STELLINGEN

behorend bij het proefschrift

Metal-ligand interactions
in xylem transport:
behaviour of cadmium-citrate complexes

1 Door de afscherming door negatief geladen bindingsplaatsen van de celwand van planten ("Zipper-effect") zijn de positief geladen bindingsplaatsen van de celwand beperkt toegankelijk voor citroenzuur.

_Dit proefschrift, hoofdstuk 6_

2 Complexvorming tussen diverente metalen en organische zuren in het xyleemsap is van invloed op het transportgedrag van zowel het metaal als het ligand.

_Dit proefschrift, hoofdstuk 4 en 7_

3 Het door middel van bacteriële afbraak geïsoleerde houtvatmateriaal heeft ionenuitwisselingseigenschappen die vergelijkbaar zijn met die van mechanisch geïsoleerd materiaal en in vivo houtvatwanden.

_Dit proefschrift, hoofdstuk 5_

4 Bij ionenuitwisselingsexperimenten met celwanden is het van belang dat de monstervoorbereiding wordt getoetst op het openen van de "Zipper".


5 Het belang van de celwand in de metaal tolerantie van de plant is niet zo duidelijk als het wordt voorgesteld.

_W. Ernst 1976, Effects of Air Pollutants on Plants, 115-133_
De kwaliteit van de speciatieberekening wordt beperkt door het gebrek aan kwaliteit van de literatuurgegevens waarop speciatieprogramma's zijn gebaseerd.


Het feit dat tomaten (Wasserbomben) grotendeels uit water bestaan is een plantenfysiologisch gegeven en is onafhankelijk van de Nederlandse teeltmethoden zodat het geen aanleiding zou mogen zijn tot Duitse boycot-acties.

Vogels en zoogdieren eten de vruchten van bepaalde planten waardoor ze bijdragen aan de verspreiding van de zaden. De laxerende of bedwelmdende werking van sommige vruchten heeft echter een negatief effect op de grootte van het verspreidingsgebied van de zaden.

Bij het samenvoegen van de termen "assistent onderzoeker" en "onderzoeker in opleiding" tot "assistent in opleiding" is het belangrijkste woord weggelaten.

Een spreekwoord is altijd een zin.

M.H.M.N. Senden
METAL-LIGAND INTERACTIONS
IN XYLEM TRANSPORT:
BEHAVIOUR OF CADMIUM-CITRATE COMPLEXES

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METAL-LIGAND INTERACTIONS
IN XYLEM TRANSPORT:
BEHAVIOUR OF CADMIUM-CITRATE COMPLEXES

PROEFSCHRIFT

ter verkrijging van de graad van doctor
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op gezag van de Rector Magnificus Prof. ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie
aangewezen door het College van Dekanen
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door

Marcel Hyacinthus Maria Nico SENDEN
geboren te Heerlen

doctorandus in de wiskunde en natuurwetenschappen
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ABBREVIATIONS

AEC  (apparent) anion exchange capacity
Cd-cit cadmium-citric acid complexes
CEC  (apparent) cation exchange capacity
CV   coefficient of variations
Cys-Cys cystine
DABSYL 4-dimethylaminoazobenzene-4-sulfonyl
DANSYL 5-dimethylaminonaphthalene-1-sulfonyl
DFNB 1,5-difluoro-2,4-dinitrobenzene
DFS Donnan free space
DW  dry weight
EDTA ethylenediaminetetraacetic acid
FMOC 9-fluorenylmethylchloroformate
FW  fresh weight
GC  gas chromatography
GLC  gas liquid chromatography
HPLC high pressure (performance) liquid chromatography
LC  liquid chromatography
LSC  liquid scintillation counter
MS  mass spectrometry
OPA  ortho-phtaldialdehyde
PITC phenylisothiocyanate
rh  relative humidity
RI  refractive index
RP-HPLC reversed phase HPLC
SCX strong cation exchanger
SD  standard deviation
SE  standard error
SEM standard error of the mean
UV  ultra violet (absorption)
WFS water free space
### SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A^+</td>
<td>indiffusible positive charges in cell walls</td>
</tr>
<tr>
<td>A_p</td>
<td>averaged cross-sectional area of the model pipe</td>
</tr>
<tr>
<td>C_0</td>
<td>constant solute concentration at the point of entry</td>
</tr>
<tr>
<td>C_p</td>
<td>solute concentration at the distance p from point of entry</td>
</tr>
<tr>
<td>d</td>
<td>averaged cell wall thickness</td>
</tr>
<tr>
<td>F_t</td>
<td>amount of water transported to leaf in time t</td>
</tr>
<tr>
<td>H</td>
<td>constant derived from distribution frequency of vessel radii</td>
</tr>
<tr>
<td>K_{diss}</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K</td>
<td>equilibrium constant</td>
</tr>
<tr>
<td>k</td>
<td>lateral escape rate constant</td>
</tr>
<tr>
<td>L</td>
<td>length of translocation pathway</td>
</tr>
<tr>
<td>\lambda</td>
<td>molar distribution coefficient</td>
</tr>
<tr>
<td>M</td>
<td>(divalent) metal</td>
</tr>
<tr>
<td>Q</td>
<td>absolute solute quantity</td>
</tr>
<tr>
<td>r</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>R^-</td>
<td>indiffusible negative charges in cell walls</td>
</tr>
<tr>
<td>t</td>
<td>perfusion time</td>
</tr>
<tr>
<td>t_{1/2}</td>
<td>half-life of radioisotope</td>
</tr>
<tr>
<td>v</td>
<td>linear flow velocity</td>
</tr>
<tr>
<td>v/v</td>
<td>volume ratio</td>
</tr>
<tr>
<td>X_{t,L}</td>
<td>amount solute in surrounding tissue after perfusion time t at length L</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

1. General

1.1 Xylem-transport

Long-distance transport of water and solutes, taken up from the soil or synthesized by plant roots, mainly occurs through the xylem vessels. This xylem transport of solutes can be described as a bulk flow driven by leaf transpiration. The substances present in this flow, which is essentially a movement through the apoplastic of the xylem, become involved in processes like adsorption to charged sites present in the cell walls (Bell and Biddulph 1963, Ferguson and Bollard 1976, Wolterbeek et al. 1985), lateral escape from the moving solution into free spaces of the cell walls and adjacent intercellular spaces (Van Bel 1978, Wolterbeek et al. 1985), and into metabolic compartments of xylem parenchyma and other cells (Biddulph et al. 1961, Jacoby 1965).

The process of xylem transport comprises mass flow, cell wall interactions and lateral escape.

1.2 Stem xylem vessels

Xylem vessels are arranged in vascular bundles, which also contain phloem elements. The xylem system is primarily composed of dead, thick-walled conducting elements, comprising vessels, tracheids and fibers (Esau 1965, Läuchli 1976, Zimmermann 1983, Van der Schoot 1989).

The xylem vessel walls are built up mainly from cellulose molecules, arranged to a large extent in micelles (Läuchli 1976). A variable number of micelles form socalled microfibrils, which, in turn, are arranged in layers oriented at different angles, thus forming a scissorgate network (Wu et al. 1985).

The properties of the intermicellar and intermicrofibrillar spaces are modified by matrix substances, which are oriented as linear or branched polymers (Läuchli 1976). Of particular importance to transport physiology are the pectic substances. The basic structural units of these pectins, α-D-polygalacturonic acids, contain free carboxylic groups, which may form salts with various cations (Läuchli 1976).
Chapter 1

Moreover, lignification of xylem cell walls is assumed to affect the water permeability of the wall (Lüttge 1973).

Apart from the pectic substances, the walls also carry glycoproteins, bearing additional charges: here, the available free amino groups are of particular interest in view of the binding of anions (Läuchli 1976).

1.3 Xylem sap

The xylem sap may be regarded as a fast-conducting part of the apoplastic, connecting sites of water uptake (roots) with sites of transpiration (leaves), thereby functioning as a transport medium for solutes. Between plant species, the chemical composition of the xylem sap may differ both quantitatively and qualitatively (White et al. 1981a, Florijn et al. 1993). In addition, the composition of the xylem sap will depend on supply (composition/chemical state of soil/nutrient solutions). As an example may serve the observations of Arzonis and Findenegg (1986) for beet and sorghum plants, indicating that ammonium supply (replacing nitrate) results in increases in xylem concentrations of malate and citrate, the latter interpreted in the light of the necessary maintainance of charge neutrality. Xylem constituents may also be subjected to diurnal or seasonal cycles: xylem sap of willow is reported to show seasonal variations in concentrations of sugars (Sauter 1982), Sauter and Van Cleve (1992) indicated seasonal variations in the amino acid composition of xylem sap of poplar.

However, notwithstanding various variations in composition, the xylem sap contains constituents which can be grouped on basis of the order of their concentrations (White et al. 1981a, Florijn et al. 1993).

Xylem constituents like ammonium, nitrate, calcium, magnesium, potassium, sodium, chloride, phosphates, amino acids, and organic acids may be found in millimolar concentration ranges, whereas elements like iron, zinc, copper, manganese, molybdenum etc. may occur in micromolar (trace) concentrations.

In addition, industrial and agricultural activities may result in the xylem occurrence of principally non-physiological/-essential elements/chemicals, like bromacil, dichlorobenzenitril, picloram (Donovan and Vanden Born 1981, McFarlane et al. 1987) and various heavy metals (Kabata-Pendias and Pendias, 1985; Adriano 1986).
Introduction

The above given general chemical composition of the xylem sap, which is buffered at pH \( \approx 6 \) (Van Bel 1978, Wolterbeek 1986), indicates the likely occurrence of various metal-ligand complexes during transport. Although sodium, potassium, calcium and magnesium, which comprise the bulk of the xylem transported metals, may be largely (up to 50 %) transported in cationic form (Tiffin 1967), many other metals are present in the xylem in organic complexes (Tiffin 1967, Brown and Chaney 1971, White et al. 1981abc).

Iron is reported to be transported only in a Fe-citrate complex (Tiffin 1967). Furthermore, negatively charged xylem organic complexes are suggested for copper (Tiffin 1972, Van de Geijn and Pikaar 1982) and zinc (Tiffin 1972, McGrath and Robson 1984). In addition, Höfﬂner (1970) indicated organic xylem complexes for manganese. Calcium organic xylem complexes are reported by Bradfield (1976) and Ferguson and Bollard (1976).

The occurrence of organic metal complexes may be regarded as principally affecting the xylem transport behaviour of both metals and ligands, especially seen in the light of the interactions with the xylem cell walls (Ferguson and Bollard 1976, McGrath and Robson, 1984). For metals like iron, complexing may be regarded as a means to cope with solubility problems during transport at pH 6 (Tiffin 1970, Kabata-Pendias and Pendias 1985); generally it may be suggested that the formation of complexes may result in altered transport behaviour of both metals and ligands, the extent of which - for both metal and ligand - will depend on characteristics like resulting complex charges, rates of diffusion, or uptake into surrounding cells.

1.4 Xylem cell wall binding

The longitudinal transport of ions in the xylem vessels is generally suggested to occur only after saturation of the exchange sites in the xylem cell walls (Bell and Biddulph 1963, Ferguson and Bollard 1976, Petit and Van de Geijn 1978, Van de Geijn and Petit 1978, 1979). The exchange sites largely consist of the pectic substances and glycoproteins (Gillet and Lefebvre 1981, Richter and Dainty 1989b), yielding both negative and positive charges. The presence of these charges makes that the cell wall should be considered to consist of water free spaces (WFS), that is, spaces freely accessible for xylem ions without any influence of charged fixed
groups, and so-called Donnan free spaces (DFS), in which the xylem ionogenic solutes will undergo effects of the fixed cell wall charges.

Here, for the fixed negative charges, densities at average cell wall pH (3-5, see Pitman 1977, Van Bel 1978, Sentenac and Grignon 1981, Wolterbeek 1986, Richter and Dainty 1989a,b) may be given as 400-500 meq.l\(^{-1}\) DFS (COO\(^-\) groups arising from \(\alpha\)-D-polygalacturonic acids, (Pitman 1977, Van de Geijn and Petit 1979, Wolterbeek 1986, Richter and Dainty 1989a,b)); the potential charge density has been reported as about 1000 meq.l\(^{-1}\) (Wolterbeek 1986).

The potential positive charge density is about 40-50 meq.l\(^{-1}\) (Gillet and Lefebvre 1981, based on determination of K\(^+\) accumulation and analysis of the Nitella flexilis cell wall basic amino acids histidine, lysine and arginine); an experimental value of about 10 meq.l\(^{-1}\) has been reported by Richter and Dainty (1989b) from Cl\(^-\) accumulation data in Sphagnum russowii cell walls.

Based on the above, and in a first and general approximation, xylem (negative) ligands may be influenced predominantly by the cell wall basic amino acids, whereas the positively charged ions may undergo interaction with the cell wall carboxylic groups. Influences undergone by xylem complexes may largely depend on resulting charges; logically, the extent of effects will strongly depend on all relevant affinities and stabilities. Effects of complexing metal ions may be foreseen to result in decreased metal accumulation in the xylem cell walls, in turn resulting in changed metal behaviour in both longitudinal and lateral transport directions. In literature, a wealth of information is available on ligand (complexing) effects on metal longitudinal xylem transport (Tiffin 1966 1970, Bradfield 1976, White et al. 1981abc, Van de Geijn and Pikaar 1982, McGrath and Robson 1984). Information on the effects on metal lateral transport, however, is lacking.

Remarkably, and in sharp contrast to the available information on effects of organic complexers on metal behaviour, no information is available on the possible influence of (specific) metals on the xylem cell wall accumulation and associated transport behaviour of organic compounds. Effects of metals on the behaviour of organic compounds are described 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 Semechkin 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 the light of the above it seems logical to raise the question as to whether metals influence the behaviour of organic compounds, both by their presence and by the formation of complexes. The occurrence of these phenomena may lead to changes in (initial) longitudinal and lateral transfer rates and to metal-specific variations in the distribution of organic compounds in plants.

1.5 Xylem lateral escape

The elements transported in the xylem are involved in processes of lateral escape from the moving solution (Van Die and Vonk 1967, Pate 1975, Van Bel 1978). Because of the various processes involved - element characteristic diffusion and active carrier-mediated uptake (Horwitz 1958, Ehwald et al. 1973, Van Bel 1979) - the rate of lateral escape may depend on the type of compound considered (Bell and Biddulph 1963, Jacoby 1965, Van Die and Vonk 1967). The rate-limiting processes seem to be the rate of diffusion in xylem cell walls and intercellular spaces, or the uptake into cells lining the xylem pathway. The latter may be modulated by the number of cells involved in uptake (Ehwald et al. 1973) or by feed-back regulation resulting from elements initially present in the surrounding tissues (Siddiqi and Glass 1982, McDaniel and Wozniak 1982).

In the overall process of lateral escape, and especially when the diffusion through the cell walls limits the overall rates, the characteristics of the accumulation of xylem components in the cell walls should be considered as of paramount importance, possibly largely defining rate constants and available free concentrations. Here, the cell wall may be regarded as disturbing local concentrations (Helfferich 1962b, Borst-Pauwels and Severens 1984, Thibaud et al. 1984). Furthermore, the permeability of the wall phase, which depends on its electrical charge (Helfferich 1962b, Borst-Pauwels and Theuvenet 1984) and resulting Donnan effects (Dalton 1984) will be specific for each element/compound
and concentration, due to accumulation/exclusion effects. A number of experiments has been performed on the effects of cell wall accumulation of metals on their rates of lateral escape (Wolterbeek 1986); however, no precise information is available on the effects of complex formation, nor is there any information on behaviour of the organics themselves.

With respect to the transfer processes over the xylem cell walls, pits, contact cells and transfer cells may be considered to be of particular importance (Esau 1960, Gunning et al. 1968, Sauter 1966 1972 1983, Sauter et al. 1973, Kramer 1983, Van der Schoot 1989). However, contact cells have also been implicated especially in the defence reactions of the vascular system to infection by pathogens (Moreau and Catesson 1985) and/or in buffering hydrostatic oscillations in the vessels (Van der Schoot 1989). Specialized xylem parenchyma transfer cells are absent in tomato (Van Bel 1984).

2. Aim of the present investigations

The present thesis aims at gaining insight in the mutual effects of metals and organic complexers on the xylem transport behaviour of both metals and organics. Here, the simultaneous presence and possible formation of complexes between metals and organics are considered to principally affect the longitudinal movement of all relevant chemical species, by changing characteristics of interactions in processes of cell wall binding and lateral escape.

3. Objectives

The longitudinal movement of xylem constituents may be expressed by data on retention, and/or concentrations (Tiffin 1970, Bradfield 1976, Ferguson and Bollard 1976, Van de Geijn and Pikaar 1982, McGrath and Robson 1984), but, apart from the concentrations applied, these parameters should be considered as plant-dependent, probably largely determined by xylem volume flow and anatomical xylem vessel characteristics such as available absolute quantities of exchange sites. The required application of at least two plants for any determination of differences in transport may therefore lead to faulty interpretations of results, if these are based
on a retention-time or concentration basis only.

Therefore, in the present thesis, the longitudinal movement is invariably characterized by combined data on retention-times, xylem volume flow, vessel dimensions and applied concentrations. These data permitted the expression of longitudinal transport by data on cell wall binding and lateral escape only. Consequently, individual behaviour and mutual effects of simultaneous xylem presence of metals and organics were investigated invariably by quantification of cell wall sorption and lateral escape.

4. Materials, experiments and techniques

4.1 Plant species

Tomato plants (*Lycopersicon esculentum* Mill cv. Tiny Tim) were used for all experiments, since they are easy to grow throughout the year and because a considerable amount of work has been done previously with this plant. All plants used in experiments were grown in a heated glasshouse under a regime of about 18° C night and 25° C day temperature, with rh of about 70 %.

4.2. Experiments

Experiments were carried out with intact plants, root systems, stem systems, and with isolated xylem cell wall material.

Intact plants were used to get an impression of the *in vivo* effects of root organic acids on both root accumulation and xylem influx of metals; root systems were used in pressure-cell experiments to enable sampling of xylem sap and constituents at the entrance of the plant shoots. The latter data give information on the xylem chemical state at the point of import into the xylem vessels of the plant stem.

Stem systems were used consisting of stem-leaf combinations and of isolated internodia. In the stem-leaf systems, xylem flow was generated by water transpiration from the attached leaves. In the internodia, flow was brought about and controlled by applied slight suction generated by a pump. In both stem-systems, longitudinal movement was determined by measuring and calculating the rate constants for lateral escape and the absolute quantities of metal/organic acids
Chapter 1

adsorbed to the xylem cell walls.

In experiments with isolated xylem cell wall material, attention was focused entirely to the process of cell wall sorption, both for metals and for organics. Here, cell walls were used in a perfusion column set-up, and applied as a mixed exchanger. In these experiments, data were obtained on cell wall interactions, without any possible interference from processes of lateral transfer.

4.3. Metal and ligand selection

Considering the processes which are thought of as relevant in the context of longitudinal xylem transport, apart from flow, the xylem cell wall emerges as the main compartment. If lateral escape is rate-limited by cell wall diffusion, uptake into surrounding tissues does not have significant effects on the magnitude of longitudinal transport of xylem constituents. Thus, in a first approximation, xylem movement may be regarded as a physico-chemical transport phenomenon, governed by pressure relations (mass flow), chemical binding and diffusion.

In the light of the above, selections regarding metals and ligands may be primarily based on physico-chemical behaviour, rather than on roles and/or functions in plant physiology/biochemistry. As a consequence, the choices made were largely based on general xylem sap composition, tuned by practical reasons, and further based on foregoing research (Wolterbeek 1986).

Citric acid was taken as the ligand of interest, largely because it is regularly reported as a relatively strong metal complexer (Martell and Calvin 1959, Sillen and Martell 1964, O'Sullivan 1969, Sposito and Coves 1988) and because it has been found in millimolar concentration ranges in xylem sap (White et al. 1981a, Florijn et al. 1993). Cadmium was taken as the metal ion used in all experiments, because of its relatively strong complexes with citrate (Sillen and Martell 1964, White et al. 1981abc), the availability of data on physico-chemical aspects of its xylem transport behaviour (Petit and Van de Geijn 1978, Wolterbeek 1986), and because of the relatively fast and easy production of the γ-rays emitting radio-isotope $^{115}$Cd at IRL.

4.3 Radiotracers

In principle, the application of radioactive tracers in experiments on plant transport permits the determination of element movement and distribution at low
concentrations without the disturbing interference of background levels of the element of interest. With properly selected radionuclides and equipment, the measurements can be carried out in vivo. In this way, the use of large numbers of plants in successive measurements with associated problem of biological variations can be avoided.

For the measurements of ion transport described in this thesis, in many cases γ-ray emitting radionuclides were used (\(^{115}\text{Cd}, ^{68}\text{Mg}, ^{122}\text{Sb}\), produced at IRI (Wolterbeek 1986, Van der Velden 1991)), measured by γ-ray spectroscopy.

Compared to other radiotracer techniques, this approach has two distinct advantages when applied in vivo to biological systems: 1). γ-radiation is highly penetrating, compared to e.g. α- or β-radiation. Therefore, in most cases, absorption in the object under study can be neglected. 2). γ-radiation is mono-energetic, and when using high-resolution γ-ray spectroscopy many radionuclides may be examined simultaneously.

In a number of experiments, β-emitters were used, for which liquid counting was conducted by scintillation, either directly in transport fluids, or, after digestion, in the plant tissues themselves. β-emitters used were \(^{3}\text{H}\) in inulin, \(^{14}\text{C}\) in citrate, and \(^{46}\text{Ca}\).

5. Layout of this thesis

The thesis is built up of eight chapters, in which, apart from Introduction (Chapter 1), seven chapters are dedicated to presentation and discussion of various aspects of the xylem transport of both cadmium and citric acid.

Chapter 2 gives results on HPLC analysis of tomato xylem carboxylic and amino acids, in order to get a general idea about xylem organic constituents under the applied growing conditions and nutrient media.

Chapter 3 presents experiments with intact plants, which were carried out to study the effects of root citric acid on uptake and distribution of cadmium. These experiments were combined with pressure cell measurements on citric acid, showing the citric acid concentrations in xylem exudates as affected by manipulation of root citric acid concentrations.

Chapter 4 is dedicated to the determination of cadmium behaviour in the
Chapter 1

xylem as affected by the presence of citric acid. Experiments were carried out in stem-leaf systems.

Experiments with isolated xylem cell wall material are presented in Chapters 5 and 6. Here, measurements were carried out on $^{115}\text{Cd}$ (Chapter 5) and $^{14}\text{C}$-citrate (Chapter 6), both in set-ups in which the cells were used as exchange columns.

Experiments with isolated internodes are presented in Chapter 7: the transport behaviour of $^{14}\text{C}$-citrate was determined in the absence and presence of cadmium. For cadmium, tissue concentrations were determined, from which xylem concentrations could be predicted.

Last but not least, in the Discussion section (Chapter 8) the results of all previous Chapters are integrated.

References.


