

Organic acids enhance the uptake of lead by wheat roots

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Abstract The uptake and bioavailability of lead (Pb) in soil–plant systems remain poorly understood. This study indicates that acetic and malic acids enhance the uptake of Pb by wheat (*Triticum aestivum* L.) roots under hydroponic conditions. The net concentration-dependent uptake influx of Pb in the presence and absence of organic acids was characterized by Michaelis–Menten type nonsaturating kinetic curves that could be resolved into linear and saturable components. Fitted maximum uptake rates (V_{\max}) of the Michaelis–Menton saturable component in the presence of acetic and malic acids were, respectively, 2.45 and 1.63 times those of the control, while the Michaelis–Menten K_m values of 5.5, 3.7 and 2.2 μM , respectively, remained unchanged. Enhanced Pb uptake by organic acids was partially mediated by Ca^{2+} and K^{+} channels, and also depended upon the physiological function of the plasma membrane P-type ATPase. Uptake may have been further enhanced by an effectively thinner unstirred layer of

Pb adjacent to the roots, leading to more rapid diffusion towards roots. X-ray absorption spectroscopic studies provided evidence that the coordination environment of Pb in wheat roots was similar to that of $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$ in that one Pb atom was coordinated to four oxygen atoms via the carboxylate group.

Keywords Ca^{2+} and K^{+} channels · Organic acids · P-type ATPase · Uptake of lead (Pb) · Wheat (*Triticum aestivum* L.) · X-ray absorption spectroscopy

Introduction

Being one of the most frequently encountered metal contaminants in many agricultural and industrial areas, the environmental fate of Pb and its uptake by plants has been the subject of much research (Manceau et al. 1996; Wu et al. 1999). Because of its limited solubility due to the formation of insoluble precipitates, bioavailable levels of Pb in soils are generally low compared to other heavy metals, thus making it difficult to apply phytoremediation techniques to Pb-contaminated soils. Recent studies demonstrated that the addition of synthetic chelates such as EDTA, HEDTA, and DTPA to contaminated soils can rapidly and dramatically increase Pb release into the soil solution, thus promoting Pb uptake by plants (Blaylock et al. 1997; Vassil et al. 1998).

Organic acids are important plant root exudates and microbial metabolites in terms of their ability to increase the dissolution of metals from highly insoluble mineral phases in soil, thereby increasing metal mobility in the vicinity of roots and enhancing their

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availability to plants (Naidu and Harter 1998; López-Bucio et al. 2000; Clemens et al. 2002). Several studies have shown the important role that organic acids play at the soil–plant interface. For example, organic acids in pot experiments triggered uranium accumulation in Indian mustard and Chinese cabbage (Huang et al. 1998), and increased chromium accumulation in tomato (Srivastava et al. 1999). Under hydroponic conditions, organic acids furthermore increased the uptake of cadmium by tomato and durum wheat (Senden et al. 1995; Berkelaar and Hale 2003), and of lanthanum by wheat and barley (Wang et al. 2004; Han et al. 2005).

Organic acids play an important physiological role in conferring metal tolerance in that a number of mechanisms involving metal translocation or accumulation are influenced by organic acids. For example, several studies have shown the key role of organic acids in affecting plant tolerance to aluminum (Pellet et al. 1995; Ma et al. 2001). The role of organic acids in mediating the uptake of Fe^{3+} was ascribed to ferric reductase activity and the presence of cation channels (Schmidt 1999). Compared to other metals, limited information exists on the interactions between organic acids and Pb at or near the soil–root interface, and the resultant processes of Pb uptake by plant roots.

Laurie et al. (1991) discussed two possible pathways for metal uptake under the influence of complexes. One pathway involves the dissociation of a metal complex (MeL) in the diffusion layer (solution phase) after which the released free metal ion (Me) may be transported to the root cell across the plasmalemma. Another possible pathway involves the absorption of the metal complex by the root cell membrane; the complex then either undergoes dissociation in the cell membrane, with the free metal ion being transported to the cell while the ligand (L) goes back to the solution phase, or the metal is transported to root cells across the plasmalemma in the form of a complex. Chloride was found to increase Cd uptake by chard from a resin-buffered solution culture in which activities of Cd^{2+} were held constant (Smolders and McLaughlin 1996). They suggested that either CdCl_2^{2-n} species were transported across the plasma membrane, or that Cl^{1-} enhanced the diffusion of Cd^{2+} through the unstirred liquid layer adjacent to the root surface or through the apoplast to sites of Cd uptake within the root itself. However, solution culture studies in the presence and absence of EDTA indicate that Cd-EDTA may not be available for plant uptake (Checkai et al. 1987). Berkelaar and Hale (2003) showed that the free ion model (FIM) alone was likely insufficient to predict plant accumulation of metals. Hence, little agreement exists on whether free metal ions or organic

ligand-complexes can be more easily taken up by plants.

Recent biophysical studies on the interactions between lead and recombinant proteins and peptides have provided considerable insight into the biological chemistry and molecular toxicology of lead (Godwin 2001). Because lead is non-essential and toxic to plants, it is generally thought that plants are unlikely to have transporters specific for lead. The first example of a plant transporter possibly mediating Pb^{2+} uptake has been described by Arazi et al. (1999). They revealed a plasma membrane protein NtCBP4 that conferred Pb^{2+} hypersensitivity and correlated this with enhanced Pb^{2+} accumulation. Lead and calcium compete for the same binding sites on proteins that belong to a large family of ion binding compounds. Members of this family include calmodulin, S-100, calretinin, calbindin, and parvalbumin. Lead substitutes for calcium in the activation of calmodulin-dependent phosphodiesterase. CadA and ZntA have been discovered as two Pb(II)-translocating P-type ATPase (Rensing et al. 1998). Still largely unanswered in the question how lead is taken up into plant cells under the influence of organic acids, and what the exact speciation of lead is within plant cells.

