Effects of short term iron citrate treatments at different pH values on roots of iron deficient cucumber: a Mössbauer analysis

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Running title:

Mössbauer study of iron accumulation in roots at different pH

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Abstract

Alkaline pH values as well as bicarbonate greatly reduce the mobility and uptake of Fe causing Fe deficiency chlorosis. In the present work the effect of pH and bicarbonate on the uptake and accumulation of Fe in the roots of cucumber was studied by Mössbauer spectroscopy combined with physiological tests and diaminobenzidine (DAB) enhanced Perls staining. Mössbauer spectra of Fe-deficient cucumber roots supplied with 500 μM ^{57}Fe(III)-citrate at different pH values show the presence of a Fe(II) and a Fe(III) component. As the pH was increased from 4.5 to 7.5, the root ferric chelate reductase activity decreased significantly and a structural change in the Fe(III) component could be observed. While at pH 4.5 the radial intrusion of Fe reached the endodermis, at pH 7.5 Fe was found only in the outer cortical cell layers. The Mössbauer spectra of Fe-deficient plants supplied with Fe(III)-citrate in the presence of bicarbonate (pH 7.0 and 7.5) showed similar Fe components but the relative Fe(II) concentration compared to that measured at pH values 6.5 and 7.5 was larger. The Mössbauer parameters calculated for the Fe(II) component in the presence of bicarbonate were slightly different from those of Fe(II) alone at pH 6.5-7.5, whereas the ferric chelate reductase activity was similarly low. On the other hand, the Fe incorporation into the root apoplast involved only the outer cortical cell layers, as in the roots treated at pH 7.5. In Fe-sufficient plants grown with Fe(III)-citrate and 1 mM bicarbonate, Fe precipitated as granules and was in diffusely scattered grains on the root surface. The "bicarbonate effect" may involve a pH component decreasing both the FCR activity and the acidification of the apoplast and a mineralization effect leading to the slow accumulation of extraplasmatic Fe particles forming an Fe plaque and trapping Fe and other minerals in biologically unavailable forms.

Keywords: Bicarbonate; Cucumis sativus L. cv. Joker; Extraplasmatic iron; Iron accumulation; Iron deficiency; Iron plaque; Mössbauer spectroscopy; Nutrient solution.

Abbreviations: DAB, diaminobenzidine; FCR, ferric chelate reductase; HEPES, [*N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino) ethanesulfonic acid; PPFD, photosynthetic photon flux density

Introduction

Iron is a particularly important micronutrient influencing the productivity of plants, as it is an essential constituent of numerous cofactors in enzymes catalyzing redox reactions and electron transport at different levels of the metabolism. However, Fe is not readily available in natural soils. One of the most important factors controlling Fe availability in soils is pH. The solubility of Fe(III) has an average logK value of 2.70 [$Fe(OH)_3$(soil-Fe)+$3H^+ \leftrightarrow Fe^{3+}+3H_2O$]; the most soluble form is amorphous $Fe(OH)_3$, while the least soluble one is goethite (FeOOH; Lindsay, 1995). The total soluble Fe in calcareous soils is approximately $10^{-10.4}$ M, whereas plants require a concentration of about 10^{-8} M. This highlights the importance of Fe immobilization due to its precipitation leading to the lime-induced Fe chlorosis in plants (Marschner, 1995). Natural or synthetic complexes and chelates modify equilibria in soils and nutrient solutions by increasing total soluble Fe and its mobility in the rhizosphere (Lucena, 2006, Varanini and Pinton, 2006).

Furthermore, it has been shown that Fe is acquired by most plant species (Strategy I, for review see Abadía et al, 2011) by reducing Fe(III)-chelates on root cell membranes by a ferric chelate reductase enzyme (FRO2) (Jeong and Conolly, 2009). This is an obligatory step prior to Fe uptake (Chaney et al., 1972). Ferrous iron was shown to be released from the chelate as $Fe(H_2O)^{2+}$ (Kovács et al., 2009) which may temporarily exist at the reduction site due to the highly reductive and acidic environment created by the FRO2 and the proton pumps. Ferrous iron may then be taken up or be reoxidised. The uptake is facilitated by the high affinity transporter IRT1 (Vert et al., 2002). In well aerated conditions Fe(II) is quickly reoxidised and may precipitate as Fe(III)-oxides and hydroxides (Lindsay, 1995, Kovács et al., 2009).