Introduction


Chapter 1


Sauter J.J. 1983. Efflux and reabsorption of sugars in the xylem. II. Seasonal changes in sucrose
Introduction

Chapter 1


CHAPTER 2

Analysis of major tomato xylem organic acids and PITC-derivatives of amino acids by RP-HPLC and UV detection

Abstract

Major amino acids and organic acids in xylem exudates of tomato plants were separated by reversed phase high-performance liquid chromatography (RP-HPLC) and quantified by UV detection. Before separation, amino acids were converted into their phenylisothiocyanate (PITC) derivatives. In a single run, Asp, Glu, Ser, Gln, His, Thr, Ala, Tyr, Val, Met, Cys, Ile, Leu, Phe, and Lys could be separated and detected down to the pmol level. Unresolved peaks were obtained for Asn and Gly and for Arg and Pro. For organic acid analysis, exudates were pre-treated by perfusion over an prepacked Adsorbex SCX cation exchange column, to eliminate exudate amino acids. Elution recoveries for organic acids were close to 100 %. The exudate organic acids were separated by ion suppression RP-HPLC chromatography, and peaks could be resolved for L-malic acid, malonic acid, maleic acid, citric acid and fumaric acid, down to the pmol level. UV signals for exudate ascorbic acid, and succinic acid were below the limits of detection. Determination of oxalic acid and tartaric acid was impossible, due to the presence of the exudate salt peak in the chromatogram. The results indicate the potential of the methods applied, and show the applicability of RP-HPLC analysis for the determination of both amino acids and organic acids in xylem exudates.

Chapter 2

Introduction

The roles attributed to amino acids, amides (e.g. glutamine, asparagine) and organic acids are generally related to cellular nitrogen, protein and energy metabolism (Bidwell, 1979; Ranson, 1965). Released from their metabolic sites, and moving in xylem or phloem (Tiffin, 1972; Van Die and Vonk, 1967) amino acids, amides and organic acids are also indicated to function in the long distance translocation of metals in plants (Bradfield, 1976; Ferguson and Bollard, 1976; McGrath and Robson, 1984; Senden and Wolterbeek, 1990; Tiffin, 1972; Van de Geijn and Pikaar, 1982; White et al., 1981; 1981a; 1981b).

In the xylem, negatively charged organic complexes of copper and zinc are suggested (Tiffin, 1972). Iron is indicated to be transported as the citrate (Tiffin, 1970), and also for manganese are xylem organic complexes reported (Höffner, 1970), although manganese may be transported in the phloem in inorganic form (Van Goor and Wiersma, 1976).

Apart from direct analysis of metal-species, advanced computer programs on simultaneous chemical equilibria have been applied to predict the occurrence and concentrations of organic metal complexes in transport fluids of various plant species (Mullins et al., 1986; White et al., 1981). For both direct analysis and for calculations of metal species, the total analysis of metals, amino acids and organic acids is to be considered as the basis of any metal-speciation investigation in plant exudates.

The classical amino acid analysis (Spackman et al., 1958) comprises ion exchange chromatography and post-column ninhydrin derivatisation, and is generally performed automatically using dedicated instrumentation. With the introduction of other reagents (e.g. ortho-phtaldialdehyde (OPA), 9-fluorenylmethoxycarbonylchloride, 9-fluorenylmethylchloroformate (FMOC), phenylisothiocyanate (PITC), 5-dimethylaminonaphthalene-1-sulfonyl (dansyl)-chloride, 4-dimethylaminoazobenzene-4-sulfonyl (dabsyl)-chloride) and the application of (RP)-HPLC techniques, methods became available for rapid, reliable and more sensitive analysis of amino acids (Cohen et al., 1984; Hancock, 1989; Pfeiffer and Hill, 1983).

For the analysis of organic acids, since the late 1960s, gas chromatographic
Analysis of xylem sap

(GC), GC-mass spectrometric (GC-MS) and liquid chromatographic (LC) methods have been introduced. Although some organic acids are reported to have been determined in biological samples without any extensive pre-treatment, derivatization is commonly carried out to increase the volatility and thermal stability (GC analysis), or to facilitate detection of the acids (LC analysis) (Liebich, 1990).

The derivatization procedures required for the analysis of organic acids are repeatedly reported to cause problems, resulting in low and/or variable recoveries (Liebich, 1990; Stahl et al., 1972; Zaura and Metcalf, 1969). Since any step in the sample preparation procedures may involve loss of organic acid, introduction of impurities/artifacts, decrease in analytical precision and increase in analysis time (Liebich, 1990), sample preparation should be as simple as the analytical aims permit.

Although LC separation is reported not to be sufficient for general profile analysis of organic acids (Liebich, 1990), it is also used in organic acid analysis in biological samples without extensive sample preparation (Liebich, 1990). Furthermore, the application of reversed phase partition and ion exchange HPLC with UV or RI detection is reported for the analysis of major organic acids in various food products (Schneider et al., 1987; Togami et al., 1990).

The above indicates that the application of RP-HPLC with UV detection may offer the possibility to determine both amino acids and organic acids in biological samples on one single column material and with only a minimum of sample pre-treatment steps. Therefore, the present paper explores the possibilities of RP-HPLC analysis and UV detection of both major amino acids and organic acids in xylem fluid of tomato plants.

Materials and methods

Plants/exudates

Tomato plants (an inbred line of Lycopersicon esculentum Mill, cv. Tiny Tim) were used throughout. They were cultivated in hydroculture in a glasshouse at ca. 25 °C and 75 % r.h.. The hydroculture solution contained (mg.l⁻¹): KH₂PO₄, 134; K₂SO₄, 154; MgSO₄.7H₂O, 473; Ca(NO₃)₂.4H₂O, 882; KNO₃, 444; MnSO₄·H₂O, 2; H₃BO₃, 2.7; ZnSO₄·7H₂O, 0.5; CuSO₄·5H₂O, 0.08; NaMoO₄·2H₂O, 0.13; Fe, 5 (in Fe-
Chapter 2

EDTA); pH = 5.5, adjusted with H₂SO₄. Xylem exudates were collected from 6 to 8 weeks old plants by decapitation of the plant stems at approximately 5 cm above the roots. The decapitated stumps were fitted with tubing and allowed to bleed for 1-1.5 hour. This exudate is considered to approximate the in-vivo system (White et al., 1981). The samples were filtered (HV Millipore, 0.45 μm) before further use.

Sample preparation

Amino acids. Amino acid analysis was carried out after precolumn derivatisation with phenylisothiocyanate (PITC) (Heinrikson and Meredith, 1984). Standard solutions were prepared from an amino acid standard (Stock No. AA-S-18, Sigma), containing a mixture of 17 amino acids (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, (all 2.5 mM) and Cys (1.25 mM). After drying in small test tubes, samples of 10 μl were dissolved in 100 μl of coupling buffer (acetonitrile:pyridine:triethylamine:H₂O, 10:5:2:3 v/v), lyophilized for 1 hour, and dissolved again in 100 μl coupling buffer, under addition of 5 μl PITC solution. After 5 minutes reaction time at room temperature (Saunders et al., 1988) and subsequent lyophylisation, the produced PITC-derivatives were dissolved and lyophylised twice again. PITC-derivatised amino acids were dissolved in 1 ml of a 9:1 (v/v) mixture of 0.05 M sodium acetate (pH 6.8) and 0.1 M sodium acetate in 40 % acetonitrile and 10 % methanol (pH 6.8), and analysed by RP-HPLC in 10 to 20 μl quantities (250-500 pmoles of each amino acid).

To establish identities of peaks resolved, 10 μl volumes of 0.01 M solutions of all individual amino acids (see above) and of asparagine (Asn) and glutamine (Gln) were processed as described above. The resulting individual PITC-derivatives were dissolved in 600 μl of a 5:1 (v/v) mixture of 0.05 M sodium acetate (pH 6.8) and 0.1 M sodium acetate in 40 % acetonitrile and 10 % methanol (pH 6.8), and added in 2 to 15 μl (300 - 2500 pmoles) aliquots to the standard solutions.

Organic acids. Analytical grade organic acids were used to compose an organic acid mixture, containing 15.6 μM oxalic acid (Baker), 66.6 μM tartaric acid (BDH), 74.6 μM L-malic acid (Janssen Chimica), 339 μM succinic acid (Sigma), 96 μM malonic acid, 5.7 μM L-ascorbic acid, 1.7 μM maleic acid, 95.1 μM citric acid and 1.7 μM fumaric acid (all Merck). Standards were also prepared and
chromatographed individually in order to determine peak positions and UV responses for each of the acids in the mixture.

**Exudates.** For amino acid analysis, small volumes (45 μl) of filtered xylem exudate were derivatised as described above, and dissolved in 225 μl of a 9:1 (v/v) mixture of 0.05 M sodium acetate (pH 6.8) and 0.1 M sodium acetate in 40 % acetonitril and 10 % methanol (pH 6.8). Aliquots of 5 to 10 μl of the resulting solutions were used in RP-HPLC procedures.

For organic acid analysis, filtered xylem exudates were perfused over an 100 mg Adsorbex SCX column (Merck). Perfusates were used directly in RP-HPLC.

**RP-HPLC analysis**

**Amino acids.** HPLC was performed using a LiChrosphere 100 RP-18 (5 μm) 125 x 4 column (Merck), operated at 55 ± 1 °C., in a water bath (Liquitherm FV Labora Mannheim). The equipment (all LKB) consisted of a 2150 HPLC pump, 2152 HPLC-controller, 11300 Ultragrad mixer driver, and 2138 Uvicord S UV-detector. The mobile phase contained a gradient-programmed mixture of solvent A (0.05 M sodium acetate pH 6.8) and solvent B (0.1 M sodium acetate in 40 % acetonitril and 10 % methanol pH 6.8). The acidity of solvents A and B was set with phosphoric acid. All solvents were continuously degassed by Helium-purging. Gradient-elution profile: 0-2 min: 100 % A, 2-17 min: linear increase of B up to 20 % B, 17-40 min: linear increase of B up to 34 % B, 40-43 min: linear increase of B up to 100 % B, 43-50 min: 100 % B. Flowrate 0.9-1.0 ml.min⁻¹.

The eluting compounds were detected at 254 nm wavelength; peaks were plotted by a BD41 recorder (Kipp & Sons). Calibrations of concentration versus UV absorbance were carried out by single point checks.

**Organic acids.** 100 μl samples were injected onto a LiChrosphere 100 RP-18 (5 μm) 125 x 4 column (Merck), operated at 0 °C., controlled via a Cryothermostat model WK 4 (Colora) and HPLC Water jacket (Alltech). The mobile phase consisted of 0.05 M KH₂PO₄/H₂PO₄ buffer, pH 2.3 , was degassed continuously by helium purging/blanketing in pressurized solvent reservoirs of a 2156 Solvent Conditioner (LKB), and was eluted isocratically at a flowrate of 0.2-0.5 ml.min⁻¹. The eluting
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⇒ Figure 1. Reversed phase HPLC separation of PITC-derivatives of amino acids in tomato xylem exudates. Individual adjustments: injection volume 7 μl, flowrate 0.9 ml/min.
1 = 184 pmoles aspartic acid, 2 = 189 pmoles glutamic acid, 3 = 130 pmoles serine, 4 = glycine, 5 = asparagine, 6 = 354 pmoles glutamine, 7 = 153 pmoles histidine, 8 = 125 pmoles threonine, 9 = 63 pmoles alanine, 10 = arginine, 11 = proline, 12 = 14 pmoles tyrosine, 13 = 125 pmoles valine, 14 = 18 pmoles methionine, 15 = 55 pmoles cystine, 16 = 43 pmoles isoleucine, 17 = 52 pmoles leucine, 18 = 28 pmoles phenylalanine, 19 = 38 pmoles lysine.
* = reagent associated background peaks.

Compounds were detected at 214 nm wavelength. Peak areas and retention times were calculated with a MP 3000 Multi-Processor.

The linearity of the relation between concentration and UV absorbance was checked in a range of seven concentrations for L-malic acid, citric acid and maleic acid; for the other organic acids single point checks were carried out.

The reproducibility of the RP-HPLC method was determined by repeated analysis of 100 μl aliquots containing 746 μM L-malic, 476 μM citric and 2.2 μM maleic acid.

Possible interferences by the presence of amino acids were examined by direct RP-HPLC separation and analysis of 100 μl mixtures of non-derivatised amino acids (diluted Stock No. AA-S-18, Sigma, resulting in a total amino acid concentration of 2.5 mM, pH 2.5), after perfusion over an 100 mg Adsorbex SCX column (Merck).

The recoveries of L-malic acid, maleic acid and citric acid were determined, after perfusion over the Adsorbex SCX column in the presence or absence of the non-derivatised amino acid standard (Stock No. AA-S-18, Merck).

Results

Amino acids.

Fig. 1 shows a typical chromatogram obtained after RP-HPLC analysis of PITC-derivatives of xylem amino acids. Of the 19 compounds for which calibrations were carried out, 15 amino acids could be identified directly and subsequently quantified. Unidentified peaks in the chromatogram consist of reagent-associated background signals (results not shown and cf. Cohen and Strydom, 1988) and
Figure 2. A typical reversed phase HPLC chromatogram of a mixture of 9 free carboxylic acids. Individual adjustments: flow rate 0.2 ml/min.

Acids: 1 = 1.56 pmoles oxalic, 2 = 6.66 pmoles tartaric, 3 = 7.46 pmoles L-malic, 4 = 9.6 pmoles malonic, 5 = 0.57 pmoles ascorbic, 6 = 0.17 pmoles maleic, 7 = 33.9 pmoles succinic, 8 = 9.51 pmoles citric, 9 = 0.17 pmoles fumaric.
Table 1. Amino acid concentrations (µM ± SEM, n = 3-5, the latter depending on the number of individual amino acids spikes) in tomato xylem exudate. Fused peaks were obtained for Gly, Asn and for Arg, Pro. Assuming an insignificant contribution of Gly to the Gly, Asn peak (White et al., 1981), the asparagine concentration could be calculated as 140 µM.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration µM (± SEM (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>144 (7)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>127 (7)</td>
</tr>
<tr>
<td>Serine</td>
<td>103 (4)</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>209 (12)</td>
</tr>
<tr>
<td>Histidine</td>
<td>127 (12)</td>
</tr>
<tr>
<td>Threonine</td>
<td>134 (19)</td>
</tr>
<tr>
<td>Alanine</td>
<td>59 (10)</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>59 (10)</td>
</tr>
<tr>
<td>Valine</td>
<td>104 (7)</td>
</tr>
<tr>
<td>Methionine</td>
<td>26 (8)</td>
</tr>
<tr>
<td>Cystine</td>
<td>47 (4)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>42 (10)</td>
</tr>
<tr>
<td>Leucine</td>
<td>46 (7)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>26 (15)*</td>
</tr>
<tr>
<td>Lysine</td>
<td>36 (8)</td>
</tr>
</tbody>
</table>

* Phenylalanine: single value of 69 µM excluded in determination of the mean.

unidentified (non-calibrated) xylem amino-components. Table 1 presents concentrations of identified xylem amino acids, showing SEM values of about 10 %. Retention times generally showed coefficients of variations (CV) of maximally 4 %. These relatively high CV and SEM values (see Discussion section) indicate that under the conditions described, problems may arise for xylem components with only small differences in retention times, as was the case for Gly and Asn and for Arg and Pro (Fig. 1).

**Organic acids.**

Fig. 2 shows the chromatogram obtained after RP-HPLC analysis of nine (standard) carboxylic acids and indicates the satisfactory system performance under the conditions applied (retention times: 0.2 % CV, peak areas: maximally 2 %
Analysis of xylem sap

CV, results not shown).

The linear UV response \( (y) \) to a range of organic acid quantities \( (x) \) (L-malic: 1.9 - 149.1 pmoles, citric 1.2 - 95.2 pmoles, maleic 0.03 - 2.15 pmoles) could be expressed by the linear function \( y = a_1 + a_2x \), with constants; \( a_1 = 1935 \), \( a_2 = 2569 \) and \( r = 0.999 \) (L-malic), \( a_1 = -2244 \), \( a_2 = 4927 \) and \( r = 1.000 \) (citric), \( a_1 = -4169 \), \( a_2 = 2.93 \times 10^5 \) and \( r = 1.000 \) (maleic).

Table 2 presents recoveries of L-malic acid, maleic acid and citric acid after perfusion over the Adsorbex SCX column of standard organic acid solutions in the absence and presence of amino acids. The recoveries obtained indicate that the SCX pre-treatment (amino acid adsorption) prevents significant interferences of amino acids in the RP-HPLC analysis of xylem organic acids, without unacceptable signal-losses for the organic acids themselves.

<table>
<thead>
<tr>
<th>Applied concentration (µM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citric acid</td>
</tr>
<tr>
<td>48</td>
<td>75</td>
</tr>
<tr>
<td>48</td>
<td>75</td>
</tr>
<tr>
<td>48</td>
<td>1491</td>
</tr>
<tr>
<td>48</td>
<td>1491</td>
</tr>
<tr>
<td>952</td>
<td>1491</td>
</tr>
<tr>
<td>952</td>
<td>1491</td>
</tr>
<tr>
<td>952</td>
<td>75</td>
</tr>
<tr>
<td>952</td>
<td>75</td>
</tr>
</tbody>
</table>

Mean recovery ± SD (n=8) 98 ± 3 99 ± 3 97 ± 2
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Figure 3 shows a typical xylem organic acid chromatogram, without (insert) and after SCX pre-treatment of the sample solution, Table 3 presents xylem concentrations, calculated under SCX pre-treatment conditions, for L-malic acid, malonic acid, maleic acid, citric acid and fumaric acid. No significant peaks could be recovered for ascorbic acid and succinic acid. As can be deduced from Fig. 2, the ion-suppression conditions (pH 2.3) during RP-HPLC separation did not prevent oxalic acid and tartaric acid positions to coinciding with the relatively large salt peak in xylem sap, shown on the extreme left in the chromatogram (Fig. 3).

Figure 3. Reversed phase HPLC chromatogram of organic acids of tomato xylem exudate after a cation exchange sample preparation. Acids: 1 = L-malic, 2 = malonic, 3 = maleic, 4 = citic, 5 = fumaric. Arrow indicates salt peak. Insert: Chromatogram of organic acids of tomato xylem exudate without sample preparation. Individual adjustments: flow rate 0.2 ml.min⁻¹.
Table 3. Concentrations (µM) of organic acids in tomato xylem exudate. All exudates were sampled during the first h of bleeding from decapitated roots. D.L. = concentration below limit of detection.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Present results</th>
<th>White et al. (1981)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-malic acid</td>
<td>181</td>
<td>628</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>D.L.</td>
<td>-</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>43</td>
<td>&lt; 35</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>0.17</td>
<td>1206</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>D.L.</td>
<td>&lt; 25</td>
</tr>
<tr>
<td>Citric acid</td>
<td>71</td>
<td>301</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>1.9</td>
<td>&lt; 25</td>
</tr>
</tbody>
</table>

Discussion

With the introduction of derivatisation reagents other than ninhydrin, e.g. OPA, FMOC, Dabsyl/Dansyl-Cl or PITC, and the increasing application of HPLC techniques, the separation and quantification of amino acid mixtures has gained new momentum (Hancock, 1989). Although the ninhydrin method is recognized for its reliability, accuracy, reproducibility and ability to separate complex mixtures, the new reagents have led to considerable improvements with respect to analysis time and sensitivity (Lim, 1987). Although less sensitive than the OPA and FMOC method, in the present study PITC reagents were applied, primarily because of the stable and unambiguous derivatisation products formed (Cohen et al., 1984; Pfeiffer and Hill, 1983) and the possibility for product-detection by UV spectroscopy.

The 10 % SEM (~CV 20 %) values presently obtained in peak height analysis of the PITC-derivatised amino acids, are contrasted by the maximally 5 % CV values obtained by Cohen et al. (1984) from peak area calculations of eight consecutive injections of PITC-derivatised amino acid mixtures. These results indicate the importance of integration equipment, stressed even more by the good results for the organic acids, showing maximally 2 % CV values for peak area
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analysis. The relatively high CV values obtained for the retention times of the amino acids are probably due to less than optimal control of the solvent composition (Bakalyar and Henry, 1976), which, in turn, may be due to observed irregular behaviour of the low-pressure magnetic stirrer in the mixing chamber. (With respect to the above it is interesting to note that Stahl et al. (1972), during tricarboxylic acid analysis, abandoned a magnetically stirred mixing chamber in order to minimize peak broadening). Furthermore, and in contrast to the organic acid analysis equipment, mobile phase solvents were not temperature-controlled in flasks or tubings.

Problems with shifts in retention times may be overcome by operator manipulation of x and y coordinates of the chromatogram (Konstantinides et al., 1987), but the specific responses of basic and acidic amino acid derivatives to variations in solvent pH or ionic strength (Chan, 1984) severely complicate clear interpretation. Co-elution problems may be solved by multicomponent analysis of the spectra (Levine and Lehrman, 1984), but results have been reported to depend on the relative concentrations of the co-eluting compounds (Strasters et al., 1988). Furthermore, the separation of Arg and Pro in amino acid standard solutions (results not shown) and the co-elution of Arg and Pro in xylem exudates (Fig. 1) suggest that problems may be overcome by Arg-Pro and/or Gly-Asn directed and optimally controlled separation conditions.

Notwithstanding the problems discussed so far, the direct separation, identification and quantification of 15 out of 19 calibrated components down to the pmol level by single RP-HPLC analysis of PITC-derivatised amino acids (Table 1, Fig. 1) clearly indicates the potential of the method applied.

LC separations of organic acids have been carried out by cation/anion exchange and ion exclusion chromatography, in combination with RI/UV detection (McCord et al., 1984; Palmer and List, 1973; Schneider et al., 1987; Togami et al., 1990), pre-treatment of sample solutions consisted of pre-elution of neutral and/or basic sample components. In the present study, organic acids were detected by UV absorption after separation by ion suppression reversed phase chromatography; pre-treatment consisted of pre-elution of the organic acids themselves by cation exchange chromatography on small pre-packed SCX columns. The presence of salts in the final exudate solutions severely complicates
the detection of tartaric acid and of the strongly acidic oxalic acid (Fig. 3); desalting may reduce these problems.

Reproducibility and linearity of the UV response agreed with values reported elsewhere, both for LC (McCord et al., 1984; Palmer and List, 1973) and GC/GLC applications (De Silva, 1971; Harvey et al., 1970) and need no further discussion. The recoveries of L-malic acid, maleic acid and citric acid after sample pretreatment were close to 100 % (Table 2), a value which was assumed for the other exudate organic acids presented in Table 3. Detailed determinations of the sensitivity were not carried out, but the fumaric acid concentrations which could be calculated in the exudate were considerably smaller than the limits of detection by GLC analysis reported by White et al., (1981), although the latter were based on 10 \( \mu l \) sample volumes. Maleic acid could be determined down to the pmol level (Table 3), but the specific UV absorbances for L-malic acid and citric acid were about two orders of magnitude lower.

The results of White et al. (1981), who reported high concentrations (1200 \( \mu M \)) of maleic acid after GLC analysis of tomato xylem exudates, were not observed by the present RP-HPLC analysis of exudates (Table 3); further reports on the presence, or possible function and importance of maleic acid in plants could not be found.

Differences between present xylem citric acid data and the results of White et al. (1981) may be attributed to their administration of citrate to maintain soluble nutrient solution Fe, whereas in the present experiments EDTA-complexed Fe was applied. Differences between L-malic acid concentrations were of the same order of magnitude, which, apart from the above, may originate from differences in applied tomato varieties (White et al. (1981): cv Rutgers PS-VF , present paper: cv Tiny Tim), or from further inorganic differences in hydroculture solutions.

The presentation of data on L-malic acid in both standards and xylem exudates (Fig. 2, Tables 2 and 3), stems from the RP-HPLC separation power for D- and L-forms of malic acid (results not shown), which cannot be achieved by exchange chromatography; however, in biological systems D-malic acid does not occur (Stryer, 1981).