The objective of this study was to gain insight into the effects of organic acids on the uptake of Pb by wheat roots under hydroponic conditions. Relevant mechanisms were investigated by using time- and concentration-dependent Pb uptake experiments, and various cation channel blockers and the H^+ -ATPase inhibitor vanadate. Speciation calculations of Pb in the absence and presence of organic acids and diffuse double layer model were further used to verify the enhanced diffusion of Pb in the nutrient solution towards wheat roots. We additionally employed such structural sensitive characterization techniques as X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) to investigate the coordination environment of Pb in wheat roots.

Materials and methods

Plant cultivation

Wheat (*Triticum aestivum* L. cv. JM9158) seeds were obtained from the Chinese Academy of Agricultural Sciences (Beijing, China). Seeds were first surface-sterilized in 3% (v/v) H_2O_2 for 30 min, and then thoroughly rinsed with deionized water. After soaking the seeds in 2.8 mM $\text{Ca}(\text{NO}_3)_2$ for 4 h, they were al-

lowed to germinate on moisture filter paper for 4 days in the dark and subsequently transferred to hydroponic solutions containing major nutrients [one-third-strength of 1.5 mM KNO_3 , 1 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM MgSO_4 , 0.25 mM $(\text{NH}_4)_2\text{H}_2\text{PO}_4$] and micronutrients [full-strength of 11.9 μM ethylenediaminetetraacetic acid–iron (EDTA–Fe), 11.5 μM H_3BO_3 , 1.25 μM MnSO_4 , 0.2 μM ZnSO_4 , 0.075 μM CuSO_4 and 0.025 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$]. The solutions were buffered to pH 5.8 with 2 mM Mes–Tris. Plants were grown in a plant growth chamber subject to continuous aeration and with day and night temperature of 22°C (16 h) and 15°C (8 h), respectively. A relative humidity between 50 and 60% and a photon flux intensity of 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ were maintained during the entire experimental period. Fresh nutrient solutions were renewed every 3 days. The Pb treatment solutions contained the same nutrients, but without $\text{NH}_4\text{H}_2\text{PO}_4$ and EDTA–Fe, while MgSO_4 was applied at one-tenth strength in order to avoid precipitation.

Effect of molar concentration ratio of organic acids to Pb on Pb accumulation

Uniform 22-day-old wheat seedlings were removed from the nutrient solutions, rinsed with deionized water for 2 min, and placed in groups of four in the modified uptake solutions. As control we used an uptake solution containing 20 μM Pb as $\text{Pb}(\text{NO}_3)_2$ without the organic acids. Six different molar concentration ratios (2:1, 5:1, 10:1, 20:1, 50:1, 100:1) of acetic or malic acid to Pb were employed. Acetic and malic acids were chosen as representative of organic acids since they are ubiquitous in many plant exudates (Cieśliński et al. 1998; Jones 1998). After exposure to amended solutions for 4 h, the seedlings were removed and thoroughly rinsed with deionized water and desorbed. The desorbed roots were next gently blotted, weighed and digested for final determination of Pb. The desorption procedures were conducted for 15 min in ice-cold 5 mM CaCl_2 solutions containing 5 mM Mes–Tris (pH 6.0) to remove apoplastic Pb (Hart et al. 1998). These and all subsequent experiments with wheat seedlings were performed in quadruplicate.

Short-term uptake and long-term accumulation of Pb as affected by organic acids

To study the time-dependency of Pb uptake, uniform 22-day-old wheat seedlings were transferred from the hydroponic solutions to uptake solutions containing 20 μM Pb with or without 200 μM of either acetic or

malic acids. The wheat seedlings were analyzed for Pb content after uptake time periods of 5, 10, 20, 40, 60, 90 and 120 min, and 2, 4, 8, 12, 24 and 48 h. For the short-term (0–2 h) uptake experiments, subsamples of the roots were collected followed by desorption for 15 min in 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0), or without desorption prior to Pb determination. The incubation solutions for the long-term (>12 h) accumulation experiments were replaced every 12 h. All wheat roots were desorbed and digested for Pb determination.

Concentration-dependent kinetics of Pb uptake as affected by organic acids

Three parallel experiments were performed simultaneously to test the effect of external Pb concentrations on Pb uptake. Uniform 22-day-old wheat seedlings were for this purpose incubated with fresh nutrient solutions containing various concentrations of Pb (0, 5, 10, 20, 40, 60, 80, 100 μM). Acetic and malic acids were added separately in two of the experiments at molar concentration ratio of 10:1 (organic acid:Pb). After incubation for 20 min, the seedlings were harvested and the roots desorbed. Pb concentrations of the wheat roots were subsequently determined following acid digestion.

Membrane permeability

Wheat seedlings were incubated in different treatment solutions for 1–2 days. During this period the incubation solution was replaced every 12 h with a fresh solution. Treatment solutions (including the control nutrient and uptake solutions) were amended with 20 μM Pb with or without 200 μM acetic acid. At the end of the experiments, roots were thoroughly washed twice with 5 mM ice-cold CaCl_2 (5 mM Mes–Tris, pH 6.0) to remove nutrient solution from the apoplast and then blotted on filter paper. Each sample consisting of four seedlings was subsequently introduced into a 25 ml of fresh 0.2 mM CaCl_2 solution and incubated for 4 h in a water bath at 25°C with gentle shaking (Andreu et al. 2000). Aliquots of this solution were taken at 1 h intervals and the K^+ efflux to the external solution was measured using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optima 2000DV, Perkin Elmer Co., Wellesley, MA, USA).

Effects of cation channel blockers on Pb influx

To better understand the transport pathway of the increased Pb influx, we next examined the physiologi-

cal functions of the Ca^{2+} channel inhibitor of La^{3+} and the K^+ channel inhibitor of Cs^+ . The experiments were carried out again with uniform 22-day-old wheat seedlings, now incubated in nutrient solutions amended with 0.2 mM La^{3+} or Cs^+ as $\text{La}(\text{NO}_3)_3$ or CsCl , respectively. An unamended nutrient solution was used as the control. $\text{La}(\text{NO}_3)_3$ and CsCl have previously been identified as cation channel blockers by White (1997) and by Essah et al. (2003), who also reported appropriate application concentrations. The Pb concentrations in our experiments were maintained at 20 μM . Acetic and malic acids were applied at molar concentration ratio of 10:1 (organic acid:Pb). The wheat seedlings were harvested after 4 h of incubation. Roots were desorbed and acid digested prior to final determination of Pb. As before, all experiments were performed in quadruplicate.

