

Effect of lead, nickel and vanadium contamination on organic acid transport in xylem sap of cucumber

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Abstract

Three organic acids (citric, fumaric and malic) of the Krebs cycle were measured by the reversed-phase high performance liquid chromatography (RP-HPLC) technique in the xylem saps of cucumber plants grown in (i) control nutrient solutions containing iron as Fe(III)-ethylene-diamine-tetraacetate (Fe(III)-EDTA), Fe(III)-citrate or FeCl₃ and (ii) in nutrient solutions contaminated with nickel, lead or vanadium in a concentration of 10⁻⁵ M. Simultaneously, the heavy metal content of the xylem sap samples was determined by total reflection X-ray fluorescence spectrometry (TXRF). In the presence of lead or nickel contamination the transport of the organic acids became higher by factor 1.1–2.3 compared to the control plants when Fe(III)-citrate or Fe(III)-chloride was added to the nutrient solutions. In the case of plants growing in Fe(III)-EDTA containing solutions, however, the transport of the organic acids decreased by 30–40%. The effect of vanadium on the transport of organic acids was considerably smaller. Generally, the deviation in the organic acid transport between the contaminated and the control plants was proportional to the heavy metal transport in the xylem in the sequence Ni > Pb > V. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

Phytotoxicity caused by environmental pollutant heavy metals such as lead, nickel, and vanadium has recently received increasing attention. Heavy metals can be accumulated in different plant tissues or vacuoles in inactive chemical forms (e.g. lead-pyrophosphate or orthophosphate) [1]. Also, being transported in the xylem and phloem elements, they may have some influence on the metabolical processes as well. Some plants developing tolerance mechanisms against phytotoxicity may constitute a risk factor for human health when entering the food chain.

Lead, which is one of the main inorganic pollutants of our environment, causes toxic symptoms like chlorosis and sub-cellular toxicity with effect on plant respiration, mitosis, water and nutrient element uptake which is related to the fact that lead reduces cell elongation [2]. By monitoring lead concentration in vegetables, it has been established that, in only one decade, the lead concentration in comestible plant

parts related to the dry mass has increased from 0.05 to 3.0 µg/g [1]. This fact proves the great capacity of vegetable life to accumulate lead.

Up to the present it is questionable whether nickel is essential for plants, animals and human beings. It is known that the urease of the nodulated vegetables is a nickel-containing enzyme [3]. It is known that surplus of nickel has an effect on photosynthesis, transpiration, and nutrient element uptake; however, the mechanism of toxicity has not yet been discovered. Nickel tolerance is dependent on the kind of plants. For example, nickel causes visible phytotoxic symptoms in maize at a concentration of 26 µg/g, while in rye grass 50 µg/g Ni²⁺ (related to dry mass) leads to a slight chlorosis [4]. Moreover, in maize plants growing in nutrient solutions containing 20 µM nickel both the nickel concentrations determined by the AAS technique and the organic acid concentrations measured using the RP-HPLC method increased in stems as compared to the control plants. This fact led the authors to believe that there is a strong correlation between nickel and organic acids in the accumulation processes of nickel in stems. Kersten et al. [5] found nickel-

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citrate and -malate in leaves of some plants known as nickel hyperaccumulators. Tiffin [6] identified negatively charged nickel–organic acid complexes in soybean plants, while Wiersma and Van Goor [7] reported that nickel forms complexes with compounds of 1000–5000 molecular weight in the phloem elements of these plants. Since the pH of the xylem of plants is 5–6, they may be a medium for complex formation. Thus, it is possible that organic acids or peptides present in the xylem or phloem saps transport the nutrient or toxic elements. However, only a few *in vitro* experiments relating to speciation using thin-layer chromatography (TLC) [8] or thin-layer electrophoresis (TLE) [8,9] are available in the literature, perhaps due to the low metal concentration in the saps.

Among the species of vanadium, the vanadyl (VO^{2+}) ions may have a great importance for plant physiology, because a considerable amount of vanadium is emitted in the environment as vanadyl sulphate by oil-fired power plants [1]. It is well known from the literature [1] that the phytotoxic vanadium concentration in nutrient solutions and soils is usually 0.5 and 140 $\mu\text{g/g}$, respectively, but the mechanism of its phytotoxicity is not yet known.