Last but not least, the price differences between the RP-columns applied (approx. US $ 75) and ion exchange or organic acid columns (approx. US $ 1000)
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may be a practical parameter favouring the application of RP-HPLC analysis of organic acids.

In conclusion, the results of the present paper suggest that RP-HPLC separation and UV detection of organic acids and PITC-derivatives of amino acids offers good prospects for the determination of both amino acids and organic acids in xylem exudates.

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CHAPTER 3

Citric acid in tomato plant roots and its effect on cadmium uptake and distribution

Abstract
Experiments were carried out to investigate the effects of root citric acid on uptake and initial distribution of cadmium (Cd) in tomato plants (Lycopersicon esculentum Mill, cv. Tiny Tim). Cd was measured by γ-spectrometry, using 118Cd spikes. Citric acid was measured by UV-detection, and, after spiking with 14C-citric acid, by β-spectrometry. Cd was applied for 48 h periods, (1) in control experiments, (2) in the presence of citric acid, and (3) after 24 h plant pre-incubation with citric acid.

Pre-incubation resulted in two-fold increases in fast-exchangeable amounts of root citric acid, as measured by the presence of citric acid in xylem exudates of decapitated and pressurized roots.

Simultaneous application of Cd and citric acid did not change Cd accumulation in total plants and in the roots, nor did any significant change occur with respect to Cd root-to-shoot transport, and Cd concentrations in shoot tissues and xylem fluid. After citric acid pre-incubation, total plant uptake of Cd increased two-fold, without any significant change in Cd accumulation in the roots. Cd root-to-shoot transport was increased 5-6 fold, and Cd concentrations in shoot tissues and xylem fluid were increased 6-8 fold. Speciation calculations indicated that, under the conditions applied, xylem Cd may be, at least partly, complexed in citric acid.

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Chapter 3

Introduction

Plant roots are generally considered to be involved in processes of synthesis, uptake and release of organic acids (Collins and Reilly, 1968; Tonin et al., 1990).

Of the plant organic acids, citric- and malic acid have received particular attention. Synthesis and release of especially citric acid from the roots into the rhizosphere (acidification) is often considered to increase soil nutrient availability (Brown, 1966; De Vos et al., 1986; Hoffland, 1992; Landsberg, 1981; Lipton et al., 1987), both acids may function as (part of) a tolerance mechanism under conditions of high element supply (Delhaize et al., 1993; Ernst et al., 1975; Godbold et al., 1984; Miyasaki et al., 1991; Ojima et al., 1984). As a consequence, and generally speaking, the root pools of organic acid may both affect nutrient uptake and be affected by accumulated nutrients (Brown, 1966; Godbold et al., 1984).

In the xylem, many metals are reported to be present as complexed to organic acid (Brown and Chaney, 1971; Tiffin, 1967; White et al., 1981a;b). Apart from Fe, hardly anything is known about the possible mutual inductive relations between metals and organic acids in root pools and xylem fluid. For Fe, one assumes that the root content of Fe may affect the amounts of citric acid transported into the xylem (Brown, 1966; Brown and Chaney, 1971) and that Fe deficiency may specifically result in increased root citric acid concentrations (Brown and Tiffin, 1964; De Vos et al., 1986).

The accumulation of organic acids in root cell compartments of metal tolerant plant species (e.g. Cu, Ni, Zn, see Ernst et al., 1975; Godbold et al., 1984; Harmens, 1993; Kishinami and Widholm, 1987; Mathys, 1977; Thurman and Rankin, 1982) may indicate a more general character of the metal organic acid interrelationships in plants. Here, as with Fe, the supply of metal may affect root organic acid concentrations and conversely, root organic acid status may have effects on metal accumulation, translocation and distribution (Thurman and Rankin, 1982).

In the present paper, the attention is focused on the uptake and translocation of cadmium in tomato plants. In previous experiments with plant subsystems (Senden and Wolterbeek, 1990; Senden et al., 1992a), effects of citrate on
Root citric acid

Cd xylem transport characteristics were observed. The present experiments were performed to get more insight in the effects of citric acid on Cd accumulation and mobility in whole plants. Two series of experiments were carried out, in one of which we introduced increased root citrate levels (Thurman and Rankin, 1982), both by 14C-citric acid pre-incubation and by simultaneous 116Cd(NO3)2 and citric acid supply. Root citric acid pools and transport into the xylem were indicated by experiments with excised roots.

Materials and methods

Plant culture

Tomato plants (an inbred line of Lycopersicon esculentum Mill cv. Tiny Tim) were used throughout. Plants were grown in hydroculture in a glasshouse at ca. 25 °C, 75 % r.h., and a light intensity of 3600 lux. The medium consisted of (mg l–1): KH2PO4, 134; K2SO4, 154; MgSO4.7H2O, 473; Ca(NO3)2.4H2O, 882; KNO3, 444; MnSO4.4H2O, 2; H3BO3, 2.7; ZnSO4.7H2O, 0.5; CuSO4.5H2O, 0.08; Na2MoO4.2H2O, 0.13; Fe, 5 (in Fe-EDTA); pH = 6.0, adjusted with H2SO4.

Experiments

Experiments were carried out with intact plants and with detached plant root systems. Intact plants were used when 8 weeks old. Plant root systems were taken from 14 weeks old plants and used in pressure cell experiments. In intact plant experiments, harvested roots were divided into main (central), and fine roots (Wolterbeek, 1987). For all plants involved, the quantities of water transpired were recorded by weighing. Here, over the 48 h periods, plant growth was not considered nor determined in the time-differences in total weights of the plant-pot combinations.

A. Intact plant experiments

Citrate pre-incubation periods. Plants were pre-incubated in 100 ml citric acid solution (250 µM (14C)citric acid, 0.1 mM Ca(NO3)2, pH 6.0), for 24 h. After the 24 h pre-incubation, the plants were transferred to 100 ml experimental solutions (0.1
Chapter 3

mM Ca(NO₃)₂, 5 μM ¹¹⁶Cd(NO₃)₂, pH = 6.0), for 48 h.

Cadmium control. Plants were pre-incubated in 100 ml 0.1 mM Ca(NO₃)₂ (pH 6.0) solutions for 24 h. Plants were then transferred to 100 ml experimental solutions (0.1 mM Ca(NO₃)₂, 5 μM ¹¹⁵Cd(NO₃)₂, pH = 6.0), for 48 h.

Simultaneous cadmium/citrate supply. Plants were pre-incubated in 100 ml 0.1 mM Ca(NO₃)₂ (pH 6.0) solution for 24 h. After the 24 h period, plants were transferred to 100 ml solution of 0.1 mM Ca(NO₃)₂, 5 μM ¹¹⁵Cd spiked Cd(NO₃)₂ and 250 μM ¹⁴C-citric acid (pH = 6.0), for 48 h.

B. Plant root (pressure cell) experiments

Plants were pre-incubated for 24 h in 750 ml 0.1 mM Ca(NO₃)₂ solutions, with or without 450 μM citric acid. The pH of all solutions was set at 6.0. The rates of transpiration during the 24 h pre-incubation periods were determined as approximately 0.5 ml.h⁻¹.g⁻¹ fine roots FW.

After pre-incubation, the plants were decapitated and the root system was transferred to a pressure cell (Mees and Weatherley, 1957; Perry, 1969; Perry and Greenway, 1973; Salim and Pitman, 1984), into 500 ml 0.1 mM Ca(NO₃)₂ with or without 450 μM ¹⁴C-citric acid (pH = 6.0) solutions. The pressure cell was operated at 1.5 bar (0.15 MPa), for approximately 1.5 h., during which the exudate flow was about 1.0 ml.h⁻¹.g⁻¹ fine roots FW.

Xylem exudate fractions (0.5 to 4 ml each) were collected. The fractions were filtered through 0.45 μm Millipore filters and perfused through 100 mg Adsorbex SCX-columns (Merck). (Perfusion procedures: 1 ml 0.1 N formic acid, 1 ml distilled water, 1 ml methanol, 2 ml distilled water, 3 ml xylem exudate (Senden et al., 1992)). The xylem perfusate was collected in four 1 ml fractions.

Xylem perfusates were analysed by UV-detection reversed phase high performance liquid chromatography (RP-HPLC) for organic acids (Senden et al., 1992). On line β-counting (Berthold) was performed for ¹⁴C-detection in the xylem exudate fractions.

Production of radioisotopes, and measurement equipment

¹¹⁶Cd (t₁/₂ = 53.5 h.) was produced by neutron activation of Cd(NO₃)₂ in the
Root citric acid

Hoger Onderwijs Reactor at I.R.I. Delft, The Netherlands, at a thermal flux density of $0.5 \times 10^{17}$ m$^{-2}$s$^{-1}$ for 4 h., yielding a specific activity of 0.155 GBq.g$^{-1}$. [1.5-$^{14}$C]citric acid (1.85 MBq.ml$^{-1}$, 20.8 GBq.g$^{-1}$) was purchased from Amersham International U.K.

$^{115}$Cd measurements were carried out using a $\gamma$-ray 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, control of measurement sequence and spectrum analysis were carried out according to the methods described by De Bruin and Korthoven, 1972; 1974; Korthoven and De Bruin, 1977.

$^{14}$C in the aqueous samples was radioassayed in 10 ml Lumagel (Lumac, Landgraaf, The Netherlands) by liquid scintillation counting (MINAXI Tri-carb 4000 Canberra Packard). On line $^{14}$C activity measurements of the xylem exudate samples were carried out by liquid scintillation counting using Berthold LSC equipment with a 2.0 ml flow-through cell, using FLO-SCINT IV scintillation fluid (Canberra Packard) at a total flow rate of 4.0 ml.min$^{-1}$, in a 1:3 mixture of mobile phase and FLO-SCINT.

Speciation calculations

The chemical speciation of elements applied in the aqueous solutions was calculated with the help of the computer program SOILCHEM and associated data base, which was developed at the University of California, Berkeley, U.S.A. (Sposito and Coves, 1988), with full consideration for mole balances, relevant thermodynamic equilibrium constants and ion strengths.

Results

Citric acid pool

A pressure cell experiment was carried out, for which a single plant was pre-incubated in a 100 $\mu$M Ca(NO$_3$)$_2$, 450 $\mu$M citric acid solution for 24 h. Due to uptake and breakdown of citric acid, at 24 h, the applied solution had become devoid of citric acid UV responses (results not shown). After decapitation, the root system was placed in a 450 $\mu$M $^{14}$C-citric acid solution in the pressure cell; a total of 15 ml
Chapter 3

Xylem exudate was collected in 1.5 h. In the first, third and fifth fraction, HPLC determined citric acid was quantified both by UV and $^{14}$C. Fig. 1 shows the time-course of the $^{14}$C/UV ratio in xylem citric acid; UV signals (not shown) already indicated 100 % throughput (relative to applied concentrations) of citric acid in the first fraction recovered, which indicates the absence of any significant dilution in root water free spaces. The relatively rapid increase in $^{14}$C/UV-ratios up to 100 % of the applied $^{14}$C/UV ratio (Fig. 1) shows the visible root cellular citric acid pool to be small and fast-exchangeable (see Discussion section).

![Graph showing $^{14}$C/UV ratio vs recovered xylem volume (ml)]

**Figure 1.** Time course (expressed as ml recovered xylem exudate) of the $^{14}$C/UV ratio of citric acid in xylem exudates of excised root systems. After 24 h pre-incubation of intact plants in 450 μM citric acid solutions, roots were decapitated and placed in the pressure-cell. 3 ml samples were recovered from the roots during application of 450 μM $^{14}$C-citric acid. The initial $^{14}$C/UV ratio of the applied medium was taken as 100 %.

Another set of plants was used in the pressure cell after 24 h pre-incubation in 100 μM Ca(NO$_3$)$_2$, once without and once with 450 μM $^{14}$C-citric acid. During the pressure cell period, no citric acid was applied: the collected exudates were to
result directly from the initially available root citric acid pools. Fig. 2 shows the time courses in relative UV-results, both for the situation with and without citric acid pre-incubation. The data may be considered to represent the fast-exchangeable root citric acid pools, and indicate that after pre-incubation, compared to controls (Fig. 2), more time is involved in releasing the pool citric acid. Quantitative determinations yielded a two-fold larger pool: the control plant showed 406 nanomoles of exuded citric acid, whereas 830 nanomoles of citric acid were collected from the pre-incubated plant.

The general data indicate that, under the described conditions, the short-term citric acid supply leads to a two-fold higher "exudate-available" citric acid pool in the root system.

![Graph showing citric acid recovery over xylem volume](image)

**Figure 2.** Time course (expressed as ml recovered xylem exudate) of the citric acid recovery, (UV measurements, shown relative to $U_{V, o}$) in xylem exudate fractions obtained from pressurized roots. ● = without pre-incubation, ■ = after 24 h pre-incubation with 450 μM citric acid. $U_{V, o}$ values are obtained by extrapolation (dashed lines).
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Cadmium

Cadmium was applied in a) control experiments, b) in the presence of 250 μM citric acid, or c) after 24 h pre-incubation in 250 μM citric acid. Plant transpiration averaged as 0.110 ± 0.015 ml.h⁻¹ (mean ± SD, n = 6), there were no significant differences between treatments. Plant fresh weights (determined after the experiments; mean ± SD, n = 6) averaged as 0.19 ± 0.02 g (main roots), 0.12 ± 0.04 g (fine roots), and 1.84 ± 0.42 g (shoots).

Cd-control. Table 1 presents the Cd uptake data for the three experimental conditions. The uptake of Cd²⁺ by control plants resulted in 20 μg Cd g⁻¹ plant FW, of which by far the larger fraction was retained in the root system (see also Kabata-Pendias and Pendias, 1985; Wolterbeek et al., 1988). The Cd distribution between shoot and root (shoot: root Cd ratio) is about 5-10 times as high as that found by Florijn and Van Beusichem, (1993) and Florijn et al. (1993) for a number of maize varieties, which were indicated as Cd "shoot-excluders"; however, the present ratios are about 5-10 times smaller than the ratios for maize "non shoot Cd excluders" (Florijn et al., 1993; Florijn and Van Beusichem, 1993). Moreover, the present ratio is about 5 times as high as the ratios in tomato as reported by Wolterbeek et al. (1988) (see Discussion section). The data show a 100 % throughput (relative to applied concentrations) of Cd²⁺ in the xylem, which agrees with results obtained earlier by Wolterbeek et al. (1988).

Simultaneous supply of Cd and citric acid. Table 1 presents the data on Cd uptake and distribution, in experiments in which Cd was applied in the presence of 250 μM citric acid. No significant differences from control plants were observed, either for total- and root uptake or for the various mobility parameters and xylem throughput.

The absence of any effects on a total uptake or root uptake level (Table 1) may be regarded as remarkable, especially if seen in the light of the citric acid induced changes in Cd speciation in the applied solutions (Table 2). Here, the presence of citric acid results in a 8-fold reduction of free Cd²⁺; it should be noted however, that a simultaneous 5-fold reduction can be calculated in free Ca²⁺. In both situations, root Cd uptake constitutes about 85 % of total uptake; apparently,
Table 1. Cd uptake and distribution in tomato plants (n=2), as affected by citric acid. Citric acid concentration applied 250 μM.

* = significantly different from other observations (P=0.05). T = transpired water volume.

<table>
<thead>
<tr>
<th></th>
<th>Cd conc. applied</th>
<th>Cd conc. total plant</th>
<th>Cd conc. fine roots</th>
<th>Cd conc. main roots</th>
<th>(shoot/root)/T</th>
<th>xylem/medium</th>
<th>shoot/T</th>
<th>(μg.g⁻¹FW).ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg.ml⁻¹</td>
<td>μg.g⁻¹FW</td>
<td>μg.g⁻¹FW</td>
<td>μg.g⁻¹FW</td>
<td>ml⁻¹</td>
<td></td>
<td></td>
<td>(μg.g⁻¹FW).ml⁻¹</td>
</tr>
<tr>
<td>Cd control</td>
<td>0.53 ± 0.01</td>
<td>20.1 ± 3.7</td>
<td>116 ± 33</td>
<td>41 ± 6</td>
<td>0.034 ± 0.002</td>
<td>1.08 ± 0.65</td>
<td>0.44 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Cd + citric acid</td>
<td>0.55 ± 0.03</td>
<td>18.8 ± 3.1</td>
<td>84 ± 23</td>
<td>33 ± 7</td>
<td>0.035 ± 0.001</td>
<td>0.95 ± 0.01</td>
<td>0.44 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>citric acid</td>
<td>0.54 ± 0.02</td>
<td>42.6 ± 6.5*</td>
<td>86 ± 4</td>
<td>41 ± 6</td>
<td>0.193 ± 0.041*</td>
<td>7.55 ± 1.91*</td>
<td>2.72 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>pre-incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
root uptake is not affected by medium Cd speciation (Tables 1 and 2). Furthermore, the 100 % Cd xylem throughput, as calculated on a "total Cd supply" basis, also indicates Cd uptake irrespective of the medium Cd speciation (Table 1, and see Discussion section).

Cd supply after plant pre-incubation with citric acid. Table 1 presents data on Cd uptake and plant distribution in Cd applications after plant incubations with 250 μM citric acid. During the Cd uptake, control measurements were carried out in the applied media: releases of initially absorbed citric acid were not observed (results not shown). Root accumulation of Cd²⁺ was not significantly changed if compared to both earlier experiments; total plant uptake, however, showed a two-fold increase (Table 1). The calculated Cd shoot:root ratio was increased 5-6 fold, indicating significant changes in (citric acid induced) root Cd mobility. Accompanying effects were observed in the xylem: Cd was determined in concentrations of about 8 times the applied medium Cd²⁺ levels. As shown in Table 1, shoot Cd concentrations were about 6-fold increased.

Table 2. Cadmium, calcium, nitrate, and citric acid chemical forms in the applied media, calculated for pre-incubation conditions (without cadmium, (-Cd)) and for conditions of simultaneous cadmium supply (+Cd).

<table>
<thead>
<tr>
<th>metal/ligand</th>
<th>conc. applied mM</th>
<th>chemical form</th>
<th>%</th>
<th>+Cd</th>
<th>-Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcium</td>
<td>0.100</td>
<td>Ca²⁺</td>
<td>18.5</td>
<td>18.2</td>
<td>81.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>citrate-bound</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cadmium</td>
<td>0.005</td>
<td>Cd²⁺</td>
<td>12.6</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>citrate-bound</td>
<td>87.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>citric acid</td>
<td>0.250</td>
<td>cit³⁻</td>
<td>38.8</td>
<td>39.7</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca-bound</td>
<td>32.4</td>
<td>32.6</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cd-bound</td>
<td>1.8</td>
<td>---</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-bound</td>
<td>27.0</td>
<td>27.7</td>
<td>27.0</td>
</tr>
<tr>
<td>nitrate</td>
<td>0.210</td>
<td>NO₃⁻</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Discussion

The present experiments were carried out to gain more insight in possible effects of concentration and release of root citric acid on uptake and initial distribution of cadmium. In the experiments, citric acid release was simulated by simultaneous supply of both Cd and citric acid; under the present conditions, citric acid release could not be stimulated by Cd, as indicated by the absence of release of pre-absorbed citric acid during Cd$^{2+}$ supply. 
The application of citric acid increased cellular citric acid concentrations (Fig. 1). The initially less than 100 % throughput of $^{14}$C-labelled citric acid should be attributed to $^{14}$C-dilution in cellular compartments. The calculated 400 to 800 nanomoles of root citric acid which could be observed in the root exudate (Fig. 2, obtained for 10 g FW roots) is markedly lower than regularly reported values for total quantities of root citric acid (Brown, 1966; De Vos et al., 1986; Godbold et al., 1984). Based on data by Brown (1966) however, exudate citric acid may be estimated to comprise about 5 % of total root citric acid; the presently resulting 160 to 320 $\mu$g citric acid per g root fresh weight is in accordance with literature data (Brown, 1966; Godbold et al., 1984; Harmens, 1993).

The cadmium experiments were invariably performed with 5 $\mu$M Cd-solutions. These concentrations may be considered to represent contaminated soil solutions (Kabata-Pendias and Pendias, 1985); however, the present short-term experiments resulted in plant Cd concentrations generally below reported toxic Cd levels (Bazzaz et al., 1974; Kabata-Pendias and Pendias, 1985; Lamoreaux and Chaney, 1978; Marschner, 1983).

The control Cd applications resulted in shoot:root Cd ratios which were about 5 times higher than values for tomato reported by Wolterbeek et al. (1988). However, Wolterbeek et al. (1988) used 1.0 $\mu$M Ca(NO$_3$)$_2$ in solutions, whereas the present experiments were carried out in 100 $\mu$M Ca(NO$_3$)$_2$; therefore, differences in results may be due to differences in fractional Cd cell wall immobilization in the roots.

Table 2 shows the high fractional presence of Cd in citrate, under simultaneous Cd and citric acid supply. The results shown in Table 1, however, do not indicate any significant differences in root Cd accumulation, nor any change in
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shoot:root Cd ratio. Considering also the 100 % xylem throughput of applied Cd (based on total applied Cd concentrations, irrespective of the Cd speciation in solution), these data may indicate that the presence of Cd-citrates in the applied solution does not affect Cd accumulation in any way: presumably, Cd will be released from citrate at the root-solution interface (cf. plant Fe uptake from Fe-chelates, see Chaney et al., 1972; Tiffin et al., 1960).

Remarkably, the simultaneous addition of 250 μM citric acid does not result in any significant changes in initial Cd behaviour in the plant (Table 1); pre-incubation, however, resulted in marked increases in total uptake and root to shoot transport of cadmium (Table 1). Prominent differences in chemical speciation (Table 2) and plant uptake of citric acid are not to be suspected; therefore, the data may be discussed in terms of differences in time.

Based on the data shown in Fig. 1, and the various exudate and transpiration flows observed, full homogenization of absorbed citric acid in existing fast-exchangeable root citric acid pools in intact plants can be determined to take at least take several (> 5) hours. On the other hand, the data shown in Fig. 2 indicate that an increase in the fast-exchangeable root citric acid pool may be accomplished within 24 hours. The fast-exchangeable root citric acid pool may thus be changed within 5-24 hours, but the question remains whether changes in pool size have immediate effects in terms of Cd behaviour: future research should yield answers regarding the kinetics of the processes involved. Here, data on the relations between Zn and citrate in root saps and root water extracts of Deschampsia ecotypes (Godbold et al., 1984) may be noted: in 24 h Zn exposures, total citrate in root water extracts remained unaffected, whereas citrate in expressed sap (vacuolar citrate) almost doubled in concentration.

As indicated in Table 1, the present data represent shoot:root Cd ratios, but they do not give any direct clues about further Cd mobility in the shoots. The xylem Cd may be present in complexed forms (White et al., 1981a; b; Wolterbeek et al., 1988); speciation calculations based on a presumed xylem presence of 5 μM Cd (Table 1), in the matrix of a tomato xylem contents as reported by White et al., (1981) (e.g. 2.6 mM Ca, 0.94 mM Mg, 6.5 μM Zn, 5 μM Cu, 6.0 μM Mn, 6.8 μM Fe\textsuperscript{3+}, 300 μM citric acid, 600 μM malic acid), show Cd to be complexed for about 27 % in citric (11 %), maleic (13 %) and malic acid (3 %).
Root citric acid

The data above suggest that Cd may be present in the xylem in combined free and complexed forms; future experiments may be aimed more specifically at direct measurements of the effects of ligands on the xylem behaviour of cadmium.