Influence of vanadate on Pb uptake

The effect of vanadate on Pb uptake was analyzed by pre-treating 22-day-old wheat seedlings with 0.1 mM vanadate for 15 min prior to exposure to 20 μM Pb solution with or without the organic acids. As before, the molar concentration ratio of organic acids to Pb was again maintained at 10:1. After 4 h of incubation, wheat roots were harvested, desorbed and acid digested prior to Pb determination.

Speciation calculations

The chemical speciation of Pb in the incubation solutions in the presence and absence of organic acids was predicted using the computer program Visual MINTEQ, version 2.50 (Gustafsson 2006). Full consideration was given in the calculations to mole balances, relevant thermodynamic equilibrium constants and ionic strengths.

X-ray absorption spectroscopic studies

We additionally performed X-ray absorption spectroscopic (XAS) studies to obtain direct information on the coordination chemistry of Pb in the 22-day-old wheat seedlings, which had been transferred into 20 μM Pb culture solutions containing 200 μM acetic acid. X-ray absorption spectroscopy is an element-specific and bulk-sensitive method. The seedlings for this study were harvested after 2 days of uptake. The fresh wheat roots (desorbed and undesorbed) were ground in an agate mortar and pestle under the protection of liquid N_2 . The fine root powders were next pressed into pellets (diameter of 0.84 cm and thickness

of 0.15 cm) for the XAS experiments. Instrumental conditions for measurement of the XAS spectra at the Pb L_3 -edge are described in detail elsewhere (Qin et al. 2006).

Data processing and analyses were performed with WinXAS 2.1 following standard procedures. The energy E_0 (13,035 eV) was defined as the inflection point of the absorption edge for the transform from energy to k space. The pre-edge absorption background was fitted and subtracted by using Victoreen functions. The post-edge absorption backgrounds were fitted with a spline function and subtracted from the absorption spectra. The EXAFS functions were normalized using the absorption edge jump, and next Fourier transformed into R-space with k^2 -weighting over the range from 2.2 to 10.0 Å. We chose $k = 2$ weighting for Pb since a higher k value decreased the signal-to-noise ratio. The fits were performed in R-space with the coordination number (N) fixed, while the interatomic distance (R), the energy shift (E_0), and the Debye–Waller factor (σ^2) were allowed to float. The amplitude and phase shift parameters were obtained from theoretical calculations with $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$ as reference using the FEFF 8.0 code (Rehr et al. 1992).

Determination of Pb

The Pb contents of the various plant samples were analyzed by digesting them with 3-ml concentrated HNO_3 – HClO_4 (2:1, v:v) solutions under high pressure conditions (Zhang and Shan 1997). Pb concentrations in the samples were determined using ICP-AES (Optima 2000 DV, Perkin Elmer Co.). Certified reference materials (peach leaves, GBW 08501, PR China) were used to ensure the quality of analyses. Good agreement was obtained between the data obtained with our method and the certified values.

Statistical analysis of data

The data were analyzed using the Microcal Origin 7.0 (Microcal Software, Northampton, MA, USA) and SPSS 12.0 for windows (SPSS, Chicago, IL, USA) software packages.

Results

Effect of molar concentration ratio of organic acids to Pb on the accumulation of Pb

Figure 1 shows the effects of acetic and malic acid on the accumulation of Pb by the wheat roots as a function

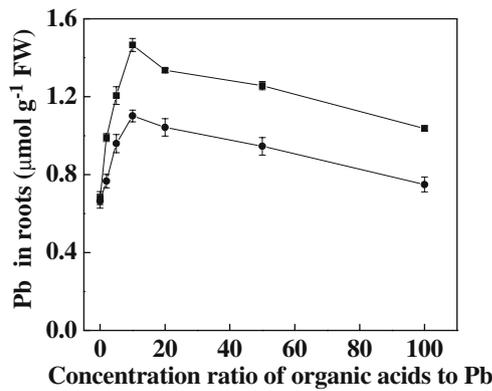


Fig. 1 Effect of molar concentration ratio (organic acids:Pb) for acetic acid (filled square) and malic acid (filled circle) on the uptake of Pb by wheat roots (*Triticum aestivum* L.). Values are means \pm 1 SE of four replicates. The data at a concentration ratio of 0 are for the controls (i.e., for nutrient solutions containing Pb but no organic acids)

of the molar concentration ratio of organic acid to Pb. While acetic and malic acids qualitatively showed similar trends in terms of enhancing Pb accumulation, acetic acid had a much greater effect than malic acid. The maximum concentrations of Pb in the roots occurred at a concentration ratio of 10:1. The Pb content of the desorbed wheat roots at that ratio, after 4 h of exposure, increased by 116 and 75% for acetic and malic acid, respectively, relative to the control ($P < 0.05$). The Pb contents of the roots decreased with a further increase in the organic acid to Pb ratio. For this reason we used a molar concentration ratio of 10:1 (organic acid:Pb) in most or all subsequent experiments.

