Effects of cadmium on the behaviour of citric acid in isolated tomato xylem cell walls

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Abstract

Effects of cadmium on the sorption of citric acid in isolated xylem cell walls were investigated. 2.5 nM to 9.5 mM [1.5-14C]citric acid solutions were perfused through columns of xylem cell wall material, isolated from tomato plants (Lycopersicon esculentum Mill, cv. Tiny Tim).

The anion exchange potential of the column was estimated by amino acid analysis as approximately 46 meq dm⁻³, whereas the apparent anion exchange capacity (AEC) was estimated as 1.65 ± 0.18 10⁻⁴ M (citric acid units). This low AEC was attributed to a 'zipper' effect, a mutual screening of fixed R⁻ and A⁺ charges.

Pre-loading with ¹¹⁵Cd²⁺ did not affect citric acid sorption, indicating the absence of Cd-effects on the availability of fixed A⁺ charges, and the absence of the formation of effective R⁻-Cd²⁺ and Donnan free space (DFS) [Cd(cit)HJ⁺ complexes.

Simultaneous application of both citric acid and ¹⁹⁵Cd²⁺, ⁴⁴Ca²⁺, or ⁸⁸Mg²⁺ resulted in increased sorption of citric acid, probably due to capacity-improvement rather than changes in valence-dependent anion sorption; this may be due to the presence of bulk [M(cit)]⁻, held in the column as [M(cit)HJ⁺ after protonation in the DFS. Sorption of citric acid was greatest in the presence of Ca²⁺, which was discussed in the light of the differences between Ca, Cd and Mg in their characteristics as co-ordinative M-complexes of citric acid. The overall results indicate the potential importance of the presence of metal ions for the xylem transport behaviour of organic acids in plants.

Key words: Cadmium, citric acid, ion exchange, ligand exchange, tomato, xylem cell walls.

Introduction

Long distance transport of nutrients taken up from the soil by plant roots occurs through the xylem vessels. The substances present in this flow become involved in such processes as adsorption to the mainly negatively charged sites present in the cell walls (Bell and Biddulph, 1963; Ferguson and Bollard, 1976; Wolterbeek et al., 1984), lateral escape from the moving solution into adjacent intercellular spaces and free spaces of the cell wall (Van Bel, 1978; Wolterbeek et al., 1984), and uptake by metabolic compartments of xylem parenchyma and other cells (Biddulph et al., 1961; Jacoby, 1965).

Generally, the transport of metals along the xylem vessels probably occurs only after saturation of the exchange sites in the xylem walls (Ferguson and Bollard, 1976; Petit and Van de Geijn, 1978; Van de Geijn and Petit, 1978, 1979). The adsorption processes in xylem cell walls may be important not only with respect to stem retention times (Ferguson and Bollard, 1976) and longitudinal movements, but should also be seen as relevant for the process of lateral transfer (Senden and Wolterbeek, 1990).

During the last several decades, much attention has been paid to the influence of the presence of organic compounds on the transport behaviour of metals in the xylem vessels. The uptake and synthesis of organic compounds in the roots (Collins and Reilly, 1968; Tonin et al., 1990), and the presence of amino acids and cation–organic complexes in xylem fluid have been reported to

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Abbreviations: A⁺-fixed cell wall positive charges; AE-anion exchange; AEC-anion exchange capacity; DFS-Donnan free space; DW-dry weight; FMOC-9-fluorenylmethyl-chloroformate; M-divalent metal; OPA-ortho-phthakjialdehyde; R⁻-fixed cell wall negative charges; RP-HPLC-reversed phase high performance liquid chromatography; WFS-water free space.
seriously affect the movement of metals in the xylem, both in longitudinal and lateral directions (Tiffin, 1966; 1970; Bradfield, 1976; White et al., 1981a, b, c; van de Geijn and Pikaar, 1982; McGrath and Robson, 1984; Senden and Wolterbeek, 1990). In this context, citric acid has received considerable attention, probably because it is generally a substantial fraction of the organic acids in the xylem (White et al., 1981a; Senden et al., 1992a), and because it is a relatively strong metal complexer (Sillen and Martell, 1964).

Remarkably, and in sharp contrast with the wealth of information on effects of organic compounds on metal behaviour, there exists hardly any literature on the possible influence of the presence of (specific) metals on the xylem transport behaviour of organic compounds. In the light of the above, it seems only logical to raise the question as to whether cations influence the behaviour of organic compounds, for the occurrence of these phenomena may lead to changes in (initial) longitudinal and lateral transfer rates and to ion-specific variations in the distribution of organic compounds in plants.