The *in vivo* ferric chelate reductase (FCR) activity of Fe-deficient sugar beet plants was tested at different pH values and it was found to be much higher at pH 6 or below than at pH 6.5 or above (Susín et al., 1996, Chang et al., 2003). In contrast, in tomato grown at high external pH (7.5) an increased expression of four Fe uptake genes (LeIRT1, LeIRT2, LeFRO1, LeNRAMP1) was found, which was accompanied by higher FCR activity compared to pH 5.0 (Zhao and Ling, 2007). Neutral or alkaline pH normally occurs and is maintained in calcareous soils, where the combination of nitrate nutrition with high bicarbonate concentration is typical (Mengel, 1994). Bicarbonate is a strong buffer neutralizing protons released by the plasma membrane P_3A –type H^+/ATPases (Hell and Stephan, 2003, Kosegarten et al., 2004, Kim and Guerinot, 2007) leading to an increase of pH in the root apoplast and a concomitant inhibition of the FCR activity (Mengel, 1994). These processes may lead to high Fe concentration in roots of chlorotic plants, where Fe is accumulated outside the cytoplasm of the root cells, both in apoplastic and extraplasmatic localizations. The high extraplasmatic accumulation of Fe at the surface of the rhizoplane may originate from nearby soil components whereas the apoplastic portion is quite low (lower than 50 mg kg^{-1} root dry matter) (Strasser et al., 1999). The extraplasmatic accumulation of Fe is frequently referred to as Fe plaque, which is thought to greatly modify the mobility and uptake of metals in flooded or aquatic environments (Hansel et al., 2001).

The mechanism of Fe accumulation in apoplastic and extraplasmatic places influenced by pH has not been understood yet. The present study is aimed to reveal some of the possible biochemical consequences of pH changes in the rhizosphere, using Mössbauer spectroscopy combined with physiological tests and diaminobenzidine (DAB) enhanced Perls staining, in order to elucidate the importance of apoplastic Fe in plant nutrition.

Materials and methods

Plant material

Iron deficient cucumber (*Cucumis sativus* L. cv. Joker) was grown without Fe (during the whole growth period) in unbuffered, modified Hoagland nutrient solution of the following composition: 1.25 mM KNO_3; 1.25 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.25 mM KH_2PO_4; 11.6 μM H_3BO_3; 4.5 μM $MnCl_2 \cdot 4H_2O$; 0.19 μM $ZnSO_4 \cdot 7H_2O$; 0.12 μM $Na_2MoO_4 \cdot 2H_2O$; 0.08 μM $CuSO_4 \cdot 8H_2O$ in a climate-controlled growth chamber (14/10 h photoperiod with 120 μmol m^{-2} s^{-1} PPFD light/dark periods, 24/22 °C temperature and 70/75% relative humidity). Three plants were grown together in plastic pots containing 400 ml nutrient solution. The solution was renewed every other day, and 14-16 day-old plants were used for the analysis. The pH of the fresh nutrient solution was 4.70±0.01, and decreased to 3.84±0.23 after 2 days of plant growth.

Iron sufficient cucumber plants were also grown using the same modified Hoagland solution as described above, but containing 10 μM ^{57}Fe(III)-citrate and 1 mM $KHCO_3$ for 16-18 days (control plants were grown without $KHCO_3$). Plants were grown individually (1 plant in a 400 ml pot as they grew much larger than the Fe-deficient ones). The pH of the fresh control and bicarbonate containing solution was 4.38±0.11 and 6.95±0.11, and was 5.86±0.02 and 6.85±0.11, respectively, after 2 days of plant growth.