Short-term uptake and long-term accumulation of Pb as affected by organic acids

Compared to seedlings grown in the control solution, Pb concentrations of wheat roots grown in nutrients solutions with the organic acids were found to be consistently greater for both the short- and long-term experiments. Figure 2a, b show the short-term (0–120 min) Pb uptake by wheat roots without and with CaCl₂ desorption, respectively. The uptake patterns were similar for the control and the organic acid treatment solutions. Acetic and malic acid both noticeably enhanced the short-term uptake of Pb. For wheat roots without CaCl₂ desorption, Pb uptake was biphasic in that a relatively fast initial phase was followed by a much slower and more linear second phase (Fig. 2a). The initial rapid phase (i.e., during the first 20 min) was presumably due to diffusion of Pb into free space and binding to root cell walls. The slower

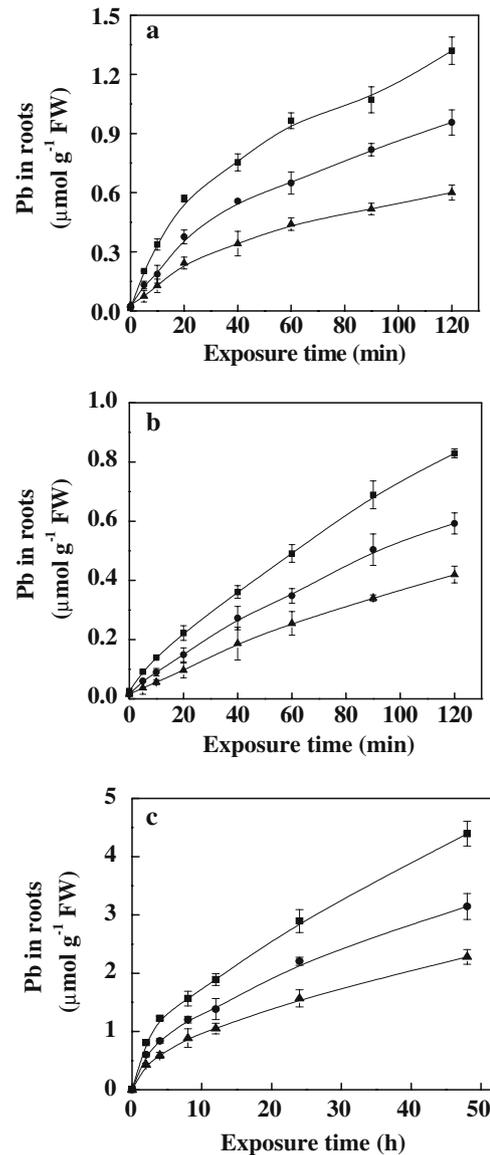


Fig. 2 Time-dependent uptake of Pb by wheat roots (*Triticum aestivum* L.) from solutions containing Pb but no organic acids (filled triangle), Pb and malic acid (filled circle), and Pb and acetic acid (filled square). Acetic and malic acids were applied at a molar concentration ratio of 10:1 (organic acid:Pb). Values are means \pm 1 SE of four replicates. Results are for the short-time (\leq h) uptake experiments without a and with b desorption, and for the long-time (up to 48 h) accumulation experiments with desorption (c)

linear phase of Pb uptake between 20 and 120 min may correspond to transport across the plasma membrane. The second phase could also be due to slower diffusion into the apoplast and external binding to cells in the cortex.

Our results for the three uptake experiments with CaCl₂ desorption resemble previous findings by Lasat et al. (1996). The linear nature of Pb uptake by de-

sorbed wheat roots suggests that unidirectional Pb influx into the root symplast occurred for at least 120 min (Fig. 2b). The curves intercepted the axis slightly above the origin, thus indicating the presence of relatively small amounts of rapidly bound Pb that were not removed during the 15-min desorption process. This undesorbed fraction probably consisted of Pb bound to reactive sites within the apoplast. After 2 h of exposure, Pb concentrations in the desorbed wheat roots of the acetic and malic acid treatments were, respectively, 2.1 and 1.5 times greater than that of the control ($P < 0.05$).

Figure 2c shows the long-term Pb accumulation by wheat roots. After about 4 h of rapid uptake, Pb continued to be accumulated for at least 48 h, but at a slower rate. The accumulation did not reach a final value after 48 h since the wheat seedlings were still growing. Compared with Pb accumulation from the control solution, the Pb content of desorbed wheat roots after 48 h of exposure was increased by 93 and 40% for acetic and malic acid, respectively (Fig. 2c, $P < 0.05$).

Concentration-dependent Pb uptake as affected by acetic and malic acids

Data from the relatively short uptake period (<20 min) were used to investigate uptake while minimizing the possibility of Pb efflux across the plasma membrane back into the external solution. Figure 3a shows that the concentration-dependent Pb uptake data for the wheat seedlings are characterized by smooth, nonsaturating curves that become linear at Pb concentration greater than about 20 μM . The organic acids increased Pb uptake over the entire range of applied Pb in the uptake solutions. The uptake kinetics of Pb in the presence and absence of acetic and malic acids were biphasic and could be described well with a modified Michaelis–Menten type equation involving linear and saturable components as follows (see also Fig. 3b):

$$V = aC + \frac{V_{\max}C}{K_m + C}, \quad (1)$$

where V is the uptake rate, C is the Pb concentration in the nutrient solution, a is parameter characterizing the linear part of the uptake rate, and V_{\max} and K_m are the maximum uptake rate and Michaelis–Menten rate constant of the saturable (second) component of (1). The r^2 values for the acetic and malic acid treatments and for the control were 0.993, 0.996 and 0.998, respectively. The linear component presumably represents cell-wall-bound Pb remaining after desorption,

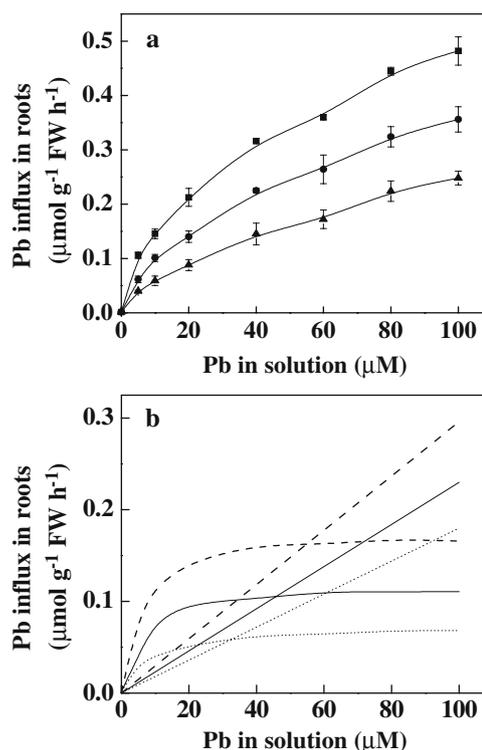


Fig. 3 Concentration-dependent uptake kinetics of Pb by wheat roots (*Triticum aestivum* L.) from solutions containing Pb but no organic acids (filled triangle), Pb and malic acid (filled circle), and Pb and acetic acid (filled square). Acetic and malic acids were applied at a molar concentration ratio of 10:1 (organic acid:Pb). **a** Measured overall kinetic curves for Pb uptake influx; values are means \pm 1 SE of four replicates; **b** Deconvolution of the overall kinetic curves into linear and saturable components according to Eq. 1. The uptake curves are for Pb (dotted line), Pb and malic acid (solid line), and Pb and acetic acid (dashed line)