Effects of metals on the behaviour of organic compounds are described and used in various ways in chemistry, but the closest association with what one may expect in xylem vessels can be found in chromatography. Since the early publications on ligand exchange (Stokes and Walton, 1954; Helfferich, 1961, 1962a), the differences in ligand complex-strengths with metal ions are widely used to separate a large range of organic compounds (Walton, 1973; Navratil et al., 1975; Davankov and Samechkin, 1977; Takayanagi et al., 1985; Kato et al., 1986; Nielen et al., 1987; Matejka and Weber, 1990; Sud et al., 1992). Here the performance of the (fixed) metals consists of the very selective attraction of specific organic compounds, by which ion exchange and co-ordination chemistry are combined.

In plants, a single observation has been reported by Senden et al. (1992b), indicating a cadmium-induced increase in citric acid accumulation in xylem cell walls. Since xylem citric acid predominantly occurs in metal complexed forms (White et al., 1981b), variations in citric acid transport behaviour may be dominated by the xylem metal constituents.

The present paper further addresses the possible effects of metal ions on the behaviour of organics in xylem vessels. Xylem cell walls were isolated from tomato plants (Lycopersicon esculentum Mill, cv. Tiny Tim), and used in column experiments (Senden et al., 1992b) in which the accumulation of citric acid was determined. A number of experiments was carried out with magnesium and calcium to investigate the general characteristics of the effects observed. The use of isolated cell wall material means that the results obtained may be interpreted only in terms of metal effects on the sorption of citric acid: further experiments are needed to elucidate possible consequences for longitudinal transport and lateral transfer.

**Materials and methods**

**Plants**

Tomato plants (an inbred line of Lycopersicon esculentum Mill, cv. Tiny Tim) were used throughout. They were cultivated in a glasshouse at c. 25 °C and 75% r.h. Stem internodes of 8-week-old plants were used for the preparation of isolated xylem cell wall material (wood powder).

**Xylem cell wall material**

Xylem cell wall material was prepared from the stem internodes by bacterial degradation. Stem internodes were submerged in tap water at 20 °C for 40 d, after which the unaffected xylem cell wall parts of the internodes were isolated by rinsing in tap water (Senden et al., 1992b). Purification was carried out according to Ritchie and Larkum (1982) by soaking and washing the xylem material in 0.5% (v/v) Triton X-100, acetone and distilled water for 4 d. The xylem cell wall material was dried, powdered and sieved into particles with diameters ranging from 0.125 to 0.250 mm. A 100 mg portion was shaken in 50 cm³ distilled water, floating pieces were removed and the remainder was packed between two quartz filters in a silicone coated glass tube (6 mm diameter, 50 mm length).

The isolated xylem cell wall material was protonated by elution of 0.1 M HCl through the column. The H⁺-form was converted by 1.0 M NaCl into the Na⁺-form, which was taken as the standard state. Cl⁻ was removed by briefly rinsing with distilled water. The acidity of the standard state effluent was pH 6.3 ± 0.1.

**General column characteristics**

The total void water volume of the xylem cell wall column was determined as 0.63 cm³ (Senden et al., 1992b). Apart from the packed xylem cell wall material, the total tube volume below the packed xylem cell wall material was measured separately as 0.39 cm³, leaving 0.24 cm³ for the water free space (WFS) volume of the 100 mg dry weight xylem cell wall material. The Donnan free space (DFS) volume of the column was set at 0.15 cm³ g⁻¹ DW, based on data reported by Wolterbeek (1987a).

**Xylem cell wall amino acid analysis**

1.6 mg xylem cell wall material was hydrolysed in the gas-phase at 166 °C for 2 h; OPA- and FMOC-derivatives of the amino acids were analysed by RP-HPLC (Eurosequence BV, Groningen, The Netherlands).

**Production and measurement of radioisotopes**

\[1.5^{14}C\text{Citric acid } (1.85 \text{ MBq cm}^{-3}, \text{20.8 GBq g}^{-1}) \text{ and } 4^{20}\text{CaCl}_{2} (83.2 \text{ MBq cm}^{-3}, \text{756.4 GBq g}^{-1}) \] were purchased from Amersham International UK.

\[113^{23}Cd (t_{1/2} = 53.5 \text{ h}) \] was produced by neutron activation of \(\text{Cd(NO}_{3})_{2} \) in the Hoger Onderwijs Reactor at Interfaculty Reactor Institute (I.R.I.), Delft, The Netherlands, at a thermal flux density of \(5.10^{16} \text{ m}^{-2} \text{ s}^{-1} \) for 1 h. The specific activity obtained was 0.8 GBq g⁻¹.

\[^{24}Mg (t_{1/2} = 20.9 \text{ h}) \] was produced by neutron activation of \(^{6}Li \text{ enriched Li-Mg alloys, }^{6}Li (\text{purchased from the Oak Ridge National Laboratory, USA}) \), in the Hoger Onderwijs Reactor at I.R.I., Delft, The Netherlands, after which \(^{24}Mg \text{ (as MgCl}_{2} \)
could be obtained by radiochemical separation (Kolar et al., 1991). The $^{28}\text{Mg}$ specific activities of the preparations were 20–30 MBq g$^{-1}$.