References


Chapter 3

Amsterdam, The Netherlands.


Root citric acid
detection. Plant and Soil 142, 81-89.
CHAPTER 4

Effect of citric acid on the transport of cadmium through xylem vessels of excised tomato stem-leaf systems

Abstract

The positive effects of citric acid on the transport of Cd through the xylem vessels of tomato stems has been quantitatively studied by measuring the rate constant \(k\) of the lateral escape from the vessels to surrounding tissues and the amount of Cd adsorbed to the xylem cell walls. These two transport parameters were shown to decrease after addition of citric acid to the Cd solution, probably due to the formation of uncharged and negatively charged Cd-citrate complexes, which show little binding to the mainly negatively charged xylem cell walls. Reduced affinity of metal complexes to the cell walls can explain the long term positive effects of citric acid on longitudinal transport of cadmium and other metals in the xylem.

M.H.M.N. Senden and H.Th. Wolterbeek
Chapter 4

Introduction

Long-distance transport of elements taken up from the soil mainly occurs through the xylem vessels. It can be described as a bulk flow mainly driven by transpiration. The substances present in this flow, which is essentially a movement through the apoplast of the xylem, become involved in processes as:

Adsorption to mainly negatively charged sites present in the walls (Bell and Biddulph 1963, Ferguson and Bollard 1976, Wolterbeek et al. 1984),

Lateral escape from the moving solution in the vessels into the adjacent intercellular spaces and walls (free space) and eventually to cells of other tissues (Van Bel 1978).

Although Cd is considered to be a nonessential element for plants, it is effectively absorbed by both root and leaf systems. Its long-distance translocation as metal ion is limited, probably due to the binding of cations to exchange sites located in the xylem cell walls (Kabata-Pendias and Pendias 1985, and more in general: Bell and Biddulph 1963, Ferguson and Bollard 1976, Wolterbeek et al. 1984). The possible formation of complexes or metal chelates in soils and in plants, however, may result in easy availability of soil-Cd and effective transport of Cd-organic complexes in plants (Tiffin 1970, 1972, White et al. 1981a, b, c, Kabata-Pendias and Pendias 1985). Increased efficiency of longitudinal Cd transport in xylem vessels will lead to elevated levels of Cd in the leaves, with possible phytotoxic effects of Cd on photosynthesis, transpiration, etc. (Bazzaz et al. 1974, Lamoreaux and Chaney 1978, Marschner 1983, Kabata-Pendias and Pendias 1985).

White et al. (1981a, b, c,) reported the presence of metal complexes with carboxylic and amino acids in xylem fluid of soybean and tomato plants. Increased longitudinal transport is often qualitatively expressed by reduction of the retention time of the substance in the stem, and is generally attributed to reduced binding of the metal concerned to the fixed negative charges of the xylem walls (Tiffin 1970, Bradfield 1976, Ferguson and Bollard 1976, Van de Geijn and Pikaar 1982, McGrath and Robson 1984).

Among the compounds mentioned above especially citric acid has attracted much attention, because it occurs in relatively high concentrations in xylem fluid
and forms stable complexes with many divalent cations (Tiffin 1970, White et al. 1981a, b, c, McGrath and Robson 1984). Formation of anionic or uncharged Zn-citrate complexes resulted in more Zn passing through the excised stem of Pinus radiata (McGrath and Robson 1984). Citric acid is reported to have the same effect on the transport of Ca in apple shoots (Bradfield 1976, Ferguson and Bolland 1976), Fe in soybeans (Tiffin 1970) and Cu in papyrus stems (Van de Geijn and Pikaar 1982).

Another, probably related, phenomenon is the lateral escape of the metal considered (Van Bel 1978), the rate of which may change too, due to the formation of organic complexes. This rate also quantitatively affects the quantitative upward movement of elements (Van Bel 1978, Wolterbeek 1986).

In the present paper experiments are described in which quantitative effects of complexation on the upward movement of Cd through the xylem of tomato stems were investigated. The influence of citric acid on the transport of Cd through the xylem vessels was described by the changes of the values of both the amount of Cd adsorbed to the xylem vessel walls and of the escape rate constant.

Materials and methods

Plants and plant culture

Tomato plants (an inbred line of the red cherry tomato Lycopersicon esculentum Mill, cv. Tiny Tim) were used throughout. They were cultivated in a glasshouse at ca 25 °C and 75 % r.h..

Uptake experiments were carried out with plant sub-systems, consisting of a stem part with one fully expanded leaf. 24 hour prior to the experiment the stem segment to be used (only one internodium) with one petiole (length stem-internodium with petiole ca. 200 mm) was cut under water and conditioned in tap water to laboratory conditions (30 °C, 70 % r.h.). From the composite tomato leaf all leaflets except the top three ones were cut, in order to ensure a homogeneous element distribution.

Element solutions

Two different element solutions were used. In Cd²⁺ transport experiments,
the solutions applied contained the radiotracers $^{115}$Cd (0.4 mM, in CdSO$_4$) and $^{122}$Sb (0.3 µM, in Sb(SO$_4$)$_2$), formed by adding SbCl$_3$ to a medium containing SO$_4^{2-}$). The acidity of the solution was set to pH 6 with KOH.

In experiments where the effect of complex formation on metal transport was determined, a citric acid solution (2.5 mM, pH 6) was applied which also contained $^{115}$Cd and $^{122}$Sb (concentrations mentioned above).

Citric acid forms stable complexes with Cd (White et al, 1981a, b, c). The dissociation constants pK$_1$, pK$_2$ and pK$_3$ of citric acid are reported as 3.08, 4.39 and 5.49 respectively (Martell and Calvin 1959). For calculations of Cd-citrate complexes the values for the association constant (log K) for the formation of Cd citrate$^-$ and Cd H citrate were taken as 3.98 and 2.28 respectively (Sillén and Martell 1964). Under the conditions applied only 6-8 % of the total Cd was present as Cd$^{2+}$.

The radiotracer $^{122}$Sb was added to both the solutions to permit the calculation of the total cross-sectional area of the xylem vessels involved in fluid and element transport (see section Measurements and calculations).

**Production of radioisotopes and measurement equipment**

The radiotracers $^{115}$Cd ($t_{1/2} = 53.5$ h, γ-ray energy, 527 keV) and $^{122}$Sb ($t_{1/2} = 2.8$ d, γ-ray energy, 564 keV) were produced by neutron activation of SbCl$_3$ and CdSO$_4$ (see section Element solutions) in a thermal neutron flux density of 0.5·10$^{17}$ m$^{-2}$·s$^{-1}$, for 4 hours. The activities used were 11.1 MBq and 0.4 MBq for $^{115}$Cd and $^{122}$Sb respectively.

Measurements were carried out using a γ-ray spectrometer with a semiconductor Ge(Li)-detector, coupled to a DEC PDP-11/44 computer through a CAMAC interface. Calibration, spectrum accumulation, control of measurement sequence and spectrum analysis were carried out according to the methods described by De Bruin and Korthoven (1972, 1974) and Korthoven and De Bruin (1977).

**Set up**

The plant sub-system (stem-leaf) was placed in a lead shielding (Fig. 1) which permitted the measurements of the radioisotopes in the leaf parts above the detector, with only minor interference from the activity in the solution and the
shielded plant parts. The container with the radioactive solution was placed on an electronic balance to monitor the amount of water absorbed by the plant. One hour counting intervals permitted semi-continuous measurements of transport of elements into the leaf.

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**Figure 1.** Schematic representation of experimental set-up and position of the plant sub-system. 1: fixed tomato leaf with petiole and stem part, 2: Ge(Li)-detector, 3: lead shield thickness 5-7 cm, 4: fixation of the stem part, 5: perspex guidance tube, 6: guidance tube holder and free keg covering, 7: top of keg, 8: keg, 9: electronic balance, 10: tube 1.5 mm for application and removal of solutions, 11: taps, 12: syringe.

**Measurements and calculations**

The amount of water transported to the leaf in time \( t \) could be described by the function \( F(t) = \alpha (1 - e^{-\beta t}) \) with constants \( \alpha \) and \( \beta \) (Wolterbeek et al. 1984).

The total cross-sectional area of xylem vessels (\( A_p \)) was calculated from the length of the stem segment and the rate of transport of the negatively charged \(^{122}\text{Sb(SO}_4)_2^-\) (indicating xylem volume), which does not interact with the negatively charged xylem cell walls (Wolterbeek et al. 1984).

During the experiments repeated one hour countings were made of the absolute quantity of element accumulated in the leaf at time \( t \) at a distance \( L \) from...
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the point of element introduction into the stem.

The lateral escape from xylem vessels was described as a first order process and expressed by the rate constant \( k \) (h\(^{-1}\)). The rate constant \( k \) was calculated by fitting a mathematical accumulation function to the data obtained, with help of an iterative non-linear regression-program (for function and full procedures, see Wolterbeek et al. 1984).

The amount of Cd adsorbed to the xylem cell walls was estimated from the value of \( t_1 \) (time needed for the fluid to reach the leaf and for full equilibration of exchange sites) and the amount of Cd introduced into the plant sub-system during this period of time (Wolterbeek et al. 1985b).

The amounts of Cd adsorbed to the xylem cell walls, were interpreted in terms of concentrations of fixed Cd, which could be derived by assuming a cell wall thickness of 2 \( \mu \text{m} \) (Wolterbeek et al. 1985a, b).

Results

Fig. 2 shows the Cd concentration in the xylem fluid transported into the leaf, relative to its concentration in the applied solution. Several experiments were conducted under identical conditions and the differences between measurements show the biological variability between plant sub-systems. The dotted line parts indicate the time required for full saturation or equilibrium of the exchange sites with Cd, starting from the time of first appearance of Cd in the leaf. The dotted line starts after an initial "zero" period needed for the xylem fluid to reach the leaf. Although obtained in the measurements no data points are presented for this experimental period, since up to the time needed for reaching equilibrium, data were not processed in terms of relative concentrations. This period of time, and especially that prior to first detection of Cd in the leaf, not only depends on Cd concentrations applied, but also on the xylem volume flow. The full lines drawn show the experimental periods in which the transport could be described by a function reported earlier (Wolterbeek et al. 1984), yielding the value of \( k \). The decrease with time of the relative Cd concentrations (Fig. 2) should be attributed to the characteristics of the xylem volume flow which also decreases with time. Consequently, the maximal values of the relative Cd concentrations logically appear
as soon as a steady state situation is reached with respect to adsorption processes.

Fig. 2 indicates that the differences with respect to the time prior to the first Cd detection in the leaf and with respect to the values of relative Cd concentrations in the xylem are the results of the mere absence or presence of citric acid in the solution applied. However, the times necessary for the exchange processes to reach an equilibrium situation are the resulants of both Cd concentrations and xylem volume flow. Thus, differences with respect to Cd adsorbed cannot be expressed on a time basis, but only on an 'amount of Cd absorbed' basis. Furthermore, differences in the relative Cd concentrations in the xylem fluid actually depend a.o. on differences in the xylem volume flow (Horwitz 1958), so that the only reliable means of expressing rates of lateral escape is by calculation of the escape rate constant $k$.

Table 1 presents the values for the escape rate constant $k$ and adsorbed Cd, averaged for a number of experiments. The results clearly show reduced values for these two transport parameters in the presence of citric acid, indicating that citric acid not only affects the adsorption of Cd to the xylem cell walls but also its rate of removal from the vessels in lateral direction.

**Table 1.** Average values ($\pm$ SE) of $k$ and Cd adsorbed (Cd ads.) for rate constant of lateral escape and the amount of Cd adsorbed during transport of Cd through xylem vessels in tomato.

<table>
<thead>
<tr>
<th>Applied Cd$^{2+}$ (mM)</th>
<th>Applied citrate (mM)</th>
<th>$k$ (h$^{-1}$)</th>
<th>Cd ads. ((meq.l$^{-1}$))</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35 ± 0.05</td>
<td>---</td>
<td>1.89 ± 0.42</td>
<td>153 ± 82</td>
<td>3</td>
</tr>
<tr>
<td>0.32 ± 0.14</td>
<td>2.5</td>
<td>0.73 ± 0.38</td>
<td>25 ± 15</td>
<td>4</td>
</tr>
</tbody>
</table>

The values of $k$ and Cd ads. under two different conditions were significantly different at $P < 0.05$ (Student's t-test)
Figure 2. Cd concentration in xylem fluid entering the leaf. Amounts are processed with respect to volume flow and are expressed as percentage of applied concentration. □ and ■ are duplicate experiments with two plant sub-systems in which both Cd and citric acid were applied. ○ and ● are examples of experiments in which only Cd is applied. The dotted line parts indicate the increase of the Cd-concentration with time up to the time t₁. The time t₁ comprises a "zero Cd" period, expressing the time needed for the xylem fluid to reach the leaf and a "dotted line" period, which represents the time needed for full equilibration of exchange sites. Note that these times are variables, depending on the rate of xylem flow and chemical Cd form.

Discussion

Results shown in Fig. 2 indicate that, apart from the metal concentrations applied, the retention time in the stem should be regarded as a plant dependent parameter, probably largely determined by xylem volume flow and anatomical xylem
vessel characteristics such as available absolute quantity of exchange sites.

Therefore, the required application of at least two plants for the determination of differences in transport may lead to faulty interpretations of results, if these are expressed on a retention-time basis only. In the present paper, the values for the retention-times (t, see Fig. 2) are used in combination with xylem volume flow, applied Cd concentration and xylem vessel characteristics (Wolterbeek et al. 1984), yielding concentrations of Cd actually adsorbed at the vessel walls (Table 1).

The presence of citric acid not only resulted in reduced amounts of Cd adsorbed to the xylem cell walls but also in smaller values for the rate constant of the lateral escape k (Table 1). The k values were invariably calculated on basis of the total Cd concentration present in the xylem fluid, thus k values observed in the presence of citric acid will be a combination of the escape rates of free and complexed Cd. The free Cd²⁺ concentration in the applied solution in the presence of citric acid could be calculated as approximately 6-8 % of the Cd²⁺ concentration in situations without citric acid. However the complex relations between the various Cd chemical forms (ionic, complexed to cell walls and citrate) existing in the xylem channels do not permit a simple control experiment with strongly reduced free Cd²⁺ solution concentration. Furthermore, the time required for saturation of the exchange sites using a 0.02 mM Cd²⁺ solution amply exceeds the practical experiment time available in short-term uptake studies using plant sub-systems under conditions described.

Essentially, the rate of lateral escape of Cd²⁺ from the xylem vessels may depend on concentration: results of Wolterbeek et al. (1985b) indicate that the value of escape rate constant may be inversely related with the Cd²⁺ concentration. Therefore, the reduced value of k observed in the presence of citric acid shows the reduction of the rate of lateral escape of Cd due to complex formation.

The quantitative results of the present study clearly show that complex formation affects cation transport in the xylem, not only by reduced adsorption to the vessel walls but also by a decreased rate of lateral escape.
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References


Cadmium in stem-leaf systems


Wolterbeek, H.Th., J. Van Luijpen and M. De Bruin (1985a). Actual escape area and the lateral escape from the xylem of the alkali ions Na\(^+\), K\(^+\), Rb\(^+\) and Cs\(^+\) in tomato. Physiologia Plantarum 65, 467-475.


CHAPTER 5

Cadmium - citric acid - xylem cell wall interactions in tomato plants

Abstract.

Mutual interactions between cadmium ions, citric acid and xylem cell walls were examined. Cadmium and citric acid were measured as $^{115}\text{Cd}$ and $[1.5-^{14}\text{C}]$ citric acid respectively. Xylem cell walls were obtained by bacterial demolition of tomato stem sections (Lycopersicon esculentum Mill, cv. Tiny Tim), and applied as ion-exchange columns. The xylem column material carried 2.4 l H$_2$O kg$^{-1}$ dry weight, and was temporarily capable of buffering perfusates at pH 5.7. Sorbed cadmium and citric acid were determined from H$_2$O and HCl rinses after perfusion periods. In all experiments total cadmium and/or citric acid recoveries were better then 98 %, indicating both the effectiveness of the rinses applied and the possibility of full regeneration of the xylem column. The results indicate that the presence of 2.45 mM citric acid causes an approximately 50 % reduction of adsorbed cadmium levels, irrespective of the applied total cadmium concentrations (0.04 - 0.4 mM Cd(NO$_3$)$_2$.4H$_2$O). This reduction is probably related to a corresponding reduction to approximately 2 % of the control applied free Cd$^{2+}$ concentration, the latter also independent of the total cadmium concentrations. Furthermore, without inducing positively charged citrate complexes in the applied solution, the presence of cadmium resulted in increased levels of citric acid absorbed in the xylem column. The Donnan Free Space accumulation of citric acid in the presence of Cd(NO$_3$)$_2$.4H$_2$O, observed in the experiments described, could be expressed by its distribution coefficient, as approximately fifteen times the control accumulation. These data indicate that the xylem column may operate as a ligand exchanger, suggesting the importance of metal ions for the longitudinal and lateral movement of organic complexing compounds in the xylem.

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Introduction

Cadmium is considered as a relatively rare element, with soil concentrations ranging from 0.07 to 1.0 mg.kg\(^{-1}\) dry weight (Kabata-Pendias and Pendias 1985). Cadmium is also known to be one of the most toxic heavy metals released in the environment and its continuous introduction in the biosphere as a result of industrial activities presents a hazard to man and the ecosystem (Friberg et al. 1974, Polar and Küçükcezzar 1986). Although cadmium is considered to be a non-essential element for plants, it is effectively absorbed by both root and leaf systems (Page et al. 1972, John 1973, Bingham et al. 1975, Kabata-Pendias and Pendias 1985). Resulting elevated levels of cadmium in the leaves may cause severe phytotoxic effects on growth, photosynthesis, transpiration, CO\(_2\)-fixation and dark respiration (Page et al. 1972, Bazzaz et al. 1974, Lamoreaux and Chaney 1978, Marschner 1983, Kabata-Pendias and Pendias 1985).

Long-distance transport of cadmium taken up from the soil by plant roots mainly occurs through the xylem vessels. It can be described as a bulk flow, mainly driven by transpiration. The substances present in this flow, which is essentially a movement through the apoplast of the xylem, become involved in processes as adsorption to mainly negatively charged sites present in the cell walls (Bell and Biddulph 1963, Fergusson and Bollard 1976, Wolterbeek et al. 1984) and lateral escape from the moving solution in the vessels into adjacent intercellular spaces and free space of the cell wall (Van Bel 1978, Wolterbeek et al. 1984), uptake by metabolic compartments of xylem parenchyma and other cells (Biddulph et al. 1961, Jacoby 1965).

In-vivo studies of long-distance transport of elements in plants, although involving extensive mathematical treatment of data obtained to account for all processes mentioned above (Wolterbeek 1986), generally do not yield results which show distinct contributions of adsorption, escape and uptake to overall longitudinal transfer phenomena.

For this reason, in most cases attention is devoted to a particular effect, both in in-vivo systems and in isolated organs or tissues (Ferguson and Bollard 1976, Van Bel 1978, Sentenac and Grignon 1981). Generally, element transport along
xylem vessels is indicated to occur only after saturation of the exchange sites (Ferguson and Bollard 1976, Petit and Van de Geijn 1978, Van de Geijn and Petit 1978, 1979), although, according to Hefferich (1962) no simple quantitative relation exists between the relative strength of the interaction between ion species and fixed groups and the relative mobility of the species. Apparently, effects of charged cell wall sites may be seen particularly in the light of disturbances of local ionic concentrations (Thibaud et al. 1984), with possible consequences for the magnitude of transport across walls (Dalton 1984, Wolterbeek 1987). Adsorption processes in xylem cell walls may therefore not only be important with respect to stem retention times (Ferguson and Bollard 1976, Senden and Wolterbeek 1990) and longitudinal movements, but should also be seen as relevant for the overall process of lateral transfer.

The isolation and purification of xylem cell walls (Van Bel 1978, Wolterbeek 1986) permits experiments specifically aimed at further clarification of adsorption behaviour of ions, in relation to the characteristics of xylem wall material. However, ion behaviour in xylem vessels is indicated to be strongly influenced by the presence of organic compounds (Senden and Wolterbeek 1990). 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 compound complexes in xylem fluid (Tiffin 1966, 1970, White et al. 1981a,b,c, McGrath and Robson 1984) may seriously affect the movement in the xylem vessels. On the other hand, the question may be raised as to whether cations influence the behaviour of organic compounds, possibly resulting in changes in (initial) longitudinal and lateral transfer rates and leading to ion-specific variations in distribution of organic compounds in plants.

In the present paper, experiments were carried out with isolated xylem walls of tomato (Lycopersicon esculentum Mill, cv. Tiny Tim), cadmium ions and citric acid. Citric acid was selected from the organic xylem constituents, because it may be regarded as important with respect to observations discussed above, based on its reported effect on the xylem transport of several ions in various plant species (Tiffin 1970, Bradfield 1976, Ferguson and Bollard 1976, Van de Geijn and Pikaar 1982, McGrath and Robson 1984, Senden and Wolterbeek 1990). The xylem material was applied as a cation exchange column (Van Bel 1978), in
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experiments where adsorptive behaviour of cadmium and citric acid, measured as the radiotracers $^{115}$Cd and [1.5-14C]citric acid was examined. A number of xylem wall characteristics, relevant for adsorption processes, were determined. Furthermore, mutual interactions between cadmium and citric acid were measured in experiments where both compounds were eluted through the columns filled with xylem material.

Materials and methods

Plant material

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

Xylem cell wall material

Xylem cell wall material was separated from the stem internodes by bacterial demolition. Stem internodes were submerged in tap water at 20 °C for 40 days, after which the unaffected xylem cell wall parts of the internodes were isolated by rinsing in tap water.

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 days.

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 ml distilled water, floating pieces were removed and the remainder was packed between two quartz filters in a plastic tube (6 mm diameter, 50 mm length).

The isolated xylem cell wall material was protonated by elution of 100 mM HCl through the column. The $\text{H}^+$-form was converted by 1.0 M NaCl into the $\text{Na}^+$-form, which was taken as the standard state. $\text{Cl}^-$ was removed by shortly rinsing with distilled water. The acidity of the standard state effluent was pH 6.3 ± 0.1.
Cd - isolated cell walls

In the course of the experiments, the column was renewed 3 times, due to breakdown of tube materials, but all columns were made and prepared as described above.

Element solutions

In the experiments described a number of solutions containing [1.5-\(^{14}\)C]citric acid, \(^{115}\)Cd and/or \(^{82}\)Br were applied. In all experimental solutions pH was set with KOH.

Determination of the column void volume was carried out with help of 0.16 mM KBr solutions containing \(^{82}\)Br, for which acidity was set to pH 6.

Citric acid buffer solutions applied consisted of citric acid-NaOH mixtures, containing 1.0 mM citric acid and NaOH ranging from 1.7 to 3.3 mM, with pH values 4.5, 6.4 and 7.8 (Perrin and Dempsey 1974).

The apparent adsorption capacity of the xylem cell wall material was determined with 5.7 mM Cd(NO\(_3\))\(_2\).4H\(_2\)O solutions labeled with \(^{115}\)Cd (pH 5.1).

Adsorption-time relations were determined with 0.1 and 1.0 mM Cd(NO\(_3\))\(_2\).H\(_2\)O solutions labeled with \(^{116}\)Cd.