Short-term treatments of Fe-deficient plants with Fe(III)-citrate at different pH values

Iron deficient plants (at 14-16 day-old stage) were transferred to fresh nutrient solution (see above) containing 500 μM Fe(III)-citrate buffered to pH 4.5, 5.5 or 6.5 (using 5 mM 2-(*N*-

morpholino) ethanesulfonic acid; MES), and pH 7.5 (using 5 mM *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid); HEPES). In other experiments MES and HEPES was replaced by 1 and 3 mM $KHCO_3$ and the pH was adjusted to 7.0 and 7.5 by KOH, respectively. These treatments were applied for 30 min under continuous shaking at 125 rpm. The pH in the solutions was checked after the experiments (mainly because MES does not have good buffer capacity at pH 4.5) and the change was less than 0.1 pH units. For Mössbauer experiments ^{57}Fe(III)-citrate was freshly prepared from enriched $^{57}FeCl_3$ (ca. 90% ^{57}Fe) and citric acid stock solutions mixed at a 1:1.1 molar ratio. For all other assays and analyses Fe(III)-citrate was prepared the same way.

Mössbauer spectroscopy

Whole roots of both Fe-deficient and Fe-sufficient plants were excised and thoroughly blotted with filter paper to remove traces of nutrient solution from the surface. No rinsing was applied, as it may remove any soluble Fe forms from the apoplast (data not shown). The samples were immediately immersed and then stored in liquid nitrogen to preserve the *in vivo* chemical conditions until the measurement was finished.

^{57}Fe Mössbauer transmission spectra were taken in order to explore the chemical forms in which Fe is incorporated. Samples were measured by a conventional constant acceleration type Mössbauer spectrometer (WissEl, Germany) in transmission geometry in a liquid nitrogen bath cryostat at 80 K. A ^{57}Co(Rh) source of ~10^9 Bq activity was used and the spectrometer was calibrated with α-iron at room temperature. The spectrum evaluation was carried out using the MOSSWIN 4.0 program (Klencsár et al., 1996). The Mössbauer parameters obtained from the spectrum give information on the valence state and the coordination number of the resonant atom. The quantitative analytical information for the species was obtained from the relative spectral areas (the area of each subspectrum devided by the total spectral area) as the recoil-free fraction

of the studied components were suggested to be close enough to each other (Greenwood and Gibb, 1971, Rodriguez et al., 2005, Goodman et al., 1982).

Determination of Fe concentration in the roots

For measuring the total Fe concentration, whole roots were blotted with filter paper (without rinsing so that the results are comparable with those of the Mössbauer spectroscopy), dried at 80 ºC and then digested. Ten ml HNO_3 (65 v/v %) was added to 1 g of sample for overnight incubation at RT. Then, the samples were pre-digested for 30 min at 60 °C. Finally, 3 ml H_2O_2 (30 m/m %) was added for 90 min, boiling at 120 °C. The solutions were made up to 50 ml, homogenised and filtered through MN 640W filter paper. The Fe concentration of the filtrate was determined by ICP-MS (OPTIMA 3300 DV ICP-OA spectrophotometer, Perkin-Elmer).

Fe-chelate reductase assay

The assay was designed after Kovács et al. (2009). Iron-treated (+Fe or +Fe+$KHCO_3$) or Fe-deficient roots (approximately 0.1 g) were excised, rinsed with 0.5 mM $CaSO_4$, blotted and transferred to 5 ml of a buffered solution containing 500 μM Fe-EDTA, 400 μM BPDS, 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$ and 0.5 mM KH_2PO_4 and continuously shaken at 100 rpm for 15 minutes in darkness. The pH of the solution was buffered by 5 mM MES (pH 4.5, 5.5, 6.5), 5 mM HEPES (pH 7.5), 1 and 3 mM $KHCO_3$ (pH 7.0 and 7.5), respectively, adjusted by adding KOH. Separate samples were made without plants to exclude any non-specific Fe reduction. At the beginning and after incubation, the solution was immediately sampled and the absorbance was measured at 535 nm. Roots were weighed and the Fe-chelate reducing activity was calculated using the specific extinction coefficient of $[Fe(II)\text{-}BPDS_3]^{4-}$ (22.14 mM^{-1} cm^{-1}).