Organic acids are attributed multiple functions like participation in cation transport [10], control over the availability of the nutrient elements [11], influence on the nitrogen assimilation [12] and implication in the metabolism of sugars and amino acids. Thus, studying the interaction between the heavy metal and organic acid transport in xylem saps may help us to understand the physiological processes taking place in plants.

For the determination of elements in xylem fluids, atomic absorption (FAAS [13,14], GF-AAS [15,16]), multi-elemental ICP-AES [17,18] or total reflection X-ray fluorescence (TXRF) spectrometry [19,20] are the most powerful tools, while the most common technique to determine the organic acids in different plant parts is HPLC [21–23]. In this study a direct reversed-phase high performance liquid chromatography (RP-HPLC) technique was used for the determination of organic acids in xylem sap of cucumber. To follow the transport processes of metals in the xylem channels of cucumber TXRF spectrometry was applied due to its excellent analytical capabilities: low volume sample demand, negligible matrix effect, simple quantification by internal standardization, simultaneous determination of 20–25 elements, absolute detection limits in the pg range.

These measurements constitute a part of our research program focused on the transport processes and speciation of heavy metals in plants exposed to pollutants of the environment.

2. Experimental

2.1. Growth of the plants and sampling of xylem fluid

Cucumber seeds (*Cucumis sativus* L. var. Budai korai) were germinated in Petri dishes on wet filter paper for one

day in darkness at 26°C. These seedlings were planted on plastic net placed between polystyrene disks which were put into PVC cups containing 200 ml 0.5 mM CaSO_4 solution. Then they were covered with wet filter paper to keep the plants in a dark, moisture-filled atmosphere at 26°C for one more day. After this step the plants were transferred into modified Hoagland nutrient solutions with the following chemical composition: 1.25 mM KNO_3 , 1.25 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM MgSO_4 , 0.25 mM KH_2PO_4 , 11.6 μM H_3BO_3 , 4.5 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 μM Fe(III)-EDTA, Fe(III)-citrate or FeCl_3 , 0.19 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.08 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The nutrient solutions were replaced three times per week. For each change they were freshly prepared from stock solutions. The nutrient solutions were maintained in a controlled environment at 22–26°C and illuminated by daylight with metal halogen lamps and fluorescent tubes with light intensity of 75 W/m^2 for 12 h per day. At these conditions 36 cucumber plants were simultaneously grown in this ‘heavy metal-free’ modified Hoagland nutrient solution. At the first leaf stage, three plants were transferred separately into each heavy metal contaminated nutrient solution containing $\text{Pb}(\text{NO}_3)_2$, NiSO_4 , or VOSO_4 in a concentration of 10 μM and iron added as Fe(III)-citrate, Fe(III)-EDTA or Fe(III)-chloride. The control plants remained in the ‘heavy metal-free’ solutions. After a growing period of 32 days, the stems of the plants were cut 5 mm above the root neck and the xylem saps were collected 60 min after the freshly cut surfaces into polyethylene vials. After determination of their weights the xylem sap samples were stored at –22°C. Subsequently, the roots were spindried to remove the rest of the nutrient solutions from the surface and their fresh weight was measured.

2.2. Sample preparation

Before the HPLC analyses, the xylem saps were filtered through a Minisart RC 15 membrane filter (0.2 μm diameter) supplied by SARTORIUS (Göttingen, Germany).

For TXRF investigations, 190 μl of each sample was spiked with 10 μl Ga standard solution to a final concentration of 1 $\mu\text{g/ml}$. From these 200 μl spiked and mixed solutions 25 μl were dropped onto quartz-glass carriers and dried in a clean box applying a ceramic coated hot plate at 80°C for 30 min. From each solution three parallel samples were prepared and analysed.

2.3. Instrumentation and analytical procedures

The HPLC analyses were performed on a GBC instrument (GBC LC 1140 system organiser, GBC LC 1150 pump, GBC LC 1206 multiwavelength UV–Vis detector) using a reversed-phase Nucleosil C18 (250 \times 4 mm, 5 μm particle size) column connected with a Nucleosil C18 (30 \times 4 mm, 5 μm particle size) guard column. The mobile phase was a buffer solution containing 0.01 M KH_2PO_4 adjusted to pH 2.2 with H_3PO_4 [24] and was filtered through a NALGENE