$^{113}\text{Cd}$ (γ-rays at 527 keV) was measured using a γ-spectrometer with a semiconductor Ge(Li)-detector (Canberra Model 7339FW) coupled to a DEC PDP-11/44 computer through a CAMAC interface. Calibration, spectrum accumulation and analysis were carried out according to the methods described by De Bruin and Korthoven (1972, 1974) and Korthoven and De Bruin (1977). $^{28}\text{Mg}$ (calibrated γ-rays at 400, 942, 1342, 1372, and 1589 keV) was determined using measurement equipment as described for $^{113}\text{Cd}$, but calibration, spectrum accumulation and analysis were carried out on network APOLLO computers (Hewlett Packard), according to methods described by Blaauw and Lindstrom (1993).

$^{14}\text{C}$ and $^{45}\text{Ca}$ samples were mixed with 10 cm$^3$ Lumagel scintillation liquid (Lumac, Landgraaf, The Netherlands) and measured by a liquid scintillation counter (MINAXI Tri-Carb 4000 Canberra, Packard).

Speciation calculations

The chemical speciation of elements in aqueous solutions was calculated with the help of the computer program SOILCHEM, developed at the University of California, Berkeley, USA (Sposito and Coves, 1988), with full considerations for mole balances, relevant thermodynamic equilibrium constants and ion strengths.

Experiments

During experiments, spiked 20 cm$^3$ solutions ($^{14}\text{C}$, $^{45}\text{Ca}$, $^{113}\text{Cd}$, or $^{28}\text{Mg}$, in citric acid, CaCl$_2$, Cd(NO$_3$)$_2$, or MgCl$_2$, respectively) were perfused through the xylem cell wall column. After perfusion, the column was washed with 5 cm$^3$ distilled water, 5 cm$^3$ HCl (0.1 M), and 5 cm$^3$ distilled water, respectively. All perfused fractions (volumes ranging from 0.5 to 2.0 cm$^3$) were collected in a fraction collector. Control citric acid perfusions were carried out with $^{14}\text{C}$-citric acid solutions ranging from 2.5 nM to 9.5 mM. After column pre-loading with 1.0 mM Cd solutions, $^{14}\text{C}$-citric acid solutions were perfused in 9.5 μM and 9.5 mM concentrations. Cd measurements in perfused fractions were performed by neutron activation analysis. Perfusions were also carried out with citric acid solutions containing Cd, Ca or Mg (applications: 9.5 μM citric acid with 0.9 mM Cd (Senden et al., 1992b), 1.0 mM Mg or 2.0 mM Ca, 2.5 mM citric acid with 1.0 mM Cd, and 9.5 mM citric acid with 1.0 mM Cd). Here, all experiments were carried out twice: first to measure the citric acid itself and second to measure the metal behaviour during citric acid perfusion.

Results

Cell wall fixed charges

The tomato xylem cell walls generally behave as a cation exchanger of 1000 meq dm$^{-3}$ Donnan free space (DFS), (Wolterbeek, 1986; Senden et al., 1992b), predominantly due to indiffusible ionized carboxylic groups ($R^-$) of polygalacturonic acids. However, the amino acid side-chains of the cell wall glycoproteins may provide additional ion exchange sites ($A^+$), which, in general, are of interest in relation to the possible binding of anions (Läuchli, 1976), and which may be of particular importance with respect to the accumulation of citric acid in the xylem cell walls. The anion exchange capacity ($AEC$) of plant cell walls is attributed to the presence of the basic amino acids arginine, lysine and histidine, and is reported to be numerically equivalent to the total mol concentrations of these three amino acids (Gillet and Lefebvre, 1981; Richter and Dainty, 1989b), because of their net positively charged lateral chains at cell wall pH.

Table 1 presents the tomato xylem cell wall amino acid contents, as determined from RP-HPLC analysis of OPA- and FMOC-amino acid derivatives. The data indicate an $AEC$ of 46 meq dm$^{-3}$, based on DFS volumes, and on arginine, lysine, and histidine only. The $AEC$ value found is in agreement with values reported by Gillet and Lefebvre (1981) for cell walls of Nitella flexilis and by Richter and Dainty (1989b) for Sphagnum russowii cell walls.

Citric acid sorption

Based on the simultaneous presence of fixed negative and positive charges in the xylem cell walls the sorption of citric acid may be regarded as governed by both attraction and exclusion processes. Following the Donnan equilibria given by Helfferich (1962a), anion sorption in negatively charged exchangers obeys adsorption rules which may be generally expressed by Freundlich isotherms, giving

$$y = ax^b$$

where $y$ is the sorbed DFS anion concentration in the cation exchanger, $x$ is the anion concentration in the bulk solution, and $a$ and $b$ are constants, theoretically depending on, e.g. salt composition and activity coefficients (Helfferich, 1962a).