while the saturable component represents true uptake across the plasma membrane. Values of V_{\max} for the acetic and malic acid treatments were found to be 179.2 and 120.5 $\text{nmol g}^{-1} \text{fw h}^{-1}$, respectively, which were 2.45 and 1.63 times that of the control, respectively. The corresponding K_m values were within the same order of magnitude (5.5, 3.7 and 2.2 μM , respectively). These results indicate that acetic and malic acids significantly enhanced Pb influx into the root symplast.

Membrane integrity

In order to test whether organic acids at high concentrations and Pb exert toxic effect on the membrane integrity of wheat roots, the membrane integrity was examined by measuring the K^+ efflux to the external solution. Results indicate that the K^+ concentration of the external solution remained almost the same when the plants were exposed to the nutrient solutions alone,

or to the nutrient solutions with 200 μM acetic acid or with 20 μM Pb or with 200 μM acetic acid and 20 μM Pb together, over culture time intervals of 1–2 days on the base of fresh wheat root weight (Fig. 4). There were no significant differences between the K^+ contents of the external solution for the different plant culture conditions. These results suggest that plasma membrane integrity was not adversely affected by the application of 200 μM acetic acid or 20 μM Pb. We hence conclude that the observed Pb uptake enhancement in wheat roots in the presence of organic acids was not due to toxic action of either Pb or the organic acids.

Effects of cation channels blockers and vanadate on Pb uptake rate

Figure 5 shows the effects of the Ca^{2+} channel inhibitor of La^{3+} , the K^+ channel inhibitor of Cs^+ , as well as vanadate on Pb uptake. Results indicate that La^{3+} and Cs^+ significantly inhibited the Pb influx ($P < 0.05$). If we designate the Pb contents of wheat roots with no organic acid, and with acetic and malic acids, as the control values, La^{3+} decreased the Pb contents of the wheat roots by 74, 72 and 71%, respectively. Analogously, Cs^+ decreased the Pb contents by 62, 59 and 57%, respectively. Pre-treatment of wheat roots by vanadate, a H^+ -ATPase inhibitor, for 15 min, caused the Pb contents of wheat roots to decrease by 55, 37, and 51%, respectively (Fig. 5, $P < 0.05$).

We note here that, except for the ion channels (White 1997), two other possible mechanisms involved in the passage of ions through the plasma membranes of cortical and xylem parenchyma are ion pumping and the presence of carriers.

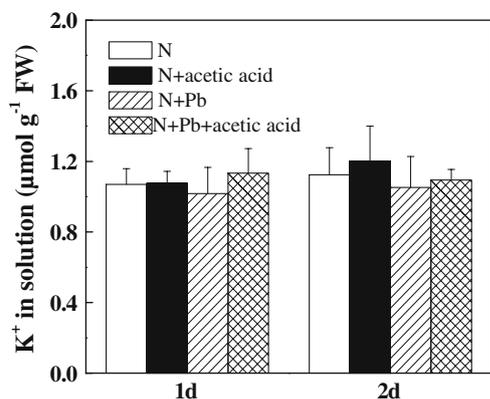


Fig. 4 Potassium concentration of the external solution after treatment for 1 and 2 d with and without the presence of 200 μM acetic acid and 20 μM Pb. N represents the nutrient solution. Values are means \pm 1 SE of four replicates

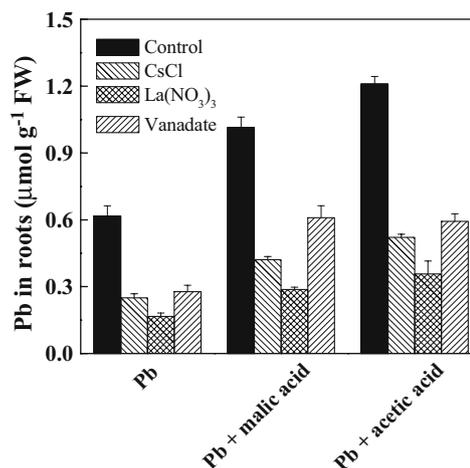


Fig. 5 Effects of cation channel inhibitors and vanadate on the influx of Pb in wheat (*Triticum aestivum* L.) roots as influenced by organic acids. Values are means \pm 1 SE of four replicates

Speciation of Pb in the absence and presence of organic acids

The speciation of Pb in the absence and presence of acetic and malic acids as computed with the Visual MINTEQ program is shown in Table 1. The results indicate that free Pb^{2+} dominated the nutrient and uptake solutions, and that organic acids decreased the free Pb^{2+} concentration as compared to the control. Malic acid decreased free Pb^{2+} concentration more than acetic acid. Moreover, as the molar concentration ratio of organic acids to Pb increased from 2:1 to 100:1, the percentage of free Pb^{2+} decreased from 94.6 to 61.7% for acetic acid and from 91.5 to 31.7% for malic acid. The Pb-acetate and Pb-malate contents were very low (only 5.19 and 18.5%, respectively) when the molar concentration ratio of acetic and malic acids to Pb was set at 10:1. However, the Pb-acetate and Pb-malate contents increased when the ratio of organic acids to Pb increased from 2:1 to 100:1.