Table 1

Amounts of organic acids n_{acid} (in nmol/(g_{FRW} h)) transported in xylem fluid of cucumber plants grown in control and heavy metal contaminated nutrient solutions during a bleeding period of 1 h related to 1 g fresh root weight (FRW), and f the molar ratio of organic acids transported in contaminated and control plants^a

n_{acid}	Control plants	Pb addition	f	Ni addition	f	V addition	f
Fe(III)-EDTA							
Citric	133 ± 12	82.6 ± 4.9	0.62	80.9 ± 3.6	0.61	159 ± 4	1.20
Fumaric	0.79 ± 0.04	0.47 ± 0.02	0.69	0.38 ± 0.01	0.48	0.66 ± 0.03	0.84
Malic	128 ± 13	87.6 ± 5.6	0.59	71.8 ± 2.5	0.56	105 ± 4	0.82
Fe(III)-citrate							
Citric	74.4 ± 1.6	95.6 ± 8.7	1.29	120 ± 4.4	1.62	84.6 ± 2.7	1.14
Fumaric	0.28 ± 0.01	0.42 ± 0.04	1.50	0.65 ± 0.05	2.32	0.28 ± 0.05	1.00
Malic	72.3 ± 0.7	101 ± 10	1.39	63.7 ± 5.3	0.88	71.9 ± 2.4	0.99
Fe(III)-chloride							
Citric	66.1 ± 6.9	115 ± 7	1.74	122 ± 8	1.85	70.8 ± 8.3	1.07
Fumaric	0.26 ± 0.03	0.31 ± 0.03	1.19	0.42 ± 0.02	1.62	0.25 ± 0.09	0.96
Malic	45.9 ± 4.7	79.5 ± 5.1	1.73	50.7 ± 6.5	1.11	37.8 ± 8.0	0.82

^a The standard deviation values were determined on the basis of three independent measurements of the mixed xylem sap of three plants.

nylon membrane filter (0.45 µm diameter) supplied by NALGE Co. (Rochester, USA). Column effluents were monitored at 210 nm. The multilevel calibration method was used for the quantitative determination of the organic acids. The standard solutions were prepared by dissolving analytical-grade malic, citric and fumaric acids supplied by REANAL (Budapest, Hungary) in bidistilled water. The stock solution was diluted to give concentrations ranging from 10 to 200 µg/ml for citric and malic acids as well as 0.1–2 µg/ml for fumaric acid. The calibration curves were linear in the above-mentioned concentration range ($r \geq 0.997$). Each sample was analysed three times.

The TXRF analyses were carried out by an EXTRA IIA equipment (ATOMIKA Instruments GmbH, Oberschleissheim, Germany) using a Mo-microfocus tube (50 kV, 38 mA), a high-energy cut-off filter (quartz-glass mirror) and an attenuation filter (200 µm Mo, 240 µm Al). The integration time amounted to 1500 s for each sample. Ga standard solution (Merck, Darmstadt, Germany) was used for internal standardization. The absolute detection limits ranged between 0.91 and 1.61 ng for lead, between 1.08 and 1.58 ng for nickel and between 6.54 and 7.16 ng for vanadium depending on the iron compound added to the nutrient solutions.

3. Results and discussion

The organic acid composition of the xylem fluid may depend on the kind of plant investigated. The predominant organic acids present in the xylem fluid of various plants (e.g. tomato, soybean, beech, etc.) are malic, citric, succinic and fumaric acids, which are intermediates of the Krebs cycle [23,25,26]. Using the RP-HPLC technique in xylem sap of cucumber plants only three organic acids of the Krebs cycle, namely citric, fumaric and malic acid, were detectable. The

amounts of the transported citric and malic acids exceeded the amount of fumaric acid by approximately two orders of magnitude (Table 1). The time demand for their reliable separation amounted to 12 min. The detection limits were 1.82 µg/ml for citric acid, 1.58 µg/ml for malic acid and 0.02 µg/ml for fumaric acid. The recovery of citric, malic and fumaric acids was 98.34, 97.94 and 98.61%, respectively. The values obtained for the capacity factor and resolution of the separation for these acids were as required [27] in the chromatographic separations except for the first eluting acid, the malic acid. To optimize the separation we took into consideration the eluent type, pH, flow rate and temperature of the eluent, but in spite of them the capacity factor and resolution of malic acid were 0.7. This fact may have been due to the presence of small inorganic anions in the saps, such as nitrate anions, which have significant UV absorbency (Fig. 1). Comparing the amounts of transported acids in the xylem sap of control and lead contaminated plants there were considerable differences, depending on the chemical form of the iron in the nutrient solutions. In the case of plants growing in nutrient solutions containing Fe(III)-citrate or FeCl₃ the increment of the organic acid transport can be characterized by a factor 1.2–1.7 (Table 1), while the transport of acids was hindered by 40 to 50% as related to the uncontaminated plants when Fe(III)-EDTA was used. This is in good agreement with our previous results [28] taking into consideration that the samples were obtained from biological systems. Depending on the iron form in the nutrient solutions the lead transport in the xylem channels changes in the following sequence: FeCl₃ > Fe(III)-citrate ≫ Fe(III)-EDTA (Table 2). This could be due to the fact that the complex formation between lead and the organic ligand of the iron(III) complexes may be responsible as we reported earlier [29]. It should be noted, however, that the nutrient solution also contained lead in a concentration of 0.017 µM, due to the impurities of the chemicals applied for the preparation of the

