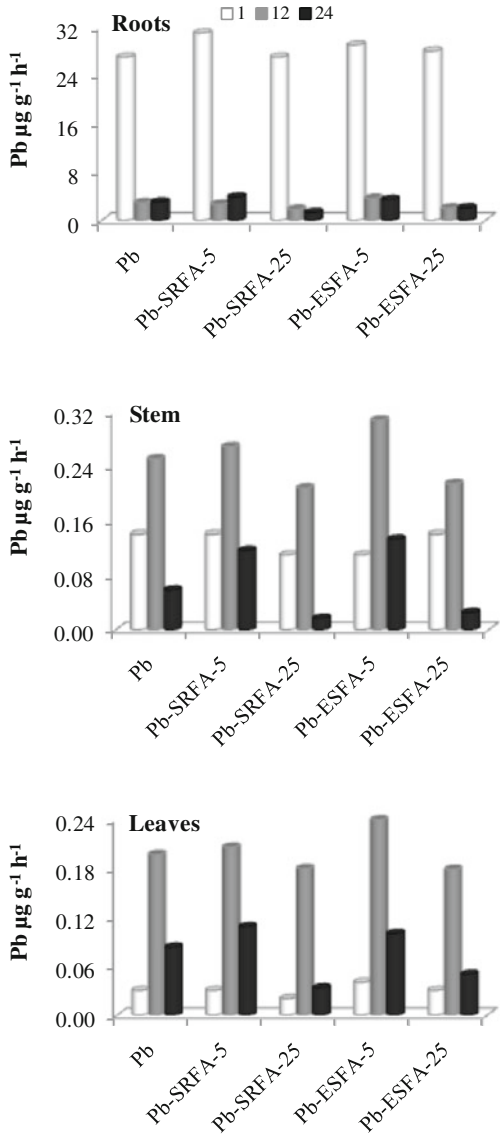

Fig. 1 Effect of SRFA and ESFA on rate of Pb uptake and translocation (in micrograms per gram per hour dry weight) in *V. faba* roots, stem, and leaves. Values are means of 12 replicates

was maximum during first hour for root, whereas the maximum rate of translocation towards stem and leaves occurred between 2–12 h for all the treatments. This showed that Pb uptake by *V. faba* roots and translocation to shoots tissues was not linear during the first 24 h of incubation but occurred in phases. The application of fulvic acids increased the rate of Pb uptake and translocation at low level and decreased it at high level of application. However, the trends/phases of Pb uptake and translocation remained the same for all treatments. Data regarding short-term metal uptake kinetic are scarce however, Nedelkoska and Doran (2000) and Sgherri et al. (2007) also showed a triphasic kinetic profile for cadmium, nickel, and copper uptake during 24 h.

Moreover, the precise properties of a given HS sample depend on their source and the specific conditions of extraction. The different behavior of applied FAs with respect to Pb accumulation and kinetic of uptake can be due to their different sources since SRFA was extracted from water and ESFA from soil.

Effect of organic ligands on lipid peroxidation and H_2O_2 induction by Pb

The effect of each FA alone was investigated on plant physiology. The high concentrations of both FAs (25 mg l^{-1}), applied alone without Pb for 24 h, did not affect lipid peroxidation and H_2O_2 production compared to the control (Figs. 2 and 3).

The effect of FAs on Pb-induced generation of H_2O_2 and lipid peroxidation is presented in Figs. 2 and 3. Application of Pb alone caused overproduction of H_2O_2 which resulted into lipid peroxidation in both roots and leaves. In roots, Pb-induced H_2O_2 production and lipid peroxidation started immediately after Pb exposure at 1 h and continued up to 12 h. The increase in H_2O_2 and TBARS contents by Pb was, respectively, 64% and 55% at 1 h and 39% and 52% at 12 h. In