The cadmium elution through the xylem cell wall column and effects of the presence of citric acid were examined with 0.04, 0.08 and 0.4 mM \(^{116}\)Cd spiked Cd(NO\(_3\))\(_2\).4H\(_2\)O solutions with or without 2.45 mM \(^{14}\)C labeled citric acid.

Effects of the presence of cadmium on citric acid behaviour towards the wood powder were determined by application of 0.0095 mM \(^{14}\)C labeled citric acid solutions, with and without 0.87 mM Cd(NO\(_3\))\(_2\).4H\(_2\)O.

Production of radioisotopes and measurement equipment

The radiotracers \(^{82}\)Br (t\(_{1/2}\) = 35.4 h, \(\gamma\)-ray measured at 776 keV) and \(^{116}\)Cd (t\(_{1/2}\) = 53.5 h, \(\gamma\)-ray measured at 527 keV) were produced by neutron activation of KBr and Cd(NO\(_3\))\(_2\).4H\(_2\)O in the Hoger Onderwijs Reactor at IRI, Delft, at a thermal neutron flux density of 0.5 \(\cdot\) 10\(^{17}\) m\(^{-2}\).s\(^{-1}\) for 0.25 hours. The activities were 150 GBq.g\(^{-1}\) for \(^{82}\)Br and 0.3 - 0.5 GBq.g\(^{-1}\) for \(^{116}\)Cd respectively.

[1.5-\(^{14}\)C]citric acid (1.85 MBq.ml\(^{-1}\), 20.8 GBq.g\(^{-1}\)) was purchased from Amersham International, U.K.

\(^{82}\)Br and \(^{116}\)Cd measurements were carried out using a \(\gamma\)-ray 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 were carried out according to the methods described by De Bruin and Korthoven (1972, 1974) and Korthoven and De Bruin (1977).

$^{14}$C was radioassayed in 10 ml Lumagel (Lumac, Landgraaf, The Netherlands) by liquid scintillation counting (MINAXI Tri-Carb 4000 Canberra Packard).

Speciation calculations

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

Experiments

During experiments spiked solutions ranging in volume from 1 to 40 ml were perfused over the xylem cell wall column. After perfusion the column was washed with 3 - 5 ml distilled water, 3 - 5 ml 100 mM HCl and 3 - 5 ml distilled water respectively. All perfused fractions (fraction volumes ranging from 0.05 to 2.0 ml) were collected in a fraction collector. Acidities of applied solutions and recovered fractions were measured with a micro pH-electrode (Orion Research Model 601 A).

For determination of cadmium adsorption with time, cut pieces of xylem cell wall material were submerged in 0.1 or 1.0 mM $^{115}$Cd labeled cadmium solutions, with pH 4.00 and 3.65 respectively. After various times, the xylem cell wall material was separated from the solution, dried and measured by $\gamma$-ray spectrometry (Wolterbeek 1986).
**Results**

**General column characteristics**

**Xylem wall water.** The total void volume of the xylem cell wall column was determined with help of the radiotracer $^{82}\text{Br}$ (in KBr). Xylem cell walls have only few positively charged binding sites, resulting in the absence of retention or accumulation of $\text{Br}^-$ in the column material (Wolterbeek 1986). From the $^{82}\text{Br}$ fractions recovered, the total void volume (i.e. column Water Free Spaces including tube volumes) could be calculated. In experiments, 1.0 ml 0.16 mM $\text{K}^{82}\text{Br}$ solution was applied to the column. After KBr perfusion, the column was washed with 3 ml distilled water, to wash out any possibly retained activity, followed by rinses with 3 ml 100 mM HCl and 3 ml distilled water respectively. Fig. 1. presents the course of the $\text{K}^{82}\text{Br}$ perfusion showing the amounts of $^{82}\text{Br}$ in relation to the total volume of recovered solution.

![Graph](image)

**Figure 1.** $\text{K}^{82}\text{Br}$ perfusion through the xylem cell wall column. The horizontal arrow indicates the 100 % bromine level during application of the solution. Insert (scale enlarged): the $\text{Br}^-$ concentrations yielded during the first 0.8 ml of the perfused solution. * and • are different experiments.
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From the time-courses of the $^{82}$Br$^-$ fractions the void volume of the columns were calculated as 0.63 ± 0.04 (ml ± SE). The total of inner tube volumes below the packed xylem wall material was measured separately as 0.39 ml, leaving approximately 0.24 ml for Br$^-$ available spaces in 100 mg dry weight of the xylem material itself. This value is in full agreement with data on wall water values obtained with cut pieces of xylem wall material, as reported by Wolterbeek (1987), indicating the absence of effects on wall water characteristics by powdering.

Apparent xylem wall cation exchange capacity. The apparent xylem cell wall cation exchange capacity (CEC) was determined by perfusion of 25 ml 5.7 mM Cd(NO$_3$)$_2$.4H$_2$O solution labeled with $^{115}$Cd (pH 5.1). Fig. 2 presents the fraction of initially administered cadmium in various recovered perfusate volumes. Total cadmium-recovery after H$_2$O and HCl rinses (calculated from the summation of all fractions collected) was 101 ± 2 %. From the 6.8 μeq cadmium recovered by rinsing with 5 ml 100 mM HCl a CEC value of 0.068 eq.kg$^{-1}$ dry weight could be derived. Considering a Donnan Free Space (DFS) volume of 0.15 l.kg$^{-1}$ dry weight (Wolterbeek 1987) the DFS apparent CEC can be estimated as approximately 450 meq.l$^{-1}$, a value which is in full accordance with data reported elsewhere (Pitman 1977, Van de Geijn and Petit 1979, Van de Geijn, Petit and Roelofsen 1979, Wolterbeek 1987).

Xylem wall water acidity. Xylem fluid in plants is reported to behave as a rather strictly pH buffered fluid (Fergusson and Bollard 1976, Van Bel and Hermans 1977, Van Bel 1978, De Boer 1985). Perfusion experiments were carried out with citric acid-NaOH buffer solutions (pH values 4.5, 6.4, and 7.8 respectively) to detect any column system ability to direct and set perfusate pH values. Micro-electrode pH determinations in recovered fraction volumes yielded results as shown in Fig. 3, indicating a temporary perfusate pH of approximately 5.7 and showing the buffering ability of the wood material. To prevent any significant changes in pH values of fluids administered in following perfusion experiments, the acidity of the relevant solutions applied were set to 5.7.
Cd - isolated cell walls

Figure 2. Per cent recovery of applied cadmium in successive perfused volumes. Successive treatments: (1) loading with $^{115}\text{Cd}$ spiked Cd(NO$_3$)$_2$.4H$_2$O solution, (2) wash with distilled water, (3) wash with 100 mM HCl, (4) wash with distilled water. Total recovery 101 ± 2 %.

Figure 3. Change of pH during perfusion with citric acid-NaOH buffers through the xylem cell wall column. The acidity is related to the volume of the buffers perfused through the column. The pH values of the applied citric acid-NaOH buffers were 7.8 (——), 6.4 (—•—) and 4.5 (•••).
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Cadmium-xylem cell wall equilibrium. In separate experiments with cut pieces of xylem cell wall material (cf Wolterbeek 1987) cadmium adsorption was followed in time (Fig. 4). The results indicate that the time needed for equilibrium between free and adsorbed cadmium ions does not depend on concentration. Furthermore, eventual full equilibration values for adsorbed cadmium concentrations do not drastically differ from 2.5 h values, suggesting fast equilibration, but the quasi-plateaus observed also indicate possible spatial effects (Demarty et al. 1978, Sentenac and Grignon 1981) and/or heterogenous cell wall charge characteristics (Morvan et al. 1979, Van Cutsem and Gillet 1981, 1982). Flow rates in all column experiments (0.26 ml.min⁻¹) implicate experiments lasting for approximately 1 to 1.5 h, but also indicate that only 55 s were needed for total column fluid volume replacements.

![Graph showing adsorption of cadmium to isolated xylem cell wall material](image)

**Figure 4.** The adsorption of cadmium to isolated xylem cell wall material in relation to the adsorption-time. The applied solutions contained 0.1 (•••) and 1.0 (+−+) mM Cd(NO₃)₂·4H₂O.
**Perfusion experiments**

In a series of experiments the behaviour of cadmium towards the xylem material was determined, with cadmium applied as Cd$^{2+}$ and as cadmium-citrate and with total cadmium concentration varying from 0.04 to 0.37 mM. Fig. 5 presents data for two cadmium concentrations, showing the cadmium contents in the fractions recovered during cadmium application and the H$_2$O and HCl washing periods. In Fig. 5, the variations in positions of cadmium peaks from H$_2$O and HCl rinses correspond to small variations in total volumes of cadmium solutions applied. The results show that fractional cadmium recovery approaches 100 % during cadmium application, both in cationic and in complexed cadmium experiments. Application of citric acid resulted in reduced total volumes perfused before stabilization of cadmium recovery values, most prominent with small cadmium concentrations applied and indicating smaller steady state levels of adsorbed cadmium under citric acid conditions. After the first H$_2$O wash, meant to clear the column from non-adsorbed cadmium, the subsequent 5 ml HCl wash released all cadmium adsorbed (Fig. 5); the results clearly show the differences in apparent equilibrium levels of adsorbed cadmium between cationic and complexed cadmium situations.

Tab. 1 presents the quantities of cadmium determined in various fractions recovered, which were pooled for the three perfusion periods. Several experiments were carried out twice, to check the absence of extravagant variations in results. Mass balances show the total cadmium yield after experiments as invariably close to 100 %, indicating the effectiveness of the desorption rinses applied. The cadmium recoveries during H$_2$O rinses may be partly due to disturbances of the equilibria reached during the application periods, but were invariably higher in the presence of citric acid. Cadmium recoveries by HCl rinses were 141 ± 2 µg and 183 ± 4 µg for the 0.08 and 0.04 mM cadmium concentrations applied (cationic applications). These smaller amounts of cadmium adsorbed, at the higher cadmium concentrations applied, probably result from differences in total volumes perfused (0.04 mM: approx. 40 ml, 0.08 mM: approx. 20 ml), which should be associated with possible effects of variations in experiments duration (cf. Fig. 4).
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Figure 5. Perfusion of cadmium through the xylem cell wall column. The solutions contained 0.08 mM cadmium (A) and 0.4 mM cadmium (B) in absence (---) and in presence (-----) of 2.5 mM citric acid. Successive treatments: (1) loading with applied solution, (2) wash with distilled water, (3) wash with 100 mM HCl, (4) wash with distilled water. The horizontal arrow indicates the 100% cadmium level during loading with the applied solution.
Cd - isolated cell walls

Tab. 1 further shows that the presence of citric acid invariably resulted in values for cadmium adsorption equilibria reduced by a factor 2, irrespective of cadmium concentrations applied. However, the relative reductions in free Cd\(^{2+}\) concentrations by citric acid applications could also be calculated as independent of the total cadmium concentrations in the solutions used in the experiments (Tab. 2). These results indicate the non-linearity of the relation between free Cd\(^{2+}\) concentrations in the solution and cadmium concentrations adsorbed in the xylem material.

**Table 1.** Values (± SE) of the amount of cadmium (Cd) recovered after perfusion on the xylem cell wall column. Total recovery is presented as 100 times the ratio between the total of fraction recoveries and the amount of cadmium applied.

<table>
<thead>
<tr>
<th>n</th>
<th>applied Cd (mM)</th>
<th>applied citric acid (mM)</th>
<th>cadmium recovered (µg)</th>
<th>Cd application period</th>
<th>5 ml H(_2)O washing period</th>
<th>100 mM HCl washing period</th>
<th>total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.043 ± 0.003</td>
<td>-</td>
<td>3.8 ± 0.1</td>
<td>3.0 ± 0.5</td>
<td>183 ± 4</td>
<td>100 ± 4</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.039</td>
<td>2.45</td>
<td>91</td>
<td>4.5</td>
<td>81</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.083 ± 0.005</td>
<td>-</td>
<td>55 ± 2</td>
<td>8 ± 1</td>
<td>141 ± 2</td>
<td>100 ± 1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.081</td>
<td>2.45</td>
<td>86</td>
<td>13</td>
<td>81</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.38</td>
<td>-</td>
<td>577</td>
<td>36</td>
<td>221</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.37 ± 0.03</td>
<td>2.45</td>
<td>652 ± 59</td>
<td>47 ± 16</td>
<td>106 ± 6</td>
<td>99 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6 presents results of [1.5-\(^{14}\)C]citric acid measurements, showing the behaviour of citric acid towards the xylem cell walls, in relation with the presence of cadmium ions. The presence of 0.87 mM Cd(NO\(_3\))\(_2\).4H\(_2\)O in a 0.0095 mM citric acid solution resulted in changes in the perfusion characteristics of citric acid, showing most clearly during the HCl wash period (see insert Fig. 6).
Figure 6. Per cent recovery of citric acid in successive perfused volumes. The applied solutions contained 0.0095 mM [1.5-13C]citric acid (•-•) and 0.0095 mM [1.5-14C]citric acid in the presence of 0.87 mM Cd(NO₃)₂·4H₂O (—•). Successive treatments: (1) loading with applied solution, (2) wash with distilled water, (3) wash with 100 mM HCl, (4) wash with distilled water. Insert, scale enlarged for per cent recoveries of citric acid during washing procedures (2), (3) and (4) as described above.

The increased quantities of citric acid in the perfusate during HCl rinsing should be interpreted as resulting from accumulation of citric acid in the charge-influenced water spaces (Donnan Free Space) of the xylem column. This accumulation may be due to shifted chemical speciation characteristics of citric acid in the applied solution (Tab. 2), but may be seen particularly in the light of the possibility of ligand exchange in the xylem column's charged-influenced phases (Helfferich 1961).
Table 2. Chemical cadmium (Cd) and citric acid (cit) species at pH 5.7, for various total concentrations. Species are presented as percentage of the total concentration, the values given to the nearest full percentage.

<table>
<thead>
<tr>
<th>total concentration (mM)</th>
<th>percentage of total [Cd] or [cit]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cdclt^-</td>
</tr>
<tr>
<td>Cd</td>
<td>cit</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.00</td>
<td>2.5</td>
</tr>
<tr>
<td>0.04</td>
<td>2.5</td>
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<tr>
<td>0.08</td>
<td>2.5</td>
</tr>
<tr>
<td>0.40</td>
<td>2.5</td>
</tr>
<tr>
<td>0.00</td>
<td>0.0095</td>
</tr>
<tr>
<td>0.87</td>
<td>0.0095</td>
</tr>
</tbody>
</table>

* Trace amount < 0.5 %.
Chapter 5

Discussion

The present paper deals with the effects of citric acid on the behaviour of cadmium ions towards tomato wood powder, measured in ion-exchange xylem column experiments. Further, attention was paid to possible effects of the presence of cadmium on the perfusion behaviour of citric acid. The xylem material applied was obtained by bacterial demolition of stem internodes, which is different from the mechanical isolation procedures reported earlier (Van Bel 1978, Van Bel et al. 1981, Wolterbeek 1986). The first series of experiments was carried out to determine possible effects of the isolation procedure and the method of preparation of the material on the xylem cell exchange characteristics. Both the amounts of water carried per unit weight ($^{82}$Br experiments, Fig. 1), and the apparent cation exchange capacity (Fig. 2) were in full agreement with data reported elsewhere (Wolterbeek 1987), coinciding for isolated material (Wolterbeek 1987), as well as for in-vivo measurements (Van de Geijn and Petit 1979, Van de Geijn, Petit and Roelofsen 1979, Wolterbeek et al. 1985b). These data indicate that results obtained with the xylem column may be interpreted as comparable with exchange results reported for in in-vivo situations, although it should be kept in mind that in stems, lateral escape from xylem vessels (Wolterbeek et al. 1984, 1985a) will attenuate concentrations of elements moving in longitudinal direction, which did not occur in the present column experiments. However, the absence of complications produced by adsorption and escape phenomena was considered to be advantageous rather than an unrealistic simplification.

Buffering characteristics of the column material (Fig. 3) were checked and found to be in agreement with data concerning pH-buffered xylem fluids (Ferguson and Bollard 1976, Van Bel and Hermans 1977, Van Bel 1978, De Boer 1985), and may be related to the weak acid characteristics of the galacturonic acid (pectins) constituents of the xylem walls (Helfferich 1962, Sentenac and Grignon 1981). However, differences between pH changes in wood powder and in internodes (Van Bel and Hermans 1977) suggest that in living tissues not only the cell walls may be responsible for the specific pH values during perfusion, but that xylem parenchyma cells ($H^+$ pumps) may also modify pH conditions in xylem cell walls and xylem sap.
Cd - isolated cell walls

(Clarkson and Hanson 1986, Sentenac and Grignon 1987, Toulon et al. 1989).

 Principally, in perfusion experiments information on adsorption may be obtained from both the contents of initial fraction volumes and the H₂O and the HCl rinses. The adsorption vs. time relations observed (Fig. 4), showing a quasi-plateau and slow further adsorption after rapid initial binding, indicate that more reliable information is obtained from the H₂O and especially the HCl fractions. Therefore, Tab. 1 invariably presents adsorption data from cadmium concentrations in fraction volumes during HCl washing periods. Furthermore, Tab. 1 indicates excellent total recovery and reproducibility, irrespective of concentrations used or total volumes perfused.

 Considering data from Fig. 2 and Tab. 1, perfusion experiments yielded cadmium-adsorption values (Tab. 1, HCl rinses) 2-4 times smaller than the apparent CEC value (Fig. 2), which indicates the absence of saturation effects on cadmium behaviour observed. The relatively small differences in cadmium-adsorbed values with increasing applied concentrations (control experiments, Tab. 1) should be regarded to result from differences in total volumes perfused (time relations, see Fig. 4) and from changes in relative accumulation values: the absolute value of the Donnan potential is reported to be inversely related to the solution’s ionic strength and applied element concentrations (Helfferich 1962).

 The presence of citric acid, leading to free applied Cd²⁺ concentrations of 2 % of the control values (Tab. 2), resulted in 50 % reductions of adsorption values (Tab.1). In both control and citric acid situations, cadmium levels in perfusate fractions approached equilibrium (100 %) concentrations (Fig. 5). Therefore, the reduced adsorption observed should attributed to the presence of citric acid, the latter however without effects on eventual cadmium concentrations in the perfusate. These observations make it hard to imagine the phenomenon of metal accumulation in the xylem cell walls as significantly contributing to tolerance mechanisms in plants (cf. Thurman and Collins 1983). The absence of Zn in Zn-citrate perfusions in pine stem sections internodes as reported by McGrath and Robson (1984) and interpreted as being due to the dynamic nature of the Zn-citrate and Zn-cell wall equilibria, are in contrast with the present results, probably due to lateral escape processes, concerning both Zn and Zn-citrate complexes.

 The results presented in Fig. 6 indicate that the behaviour of citric acid
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towards the wood powder may be influenced by the presence of cadmium ions. The increased quantities of citric acid recovered during HCl washes show its accumulation in the xylem column. Based on the available total column free water spaces (\(^{43}\)Br measurements, see Fig. 1), molar distribution coefficients (defined as the ratio between concentrations in the sorbent and in the applied solution, see Helfferich 1962) could be calculated as 1.0, and 1.23 for the control 0.0095 mM citric acid and 0.0095 mM citric acid in the presence of 0.87 mM Cd(NO\(_3\))\(_2\).4H\(_2\)O respectively. Recalculating the citric acid molar distribution coefficients after correction for non-accumulating tube-volumes, and considering only the DFS volume as accumulating phase, values were determined as 1.0 and 14.7 for the control 0.0095 mM citric acid and the 0.0095 mM citric acid in the presence of 0.87 mM Cd(NO\(_3\))\(_2\).4H\(_2\)O respectively.

White et al. 1981b growing tomato plants in normal nutrient solutions, calculated xylem citrate to be complexed for about 98 % of its total concentration, predominantly to Ca and Mg. This value is fully consistent with the fraction complexed citric acid as applied in experiments with 0.0095 mM citric acid in the presence of 0.87 mM Cd(NO\(_3\))\(_2\).4H\(_2\)O (Tab. 2, Fig.6). However, the virtual absence of any positively charged citrate complexes in the applied solution (Tab. 2) and the results shown in Fig. 6 indicate that citrate complexes may be formed within the exchange material itself: the xylem column material, containing Cd\(^{2+}\), may operate as a ligand exchanger (Helfferich 1961, 1962), by which ion exchange and coordination chemistry are combined.

These results indicate that the xylem cell walls may be a rather flexible exchanger. Displacement of complexing cations from the cell walls by ion exchange with other cations in the xylem fluid may severely alter it's ligand exchange characteristics: differences in ligand complex-strengths with metal ions are the basis of ligand exchange (Helfferich 1961, Takayanagi et al. 1985, Nielen et al. 1987). Further study is needed to get insight in the occurrence and extent of ligand exchange in the xylem vessels. Ligand exchange may emphasize the importance of metal ions for the characteristics of longitudinal movement and lateral escape from transport vessels of organic compounds in plants.
Cd - isolated cell walls

References


John M.K. 1973. Calcium uptake by eighth food crops as influenced by various soil levels of cadmium. Environmental Pollution 4, 7-15.

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CHAPTER 6

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

Abstract

Effects of cadmium on the sorption of citric acid in isolated xylem cell walls were investigated. 2.5 nM to 9.5 mM \([1.5-^{14}\text{C}]\text{citric acid}\) 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.l\(^{-1}\), 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 \(^{115}\text{Cd}^{2+}\) 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\(^-\)-Cd\(^{2+}\) and Donnan free space (DFS) [Cd(cit)H\(_2\)]\(^+\) complexes.

Simultaneous application of both citric acid and \(^{115}\text{Cd}^{2+}, ^{45}\text{Ca}^{2+}, \text{or} ^{28}\text{Mg}^{2+}\) 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)H\(_3\)]\(^+\) after protonation in the DFS. Sorption of citric acid was greatest in the presence of Ca\(^2+\), which was discussed in the light of the differences between Ca, Cd and Mg in their characteristics as coordinative 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.

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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 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
also 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 distributions 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 (e.g. 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 Semechkin 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 coordination 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 in the absence and presence of cadmium ions. 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.
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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 ca. 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, powderd and sieved into particles with diameters ranging from 0.125 to 0.250 mm. A 100 mg portion was shaken in 50 ml 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 ml (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 ml, leaving 0.24 ml 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 ml.g⁻¹ DW, based on data reported by Wolterbeek (1987a).
**Citric acid in cell walls**

**Xylem cell wall amino acid analysis**

1.6 mg xylem cell wall material was hydrolysed in the gasphase 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**

[1.5-14C]Citric acid (1.85 MBq.ml⁻¹, 20.8 GBq.g⁻¹) and 45CaCl₂ (83.2 MBq.ml⁻¹, 756.4 MBq.g⁻¹) were purchased from Amersham International U.K.

115Cd (t1/2 = 53.5 h) was produced by neutron activation of Cd(NO₃)₂ in the Hoger Onderwijs Reactor at Interfaculty Reactor Institute (I.R.I.), Delft, The Netherlands, at a thermal flux density of 0.5·10¹⁷ m⁻².s⁻¹ for 1 h. The specific activity obtained was 0.8 GBq.g⁻¹.

26Mg (t1/2 = 20.9 h) was produced by neutron activation of 6Li enriched Li-Mg alloys, 6Li: purchased from the Oak Ridge National Laboratory, USA), in the Hoger Onderwijs Reactor at I.R.I., Delft, The Netherlands, after which 26Mg (as MgCl₂) could be obtained by radiochemical separation (Kolar et al. 1991). The 26Mg specific activities of the preparations were 20-30 MBq.g⁻¹.