The chemical binding of citric acid in positively charged uniform exchangers may be deduced from saturation

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (mM DFS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>59</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>30</td>
</tr>
<tr>
<td>Serine</td>
<td>37</td>
</tr>
<tr>
<td>Histidine</td>
<td>6</td>
</tr>
<tr>
<td>Glycine</td>
<td>51</td>
</tr>
<tr>
<td>Threonine</td>
<td>27</td>
</tr>
<tr>
<td>Alanine</td>
<td>47</td>
</tr>
<tr>
<td>Arginine</td>
<td>8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
</tr>
<tr>
<td>Cys-Cys</td>
<td>n.d.</td>
</tr>
<tr>
<td>Valine</td>
<td>35</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>23</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>28</td>
</tr>
<tr>
<td>Leucine</td>
<td>42</td>
</tr>
<tr>
<td>Lysine</td>
<td>32</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>70</td>
</tr>
</tbody>
</table>

* n.d. = not detected.
equilibrium arguments, and expressed by a Langmuir isotherm, as

\[ z = \frac{cy}{d+y} \]

where \( z \) is the chemically bound DFS citric acid concentration, \( y \) is the free (DFS) cell wall citric acid concentration, \( c \) is the apparent AEC (citric acid values), and \( d \) represents the apparent dissociation constant.

Combination of the equations shown above yields the total citric acid sorption \( S \) as

\[ S = y + z = ax^b + \frac{ca^x}{d+ax^b}. \]

Figure 1 presents the citric acid sorption data for a range of bulk citric acid concentrations applied. The cell wall citric acid concentrations are given in M, on a DFS volume basis. Here, it should be noted that the concentrations are based on the HCl rinses of the xylem column material, indicating relatively strongly retained citric acid fractions. The insert in Fig. 1 shows the same data, expressed in molar distribution coefficients (\( \lambda \)), which may be defined as the ratio between cell wall and bulk solution citric acid concentrations (\( S/x \)). The initially high \( \lambda \)-values indicate the effects of the presence of positive charges, the rapid drop in magnitude and small values at higher bulk citric acid concentrations applied suggest the saturation of positively charged exchange sites and the exclusion effects of the presence of fixed negative cell wall charges.

The solid line in the main figure is drawn from the fitted \( S \) values, by which the various constants (±SD) were derived as 1.30±0.26 (a), 1.48±0.05 (b), 1.65±0.18 \( 10^{-4} \) (c, in M units) and 2.3±2.4 \( 10^{-6} \) (d, in M units), respectively. The dashed lines represent the \( y \)- and \( z \)-curves, following the equations shown above. The data indicate an apparent AEC (citric acid values) of 0.165 mM (based on the DFS volume), which is only about 0.4% of the capacity suggested by the amino acid analysis. This low figure may be attributed to the inaccessibility of positive charges due to the screening by negative charges, a phenomenon which we like to refer to as the 'zipper effect' (see Discussion section, and K⁺ data reported by Gillet and Lefebvre 1981). The value of the apparent dissociation constant (\( d \)) was derived in the order of \( 10^{-6} \) M, but is statistically ill-defined and needs closer examination in future experiments.

To a first approximation, the \( a \) and \( b \) values may be regarded as empirical constants (Ritchie and Larkum, 1982), but the \( b>1 \) value agrees with theory, which predicts \( b>1 \), due to the involvement of a Donnan-type equilibrium (see Discussion section, and Helfferich, 1962a).

As stated already, the Fig. 1 data were determined from HCl rinses. The HCl rinses were preceded by H₂O rinsing of the column material, by which the citric acid content of the column water free spaces was eluted (Senden et al., 1992b). Based on the \( ^{14} \)C counting, H₂O rinsing gave a ratio 0.97±0.07 (\( n = 8 \)) for citric acid appearance in H₂O eluted fractions and bulk solution, a value which is in close agreement with the concept of full accessibility of the column water free spaces (Dainty and Hope, 1959; Wolterbeek, 1986).

Total citric acid recoveries in the experiments described above averaged 99±2%, indicating both the effectiveness of the rinses applied, and the possibility of full regeneration of the xylem column.