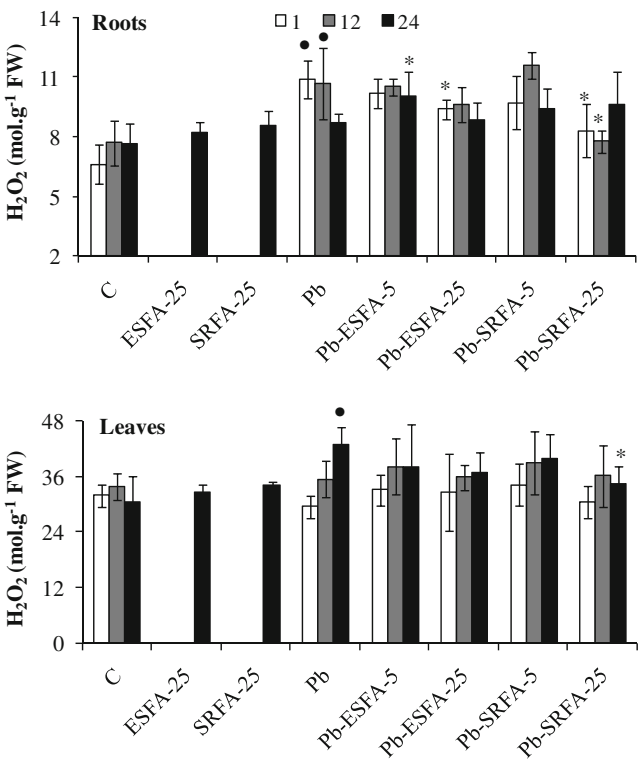

Fig. 2 Effect of SRFA and ESFA on Pb-induced production of H_2O_2 (in micromolars per gram fresh weight) in *V. faba* roots and leaves. Values are means of 12 replicates. An *asterisk* indicates significant differences at $P<0.05$ for Pb–FA treatments compared to Pb alone whereas a *solid dot* indicates significant differences at $P<0.05$ for Pb and FAs alone compared to the control

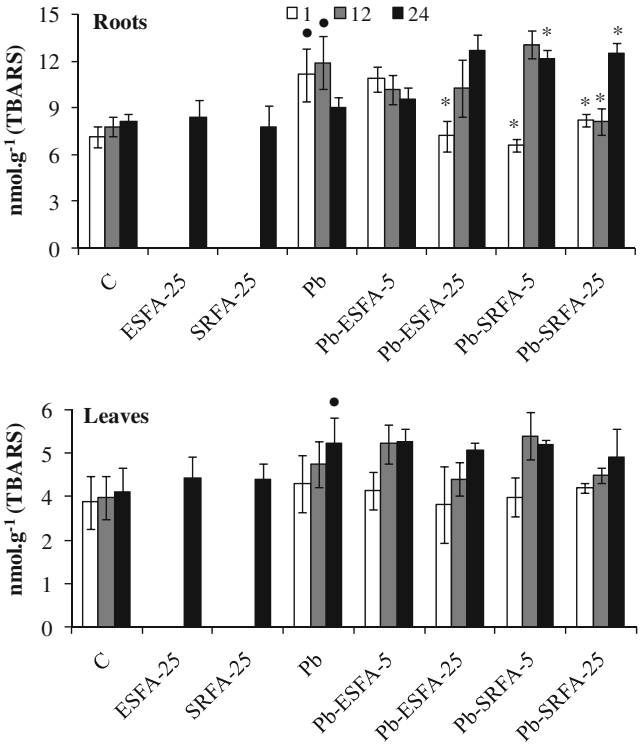

Fig. 3 Effect of SRFA and ESFA on Pb-induced production of lipid peroxidation (TBARS in nanomoles per gram fresh weight) in *V. faba* roots and leaves. Values are means of 12 replicates. An *asterisk* indicates significant differences at $P<0.05$ for Pb–FA treatments compared to Pb alone whereas a *solid dot* indicates significant differences at $P<0.05$ for Pb and FAs alone compared to the control

leaves, the Pb-induced increase in H_2O_2 and TBARS contents started after 12 h but the effect was only significant ($P<0.05$) at 24 h (34% and 36%, respectively, for H_2O_2 and TBARS compared to the control). The presence of Pb across the root–cell membrane, even in small amounts, is known to induce oxidative stress through overproduction of ROS (Islam et al. 2008; Singh et al. 2010). This overproduction of ROS is one of the earliest responses of plant cells to heavy metal toxicity, and it is due to the imbalance between the generation and the neutralization of ROS by antioxidant mechanisms. These free radicals react (oxidize) with various cell components including DNA, proteins, and lipids/fatty acids and cause DNA damage, mitochondrial malfunction, and cell membrane damage (Aarti et al. 2006; Mishra et al. 2006; Pourrut et al. 2008). Lead ions are known to induce lipid peroxidation, decrease the level of saturated fatty acids, and increase the content of unsaturated fatty acids of membranes in several plant species (Singh et al. 2010).

In roots, Pb-ESFA-5 prolonged the Pb induction of H_2O_2 to 24 h without any effect on lipid peroxidation (Figs. 2 and 3). Application of Pb-SRFA-5 did not affect H_2O_2 production but delayed the start of lipid peroxidation from 1 to 12 h which then continued up to 24 h. Lead-induced H_2O_2 production and lipid peroxidation were reduced significantly ($P<0.05$) at 1 h by Pb-ESFA-25 and at 1 and 12 h by Pb-SRFA-25. In contrast, at 24 h, Pb-SRFA-25 increased H_2O_2 contents and TBARS values. In leaves, addition of FAs in the presence of Pb at both levels of application did not sufficiently modify Pb-induced H_2O_2 production and lipid peroxidation, except for Pb-SRFA-25, which significantly ($P<0.05$) reduced H_2O_2 production induced by Pb at 24 h.