Effect of acetic and malic acids on the thickness of unstirred layer of Pb adjacent to roots

Plant roots generally have a negatively charged surface due to the presence of O^- and COO^- groups, which leads to the formation of an unstirred layer adjacent to the root surface. The unstirred layer of a nutrient solution contains an excess of ions called counterions (e.g., i^+ in Fig. 6) that carry charge equal in magnitude and opposite in sign to that exhibited by the root surface. The thickness of the unstirred layer varies inversely with the ionic strength of the nutrient solution. The characteristic thickness is given by $0.28 \times I^{-0.5}$ nm,

Table 1 Pb chemical speciation (% of total Pb) of nutrient solutions in the presence and absence of organic acids as calculated with Visual MINTEQ using our initial solution composition

	Pb	Acetic acid:Pb ^a						Malic acid:Pb ^a					
		2:1	5:1	10:1	20:1	50:1	100:1	2:1	5:1	10:1	20:1	50:1	100:1
Pb ²⁺	95.7	94.6	93.1	90.7	86.2	75.1	61.7	91.5	85.9	78.0	66.0	46.0	31.7
PbOH ⁺	1.33	1.31	1.29	1.25	1.19	1.02	0.83	1.27	1.18	1.07	0.90	0.61	0.40
PbSO ₄ (aq)	1.55	1.53	1.51	1.46	1.38	1.17	0.93	1.49	1.38	1.24	1.03	0.67	0.42
PbNO ₃ ⁺	1.46	1.44	1.41	1.37	1.30	1.12	0.91	1.35	1.26	1.17	0.96	0.64	0.42
Pb-Ac ⁺		1.09	2.67	5.19	9.83	21.2	34.1						
Pb(Ac) ₂ (aq)				0.02	0.09	0.48	1.53						
Pb-(Mal) ₂ ²⁻									0.01	0.04	0.13	0.57	1.50
Pb-Mal(aq)								4.39	10.3	18.5	31.0	51.5	65.6

Results are for a pH of 5.8 and a temperature of 25°C. Pb was maintained at 20 μM

Ac acetic acid, Mal malic acid

^a Different molar concentration ratio of organic acids to Pb

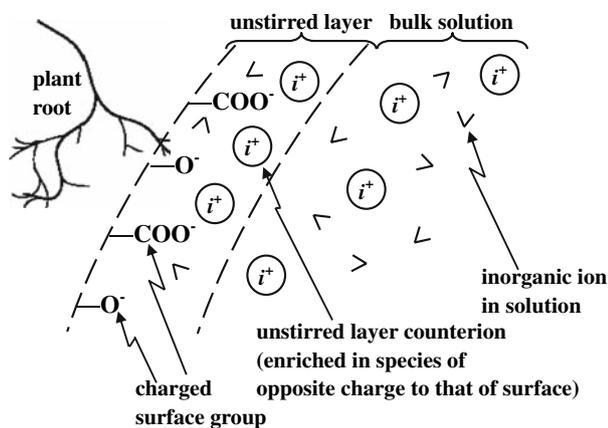


Fig. 6 Schematic showing how a negatively charged root surface attracts cations, including Pb²⁺ (e.g., i⁺), in the unstirred layer of a nutrient solution

where I is the nutrient solution's ionic strength in molar units (e.g., Schwarzenbach et al. 2003). The thickness of the unstirred layer was 6.56 nm for the nutrient solution without Pb and without organic acids. When the molar concentration ratio of acetic or malic acid to Pb increased from 2:1 to 10:1, the thickness of the unstirred layer decreased from 6.42 to 6.30 nm for acetic acid and from 6.16 to 5.92 nm for malic acid. These results suggest that the unstirred layer was thinner in the presence of organic acids, thus creating a situation in which the counterions (including Pb²⁺) are more readily absorbed or taken up by wheat roots. This finding is consistent with the observed enhanced uptake of Pb by wheat (Fig. 1). However, when the molar concentration ratio of organic acids to Pb further increased and the thickness of the unstirred layer further decreased, the uptake of Pb by wheat roots eventually decreased. We postulate that this is due to the adverse

effect of higher organic acid concentrations on the plasma membrane.

Coordination environment of Pb in wheat roots

Normalized XANES spectra of Pb for the root samples and model compounds are shown in Fig. 7. A sharp peak occurred in Pb L₃-XANES, which was caused primarily by 2p to 5d electronic transition of Pb. Better spectral agreement was obtained between root samples (inflection at 13049.3 eV) and Pb(CH₃COO)₂·3H₂O (inflection at 13048.5 eV). For Pb(NO₃)₂ the peak shifted to a lower energy at 13042.3 eV and showed greater intensity. The XANES spectra of the root samples also exhibited a clear maximum at 13085.3 eV. Similar maxima were observed for Pb(CH₃COO)₂·3H₂O, but to a lesser extent, while the Pb(NO₃)₂ and β-PbO spectra did not show such features. These distinct differences in the XANES spectra arise from different local geometries and occupancies of the final electronic states. The similarities in the XANES structure for root samples and Pb(CH₃COO)₂·3H₂O demonstrate that their local atomic configurations surrounding the central Pb atoms are similar. In Pb(CH₃COO)₂·3H₂O, Pb is bound with carboxyl functional groups, which indicates that Pb in roots are coordinated via similar chemical environments. Similar results were obtained by Sarret et al. (1998).