**Citric acid sorption in Cd-preloaded columns**

Figure 1 shows the importance of the \( z \)-curve at low concentrations, and the importance of the \( y \)-curve at high concentrations.
Fig. 2. Cadmium recovery (percentage of applied concentration) in fractions obtained during 20 cm$^3$ distilled water wash, after initial column loading with 20 cm$^3$ 1.0 mM Cd(NO$_3$)$_2$.

citric acid concentrations. Consequently, Cd effects were studied for 9.5 $\mu$M (z-domination) and for 9.5 mM (y-domination) citric acid applications. Before citric acid administration, the column was preloaded with Cd, by percolation of 20 cm$^3$ 1.0 mM Cd(NO$_3$)$_2$ solutions. The column Cd retention was checked by prolonged (up to 20 cm$^3$) H$_2$O rinsing; Fig. 2 shows the absence of appreciable losses in excess of the expected elution of column WFS Cd. Perfusion by 9.5 $\mu$M citric acid did not markedly affect Cd behaviour: Cd elution during the 20 cm$^3$ citric acid perfusion did not exceed 2% of the total Cd present in the DFS (results not shown), and the Cd recovery during HCl rinsing was comparable to control conditions (Table 2).

However, Cd was largely eluted during the perfusion of 9.5 mM citric acid, resulting in a recovery during HCl rinsing of only 2% of the initial DFS Cd. Remarkably, under these conditions, during HCl rinses, Cd and citric acid eluted in a 1:1 molar ratio (Table 2), suggesting 1:1 complexes formed in the column DFS (see Discussion section). The overall recoveries of both Cd and citric acid were invariably close to 100% (Table 2), indicating the accuracy of the experiments performed.

The citric acid elution data (Table 2, HCl rinses) could be transformed into molar distribution coefficients ($\lambda$-values), and compared with values obtained in the absence of Cd. Figure 3 shows the $\lambda_{\text{Cd-preloaded}}/\lambda_{\text{control}}$ ratio's for citric acid applications in both 9.5 $\mu$M and 9.5 mM concentrations. The results indicate the absence of any significant Cd-effect for 9.5 $\mu$M citric acid applications (z-domination), but shows a 4-fold increase in citric acid sorption in the presence of Cd for 9.5 mM citric acid applications (y-domination). The first mentioned outcome suggests that Cd may not affect the $c$ and $d$ values of the $z$-equation, in other words, apparently Cd neither opens the 'zipper' (which is formed by the close association of fixed negative and positive charges, Gillet and Lefebvre, 1981), nor forms complexes between $R_2Cd$ and citric acid (Wolterbeek et al., 1987; where R denotes the fixed negative charges), thus does not increase the cell wall's apparent $AEC$. Moreover, under the 9.5 $\mu$M citric acid conditions, Cd preloading does not even significantly affect the $y$-curve characteristics. The latter reasoning may be deduced from the simultaneous presence of the $a$–$d$ constants in the $S$-equation, the absence of any significant Cd presence in the applied and percolating solutions.

Table 2. Citric acid (cit) and Cd data in perfusion experiments with Cd-preloaded xylem column material

<table>
<thead>
<tr>
<th>Cd preloading solutions</th>
<th>Citric acid solutions</th>
<th>Cd elution during citric acid perfusion</th>
<th>Elution during HCl rinsing</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
<td>cit</td>
</tr>
<tr>
<td>1.02</td>
<td>—</td>
<td>—</td>
<td>268$^*$</td>
<td>14.6 10$^{-3}$</td>
</tr>
<tr>
<td>1.07</td>
<td>9.5 10$^{-3}$</td>
<td>6.4</td>
<td>294</td>
<td>5.20</td>
</tr>
<tr>
<td>1.01</td>
<td>9.5</td>
<td>313</td>
<td>5.20</td>
<td>5.20</td>
</tr>
</tbody>
</table>

$^*$ Data obtained after 20 cm$^3$ H$_2$O wash (not the regular 5 cm$^3$ H$_2$O rinsing).
citric acid solutions, and the buffering of negative charge concentrations in the DFS (Wolterbeek, 1987b).

At first sight, the discussion above may be regarded as in contradiction with the results obtained for the 9.5 mM citric acid concentrations. However, the severe Cd losses during perfusion of the 9.5 mM citric acid (Table 2) suggests the formation of complexes between Cd and citric acid, thereby making perfusion conditions different from those during the 9.5 μM citric acid runs.

Based on the above, the differences in responses between the low and the high citric acid concentration applications may be explained by the differences in amounts of complexes between Cd and citric acid in the fluid percolating through the xylem column material.