This is the first time that the influence of FAs was studied on H_2O_2 induction and lipid peroxidation. Marcato-Romain et al. (2009b) suggested a protecting effect of soil organic matter on Pb-induced genotoxicity to *V. faba*. On the basis of our results (Tables 2 and 3), it is assumed that Pb-induced toxicity correlates with the concentration of free Pb^{2+} ions in plants. Recently, Shahid et al. (2011c) calculated a linear relationship between free Pb^{2+} ions concentration in nutrient solution and genotoxicity in the presence of EDTA. Application of FAs at high levels complexed Pb^{2+} ions in the nutrient solution and reduced its uptake and consequently alleviated or delayed the toxicity. Pourrut et al. (2008) previously demonstrated that Pb-induced toxicity is antagonized by the Ca^{2+} ions due to the great inhibition of Pb entry into the roots, suggesting an important role of Pb level in the oxidative stress induction in plants.

The other possible hypothesis of reduced/delayed Pb toxicity when FAs were added at high level can be the minimum threshold level of Pb^{2+} ions required inside the plant to induce the production of H_2O_2 and lipid peroxidation. Moreover, the plant detoxification mechanism is proposed to be more efficient at lower than higher plant Pb level. Mishra et al. (2006) reported increased levels of protein accumulation involved in the maintenance of cell redox status or in the sequestration of the metal like phytochelatins under low Pb levels. This decreased uptake of Pb with the more efficient detoxification mechanism could be due to the reduced or delayed Pb-induced phytotoxic effects in the presence of higher than lower levels of FAs. At low level of FAs application, despite the increased Pb uptake, the Pb-induced toxicity remained slightly lower than that due to Pb alone. Under diluted conditions, a portion of Pb could be taken up by plants as Pb–FA complex due to fiber FA structure (Kleber and Johnson 2010). Inside the plants, this complex either remains stable, and in this way slightly reduces Pb toxicity, or dissociates after some times, and thus, delays the toxicity. Shahid et al. (2011c) concluded that only stable organic–metal complexes could decrease Pb genotoxicity.

Effect of organic ligands on chlorophyll contents

The application of FAs alone has no effect on pigment contents compared to the control except for carotenoid

contents, which decreased significantly ($P<0.05$) by 9% in the presence of Suwana river FA (Fig. 4). Humic substances are, generally, known to increase plant tolerance against metal stress and enhance pigment contents and plant growth (Piccolo et al. 1992; El-Ghamry et al. 2009). However, in the present study, the HSs did not enhance *V. faba* pigment contents, which can be due to the short duration of the experiment.

The pigment composition remained unaffected after 1 h in all the treatments compared to the control (Fig. 4). This is due to slow Pb translocation which takes more than 1 h to reach the threshold level in leaves as indicated by Pb uptake kinetic (Fig. 1). The application of Pb alone reduced pigment contents at 12 and 24 h, but the effect was only significant ($P<0.05$) after 24 h, except for carotenoids content where the effect was also significant ($P<0.05$) at 12 h. Lead is known to stimulate the degradation of pigments (Islam et al. 2008; Shahid et al. 2011b).