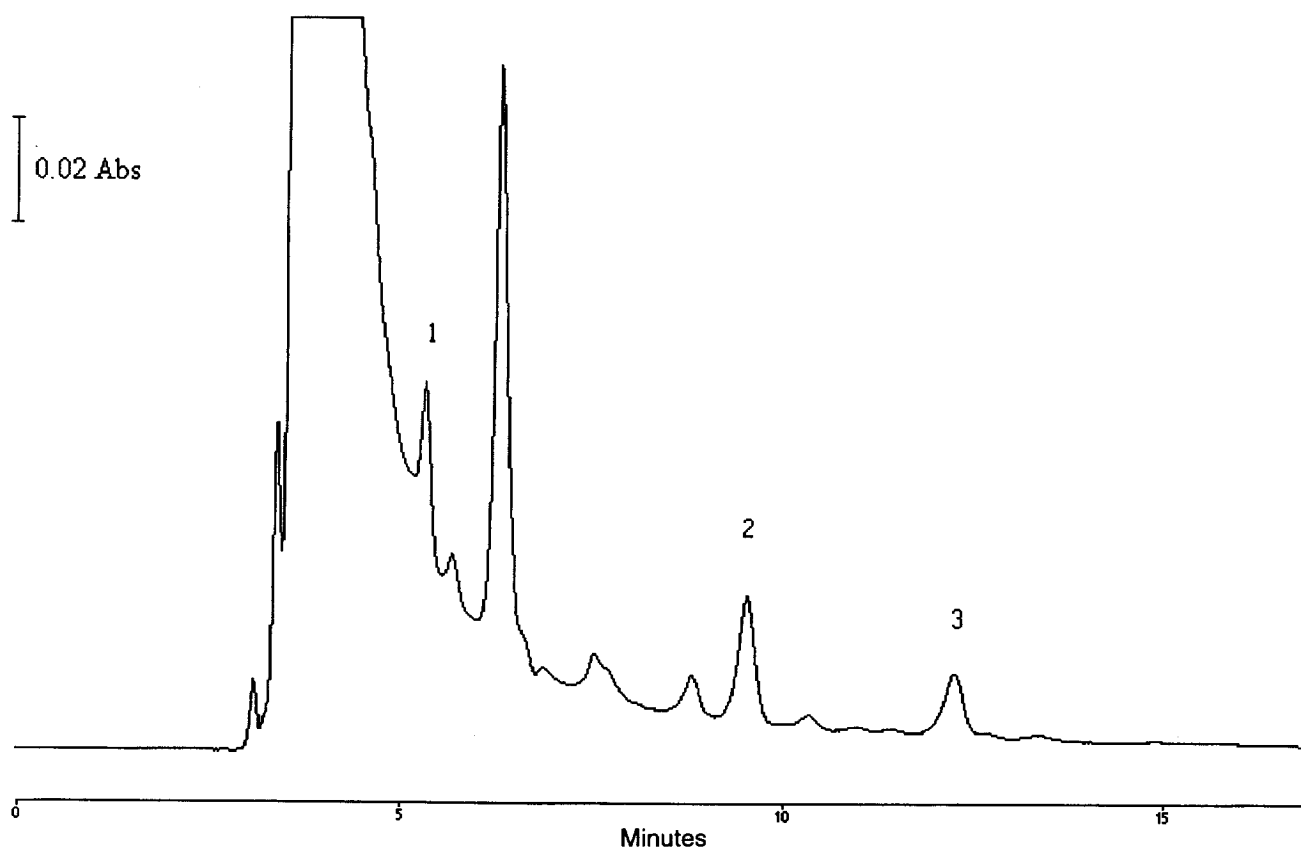


Fig. 1. Chromatogram of xylem sap of cucumber grown in control nutrient solution containing Fe(III)-citrate: (1) malic acid; (2) citric acid; (3) fumaric acid. Chromatographic conditions: 250 × 4 mm Nucleosil 5 C18 column; 30 × 4 mm Nucleosil 5 C18 guard column; eluent: 0.01 M $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ at pH 2.2; flow rate: 0.75 ml/min; UV detection at 210 nm.