**Citric acid sorption during perfusion with Cd, Ca, or Mg.**

In a series of experiments, 20 cm³ 9.5 μM citric acid + Cd (1.0 mM), Ca (2.0 mM) or Mg (1.0 mM) with chloride or nitrate (see Materials and methods section) solutions were perfused through the xylem column (‘+M’ conditions). For Cd additions, 2.5 mM and 9.5 mM citric acid experiments were also carried out. Table 3 gives the metal and citric acid speciation in the applied solutions, indicating full incorporation of citric acid in M-complexes, except, naturally, for the 9.5 mM citric acid application. Table 4 presents the concentrations of metals and citric acid in the applied solutions and in the DFS, the latter concentrations being obtained by HCl rinsing. The high recoveries (Table 4) again indicate the experimental accuracies. Figure 4 shows the results for the citric acid runs in the presence of Cd, presented as λ-values. The λ-values decreased with increasing citric acid concentrations (both control and +Cd conditions), showing the diminishing effect of the z-curve sorption. The λ_{Cd}/λ_{control} ratio's however, increased with increasing citric acid concentrations, which may be interpreted as due to (a) the progressive increase in domination of the Cd-cit affected y-curve in total citric acid sorption, (b) an (unexpected) 'zipper opening' action of Cd-cit complexes, or (c) an increase in citric acid sorption by the formation of complexes by R~ and Cd-cit (contrasted with pre-loading situations, see Discussion section). The λ_{Cd}/λ_{control} ratio value of 7.3 for the 9.5 mM citric acid additions may need further examination in future experiments, because of the expected reduced Cd presence in the DFS: under the 9.5 mM citric acid conditions, the low concentration of Cd in the DFS may be determined from the amount of free Cd in the applied solutions. WFS free Cd was calculated as approximately 10 μM (Table 3), a value from which a DFS Cd concentration may be expected of approximately 40 mM (based on K_{dis} = 10^{-4} M and fixed R~ = 0.4 M, Wolterbeek, 1986, 1987a).

Table 4 presents the citric acid λ-values and λ_{Cd}/λ_{control} values for perfusions in the presence of Cd, Ca and Mg. The similar results obtained for Cd and Mg may support the observations made above with respect to the minor importance of Cd preloading: the formation strengths for Mg-cit and Cd-cit (and Ca-cit) complexes are of closely comparable magnitudes (Sposito and Coves, 1988),

<table>
<thead>
<tr>
<th>M</th>
<th>Applied solution concentrations (mM)</th>
<th>DFS concentrations (mM)</th>
<th>Total recoveries after H₂O and HCl rinses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>cit</td>
<td>M</td>
<td>cit</td>
</tr>
<tr>
<td>Cd</td>
<td>1.0 2.5 × 10^{-6} n.m.</td>
<td>119 10^{-6}</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>0.9 9.5 × 10^{-3} 334</td>
<td>44 10^{-3}</td>
<td>98 98</td>
</tr>
<tr>
<td></td>
<td>1.0 9.5 n.m. 10.4</td>
<td></td>
<td>99 99</td>
</tr>
<tr>
<td>Mg</td>
<td>1.0  —</td>
<td>349</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0 9.5 × 10^{-3} 375</td>
<td>44 10^{-3}</td>
<td>103 100</td>
</tr>
<tr>
<td>Ca</td>
<td>2.0 9.5 × 10^{-3} 371</td>
<td>68 10^{-3}</td>
<td>100 98</td>
</tr>
</tbody>
</table>

**Table 3. Citric acid and metal speciation in applied solutions (pH 5.7) in experiments with simultaneous addition of citric acid and Cd, Ca or Mg.**

M = metal, cit = citric acid. Fractional presence rounded off to the nearest full %.

<table>
<thead>
<tr>
<th>M</th>
<th>Applied solution concentration (mM)</th>
<th>M²⁺</th>
<th>M²⁺ cit⁻</th>
<th>M²⁺ (cit)⁺</th>
<th>MHcit⁺</th>
<th>MHcit⁺ cit⁻</th>
<th>H₂cit⁻</th>
<th>H₂cit⁻ cit⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>cit</td>
<td>M</td>
<td>cit</td>
<td>M</td>
<td>cit</td>
<td>M</td>
<td>cit</td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>1.0 2.5 × 10^{-6} 100</td>
<td>92</td>
<td>*</td>
<td>5</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9 9.5 × 10^{-3} 99</td>
<td>1</td>
<td>91</td>
<td>*</td>
<td>*</td>
<td>4</td>
<td>*</td>
<td></td>
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<td></td>
<td>1.0 9.5</td>
<td>1</td>
<td>68</td>
<td>7</td>
<td>28</td>
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<td>Mg</td>
<td>1.0 9.5 × 10^{-3} 99</td>
<td>1</td>
<td>89</td>
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<td>5</td>
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<tr>
<td>Ca</td>
<td>2.0 9.5 × 10^{-3} 99</td>
<td></td>
<td>91</td>
<td>*</td>
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* Trace amount <0.5%.
The presence of glycoproteins (Lauchli, 1976), more especially AEC is generally attributed to the plant's cell wall. The co-ordination chemistry of the metal-citrate complexes may provide a further clue to the elucidation of the issues raised above (see Discussion section).