The k^2 -weighted EXAFS spectra and Fourier transforms for the wheat root samples and reference compounds are presented in Fig. 8a, b. Compared to the undesorbed roots, the k^2 -weighted EXAFS spectrum for the desorbed roots showed more noise above 6.2 Å⁻¹ due to lower Pb contents in the desorbed roots. The first two oscillations for the root samples at 3.6 and 5.5 Å⁻¹ showed a fingerprint feature, and are

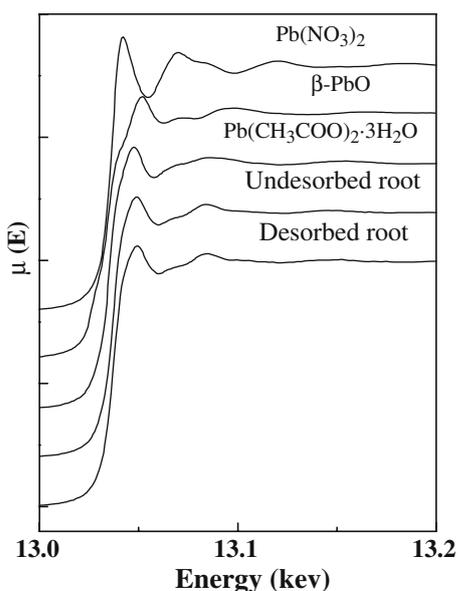


Fig. 7 Normalized Pb L_3 -edge XANES for desorbed and undesorbed wheat (*Triticum aestivum* L.) roots and model compounds

similar to those for $Pb(CH_3COO)_2 \cdot 3H_2O$. The Fourier transforms reflect a relative radial distribution function of lead(II) with neighboring atoms located in the local coordination shells. The dominant peak at 1.62 Å arose from the first shell of Pb–O. However, this peak for model compound $Pb(NO_3)_2$ was shifted 0.3 Å higher than the others. The second weak peak around 3.3 Å for the root samples probably arose from the second-shell due to metal–metal bonding. For $Pb(CH_3COO)_2 \cdot 3H_2O$ the second peak may be due to the effect of Pb–Pb and/or Pb–C bonds. In addition, a second weak peak around 3.7 Å for $Pb(NO_3)_2$ was due

to the second-shell of Pb–N. The similarities between the spectra of the root samples and $Pb(CH_3COO)_2 \cdot 3H_2O$ further support the binding of lead(II) to wheat roots through a similar mechanism, i.e., Pb was most likely coordinated to oxygen atoms via carboxylic groups.

The fitted results are listed in Table 2 for the first shell. The data indicate that the nearest-neighbor distance of the Pb–O bond in wheat roots is similar to those for $Pb(CH_3COO)_2 \cdot 3H_2O$ but deviates from those of $Pb(NO_3)_2$. No XAS study was carried out for wheat roots treated with malic acid since we were unable to obtain lead malate reference compounds.

Discussion

Organic acids may influence metal solubility and uptake through their indirect effects on microbial activity, rhizosphere physico-chemical properties, and root growth dynamics, as well as more directly through acidification, complexation, precipitation and oxidation-reduction reactions in the rhizosphere (Cieřliński et al. 1998). Still, relatively little information exists on the effects of organic acids on the uptake of Pb by plants. The results of our experiments clearly demonstrate that organic acids enhance Pb uptake by wheat roots (Figs. 1, 2). The significant greater V_{max} values in the presence of acetic and malic acids reflect the enhanced net influx of Pb into symplasm.

In the uptake experiments, binding to the cell wall could have confounded the estimation of Pb transport into the cytosol. In principle, extracellular absorption must not be considered true uptake by plants.

Fig. 8 Pb L_3 -edge EXAFS data (a) and Fourier transforms of the EXAFS data (b) along with fits to the data. The solid line represents the data, while the dotted line was fitted to the data. Results of the data analysis are presented in Table 2

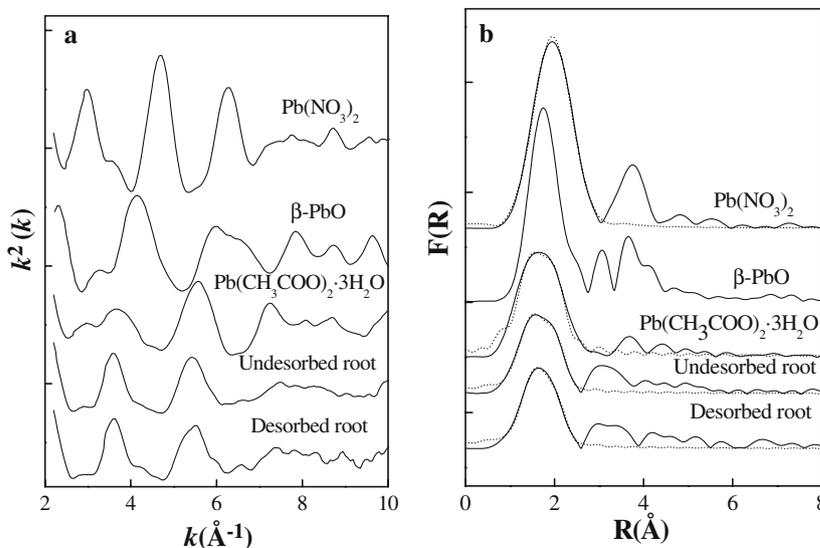


Table 2 Results of fits to the EXAFS data

Samples	First Pb–O shell			Second Pb–O shell		
	N	R (Å)	σ^2 (Å ²)	N	R (Å)	σ^2 (Å ²)
Undesorbed roots	4.13	2.36	0.008			
Desorbed roots	4.02	2.26	0.011			
Pb(CH ₃ COO) ₂ ·3H ₂ O	4	2.30	0.006	4	2.39	0.007
Pb(NO ₃) ₂	6	2.63	0.002	6	2.72	0.008

Errors of approximately 20% in the coordination number and 0.01 Å in the bond distance

Therefore, removal of Pb from the root surface is essential for accurate measurement of uptake of metals by plants. Desorbed Pb could have originated from the cell walls and/or from the cytosol via efflux across the plasma membrane back into the external solution. In our experiments, desorption was performed in an ice-cold water bath. Under such conditions the active Pb efflux from cytoplasm is presumably inhibited. Based on our experimental results we conclude that most of the desorbed Pb originated from the root cell walls.