115Cd (γ-rays at 527 keV) was measured using a γ-ray 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). 26Mg (calibrated γ-rays at 400, 942, 1342, 1372 and 1589 keV) was determined using measurement equipment as described for 115Cd, but calibration, spectrum accumulation and analysis were carried out on network APOLLO computers (Hewlet Packard), according to methods described by Blaauw and Lindstrom (1993).

14C and 45Ca samples were mixed with 10 ml 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
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California, Berkeley, U.S.A. (Sposito and Coves 1988), with full considerations for mole balances, relevant thermodynamic equilibrium constants and ion strengths.

Experiments

During experiments, spiked 20 ml solutions (\(^{14}\)C, \(^{40}\)Ca, \(^{115}\)Cd, or \(^{28}\)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 ml distilled water, 5 ml HCl (0.1 M), and 5 ml distilled water respectively. All perfused fractions (volumes ranging from 0.5 to 2.0 ml) were collected in a fraction collector. Control citric acid perfusions were carried out with \(^{14}\)C-citric acid concentrations ranging from 2.5 nM to 9.5 mM. After column pre-loading with 1.0 mM Cd solutions, \(^{14}\)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 nM 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, 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 capacity 1000 meq.1\(^{-1}\) 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 especially 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
Citric acid in cell walls

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.l\(^{-1}\), 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.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration mM</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.

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
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\[ y = ax^b \]

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 (Hefflerich 1962a).

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

\[ S = y + z = ax^b + \frac{cax^b}{d + ax^b} \]

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 Figure 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.

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Figure 1. Citric acid accumulation (S) in tomato xylem cell wall DFS, as a function of the total citric acid concentrations (x) applied. The fitted solid line represents the function
\[ S = a \cdot x^b + c \cdot a \cdot x^d \cdot (d + a \cdot x^e)^{-1}, \]
the dashed lines show \( y = a \cdot x^b \) and \( z = c \cdot a \cdot x^d \cdot (d + a \cdot x^e)^{-1}. \) The insert shows the citric acid molar distribution coefficients (\( \lambda \)) in relation to the total citric acid concentration applied, \( \lambda = \frac{S}{x}. \)

Applied citric acid concentrations 2.5 nM, 9.5 \( \mu \)M, 3.0 \( 10^{-2} \) mM, 4.75 \( 10^{-1} \) mM, 2.5 mM, 6.0 mM and 9.5 mM
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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 it 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 Hefferich 1962a).

As stated already, the Figure 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 ¹⁴C 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 1966).

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 citric acid concentrations. Consequently, Cd effects were studied for 9.5 μM (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 ml 1.0 mM Cd(NO₃)₂ solutions. The column Cd retention was checked by prolonged (up to 20 ml) H₂O rinsing; Figure 2 shows the absence of appreciable losses in excess of the expected elution of column WFS Cd. Perfusion by 9.5 μM citric acid did not markedly affect Cd behaviour: Cd elution during the 20 ml 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).

Figure 2. Cadmium recovery (percentage of applied concentration) in fractions obtained during 20 ml distilled water wash, after initial column loading with 20 ml 1.0 mM Cd(NO₃)₂.
Table 2. Citric acid (cit) and Cd data in perfusion experiments with Cd-preloaded xylem column material. All data are given in mM concentration units. The data for elution are based on DFS volumes.

<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>-</td>
</tr>
<tr>
<td>1.07</td>
<td>$9.5 \cdot 10^{-3}$</td>
<td>6.4</td>
<td>294</td>
<td>$14.6 \cdot 10^{-3}$</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 ml H$_2$O wash (not the regular 5 ml H$_2$O rinsing).
  n.m. = not measured
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 (λ-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 μM and 9.5 mM concentrations. The results indicate the absence of any significant Cd-effect for 9.5 μM citric acid applications (z-domination), but shows a four-fold increase in citric acid sorption in the presence of Cd for 9.5 mM citric acid applications (y-domination). The first mentioned outcomes suggest 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 $R_2$Cd 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 μM 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 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) suggest 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 in cell walls

Figure 3. Citric acid (cit) molar distribution coefficients (\(\lambda\)) in tomato xylem cell wall DFS. Cit_{DFS} was recovered by 5 ml 0.1 M HCl rinses. Applied citric acid concentrations were 9.5 \(\mu\)M and 9.5 mM in control and Cd-preloaded columns respectively.

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

In a series of experiments, 20 ml 9.5 \(\mu\)M citric acid + Cd (1.0 mM), Ca (2.0 mM) or Mg (1.0 mM) with chloride or nitrate (see M & M-section) solutions were perfused through the xylem column (+M* conditions). For Cd additions, 2.5 nM 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 \(\lambda\)-values. The \(\lambda\)-values decreased with increasing citric acid concentrations (both control and +Cd conditions), showing the diminishing effect of the z-curve sorption. The \(\lambda_{+Cd}/\lambda_{control}\) 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 \(\lambda_{+Cd}/\lambda_{control}\) 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 \(\mu\)M (Table 3), a value from which a DFS Cd concentration may be expected of approximately 40 mM (based on \(K_{diss} = 10^{-4}\) M and fixed R\(^-\) = 0.4 M, Wolterbeek 1986, 1987a).

Figure 4. Citric acid (cit) molar distribution coefficients (\(\lambda\)) in tomato xylem cell wall DFS. Cit_{DFS} was recovered by 5 ml 0.1 M HCl rinses. Applied citric acid concentrations were 2.5 nM, 9.5 \(\mu\)M and 9.5 mM in the absence or presence of 1.0 mM Cd(NO\(_3\))\(_2\).
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</th>
<th>Fractional presence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M cit</td>
<td>M²⁺</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5×10⁻⁶</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>9.5×10⁻⁵</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>92</td>
</tr>
<tr>
<td>Cd</td>
<td>1.0</td>
<td>2.5×10⁻⁶</td>
</tr>
<tr>
<td>Cd</td>
<td>0.9</td>
<td>9.5×10⁻⁵</td>
</tr>
<tr>
<td>Cd</td>
<td>1.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Mg</td>
<td>1.0</td>
<td>9.5×10⁻⁵</td>
</tr>
<tr>
<td>Ca</td>
<td>2.0</td>
<td>9.5×10⁻⁵</td>
</tr>
</tbody>
</table>

* Trace amount < 0.5%
Table 4. Metal (M) and citric acid (cit) data, for experiments in which citric acid and Cd, Ca and Mg were perfused simultaneously. Recoveries rounded off to the nearest full percentage.

n.m. = not measured.

<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></td>
<td>M</td>
<td>cit</td>
<td>M</td>
</tr>
<tr>
<td>Cd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.5 \cdot 10^{-6}</td>
<td>n.m.</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>9.5 \cdot 10^{-3}</td>
<td>334</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>9.5</td>
<td>n.m.</td>
</tr>
<tr>
<td>Mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>9.5 \cdot 10^{-3}</td>
<td>375</td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td></td>
<td>394</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>9.5 \cdot 10^{-3}</td>
<td>371</td>
</tr>
</tbody>
</table>
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Table 5 presents the citric acid $\lambda$-values and $\lambda_{+M}/\lambda_{\text{control}}$ 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), whereas Mg affinity for the cell wall’s fixed negative charges is reported as an order of magnitude lower than the corresponding values for Cd and Ca (O’Sullivan 1969, Sentenac and Grignon 1981, Wolterbeek 1986). At first sight, additional support is delivered by the higher citric acid $\lambda$-values observed for Ca-situations (Table 5), which may be due to the differences in applied metal concentrations in the WFS (Table 4): Ca concentrations in the DFS were fully comparable to the ones for Cd and Mg.

However, although the data indicate that differences in sorption behaviour of citric acid may originate from metal-related differences in WFS citric acid chemical speciation rather than being brought about by pre-sorbed Cd, Ca or Mg in the cell wall, possible differences in coordination chemistry of the metal-citrate complexes may provide a further clue to the elucidation of the issues raised above (see Discussion section).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Citric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{+M}$</td>
</tr>
<tr>
<td>Cd</td>
<td>4.6</td>
</tr>
<tr>
<td>Ca</td>
<td>7.2</td>
</tr>
<tr>
<td>Mg</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Table 5. Citric acid $\lambda$-values in perfusions in the presence of Cd, Ca and Mg. Applied citric acid 9.5 $\mu$M, applied Cd, Ca and Mg concentrations 0.9, 2.0 and 1.0 mM respectively.
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 -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 Cl$^-$ association with positive charges may introduce citrate vs Cl$^-$ ligand exchange, the total citric acid behaviour thereby becoming dependent on relative affinities. Effective Cl$^-$ absorption was reported by Richter and Dainty (1989a,b), where there was relatively easy 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 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 $^{86}$Br absorption in tomato xylem cell walls ($^{86}$Br$^-$ vs Cl$^-$ exchange, Wolterbeek 1987b, Wolterbeek et al. 1987). Furthermore, Ritchie and Larkum (1982) reported full Freundlich-like $^{36}$Cl$^-$ absorption (applied 0.1 to 1000 mM Cl$^-$) in 1.0 M NaCl pre-treated Enteromorpha intestinalis cell walls. These data suggest that, generally, it may be relatively difficult to make the wall's anion
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exchange potential available for Cl\(^-\). In the present paper, possible 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\(_n\)Y\(_m\) salt in an exchanger of univalent fixed ionic groups should theoretically yield b as being equal to \((n+m)/m\), provided that \(Y_{DFS} << R^-\). 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 \(|z_Y|/R^-n/m\), \(z_Y\) 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 straight-forward 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 \(\times\) 10\(^{-6}\) M citric acid (Figure 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-neutralising 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, 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, Figure 3) underline the importance of complex formation in the applied solution, and, at the same time, stress the 1 to 1 molar ratio of sorbed Cd-cit complexes. The simultaneous supplying of Cd, Ca or Mg and citric acid (Tables 3,4,5, Figure 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 M&M section). S-curve citric acid fitting of the Figure 4 data for "+Cd" conditions, under assumed constant c and d values, actually yielded increases in the a constant (a ≈ 5.9) rather than in the b constant (b ≈ 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 valencies (Helfferich 1962a). The above indicates that the absence of excess Cd$^{2+}$ under Cd pre-loading conditions and 9.5·10$^{-5}$ M citric acid applications (Tables 2,4, Figure 3) may have resulted in the relatively high λ-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 λ-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 ("+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
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characteristics of complexes between citric acid and Cd, Mg or Ca.

Generally, citric acid tends to form monomeric tridentate species, by coordinating 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 β-carboxyl group. However, citric acid’s versatility is shown by it’s possible bidentate (central carboxylic and the hydroxyl group) or polymeric complexing (Pedrosa de Jesus 1987, Killa et al. 1991). The nature of the citric acid complexes is reported as bidentate for Ca, and tridentate for Cd or Mg (Johnson 1965, Francis et al. 1992), with comparable complex stabilities (Sposito and Coves 1988). Consequently, in the DFS, ongoing protonation of complexes ( [M(cit)]⁻ + H⁺= [M(cit)H]⁰, [M(cit)H]⁰ + H⁺= [M(cit)H₂]⁺, Amico et al. 1982), and the subsequent progressive coordinative unsaturation of the M-cit complexes, may leave the Cd and Mg complexes more unstable than Ca-cit, due to their higher degree of unsaturation (Helfferich 1962b). Furthermore, and apart from the Ca, Mg, Cd indistinguishable electrostatic attraction of [M(cit)H₂]⁺ complexes, when also considering possible covalent [RM(cit)H₂] 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 [M(cit)]⁻, which undergoes protonation on entering the wall DFS, in turn followed by electrostatic and/or covalent attraction of [M(cit)H₂]⁺.

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 et al. 1995).
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CHAPTER 7

Effects of cadmium on the longitudinal and lateral xylem movement of citric acid through tomato stem internodes

Abstract

Short-term experiments (2.5 h) were performed to gain insight in the effects of cadmium (Cd) on the longitudinal and lateral xylem movement of $^{14}$C-citric acid through tomato stem internodes (*Lycopersicon esculentum*, Mill, cv. Tiny Tim). The behaviour of citric acid was expressed by the concentrations of citric acid sorbed to the xylem walls (apparent capacity AEC), and by the rate constant $k$ for citric acid lateral escape.

For 0.05 mM citric acid applications, the presence of 1.0 mM cadmium resulted in approximately two-fold increases in values for apparent AEC and $k$, for the 9.5 mM citric acid concentrations, cadmium resulted in less than two-fold increase in AEC; but here $k$ did not change.

In the presence of Cd, the stem-length profile of absorbed citric acid was shown to be in accordance with stem-length profiles of Cd xylem concentrations, rather than with the xylem concentrations of citric acid itself. These results were discussed in the light of the expected presence of Cd-citric acid complexes in the xylem sap; effects on $k$ values were discussed with consideration for the possible processes of escape rate limitation in the stem.

M.H.M.N. Senden, A.J.G.M. van der Meer and H.Th. Wolterbeek
Chapter 7

Introduction

Organic and amino acids in the xylem are generally indicated to affect the behaviour of many metal ions during transport through the xylem channels (White et al. 1981a,b,c, Senden and Wolterbeek 1990). Among the organic acids, especially citric acid is considered as being of relevance, primarily due to the formation of relatively stable metal complexes (Tiffin 1970, Bradfield 1976, Ferguson and Bollard 1976, Van de Geijn and Pikaar 1982, McGrath and Robson 1984, White et al. 1981a, Senden et al. 1992a).

The effects of the formation of complexes on metal behaviour are mostly indicated by changes in the metal longitudinal bulk movements, the latter expressed by data on stem retention, general movement efficiency (Tiffin 1970 1972, White et al. 1981a,b,c, Kabata-Pendias and Pendas 1985), and apparent exchange capacity (Wolterbeek 1986, Senden and Wolterbeek 1990, Senden et al. 1992b). A related measure of the effects of complexation on the behaviour of the metal is the lateral escape (Senden and Wolterbeek 1990), expressed by the escape rate constant, which, for cadmium, was reported to be reduced due to the formation of cadmium-citric acid complexes (Senden and Wolterbeek 1990).

Remarkably, hardly any information exists on the effects of metals on the behaviour of xylem organic solutes. Effects of metals on the behaviour of organic solutes are described and used in various ways in chemistry, most especially in ligand exchange chromatography (Helfferich 1961, 1962, Navratil et al. 1975, Davankov and Senechkin 1977, Matejka and Weber 1990, Sud et al. 1992), but in plant transport physiology the only information available is reported by Senden et al. (1992b, 1994). In the latter papers, cadmium, calcium and magnesium were shown to affect the sorption of citric acid into columns of isolated tomato xylem cell walls: the total citric acid sorption increased up to 7-fold the control values by metal treatments.

The present paper describes perfusion experiments with isolated stem internodes of tomato plants (Lycopersicon esculentum Mill, cv. Tiny Tim). The experiments were set up to investigate the persistence of effects of Cd on citric acid sorption in isolated xylem cell walls (see Senden et al. 1994), in a plant system which also allows lateral escape. The data indicate the effects of cadmium on both
longitudinal movement (expressed by values on apparent anion exchange capacity, AEC) and lateral escape (expressed by the lateral escape rate constant) of citric acid during its transport through the xylem.

Materials and Methods

Plant material

Tomato plants (an inbred line of the red cherry tomato Lycopersicon esculentum Mill cv. Tiny Tim) were grown in hydroculture (solution composition according to Senden et al. 1992a) in a heated glasshouse at 25 °C and 75 % r.h.. Internodes (lengths and cross sections approximately 120 mm and 3.5 mm, respectively) of 12 weeks old plants were cut off under water and used throughout.

Preparation for perfusion

Silicone tubes were fitted around the morphological basal and apical ends of the internode, which was wrapped in aluminium foil to prevent stem-surface transpiration (Van Bel 1974). The internode was fixed and connected by the silicone tubing to a supply reservoir (morphological basis) and a pump (jp-12 Ismatec) (morphological apical end). The volume-flows during perfusions were about 0.85 ml.h⁻¹. The perfusate (controlled as 50-2500 µl) was collected in a fraction collector (Retriever II ISCO), under permanent time registration.

Internodes pre-perfusions

Before experiments, all internodes were standardized (Na-form) by pre-perfusion with 0.5 ml 10 mM Na₂EDTA solutions (pH = 6.0); excess Na and metal-EDTA were removed by subsequent rinsing with 0.5 ml distilled water. The applied 5 µmoles of EDTA was considered to remove metal ions from xylem vessel walls without significant effects on cell membranes in the internode tissues, assuming a) the high xylem volume flow and corresponding small quantities of EDTA escaping in lateral direction, and b) the small amount of introduced EDTA relative to a total internode Ca of about 50 µmoles (see also Van Steveninck 1965).

Determination of the average cross section Aₚ (Wolterbeek 1986) of the xylem vessels was carried out by pre-perfusion of 0.5 ml [³H]inulin (Van Bel 1978).
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Based on calculated $A_p$ values, average linear flow rates of about 0.75 mm.s$^{-1}$ were obtained.

Experiments

Perfusion experiments were carried out with 2.5 ml 0.050 mM and 9.5 mM citric acid solutions (spiked with $^{14}$C-citric acid), both in the absence and presence of 1.0 mM Cd(NO$_3$)$_2$. Before use, all solutions were set at pH 6.0 with KOH; further buffering was not considered necessary (Van Bel 1978, Senden et al. 1992b). After perfusion, xylem vessels were flushed by 1.0 ml distilled water. For the 9.5 mM citric acid applications, citric acid associated with xylem vessel walls was exchanged by direct perfusion of 2.5 ml 20 mM Ca(NO$_3$)$_2$, after which the internode was cut into pieces of 10 mm each.

For the 0.050 mM citric acid applications, internodes were cut into pieces prior to the perfusion with 20 mM Ca(NO$_3$)$_2$; in this case 0.1 ml volumes were applied to internodal sub-segments of 10 mm length. For this purpose, each of the pieces was connected by silicone tubing to a pump, similar to the routines applied in experiments with 100 mm internodes.

Cd perfusion was carried out using 2.5 ml of a 0.1 mM $^{155}$Cd spiked Cd(NO$_3$)$_2$ solution. Here, after perfusion and rinsing with 1.0 ml distilled water, the internode was cut into 10 mm pieces, and directly analysed for $^{155}$Cd. After analysis, each of the pieces was perfused with 100 $\mu$l 20 mM Ca(NO$_3$)$_2$; here both collected perfusates and the stem pieces were counted for $^{155}$Cd.

Radioisotope production and measurement

The radiotracer $^{155}$Cd ($t_{1/2} = 53.5$ h., initial specific activity 0.9 GBq.g$^{-1}$) was produced by neutron activation of Cd(NO$_3$)$_2$ in the Hoger Onderwijs Reactor at Interfaculty Reactor Institute (IRI), Delft, The Netherlands, at a neutron flux density of $0.5 \times 10^{16}$ m$^{-2}$s$^{-1}$ for 4.0 h. $^{155}$Cd measurements were carried out using a $\gamma$-ray spectrometer with a NaI-detector (Auto-Gamma Minaxi 5000 Packard, used settings 425-625 keV) coupled to a multichannel analyzer (Canberra).

$^{14}$C-citric acid (1.85 MBq.ml$^{-1}$, 20.8 GBq.g$^{-1}$) and $[^3]$H]inulin (54.1 GBq.mmol$^{-1}$, 10.4 GBq.g$^{-1}$) were purchased from Amersham International U.K.. For $^{14}$C-analysis, internode segments (50-100 mg fresh weight) were oxidized in a
Tri-Carb Sample Oxidizer Model 306 (Packard); $^{14}\text{CO}_2$ was trapped in an organic base Carbo-sorb (Packard), and mixed with $^{14}\text{C}$-Scintillator Permafluor V (Packard). $^{14}\text{C}$ and $^3\text{H}$ in aqueous samples were radioassayed in 10 ml Lumagel (Lumac Landgraaf, The Netherlands) by liquid scintillation counting (Tri-Carb 1600 TR Packard). $^{14}\text{C}$ in organic base samples (Carbo-sorb) were radioassayed in $^{14}\text{C}$-Permafluor V (Packard) by liquid scintillation counting (Tri-Carb 1600 TR Packard). $^{14}\text{C}$-recoveries (in sample treatment and oxydation procedures) were determined as 96 ± 9 % (n=24) by regular analysis of $^{14}\text{C}$ standards (Amersham International U.K.).

**Calculations**

**Apparent anion exchange capacity.** Absolute quantities of citric acid (Q) associated with the xylem vessel walls were determined from the Ca(NO$_3$)$_2$ flushes. The apparent anion exchange capacity AEC (concentration basis) was derived from the known average cross-sectional area ($A_p$) and the length of the translocation pathway (L), following Wolterbeek *et al.* (1985), assuming an average cell wall thickness $d$, as:

$$AEC = \frac{H \cdot Q}{A_p \cdot L \cdot d} \quad (1)$$

with $H = 0.013$ mm, as derived from the frequency distribution of vessel diameters (Wolterbeek *et al.* 1985). In all calculations, $d$ was taken as 2 μm (Van de Geijn and Petit 1979, Wolterbeek *et al.* 1985).

**Lateral escape rate constant.** Following the formulations by Horwitz (1958), for flow of solutes through plant transport channels, the solute concentration $C_p$ after perfusion will be related to the introduced solute concentration $C_0$ by:

$$\frac{C_p}{C_0} = e^{-\frac{k \cdot L}{L}} \quad (2)$$

with $L$ as the length of the translocation pathway, $v$ as the linear velocity of solution flow, and with $k$ as a first order reaction constant, expressing the rate at which solutes laterally escape from the moving xylem sap.
Chapter 7

The amount of solute in the surrounding tissues after perfusion time $t$ and at length $L$, $X_{t,L}$, can be expressed as

$$X_{t,L} = A_p \cdot k \cdot C_0 \cdot e^{-\frac{k \cdot L}{v}} \cdot (t - \frac{L}{v})$$  \hspace{1cm} (3)

with $A_p$ as the xylem vessel cross sectional area, and where the term $(t - L/v)$ expresses the time needed for the fluid front to reach distance $L$.

In the present 3 h. perfusion experiments, $t \gg L/v$; therefore, $X_{t,L}$ was expressed in integrated form as:

$$X_t = \int_0^L X_{t,L} dL = A_p \cdot C_0 \cdot v \cdot t \left(1 - e^{-\frac{k \cdot L}{v}}\right)$$  \hspace{1cm} (4)

with $X_t$ as the absolute amount of solute in the total internode at time $t$, from which $k$ was calculated as:

$$k = \frac{-\ln \left[1 - \frac{X_t}{(A_p \cdot C_0 \cdot v \cdot t)}\right]}{\frac{L}{v}}$$  \hspace{1cm} (5)

Speciation. The chemical speciation of Cd and citric acid was calculated with the help of the computer program SOILCHEM, developed at the University of California, Berkeley, USA (Sposito and Coves 1988), with consideration for mol balances, relevant thermodynamic equilibrium constants and ionic strengths.