The Freundlich term in the sorption of citric acid, yielding the $a$ and $b$ constants, may be discussed following Helfferich (1962a). Generalizing Helfferich’s derivations for anion sorption in a cation exchanger, shows that the application of a fully dissociated $\text{AEC}$ should theoretically yield $b$ as being equal to $(n + m)/m$, provided that $Y_{\text{desw}}R^-$.

### Discussion

The plant’s cell wall $AEC$ is generally attributed to the presence of glycoproteins (Läuchli, 1976), more especially the basic amino acids arginine, lysine, and histidine (Gillet and Lefebvre, 1981; Richter and Dainty, 1989a). The agreement of the present amino acid analysis (Table 1) with earlier literature data (Lamport and Northcote, 1960; Thomson and Preston, 1967) may be regarded as a further justification of both the cell wall isolation procedures applied (Senden et al., 1992b) and the adopted cell wall DFS volumes (Wolterbeek, 1987a). However, the amino acid composition should be interpreted as giving the anion exchange potential rather than an apparent capacity; the neutralization (screening) of fixed $\text{NH}_3^+$ by $R^-$ groups may deprive the wall of positive charges, thereby also lowering the net presence of $R^-$ groups. This effect, which we are here calling the ‘zipper’ effect, was first shown by Gillet and Lefebvre (1981), who reported about 45 mM extra negative charges available for $K^+$ cell wall absorption after 1,5-difluoro-2,4-dinitrobenzene (DFNB) treatment of *Nitella flexilis* cell walls or after alkalinization (pH 8.5) of external solutions. An almost entirely closed ‘zipper’ may account for the very low apparent $AEC$ value, as was observed for the control citric acid experiments (Fig. 1).

But, it should be noted that, in principle, the low citric acid $AEC$ may also be attributed to the HCl/NaCl cell wall pre-treatment procedures; any extensive $\text{Cl}^-$ association with positive charges may introduce citrate versus $\text{Cl}^-$ ligand exchange, the total citric acid behaviour thereby becoming dependent on relative affinities. Effective $\text{Cl}^-$ absorption was reported by Richter and Dainty (1989a, b), where there was relatively easy $\text{Cl}^-$ filling of the *Sphagnum russowii* wall’s apparent $AEC$ (up to about 65 μeq g⁻¹ DW), after initial cell wall protonation by pre-treatment with 25 mM HCl. However, in *Nitella flexilis*, the introduction of 10 mM $\text{Cl}^-$ (in KCl) did not liberate any neutralized $R^-$ (Gillet and Lefebvre, 1981), nor did 0.1 N HCl wall pre-treatment lead to any extensive $^{82}\text{Br}$ absorption in tomato xylem cell walls ($^{82}\text{Br}^-$ versus $\text{Cl}^-$ exchange, Wolterbeek, 1987b; Wolterbeek et al., 1987). Furthermore, Ritchie and Larkum (1982) reported full Freundlich-like $^{36}\text{Cl}^-$ absorption (applied 0.1 to 1000 mM $\text{Cl}^-$) in 1 M NaCl pre-treated *Enteromorpha intestinalis* cell walls. These data suggest that, generally, it may be relatively difficult to make the wall’s anion exchange potential available for $\text{Cl}^-$. In the present paper, possible $\text{Cl}^-$ effects were neglected, because of the above-mentioned earlier results with tomato xylem walls.

The Freundlich term in the sorption of citric acid, yielding the $a$ and $b$ constants, may be discussed following Helfferich (1962a). Generalizing Helfferich’s derivations for anion sorption in a cation exchanger, shows that the application of a fully dissociated $A_{\text{pH}}Y_{\text{d}}$ salt in an exchanger of univalent fixed ionic groups should theoretically yield $b$ as being equal to $(n + m)/m$, provided that $Y_{\text{desw}}R^-$. Neglecting any pressure effects (Ritchie and
Larkum, 1982), and assuming near unit values for the ionic activity coefficients (Boyd and Bunzl, 1967), the $a$ value may be given by $(z\nu/R^a)^{n/(m+z\nu)}$, $z\nu$ being the anion valence. However, the mixed citric acid speciation in both applied solutions (Table 3) and in the Donnan free space (decreased pH, Wolterbeek et al., 1987) severely complicates straightforward interpretation of the Freundlich constants. Furthermore, non-ideal Donnan behaviour of the cell walls may result in deviations from theoretically expected $a$ and $b$ values (Ritchie and Larkum, 1982). For these reasons, the Freundlich constants were handled as empirical values only.