Table 2

Amounts of nickel and lead n transported in the xylem fluid of cucumber plants grown in heavy metal contaminated nutrient solutions containing Fe(III)-EDTA, Fe(III)-citrate or Fe(III)-chloride during a bleeding period of 1 h related to 1 g fresh root weight (FRW) ^a

Contamination	n (nmol/(g _{FRW} h))	Contamination	n (nmol/(g _{FRW} h))
Fe(III)-EDTA			
Ni	4.73 ± 0.12	Pb	0.16 ± 0.01
Fe(III)-citrate			
Ni	5.90 ± 0.12	Pb	1.09 ± 0.02
Fe(III)-chloride			
Ni	8.68 ± 0.18	Pb	1.28 ± 0.02

^a The standard deviation values were determined on the basis of three independent measurements of the mixed xylem sap of three plants.

nutrient solutions. Nickel and vanadium could not be detected in the control nutrient solutions by the TXRF technique.

By studying the interaction between the transport of organic acids and nickel contamination, and relating the values to the transported acids in the control plants, the following could be established: the organic acid transport and the influence of complexing agents were similar in plants grown in nickel containing nutrient solutions and plants grown in lead contaminated nutrient solutions. In the presence of Fe(III)-

EDTA, the transport of organic acids in the nickel contaminated plants was lower compared to the control plants, the rate of the decrease being very similar to the values obtained for lead contamination. For Fe(III)-citrate, an increasing tendency was registered in the transport of citric and fumaric acids, higher than in the case of the presence of the same iron(III) compound and lead contamination. However, the transport of malic acid decreased by 12% as compared to uncontaminated plants. Organic acid transport in the xylem elements of nickel contaminated plants grown in nutrient solutions containing Fe(III)-chloride can be characterized by an increase by a factor of 1.11–1.85 for the acids investigated. The amounts of nickel transported in the xylem depends on the chemical form of iron used in the nutrient solutions (Table 2). Due to the possible complex formation with EDTA or citrate from the nutrient solutions, the amounts of nickel transported were higher by 25% in the case of Fe(III)-citrate than in the presence of Fe(III)-EDTA. The most pronounced transport of nickel was measured at FeCl_3 , 8.68 nmol with respect to a 1 h bleeding period and 1 g fresh root weight. This value is higher by 47 and 84% as compared to amounts of nickel transported in the case of Fe(III)-citrate and Fe(III)-EDTA, respectively.

Comparing the analytical data obtained for the control and vanadium contaminated plants, the organic acid content of

xylem fluid samples collected during a bleeding period of 1 h shows only small differences, independently of the iron compounds used for the preparation of the nutrient solutions. The citric acid transport slightly increased in the presence of each iron(III) compound, while in the cases of malic and fumaric acids a small reduction was observable. Vanadium could not be detected in the xylem sap samples by the TXRF method. The moderate effect observable in the transport of organic acids in the xylem fluids for vanadium contamination may be attributed to the low uptake of vanadium in the roots.

4. Conclusions

By studying the influence of heavy metal pollutants on the transport of the organic acids in xylem saps of cucumber it can be concluded that:

(i) The organic acid and toxic element transport in the plants depend on the chemical form of iron(III) used for the preparation of the nutrient solutions due to the organic ligands of the iron(III) complexes, which have a definite influence on the heavy metal uptake and transport. The more stable the complex of the heavy metals investigated with these ligands, the harder is the uptake and transport of these metals.

(ii) The presence of heavy metals in the plants seems to have an impact on the organic acid transport. Generally, greater amounts of heavy metals were transported in the xylem vessels ($\text{Ni} > \text{Pb} \gg \text{V}$); the more significant deviations were observed in the organic acid transport.

(iii) In the case of vanadium, whose presence could not be detected in the xylem saps, the organic acid transport remained practically unchanged compared to the control plants.

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