For speciation calculations in xylem fluid, the effects of the fixed wall charges were taken into account only indirectly: determinations were based on the calculated total concentrations in bulk xylem fluid, $C_p$ which, of course were affected by the fixed wall charges.
Results

AEC

Application of 9.5 mM citric acid resulted in an AEC value for citric acid of 2.94 mM (Table 1). This value was calculated from the citric acid recoveries during Ca(NO₃)₂ flushing, under assumed \( d = 2 \mu \text{m} \). Similarly, under 1.0 mM Cd(NO₃)₂ perfusion conditions, the citric acid apparent AEC was determined as 4.84 mM (Table 1).

For the 0.05 mM citric acid perfusions, duplicate AEC-values were obtained in the absence and presence of 1.0 mM Cd(NO₃)₂ as (0.015 and 0.021 mM) and (0.043 and 0.044 mM) respectively. Obviously, Cd additions resulted in an about two-fold increase in AEC values for citric acid, for both citric acid concentrations. The order of magnitude of AEC values agrees with values found in experiments with columns of isolated xylem cell walls as reported by Senden et al. (1994). The two-fold increase in response to Cd perfusion corresponds to predictions distilled from earlier column experiments (Senden et al. 1994) for 0.05 mM citric acid perfusions.

Table 1. Amounts of citric acid adsorbed to xylem cell walls (AEC) and values for lateral escape rate constant \((k)\) of citric acid solutions with and without cadmium during transport through tomato stem internodes.

<table>
<thead>
<tr>
<th>Applied citric acid (mM)</th>
<th>Applied cadmium (mM)</th>
<th>Total citric acid recovery (%)</th>
<th>( A_p ) (mM²)</th>
<th>( k ) (h⁻¹)</th>
<th>AEC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>---</td>
<td>101</td>
<td>0.08</td>
<td>0.75</td>
<td>0.015</td>
</tr>
<tr>
<td>0.050</td>
<td>---</td>
<td>101</td>
<td>0.27</td>
<td>1.17</td>
<td>0.021</td>
</tr>
<tr>
<td>0.050</td>
<td>1.0</td>
<td>94</td>
<td>0.37</td>
<td>1.88</td>
<td>0.043</td>
</tr>
<tr>
<td>0.050</td>
<td>1.0</td>
<td>93</td>
<td>0.32</td>
<td>3.09</td>
<td>0.044</td>
</tr>
<tr>
<td>9.5</td>
<td>---</td>
<td>100</td>
<td>0.31</td>
<td>0.76</td>
<td>2.94</td>
</tr>
<tr>
<td>9.5</td>
<td>1.0</td>
<td>105</td>
<td>0.28</td>
<td>0.85</td>
<td>4.84</td>
</tr>
</tbody>
</table>

Cell wall sorption versus internode length

Figure 1 shows the cell wall citric acid sorption determined directly from \(^{14}\text{C}\)-counting, in 0.05 mM citric acid experiments. The lines drawn were obtained by
fitting of $F(L) = a \cdot e^{-bL} + c$ functions, with constants a, b and c. The marked decline in citric acid sorption with stem length under Cd conditions is corresponding with the escaped quantities of Cd rather than with the cell wall sorbed Cd concentrations (Fig 2). From the Cd-distribution (Fig 2), using eqs. 2-5, the $C_p/C_o$ values for xylem Cd could be calculated (Fig 3), which, together with the citric acid $C_p/C_o$ values (Fig 3), underline the importance of the presence of Cd in the xylem sap for the chemical speciation and subsequent cell wall sorption of citric acid (Senden et al. 1994). Based on Fig 3, the fractional citric acid presence as xylem Cd-complexes calculated as 97 %, 92 % and 76 %, for $L = 0$, 50 and 110 mm respectively. Here, it should be noted that effects from both fixed charges and lateral escape are expressed in the $C_p$ values determined.

**Figure 1.** Distribution of xylem cell wall sorbed citric acid, per stem length, for control 0.05 mM $^{14}$C-citric acid concentrations. The stem length is given from basal to apical direction. The data are averaged values (± SD) from perfusions with two different stem internodes. The lines were drawn after curve-fitting by $F(L) = a \cdot e^{-bL} + c$ (control: $r^2 = 0.79$, $a = 0.16 \pm 0.07$, $b = 0.15 \pm 0.08$, $c = 0.056 \pm 0.05$; with 1.0 mM Cd: $r^2 = 0.91$, $a = 0.47 \pm 0.22$, $b = 0.010 \pm 0.008$, $c = -0.03 \pm 0.25$).
Figure 2. Distribution of $^{115}\text{Cd}$ in the stem, after 1.0 mM $^{115}\text{Cd(NO}_3)_2$ applications. The stem length is given from basal to apical direction. Solid squares: total internode $^{115}\text{Cd}$, the line drawn was fitted by $F(L) = a.e^{-bL} + c$ ($r^2 = 0.98$). Constants derived ($\pm$ SD) were 8.98±0.48 (a), 0.031±0.004 (b) and 0.66±0.24 (c). Solid circles: xylem cell wall adsorbed $^{115}\text{Cd}$, line drawn was fitted by $F(L) = a.L + b$ ($r^2 = 0.08$), with constants - 0.00064±0.00069 (a) and 0.19±0.05 (b).

Lateral escape

For 9.5 mM citric acid, no appreciable Cd-related changes in $k$ could be observed (Table 1). The absence of any response to Cd may be due to regulation of the escape by surrounding tissues rather than by the transport of citric acid through the xylem cell walls (Wolterbeek et al. 1985). As a result, effects of the presence of Cd on the lateral escape of citric acid may not be unequivocally determined by calculating rate constants $k$.

For the 0.05 mM citric acid concentrations, simultaneous application of Cd(NO$_3$)$_2$ roughly resulted in a 2.5-fold increase in the lateral escape of citric acid (Table 1). At this concentration, an increase in cell wall sorption of citric acid (AEC-
Chapter 7

values, Table 1) concurs with an increase in the rate of lateral escape.

\[ \frac{C_p}{C_o} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \quad 120 \]

\[ \text{Stem length (mm)} \]

**Figure 3.** Xylem concentrations \( C_p \) of cadmium (squares) and of citric acid (control: solid circles, with 1.0 mM Cd: solid triangles), relative to the introduced concentrations \( C_o \), in relation to stem length. Introduced concentrations \( C_o \) were 1 mM (Cd) and 0.05 mM (citric acid).

**Discussion**

The present experiments were performed to investigate the persistence of effects of Cd on citric acid sorption in isolated xylem cell walls (Senden et al. 1994) in a plant system which allows lateral escape. Transport characteristics were expressed by apparent AEC and \( k \) transport parameters independent of variations in \( A_p \), \( L \), or \( v \) between individual plants (Wolterbeek 1986, Senden and Wolterbeek 1990).
Table 2. Citric acid and cadmium speciation in applied solutions (pH 5.7) in experiments with simultaneous addition of citric acid and Cd.
Fractional presence rounded off to the nearest full %.

<table>
<thead>
<tr>
<th>applied solution concentration</th>
<th>Cd</th>
<th>cit</th>
<th>Cd(^{2+})</th>
<th>Cd(\text{cit}^-)</th>
<th>Cd((\text{cit})_2^{4-})</th>
<th>CdH\text{cit}^-</th>
<th>CdH(_2\text{cit}^+)</th>
<th>cit(^3^-)</th>
<th>H\text{cit}^{2-}</th>
<th>H(_2\text{cit}^-)</th>
<th>H(_3\text{cit}^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd mM</td>
<td></td>
<td>cit mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 (\times 10^{-2})</td>
<td>95</td>
<td>5</td>
<td>91</td>
<td>*</td>
<td>*</td>
<td>4</td>
<td>*</td>
<td>2</td>
<td>2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>1.0</td>
<td>95</td>
<td>5</td>
<td>91</td>
<td>*</td>
<td>*</td>
<td>4</td>
<td>*</td>
<td>2</td>
<td>2</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* Trace amount < 0.5%
Chapter 7

The applied Cd(NO$_3$)$_2$ concentrations are in line with Cd concentrations used elsewhere in short-term experiments (Van de Geijn and Petit 1979, Van de Geijn et al. 1979, Senden and Wolterbeek 1990). Accumulated amounts of Cd in stem segments are in the same order of magnitude as reported by Florijn (1993), De Knecht (1994). Although these levels may be suspected to affect cellular metabolism (Kabata-Pendias and Pendias 1985, Adriano 1986), time aspects of Cd toxicity (Bazzaz et al. 1974, Lamoreaux and Chaney 1978, Wolterbeek et al. 1988, De Knecht 1994) make that Cd effects on cellular metabolism were not taken into consideration in the present short-term experiments.

The AEC data in internode tissues (Table 1) clearly demonstrate the effects of Cd on cell wall sorption of citric acid, as shown earlier for columns of isolated xylem cell wall material (Senden et al. 1994). For 0.05 mM citric acid, the two-fold increase corresponds to what was predicted by the column experiments. The less than two-fold increase for 9.5 mM citric acid, however, is markedly less than the 7-fold increase in column experiments (Senden et al. 1994). This discrepancy cannot be explained at present, but effects of the relatively high acid concentrations are not expected (see Van Bel 1978, Van der Schoot 1989, Senden et al. 1992b, for pH buffering in internodes and isolated xylem cell walls).

Column and internode experiments differed with respect to their pre-perfusion protocols. Columns were pre-perfused by subsequent rinses of HCl, NaCl and H$_2$O (Senden et al. 1994); the pre-perfusions in internodes were subsequent rinses of Na$_2$EDTA and H$_2$O. The latter rinsing solutions were adopted from Van de Geijn and Petit (1979) to prevent any possible cellular damage by HCl. Furthermore, and for similar reasons, although in column experiments the exchange capacity was determined by HCl flushes, in the present experiments use was made of 20 mM Ca(NO$_3$)$_2$. At first sight, the Ca(NO$_3$)$_2$ flushing may not have been as effective as the HCl in extracting all citric acid, especially at high citric acid applications, but the flushes were collected in 7 fractions, of which the first showed about 55% of the total citric acid recovery during Ca(NO$_3$)$_2$ flushing. Furthermore, the total recovery (105%, see Table 1) does not indicate any loss of citric acid.

All k-data determined were based on application of equation 5. In principle, use of equation 2 is more convenient to yield k values, but here the accuracy of k depends on the ratio C$_p$/C$_0$. As relatively high linear velocities of flow were used,
Citric acid in stem internodes

$C_p/C_o$ ratios were near unit ratio (see Fig 3), leading to high variances in $k$. Therefore, $k$ was invariably determined from stem data rather than from solution data.

The $k$-data (Table 1) indicate that Cd(NO$_3$)$_2$ increased the rate of citric acid lateral escape by a factor of about 2.5 for 0.050 mM citric acid, but hardly for 9.5 mM citric acid. It should be noted that, in 0.050 mM additions, citric acid is present as Cd$\text{citr}^-$ for 91% (Table 2), whereas in 9.5 mM additions, only about 10% of the citric acid is complexed to Cd (Table 2: Cd$\text{citr}^-$, Cd(cit)$_2^{4-}$).

For 9.5 mM citric acid, the two-fold increment in AEC (Table 1, 9.5 mM citric acid) does not appear to correspond with any increase in $k$ value. As all escape rates ($k$-values) were based on total citric acid concentrations, effects expressed by $k$ may be obscured by the small fractional presence of complexes between citric acid and Cd in 9.5 mM citric acid solutions. Moreover, the absence of effects may also be due to a shift in the regulation of the lateral escape from the rate of transport through the xylem cell walls to the processes of uptake in the surrounding tissues (Wolterbeek et al. 1985). The basis for this reasoning is a limited rate of uptake into cells (Michaelis Menten kinetics), whereas, at high applied concentrations, relatively high intrinsic citric acid fluxes through the xylem cell walls may be expected.

The present results indicate that Cd may affect the behaviour of citric acid during its transport through the xylem vessels, both by influencing its cell wall sorption and its rate of lateral escape. The physico-chemical nature of the processes involved, and the analogous results obtained with Ca and Mg in isolated tomato xylem cell walls (Senden et al. 1994), may suggest that the present data in internodes may have more general character than applicable for Cd only.

References

Chapter 7

Chromatography 141, 313-353.


Sud D., H.S. Hothi and B.S. Pannu (1992. Role of metal ions in the ligand-exchange separation of
Citric acid in stem internodes


CHAPTER 8

Discussion

General

The xylem sap may be regarded as a fast-conducting part of the apoplastic, connecting sites of water uptake (roots) with sites of transpiration (leaves), thereby functioning as a transport medium for solutes and plant nutrients.

Over the years, much attention has been given to the control and driving forces of xylem transport. Furthermore, the analysis of xylem transported substances has been a key aspect in the study of plant nutrition.

During the last several decades, an increasing number of studies has been aimed at the determination of effects of the formation of complexes of various metal ions on their xylem transport characteristics (Tiffin 1967 1972, Höffner 1970, Brown and Chaney 1971, Ferguson and Bollard 1976, White et al. 1981abc, McGrath and Robson 1984). The underlying reasoning for undertaking these studies evolved from a.o. physico-chemical, physiological and toxicological points of view. In these studies, however, the attention is focused entirely on the effect of the ligand on the transport behaviour of the metal.

The studies described in the various chapters of this thesis consist of experiments in which the effects of ligands on the xylem behaviour of metals were studied (Chapters 3-5), balanced by a number of experiments where the effects of metals on the behaviour of ligands were investigated (Chapters 6-7). The latter experiments were induced by the absence of information in plant physiology literature on the effects of metals on the behaviour of ligands. This is in strong contrast with a growing number of applications based on the effects of metals on ligand behaviour, predominantly described in literature on ligand exchange chromatography (Stokes and Walton 1954, Helfferich 1961, 1962, Walton 1973, Navratil et al. 1975, Davankov and Semechkin 1977, Takayanagi et al. 1985, Kato et al. 1986, Nielen et al. 1987, Matejka and Weber 1990, Sud et al. 1992).

The present discussion addresses methodologies, results and conclusions.
Chapter 8
Methodology

HPLC

RP-HPLC techniques were applied for qualitative and quantitative determination of amino acids and carboxylic acids in xylem exudate (Chapter 2), amino acid analysis of xylem cell wall material (Chapter 6) and citric acid determination in experimental solutions (Chapter 3).

Amino acids were determined by RP-HPLC analysis of amino acid PITC-derivatives (Chapter 2), or OPA-derivatives (Chapter 6); organic acids were determined by ion suppression RP-HPLC.

Ion suppression RP-HPLC for organic acids was developed to permit fast and easy analysis without any derivatization procedures; UV detection yielded reliable information on all relevant xylem exudate organic acids. The direct analysis was used to avoid problems, such as low and/or variable recoveries in derivatization (Zaura and Metcalf 1969, Stahl et al. 1972, Liebich 1990).

Speciation calculations

Throughout the thesis, the presence of the Cd-citrate complex plays a central role in all experiments. In Chapter 5, apart from the data on Cd-citrate, some data are included on citrate complexes of Mg and Ca. It should be noted that, although all results are discussed in terms of presence or absence of metal-citrate complexes, none of the data were obtained by direct determination of the complex itself. Instead, all analyses were aimed at the determination of the total concentrations of the complex components (Chapter 2). Based on the obtained total concentrations, relevant chemical species were computed using the SOILCHEM-program developed by Sposito and Coves (1988).

In fact, considering the HPLC approaches described in Chapter 2 for the determination of citrate, the applied 0.05 M KH₂PO₄/H₃PO₄-buffered mobile phase is likely to prevent any possible detection of Cd-citrate complexes, due to the formation of complexes between Cd and phosphate and the protonation of citrate.

The above approach was selected because of unavoidable shifts in complex-equlibria, and because of the strong-acid flushes used in recovering metals and citrate from xylem cell wall Donnan Free Space (DFS) phases (Chapters 5,6).
Discussion

However, the 1 : 1 stoichiometric Cd-citrate complexes (Cdcit\(^-\), CdHcit, CdH\(_2\)cit\(^+\)), predicted from the SOILCHEM program is in agreement with actual data on complexes in the DFS (Chapter 6), showing Cd and citrate to be acid-flushed in an unit molar ratio, after pre-flushing of non-complexed excess metal ions.

Notwithstanding the value of the present results, future work on xylem metals, organic acids and metal-organic acid complexes, will gain improvement by the development of reliable methodologies for the direct analysis of complexes in both Water- and Donnan Free Spaces.

Radiotracers

Although for metal analysis a variety of analytical techniques is available, only radiotracers in combination with \(\gamma\), \(\beta\)-spectrometry were used, in the experiments described in the present thesis.

The application of radioisotope techniques, particularly combined with \(\gamma\)-ray spectrometry, offers the possibility of detection of total trace concentrations (irrespective of the physico-chemical form) of metals in biological systems, without any necessary destruction/digestion procedure (Chapter 4,7), and without extensive matrix effects.

Furthermore, radiotracer techniques may discriminate between newly imported and initially present quantities of elements/compounds. In root system experiments (Chapter 3), newly absorbed citrate could be distinguished from the already present citric acid pool: the determination of \(^{14}\)C specific activities (\(\beta\)-counting \(^{14}\)C, UV-detection of citrate) permitted interpretations in terms of mixing/homogenization of newly imported citrate in the root citrate compartments.

In a number of experiments with stem-leaf systems (Chapter 4), monenergetic \(\gamma\)-rays of \(^{115}\)Cd (527 keV) and \(^{122}\)Sb (564 keV) were determined simultaneously using a high-resolution \(\gamma\)-ray detector: \(^{122}\)Sb, incorporated into a negatively charged compound, was used to characterize anatomical features of the xylem transport system, whereas at the same time, \(^{115}\)Cd was used to determine xylem transport and leaf-accumulation of Cd. Here, the application of radiotracer techniques permitted all necessary determinations to be performed in one and the same plant system, under identical conditions.
Chapter 8

Transport parameters

In this thesis, the movement of ions in the xylem is invariably expressed by data on cell wall binding (CEC, AEC) and (rate of) lateral escape \( (k) \). In literature, changes in longitudinal movement are interpreted in terms of changes in cell wall binding, but these latter changes are often qualitatively determined by the retention time of the ion in the stem (Tiffin 1970, Bradfield 1976, Ferguson & Bolland 1976, Van de Geijn and Pikaar 1982, McGrath and Robson 1984). However, apart from the metal concentrations applied, the retention time should be regarded as a plant-dependent parameter, which may be largely determined by xylem volume flow and anatomical xylem vessel characteristics (Chapter 4).

For this reason, transport is presented by parameters based on a combination of data on retention times, xylem volume flow, applied ion concentrations and xylem vessel characteristics. From these data, reliable information can be gathered on both lateral escape and ion quantities actually sorbed in the vessel walls. This approach is justified by comparable outcomes of determinations on CEC, AEC and \( k \) in experiments with stem-leaf systems (Chapter 4), isolated cell wall material (Chapters 5,6) and internodes (Chapter 7).

It should be noted that determinations of \( k \) yielded data averaged over 1) the total stem-length, and 2) the total of all chemical species. Due to experimental set-ups, the lengths of stem-segments used were standardized, and did not vary markedly (< 10 %) in the various experiments. Therefore, the data do not contain any information on possible length(L)-dependent variations in \( k \). Here, due to the length-averaged determination of the vessel cross-sectional area \( A_v \), \( k \) is not calculated into any more detail. Furthermore, the stem-segments used were taken in-between nodes: therefore, no marked effects of stem-length on \( A_v \) are considered (Artschwager 1918).

For all determinations, \( k \) is averaged over all chemical species, by taking only total concentrations into consideration. Therefore, the \( k \)-value should be regarded as the result of a summation of all escape rates operating on individual species. For instance, in stem-leaf experiments (Chapter 4), \( k \) was determined on introduced total Cd concentration, whereas Cd was actually applied as a combination of Cd\(^{2+} \) (7 %) and Cd-cit (93 %). However, based on the inversed relationship between \( k \) and concentration (Wolterbeek et al. (1985)), the observed
reductions of $k$ for Cd in + cit situations should be regarded as an underestimation of the real differences between $k_{Cd}$ and $k_{Cd-cit}$ rather than an enlargement of small or even non-significant variations.

In principle, also the CEC and AEC determinations are averaged over the total stem-length: absolute sorbed quantities are converted into concentrations by using Donnan Free Space volumes calculated for the total stem-part used. Notwithstanding time-effects (Cd, Chapter 5), for Cd$^{2+}$ in internodes experiments a length-independent distribution in the xylem cell walls was observed (Chapter 7), which may be regarded as consistent with the basis (Artschwager 1918) for the length-averaging of $A_p$.

Apparently, CEC or AEC are closely related with $k$: roughly speaking, changes in cell wall sorption are directly reflected by similar changes (direction and magnitude) in the rate of lateral escape (Chapter 4 (cit effects on Cd), Chapter 7 (Cd effects on cit)). Based on applications of constant total concentrations, changes in $k$ may be regarded directly as changes in flux. When also considering approximations of flux equations in ion exchangers given by Dalton (1984), the $k$ and CEC/AEC data indicate that concentration gradients in cell walls should be directly related with total concentrations (CEC, AEC). For the Cd concentrations applied, these results agree with earlier data on Cd fluxes observed in isolated xylem cell walls (Wolterbeek 1987).

Plant sub-systems

Experiments were carried out with whole plants (Chapter 3), excised roots (Chapter 3) and internodes (Chapter 7), and with isolated xylem cell walls (Chapters 5 and 6). By these approaches, interactions between cadmium and citrate were investigated both on tissue, on organ and on organism level. Furthermore, chemical analysis was performed on xylem transport fluids, the latter primarily aimed at the chromatographic separation and eventual detection of xylem organic substances (Chapter 2).

The combination of experiments permitted relatively detailed interpretation of effects shown on higher organisation levels, whereas, at the same time, effects observed on sub-cellular level could be shown to be of "higher-level" relevance.
Chapter 8

Results

General xylem cell wall characteristics

Sorption in xylem cell walls was measured in stem-leaf systems (Chapter 4), columns of cell wall material (Chapter 5), and isolated internodes (Chapter 7). The procedures used to isolate cell walls resulted in cell wall pieces/powder in which the general characteristics of the material were maintained. Control measurements were performed on buffer capacity and Br\textsuperscript{−} retention; the CEC in powdered walls were equal to CEC values found in cut wall pieces. In addition, cell wall amino acid concentrations (Chapter 6) agree with literature data on cell wall amino acid content (Lamport and Northcote 1960, Thompson and Preston 1967).

In Cd-experiments (ca. 0.3 mM Cd), measurements in stem-leaf systems yielded values for the amount of Cd adsorbed to the cell walls of 153 ± 83 meq.l\textsuperscript{−1} (Chapter 5), which is in full agreement with the Cd adsorption data (130 meq.l\textsuperscript{−1}) from experiments with isolated xylem cell walls (Chapter 5).

Cd sorption experiments in cell wall columns with 1.0 and 5.7 mM Cd concentrations (Chapter 5 and 6) yielded 450 meq.l\textsuperscript{−1} and 536 meq.l\textsuperscript{−1}, respectively; these values are in full accordance with data from measurements in intact systems reported elsewhere (Pitman 1977, Van de Geijn and Petit 1979, Van de Geijn, Petit and Roelofsen 1979, Wolterbeek 1986).

For citric acid sorption, experimental data were obtained in internodes (Chapter 7), varying from 0.02 to 5 meq.l\textsuperscript{−1}, which is in reasonable agreement with experimentally obtained and calculated data (Freundlich/Langmuir equations, Chapter 6) in isolated cell walls (0.01 to 10 meq.l\textsuperscript{−1}).