The absence of any effect of Cd column pre-loading on citric acid sorption at 9.5 $10^{-6} \text{ M}$ citric acid (Fig. 3) indicates that no significant ligand exchange improvement was induced. Apparently, Cd sorption did not result in any significant increase in available fixed positive charges, nor did any further metal-mediated attraction of citric acid occur. This suggests that the cadmium ions are held by the fixed R$^{+}$ groups, fully charge-neutralizing them, the latter irrespective of the nature of the bonds (Zhu and Sengupta, 1992) for the absence of effects on oxalate sorption of Cu pre-loading of IRC-718 commercially available iminodiacetate columns). Here, it should again be noted that excess Cl$^-$ (pre-treatment procedures) may have hampered citric acid sorption. However, any net wall positive charge induced by Cd sorption would have strongly favoured citric acid sorption, both by differences in affinities (Blackwell and Carr, 1992a, b) and by differences in valence (Table 3, and see Helfferich (1962a) and Veuthey et al. (1986) for the reversal of initial Donnan effects). Lastly, the complex nature of citric acid behaviour may be further attributed to the possible back-diffusion of attracted negatively charged citric acid forms, due to fast protonation reactions in the more strongly acidic cell wall Donnan free space (Frost and Pearson, 1962; Wolterbeek, 1986; Burgess, 1992) and the virtual absence of H$_2$cit in the applied solutions (Table 3).

On the other hand, the remarkable effects of Cd pre-loading on the 9.5 mM citric acid sorption (Table 2; Fig. 3) underline the importance of complex formation in the applied solution, and, at the same time, stress the 1:1 molar ratio of sorbed Cd-cit complexes. The simultaneous supplying of Cd, Ca or Mg and citric acid (Tables 3, 4, 5; Fig. 4) further indicate the accumulative potential of the complexes. Most probably, the predominant bulk solution citric acid species Mcit$^-$ (Table 3) may be sorbed following Freundlich isotherm or $S$-curve characteristics, but, once present under more acidic DFS conditions, Mcit$^-$ may be further protonated into neutral MHcit and positively charged MH$_2$cit$^+$ complexes (Pedrosa de Jesus, 1987). (Note that all neutral species are removed by H$_2$O rinsing, see Materials and methods section). $S$-curve citric acid fitting of the Fig. 4 data for $^{+}\text{Cd}$ conditions, under assumed constant $c$ and $d$ values, actually yielded increases in the $a$ constant ($a\approx 5.9$) rather than in the $b$ constant ($b\approx 1.4$), possibly indicating capacity-improvement rather than changes in valence-dependent anion sorption. Here, the MH$_2$cit$^+$ formation in the DFS may have resulted in improved accumulation of citric acid relative to controls; the excess bulk Cd$^{2+}$ (Table 3), however, should be regarded as severely suppressing the extent of effects (see DFS concentrations of Cd, Ca and Mg, Table 4), due to the Donnan rules for differences in valences (Helfferich, 1962a). The above indicates that the absence of excess Cd under Cd pre-loading conditions and 9.5 $10^{-3} \text{ M}$ citric acid applications (Tables 2, 4; Fig. 3) may have resulted in the relatively high $\lambda$-values observed.

Notwithstanding the absence of marked differences in citric acid chemical speciation between Cd, Ca or Mg conditions (Table 3), the application of 2.0 mM Ca$^{2+}$ resulted in stronger increases in $\lambda$-values than was the case for 1.0 mM Cd and Mg (Table 5). These differences appear to be generated without any significant difference in total metal concentrations in the DFS (Table 4). The latter observation, combined with the unchanged $b$ values ('$+\text{Cd}$' conditions, see above), may be interpreted as contra-indicating effects of the slight differences in bulk neutral (MHcit) citric acid species (Table 3) on overall citric acid sorption. An explanation for the results presented in Table 5 may be given by the differences in characteristics of complexes between citric acid and Cd, Mg or Ca.

Generally, citric acid tends to form monomeric tridentate species, by co-ordinating to the metal ion through one terminal carboxyl group, the hydroxyl group and the central carboxyl group. Here, the complex may be protonated or deprotonated at the $\beta$-carboxyl group. However, citric acid’s versatility is shown by its possible covalent [RM(cit)H$_2$] formation (Dyson, 1978), bond strengths may be weaker for Cd and Mg than for Ca, due to differences in available M covalencies (Martell and Calvin, 1959; Fenton, 1987).

Overall, the results of the present paper indicate the positive effect of the simultaneous presence of metal ions
on the cell wall sorption of citric acid. This effect may be due to the predominant presence and sorption of bulk $[\text{M(cit)}]^-$, which undergoes protonation on entering the wall DFS, in turn followed by electrostatic and/or covalent attraction of $[\text{M(cit)H}_2]^+$. 

The present results, and the nearly 100% metal-load (Ca, Mg, see White et al., 1981a, b, c) of citric acid in the xylem fluid stress the potential importance of metals for the transport behaviour of organic acids.

Further experiments are needed to investigate whether the present results are applicable to intact plants; ongoing study has already shown the in vivo positive effects of the presence of Cd on citric acid exchange and lateral escape in tomato stem systems (Senden, unpublished).

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