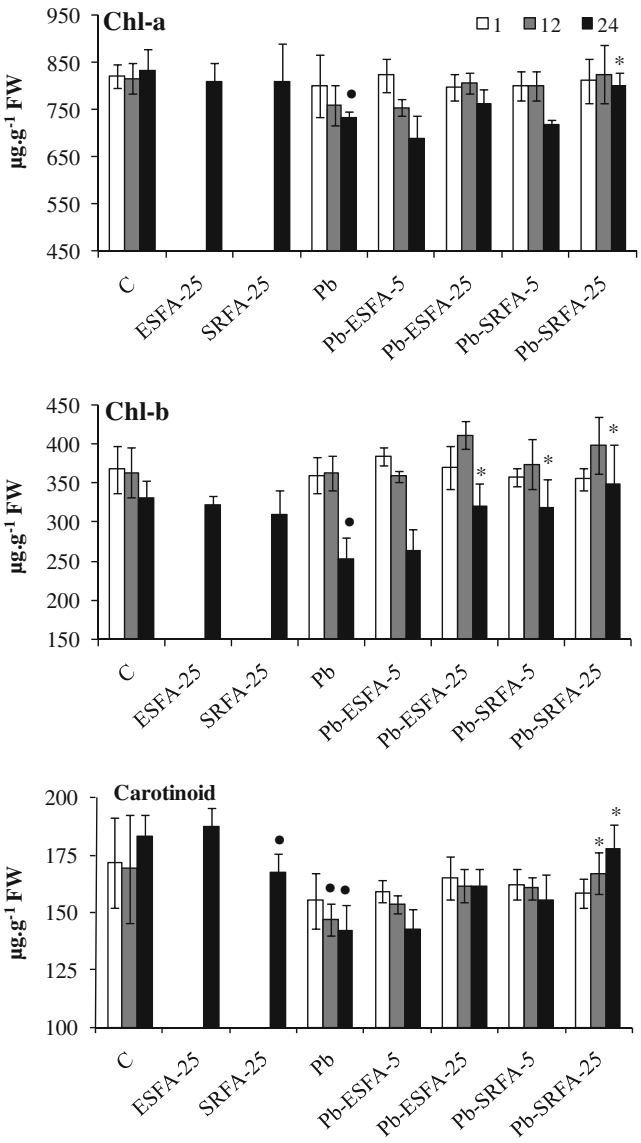

Fig. 4 Effect of SRFA and ESFA on Pb-induced reduction of pigment contents (in micrograms per grams fresh weight) in *V. faba* leaves. Values are means of 12 replicates. An *asterisk* indicates significant differences at $P<0.05$ for Pb–FA treatments compared to Pb alone whereas a *solid dot* indicates significant differences at $P<0.05$ for Pb and FAs alone compared to the control

Lead-induced degradation of pigment has been attributed to direct or indirect factors, such as distortion of chloroplast ultrastructure, inhibition of the synthesis of photosynthetic pigments and enzymes of Calvin cycle (Mishra et al. 2006), impaired uptake of essential elements like Mn and Fe, and damage of the photosynthetic apparatus or due to chlorophyll degradation by increased chlorophyllous activity (Shahid et al. 2011b). Recently, Cenkci et al. (2010) reported dose-dependent negative correlation between Pb concentration and pigment contents in *Brassica rapa* exposed to 0.5–5 mM Pb for 6 days. However, in our case, the rapidity (after 12 h) of the observed effect on the pigment content compared to other studies suggests the involvement of fast mechanisms, such as production of ROS (Aarti et al. 2006; Singh et al. 2010).

The low-level application of both FAs (5 mg l^{-1}) on the pigment contents depended on the FA type. The effect of Pb-ESFA-5 was similar to that of Pb alone. In contrast, application of Pb-SRFA-5 reduced significantly ($P<0.05$) Pb-induced Chl-b degradation at 24 h. The application of FAs at high levels (25 mg l^{-1}) inhibited the Pb-induced toxicity to pigment contents with Pb-SRFA-25 being more effective than Pb-ESFA-25. Addition of Pb-ESFA-25 significantly ($P<0.05$) reduced the degradation of Chl-b induced by Pb. In the case of Chl-a and carotenoids contents, the values were always higher than those with Pb alone. Application of Pb-SRFA-25 alleviated the Pb-induced reduction of pigment contents throughout the experiment. The values of pigments remained close to control values in this treatment.

The results show that only high levels of FAs can effectively bind and reduce Pb toxicity to chlorophyll contents. This effect was due to the reduced Pb concentration in roots and leaves in the presence of high levels of FAs. Ruley et al. (2006) also reported similar effect for LMWOAs on Pb-induced degradation of chlorophyll contents in *Sesbania drummondii.* They stated that due to low binding affinity of citric acid, only high level of this acid can efficiently bind and reduce Pb^{2+} toxicity. The mechanism of protection provided by HSs against Pb toxicity can be similar to that of the clay minerals, the adsorption of free Pb^{2+} ions to fulvic acid, and can limit the uptake and toxicity of Pb to living organisms (Babich and Stotzky 1979). Kruatrachue et al. (2002) reported that the application of HSs inhibited the Pb-induced degradation of total chlorophyll contents and growth rate of *Lemna minor.*

Conclusions and perspectives

The present study highlights the important role of natural FAs to the fate of Pb in the soil–plant systems. The FAs are capable to alleviate Pb toxicity to plants by complexing highly toxic free Pb^{2+} in solution and reducing Pb uptake. This protecting role of FAs against Pb uptake and toxicity depends on the applied concentration: only the high concentrations (25 mg l^{-1}) FAs could effectively bind and reduce metal toxicity.

Therefore, the behavior of pollutants in terms of bioavailability, uptake, and toxicity in soil can vary with concentration of HSs. Our results also proposed that Pb toxicity depends on metal binding capacity of FA. Due to the high binding constant, the SRFA bound more strongly to Pb compared to ESFA and was more effective against Pb toxicity. Therefore, understanding metal speciation along with the type and level of complexing agents in the natural systems are important for the risk assessment and/or soil or water quality criteria.

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