Based on these data, experiments in isolated cell wall tissues may be considered to represent ion cell wall interaction processes taking place in plant systems/organs.

Metal-ligand interactions

Effects of citrate on Cd transport behaviour were examined in whole plants (Chapter 3), stem-leaf systems (Chapter 4), and in isolated xylem cell wall material (Chapter 5). Changes in total accumulation and in root-to-shoot mobility of Cd were
induced by increasing the root citrate pool; furthermore, the presence of xylem citrate resulted in enhanced longitudinal xylem movement of Cd. Here, the presence of Cd in Cd-citrate complexes resulted in decreases in both cell wall binding and rate of lateral escape. These data may explain the reported decrease in stem retention of various metals (Fe, Ca, Zn, Cu) transported in the xylem in complexed forms (Tiffin 1970, Bradfield 1976, Ferguson and Bollard 1976, Van de Geijn and Pikaar 1982, McGrath and Robson 1984).

Effects of Cd on the xylem transport behaviour of citrate are described in Chapters 5-7. The formation of Cd-cit complexes is shown to result in increases in both cell wall sorption and rate of lateral escape of citrate. The general characteristics of the processes involved were indicated by a number of measurements of citrate behaviour in isolated xylem cell walls. In these experiments Cd, Ca and Mg, increased citric acid cell wall sorption as a result of metal-citric acid complexation.

"Zipper"-effect

The density of negative charges fixed in the cell wall material (CEC) at average cell wall pH (3-5) is 400-500 meq.l⁻¹_{dfs} (generally measured as COO-metals), and may be interpreted as due primarily to the presence of COO⁻ groups associated with α-D-Polygalactoronic acids in cell wall pectines (Chapter 4). The potential density of the negative charges (COO⁻ + COOH + COO-Metal) has been reported as about 1000 meq.l⁻¹_{dfs} (Wolterbeek 1986).

The potential cell wall density of positive charges is about 40-50 meq.l⁻¹_{dfs} (Chapter 6, amino acid analysis), and is interpreted as due primarily to -NH₃⁺ groups from basic amino acids in cell wall glycoproteins (Läuchli 1976, Gillet and Lefebvre 1981, Richter and Dainty 1989).

The positive cell wall charge density (AEC) estimated from citric acid measurements is about 0.165 meq.l⁻¹_{dfs} (Chapter 6), which is 0.3 % of the potentional charge density. This low AEC is attributed to a mutual screening of the fixed negative and positive charges in the cell wall structure. This phenomenon, is presently called the "zipper"-effect. The concept of mutual screening is supported by the results from measurements of K⁺-sorption (Gillet and Lefebvre 1981): here, however, effects from screening on CEC are much less pronounced. The "zipper"-
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effect makes clear that the use of the potential density of positive charges to calculate directly the cell wall AEC, may result in severe misinterpretations of the actual relations between ions in the cell wall phase. Furthermore, zipper opening processes may have significant consequences for cell wall anion sorption and related transport processes.

Conclusions

The results of the present thesis indicate that metal-ligand interactions are not only relevant for the transport behaviour of the metal ions, but may have similar consequences for the behaviour of the ligand itself. The presence of metal-organic acid complexes in the xylem fluid should be considered of main importance for the metal and/or organic acid behaviour in xylem transport. The effects of metals on ligands on cell wall level should be regarded as a physical-chemical phenomenon, directed by processes as diffusion, adsorption, exclusion and fast protonation. Simultaneous application of both citric acid and Cd\(^{2+}\), Ca\(^{2+}\) or Mg\(^{2+}\) results in increased sorption of citric acid. Sorption of citric acid was largest in the presence of Ca\(^{2+}\). The cell wall sorption (AEC) of citric acid is far below the amount that may be accepted by means of the cell wall potential density of positive charges, a phenomenon here called the "zipper"-effect. In stem experiments the relevant and significant mutual effects of citric acid and cadmium could be expressed by means of cell wall sorption and lateral escape. In stems cadmium increased citric acid cell wall sorption and lateral escape. In root-system and intact plant experiments an increase of the fast-exchangable root citric acid pool leads to an enhanced cadmium mobility and total cadmium adsorption in plants. The methods and procedures used permitted determinations of effects on various organisation levels and under various experimental conditions. Furthermore the combination of these experiments demonstrates the impact of complexation on both metal and ligand during their xylem transport.

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References


Chapter 8


Discussion

191. Commonwealth Agricultural Bureau, Slough, UK.
SUMMARY

During the past several decades, much attention has been paid to the effects of the presence of organic acids on the transport behaviour of metals in xylem vessels. These effects are generally described in terms of changes in the characteristics of the metal transport in both longitudinal and lateral directions. 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 metals on the xylem transport behaviour of organic compounds. In the light of the above, it seems logical to also raise the question as to whether cations influence the transport behaviour of organic compounds. Such effect may lead to changes in longitudinal and lateral transfer rates and to ion-specific variations in initial distribution of organic compounds in plants. In this thesis, both the effects of organic acids on metals and the effects of metals on the behaviour of organic acids in xylem transport processes are described.

Tomato plants (Lycopersicon esculentum Mill cv. Tiny Tim), were used throughout all experiments. Cadmium (and the radiotracer $^{115}$Cd) and citric acid (and $^{14}$C-labelled citric acid) were used as "model" metal and organic acid, respectively. The application of radioisotope techniques, particularly using $\gamma$-ray spectrometry, permits simultaneous detection of trace concentrations of several metals in biological systems without any necessary of destruction/digestion procedures and without extensive matrix effects.

Experiments were carried out with intact plants, plant parts (root system and stem systems) and isolated xylem cell wall material. The combination of these experiments permits a relatively detailed interpretation of effects shown on higher organisation levels, whereas, at the same time, effects observed on cell wall level could be shown to be of higher-level relevance.

In a first series of experiments, the concentrations of both amino acids and organic acids were determined in tomato xylem fluids, which were sampled from plants grown under the selected experimental conditions. For the amino acid analysis, PITC-derivatives were used in chromatography; for the xylem organic acids a rapid ion suppression RP-HPLC method was developed (Chapter 2).
Summary

In intact plants, effects were studied of root pool citric acid on accumulation and transport behaviour of cadmium (Chapter 3). Pressure cell experiments showed that a short-term supply of citric acid increases the fast-exchangeable root citric acid pool about two-fold. As a result, after citric acid preincubation, total accumulation of Cd increased two-fold without any significant effects on root Cd levels, root-to-shoot Cd mobility increased 5-6 fold and the Cd concentrations in the xylem fluid increased 6-8 fold (Chapter 3).

Stem transport was investigated in leaf-stem systems (Chapter 4, effects of citric acid on Cd) and in isolated internodes (Chapter 7, effects of Cd on citric acid). Here, stem transport parameters are expressed by the rate constant for lateral escape $k$ and the quantitative sorption into the xylem vessel walls, $CEC$ (Cd) or $AEC$ (citric acid). These parameters were selected based on the idea that other parameters (e.g. stem retention, xylem concentrations) should be regarded as plant-dependent and largely determined by (variations in) xylem volume flow and anatomical xylem vessel characteristics.

In leaf-stem systems, the presence of citric acid resulted in a 3-fold and 6-fold decrease in $k$ and $CEC$ for Cd respectively; both probably due to the formation of uncharged and negatively charged Cd-citrate complexes, which show little binding affinity to the mainly negatively charged xylem cell walls.

In internodes, experiments were performed to gain insight in the effects of Cd on the xylem longitudinal and lateral xylem movement of citric acid (Chapter 7). The presence of Cd resulted in approximate two-fold increases in values for both $AEC$ and $k$ for 0.05 M added citric acid. For the 9.5 mM citric acid applications, however, Cd resulted in less than two-fold increases in $AEC$, whereas $k$ did not show any significant change. Here, the escape of citric acid is probably limited by the uptake into surrounding tissues after then by the transport across the xylem vessel walls.

In the presence of Cd, the stem-length profile of absorbed citric acid agrees more with stem-length profiles of Cd than with the profiles of citric acid itself, which may be interpreted as indicating the importance of Cd-cit complexes for the overall xylem behaviour of citric acid under the conditions applied.

The sorption processes into the xylem vessel walls were investigated in more
detail in column experiments with isolated xylem cell walls (Chapters 5 and 6). In Chapter 5, general cell wall column characteristics, such as pH buffering capacity, potential Cd CEC, column void volumes and WFS/DFS ratio’s, are described. The negatively charged Cd-cit complexes in applied solutions resulted in 50 % decreased Cd sorption in the vessel walls under the conditions applied.

In Chapter 6, the amino acid content (especially the basic histidine, arginine and lysine) of the vessel walls was determined and discussed as the probable potential capacity of fixed positively charged cell wall groups. The overall sorption of citric acid is governed by processes of binding to positive charges and of exclusion, the latter due to the overwhelming presence of fixed negative charges. In the presence of Cd, the total citrate DFS accumulation, expressed by the distribution coefficient $\lambda$, increased 15-fold (Chapter 5). In Chapter 6, citrate DFS accumulation is presented as the amounts eluted by HCl rinsing ($H_2O$ rinsing discarded), thereby selecting the more firmly attracted citrate.

The amino acid analysis indicated an anion exchange potential of the column as approximately 46 meq.L$^{-1}$, whereas the AEC for citric acid was determined as $1.65 \pm 0.18 \times 10^{-4}$ M (citric acid units). This low AEC is attributed to a "zipper" effect, a mutual screening of fixed $R^-$ and $A^+$ charges.

Pre-loading with Cd$^{2+}$ does not affect citric acid sorption, indicating the absence of Cd-effects on the availability of fixed $A^+$ charges, and the absence of effective R$^-$-Cd$^{2+}$ and Donnan free space (DFS) [Cd(cit)H$_2$]$^+$ complexes. Simultaneous application of both citric acid and Cd$^{2+}$, Ca$^{2+}$, or Mg$^{2+}$ 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)H$_2$]$^+$ after protonation in the DFS. Sorption of citric acid was largest in the presence of Ca$^{2+}$, which is discussed in the light of the differences between Ca, Cd and Mg in their characteristics as coordinative M-complexes of citric acid.

Finally, in Chapter 8 methods, speciation, transport parameters and plant material are discussed. Special attention is given to general cell wall characteristics, metal-ligand interactions and the "zipper"-effect. Last, the general discussion also presents conclusions.
Summary

Summarizing, it can be concluded that in xylem transport the effects of metals on organic acids are of similar importance as the effects of organic acids on metals. The significance of these effects, as a result of complexation in xylem fluid, could be shown on several levels of organisation. Metals as cadmium, calcium and magnesium increase cell wall sorption and lateral escape of citric acid.
SAMENVATTING

In de bladeren van hogere planten vinden belangrijke processen zoals fotosynthese en ademhaling plaats. Voor deze processen zijn koolzuur, zuurstof en water nodig. Koolzuur en zuurstof neemt de plant via de bladeren op uit de lucht en via de wortels wordt uit de bodem water opgenomen.

Om het water en de daarin opgeloste stoffen (door de wortel opgenomen of gesynthetiseerd) over relatief lange afstanden te kunnen transporteren beschikken planten over een transportweefsel, wat functioneel analoog is aan het bloedvaatstelsel bij hogere diersoorten.

Bij hogere planten is het transportweefsel samengesteld uit het floëem (vnl. zeevaten) en het xyleem (vnl. houtvaten). In dit proefschrift is de aandacht gericht op het xyleem. Dit speelt een rol bij het lange-afstand-transport van water van de wortels naar de bladeren. Kenmerkend voor het xyleem zijn de tracheale elementen: houtvatleden en tracheïden. Daarnaast komen in het xyleem parenchymcellen en sklerenchymatische vezels voor. Houtvaten zijn een aaneenschakeling van houtvatleden (cellen) waarvan de dwarswanden vrijwel geheel zijn opgelost en daardoor vanuit de wortel doorlopen tot in de bladeren. In de houtvaten bevindt zich een waterige vloeistof; het xyleemsap, dat onder invloed van de verdamping in de bladeren omhoog wordt getransporteerd. In het xyleemsap bevinden zich naast de door de wortel opgenomen stoffen ook door de wortel gesynthetiseerde amino- en carboxylzuren. Deze carboxylzuren (anionen) hebben in het xyleem een functie bij o.a. de handhaving van het electrochemisch evenwicht. Tevens spelen bepaalde carboxylzuren d.m.v. complexvorming een rol bij het metaaltransport door het xyleem.

In de laatste decennia is er veel aandacht besteed aan de effecten van organische zuren op het transportgedrag van metalen. Er is echter nog weinig bekend over de mogelijke invloed van metalen op het gedrag van organische zuren gedurende het xyleemtransport. Het lijkt logisch dat m.n. divalent metalen, die stabiele complexen vormen met carboxylzuren van invloed kunnen zijn op het gedrag van carboxylzuren in het xyleem. Deze verandering in chemisch gedrag kan weer leiden tot veranderingen in het opwaartse (longitudinaal) en zijwaartse (lateraal) transport, hetgeen weer gevolgen kan hebben op de verdeling van carboxylzuren over de plant.
Samenvatting

De bedoeling van het onderzoek was na te gaan of er naast de effecten van het carboxylzuur citroenzuur op het transportgedrag van het metaal cadmium, ook effecten van cadmium op het transportgedrag van citroenzuur in het xyleem zouden kunnen worden aangetoond. Naast cadmium werd ook een aantal andere metalen getoetst.

De experimenten werden uitgevoerd met zowel intakte tomatenplanten (*Lycopersicon esculentum* Mill cv. Tiny Tim) als met delen daarvan (wortelsystemen, stengelsystemen). Tevens werd er onderzoek verricht op verschillende organisatie niveaus: zo werden er met betrekking tot de stengel, experimenten uitgevoerd op zowel weefselniveau (internodium) als op sub-cellulair niveau (geïsoleerd celwandmateriaal). De combinatie van deze experimenten maakt het mogelijk om een relatief gedetailleerde interpretatie te geven van effecten die zijn waargenomen op hogere organisatie niveaus.

Veel van de in het verleden waargenomen effecten van carboxylzuren op het gedrag van divalente metalen in het xyleem kunnen worden toegeschreven aan complexvorming. Het is voor de eenduidige interpretatie van belang om bij onderzoek naar processen waarbij dit een rol speelt uit te gaan van stabiele complexvormers. De keuze voor het complex cadmium-citraat was dan ook grotendeels gebaseerd op het fysisch-chemisch gedrag en niet zozeer op de plantenfysiologische of biochemische betekenis van het complex.

De effecten van metaal-ligand interacties worden gerelateerd aan 1) de binding van metalen en liganden (resp. cadmium en citroenzuur) aan de wanden van de houtvaten en 2) de zijwaartse beweging (laterale ontsnapping) van metalen en liganden uit de houtvaten. De bindings-effecten worden beschreven aan de hand van de Cation Exchange Capacity (CEC) en de Anion Exchange Capacity (AEC). De effecten op de laterale ontsnapping, worden uitgedrukt aan de hand van de snelheidsconstant van de laterale ontsnapping (k). Het voordeel van deze transportparameters is dat er rekening wordt gehouden met omgevingsfactoren (aangeboden concentraties, verdamping, stroomsnelheid van het xyleemsap) en met plantafhankelijke factoren (aantal en grootte van de houtvaten, aantal bindingsplaatsen in de houtvatwanden). Zowel de omgevingsfactoren als de plantafhankelijke factoren kunnen van invloed zijn op het transport van o.a. metalen.
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en carboxylzuren door het xyleem.

De aminozuursamenstelling van het xyleemsap (Hoofdstuk 2) en van het houtvatwandmateriaal (Hoofdstuk 6) werd bepaald door middel van twee verschillende HPLC analyse methoden. Voor de analyse van carboxylzuren van het xyleemsap moest echter een nieuwe, snelle RP-HPLC methode ontwikkeld worden, waarbij gebruik is gemaakt van ion-suppressie (Hoofdstuk 2).

Met uitzondering van de chromatografische analyses, werd in alle experimenten gebruik gemaakt van radiotracertechnieken. Radiotracertechnieken bieden in combinatie met gamma-spectrometrie voordelen bij de detectie. Zo kunnen lage (trace) concentraties van verscheidene metalen in biologische systemen gelijktijdig worden bepaald zonder ontsluiting van het plantenmateriaal. Ook ontbreken grote matrix effecten.

Voor de correcte interpretatie van de resultaten is een speciatiebepaling van de experimentele oplossingen nodig. Het is nog niet mogelijk om deze de speciatiebepaling op een directe analytische wijze uit te voeren. Ze is daarom berekend aan de hand van het SOILCHEM-specialieprogramma.

Bij intakte planten werden de effecten van de veranderingen in de citroenzuur-"pool" van de wortel op de accumulatie en het transportgedrag van cadmium bestudeerd (Hoofdstuk 3). Aangetoond werd dat een kortdurend aanbod van citroenzuur (24 h) resulteerde in een verdubbeling van de snel uitwisselbare wortel-citroenzuur-"pool". Als gevolg hiervan was bij de planten na preincubatie (24 h) met citroenzuur, de totale accumulatie van cadmium in de hele plant twee maal zo hoog, maar het cadmiumgehalte in de wortels veranderde niet significant. Tevens nam de mobiliteit van cadmium van de wortel naar de spruit 5 tot 6 maal toe en werd de concentratie van cadmium in het xyleemsap een factor 6 tot 8 verhoogd (Hoofdstuk 3). Blijkbaar leidt de verhoging van de snel uitwisselbare citroenzuur-"pool" in de wortel tot de vorming van meer cadmium-citroenzuurcomplexen, die snel naar het xyleem en vervolgens naar de bovengrondse plantendelen getransporteerd worden.

De effecten van citroenzuur op het cadmium transport in de stengel werden nader onderzocht in blad-stengel-subsystemen (Hoofdstuk 4). De aanwezigheid van
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citroenzuur veroorzaakte een 6-voudige en 3-voudige afname in respectievelijk CEC en k voor cadmium; beide reducties zijn mogelijk het gevolg van de vorming van neutrale en negatief geladen cadmium-citraat complexen, die een lage bindingsaffiniteit hebben tot de voornamelijk negatief geladen houtvatwanden.

In Hoofdstuk 7 worden experimenten met internodia (stengelstukken) beschreven. Deze experimenten werden uitgevoerd om inzicht te krijgen in de effecten van cadmium op het longitudinaal en het laterala transportgedrag van citroenzuur in het xyleem. De aanwezigheid van cadmium leidde tot een meer dan tweevoudige verhoging (factor 2,5) van zowel de AEC als de k van citroenzuur. Echter, bij het aanbod van een hoge citroenzuurconcentratie (150 maal hoger dan de xyleemsapconcentratie), resulteerde de aanwezigheid van cadmium in een minder verhoogde (factor 1,5) AEC, terwijl k niet significant veranderde. Blijkbaar wordt de laterale ontsnapping van citroenzuur, bij een hoog citroenzuur aanbod, meer gereguleerd door de opname in het omliggend weefsel dan door het transport door de houtvatwanden. In de aanwezigheid van cadmium vertoonde het stengel-lengteprofiel (verdelingspatroon van citroenzuur over de stengellengte) van het aan de houtvatwand gebonden citroenzuur meer overeenkomst met de stengellengteprofie len van cadmium, dan met de profielen van citroenzuur zelf. Deze resultaten tonen het belang aan van cadmium-citraat complexen voor het citroenzuurtransport in de houtvaten.

Door middel van perfusie-experimenten, die werden uitgevoerd met kolommen gevuld met geïsoleerd celwandmateriaal van houtvaten uit de stengel (Hoofdstuk 5 en 6), werden de binding van cadmium en citroenzuur aan de celwanden onderzocht.

Met deze experimenten (Hoofdstuk 5) werd een aantal relevante eigenschappen van het celwandmateriaal zoals, pH buffercapaciteit, potentiële CEC van cadmium en WFS/DFS ratio's bepaald. Uit deze experimenten bleek dat de toegepaste isolatieprocedure geen effect had op de fysisch-chemische eigenschappen van de celwanden. Dit houdt in dat de resultaten verkregen met het geïsoleerde celwandmateriaal vergeleken kunnen worden met resultaten afkomstig uit in-vivo omstandigheden. Uit Cd-citraat perfusie experimenten bleek dat de aanwezigheid van negatief geladen cadmium-citraatcomplexen in de aangeboden oplossingen resulteerden in 50 % afname van binding van cadmium.
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In Hoofdstuk 6 zijn perfusie-experimenten beschreven waarbij de aandacht gericht was op de binding van citroenzuur aan de celwanden en de mogelijke effecten van cadmium daarop. De totale hoeveelheid citroenzuur (neutraal en/of negatief geladen) in de celwand wordt bepaald door binding aan positief geladen celwandgroepen en door exclusie uit de Donan free spaces (DFS). De exclusie wordt veroorzaakt door de grote hoeveelheid negatief geladen celwandgroepen (polygalacturonzuur). Door middel van een HPLC aminozuur-analyse werd het gehalte aan basische aminozuren (histidine, arginine en lysine) van het celwandmateriaal bepaald. Op basis van dit gehalte werd een potentiële cation-concentratie van circa 46 meq.l⁻¹ berekend. De met perfusie-experimenten vastgestelde hoeveelheid gebonden citroenzuur (AEC) bedroeg 1.65 ± 0.18 10⁻⁴ M (citroenzuur-eenheden). Deze lage AEC (0.4 % van de potentiële cation-concentratie) wordt toegeschreven aan een "zipper"effect, waaronder een wederzijdse afscherming van negatief geladen celwandgroepen (R⁻) en positief geladen celwandgroepen (A⁺) wordt verstaan.

Het vooraf laden van de kolom (gevuld met celwandmateriaal) met Cd²⁺ had géén effect op de binding van citroenzuur, hetgeen duidt op de afwezigheid van effecten van cadmium op de beschikbaarheid van de gebonden positieve ladingen in de celwanden en op de afwezigheid van de directe vorming van effectieve $R^-$-Cd²⁺ en [Cd(cit)H₂]⁺ complexen in de DFS. Het gelijktijdig aanbieden van zowel citroenzuur als Cd²⁺, Ca²⁺ of Mg²⁺ resulteerde in een verhoogde binding van citroenzuur aan de celwanden. In de aanwezigheid van cadmium nam de accumulatie van citroenzuur in de DFS, uitgedrukt in de distributiecoëfficiënt λ, een factor 15 toe (Hoofdstuk 5). Dit wordt waarschijnlijk veroorzaakt wordt door een capaciteitsverbetering en niet zozeer door veranderingen in de valentie-afhankelijke binding van anionen. Deze capaciteitsverbetering kan veroorzaakt zijn door de aanwezigheid van een hoge concentratie [M(cit)]⁻ (M is divalent metaal), dat na protonatie in de DFS, door de kolom vast gehouden wordt als [M(cit)H₂]⁺. De binding van citroenzuur was het grootst in de aanwezigheid van Ca²⁺. Dit kan verklaard worden aan de hand van de verschillen tussen Ca, Cd en Mg als coördinatieve M-complexen van citroenzuur.

Samenvattend kan gesteld worden dat er naast effecten van citroenzuur op
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het transport van metalen, nu ook de invloed van metalen op het transport van citroenzuur door het xyleem is aangetoond. Deze effecten, die het gevolg zijn van complexvorming in het xylemsap, spelen op meerdere (organisatie) niveau's een rol. Metalen als calcium, magnesium en cadmium verhogen de celwandadsorptie en laterale ontsnapping van citroenzuur.
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CURRICULUM VITAE