Pb has been found to significantly inhibit voltage-gated Ca²⁺ channel activity in the plasma membrane of wheat roots (Huang and Cunningham 1996). The inhibition of Ca-channel activity by Pb²⁺ could result either from competitive transport of Pb²⁺ through Ca-channel or from its blockage by Pb. While monitoring Pb entry into isolated cells, Tomsig and Suszkiw (1991) observed permeation of Pb through Ca-channels. In maize and wheat plasma membrane vesicles the Ca²⁺ channel is very sensitive to La³⁺ (Tyerman and Skerrett 1999). Bregante et al. (1997) reported that 1 mM Cs⁺ was able to block in a very fast and voltage-dependent manner up to 96% of the potassium current in protoplasts of maize roots. Our results suggest that La³⁺ and Cs⁺ significantly inhibit the uptake of Pb by wheat roots (Fig. 5). It is generally recognized that Cs⁺ is a K⁺ channel blocker, while not affecting any Ca²⁺ channels. La³⁺ is a broad range Ca²⁺ channel blocker, but also blocks non-selective cation channels, anion channels and the general metabolism. Interestingly, Pb²⁺ can bind to Ca²⁺-binding sites in regulatory proteins such as calmodulin. Recently, the tobacco plasma membrane protein *NtCBP4* and the Arabidopsis gene *CNGC1* were reported to be components of a transport pathway responsible for Pb²⁺ entry into plant cells (Sunkar et al. 2000). The involvement of *CNGC1* and *CNGC2* in the transport of potassium has been reported also (Leng et al. 1999). These results support our evidence that the uptake of Pb²⁺ was mediated at least in part by K⁺ and Ca²⁺ channels.

Uptake of cationic solutes is likely to be driven largely also by the negative membrane potential across the plasma membrane, which is generated in part by metabolically dependent processes such as proton extrusion via the plasma membrane H⁺-ATPase

(Kochian 1991). The pmf generated by plasma membrane H⁺-pumping ATPase powers transport through a variety of carriers; it also influences ion channel activity through its impact on V_{max}. The plasma membrane H⁺-ATPase is a P-type ATPase. All P-type ATPase membranes are inhibited by orthovanadate (H₂VO₄⁻). Pre-treatment of wheat roots by vanadate significantly inhibited Pb uptake in our study (Fig. 5), thus suggesting that the enhanced Pb uptake into the cytosol of wheat was dependent upon physiological functions of the plasma membrane P-type ATPase.

The above discussion on the mechanisms of enhanced uptake of Pb in the presence of organic acids focuses on mostly plant-physiological aspects of the uptake problem. Interestingly, the mechanisms can also be discussed from a solution chemistry perspective. Table 1 shows the speciation of Pb in the nutrient and uptake solutions as estimated with Visual MINTEQ, version 2.50. Our results suggest that free Pb²⁺ was the dominant species, even in the presence of organic acids. In view of the size and polarity of intact Pb(II)-organic ligand complexes, absorption of these complexes is very unlikely. Also, synthetic chelates cannot slip through the plasma membrane since they are too large and too polar to move through the plasmalemma lipid bilayer (Berne and Levy 1998). However, it seems possible that diffusion of Pb-acetate or Pb-malate complexes was more rapid than free Pb²⁺ because of the high charge density of Pb²⁺, thus facilitating these complexes to move more rapidly towards the roots. Once these chelates reach the root surface, the surfaces will promote their dissociation into free Pb²⁺, which are then more readily absorbed or taken up by the roots.

The reason for acetic and malic acids exert different enhancement effects on the uptake of Pb may be explained by their different chelating capabilities. The stability constants of complexes formed between Pb and acetic acid are less than those between Pb and malic acid, which is contrary to the order of the enhancement effects obtained in our experiments. It is apparent that the lower the stability constants of the Pb(II)-organic ligand complexes, the greater the enhancement effect. This means that Pb(II)-organic ligand complexes are more easily dissociated by wheat

roots, leading to more uptake of dissociated Pb ions by the wheat roots.

Negatively charged plant root surface promote the development of an adjacent unstirred layer, and hence relatively high Pb^{2+} concentration gradient near the roots. According to Kochian et al. (1989), diffusive gradients in the unstirred layer around the roots or in the apoplast can occur and influence the uptake rate of elements, even in well-stirred solutions. Our results suggest that the unstirred layer was effectively thinner in the presence of organic acids, thus allowing Pb^{2+} to be more readily absorbed or taken up by roots. This finding supports the enhanced uptake of Pb as shown in Fig. 1.

Lead can be tetravalent or divalent. Divalent lead possesses a $6s^2$ outer shell electronic configuration. The two lone pair electrons are often stereochemically active and induce a strong deformation of divalent lead polyhedron. Another source of complexity of the coordination chemistry of lead comes from the high variability of its coordination number. All possible coordination values (between 3 and 12) have been reported in the literature. Our EXAFS data indicate that one Pb(II) atom was coordinated to four oxygen atoms and that the coordination environment of intracellular Pb in wheat roots is similar to that of $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$. It is worth noting that the coordinated oxygen atom is not from $\text{Pb}(\text{NO}_3)_2$ in solution but from carboxylate functional groups. The coordination number for root samples was particularly low compared to that of $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$ because of the high structure disorder of the Pb coordination shells in root samples. Similar observations for Pb–O coordination have been reported for organic macromolecules such as humic acids, fulvic acids or salicylate in soils (e.g., Manceau et al. 1996), for lichen cells exudates such as oxalate and parietinic acid (Sarret et al. 1998), and for lignocellulosic biomaterial (Dupont et al. 2002).

The major conclusions from this study are that (1) the maximum Michaelis–Menten influx rate (V_{max}) for Pb uptake was enhanced by the presence of organic acids; (2) the enhanced influx of Pb into wheat roots was partially mediated by Ca^{2+} and K^+ channels, and depended upon the plasma membrane P-type ATPase; (3) the enhanced uptake of Pb was attributed to the enhanced diffusion of Pb towards roots by organic acids; and (4) one Pb(II) atom was coordinated to four oxygen atoms in wheat roots such that the coordination environment of Pb in wheat roots was similar to that of $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$. This last conclusion supports earlier findings that the binding of Pb(II) in wheat roots occurs via carboxylic groups. However, we

emphasize that our findings were the result of experiments performed with solution cultures and therefore may not be directly applicable to actual field situations.

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