Microscopic analysis: Perls Stain and DAB / H_2O_2 intesification

The method of Roschzttardtz et al. (2009) with slight modifications was used. The excised root samples were incubated in fixation solution containing 2% (v/v) formaldehyde, 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 12 hours. The fixed samples were washed twice with 0.1 M phosphate buffer (pH 7.0) for 20 minutes each time. The root samples were dehydrated in successive baths of 25%, 50%, 70%, 90%, 96% ethanol and then embedded in Technovit 7100 resin (Kulzer) according to the manufacturer's instructions. Thin sections (7 µm) were made from the region 3-5 mm behind the lateral root tips, i.e. the swollen root region where the highest Fe-chelate reduction rates are located when grown under Fe deficiency (Landsberg, 1994). The sections were placed on glass slides, and then incubated in 4% (v/v) HCl and 4% (w/v) $K_4[Fe(CN)_6].3H_2O$ for 45 minutes at RT. Following washing with distilled water, the sections were incubated again in a methanol solution containing 0.01M NaN_3 and 0.3% (v/v) H_2O_2, and then washed with 0.1 M phosphate buffer (pH 7.0). For the intensification reaction, the samples were incubated in 0.1 M phosphate buffer solution (pH 7.0) containing 0.025% (w/v) DAB, 0.005% (v/v) H_2O_2 and 0.005% (w/v) $CoCl_2$ for 20 minutes. $CoCl_2$ was added to improve the visibility of the DAB reaction. The reaction was stopped by rinsing the sections with distilled water.

Statistics

ANOVA was performed with Tukey-Kramer multiple comparison post test by InStat v. 3.00 (GraphPad Software, Inc.). The term 'significantly different' means $P<0.05$.

Results

Plants treated with Fe(III)-citrate at different pH values

Mössbauer spectra of the Fe-deficient cucumber roots supplied with 500 μM ^{57}Fe(III)-citrate for 30 min at different pH (Fig. 1) show the presence of two different Fe components. According to the Mössbauer parameters (Table 1) the subspectra represent an Fe(II) and an Fe(III) component depicted as shaded and clear areas in Fig. 1, respectively. The quadrupole splitting of the Fe(II) component decreased when the pH was increased from 4.5 to 7.5 (note that the error of Mössbauer parameters of the Fe(II) component is higher at lower Fe(II) concentrations.)

The relative amount of Fe(II) estimated from the ICP measurement of roots and the Mössbauer spectra shows an abrupt decrease from 16% to 5% when the pH was increased from 4.5 to 7.5. Both the total Fe concentrations and the total Fe(II) decreased with increasing pH (Fig. 2). When the pH was increased from 4.5 and 5.5 to 6.5 and 7.5, the quadrupole splitting of the Fe(III) component increased supporting the existence of a structural change of the Fe(III) component associated to the more alkaline pH.

The FCR measurements of the roots were carried out using two different setups. When Fe-deficient root samples were subjected to Fe(III)-citrate treatment for 30 minutes at pH values 4.5-7.5 (MES-HEPES) but the enzyme activity measurements were carried out at pH 5.5 (MES) there was no significant difference between the measured FCR values (not shown). Furthermore, these values were similar to those found in the Fe-deficient controls (without the 30 min Fe supply). However, when the pH in the assay solution was the same as that used in the 30 min treatment, the FCR activity showed major decreases at pH 7.5 (Fig. 3). Moreover the FCR activities measured in Fe-deficient control roots and in those treated with Fe(III)-citrate were

different: at pH 4.5 the FCR activity significantly decreased in the Fe(III)-citrate treated roots when compared to the control.

The microscopic analysis of the root cross section using Perls/DAB staining shows a characteristic difference in the Fe distribution at pH 4.5 and 7.5 (Fig. 4, A and B). Whereas equally strong staining shows the radial intrusion of Fe, leaving stain-free only the central cylinder at pH 4.5, Fe stain was found only in the outer cortical cell layers at pH 7.5. This shows a significantly decreased mobility of Fe at alkaline pH.

Plants treated with Fe(III)-citrate at different pH values in the presence of bicarbonate

The Mössbauer spectra of the Fe-deficient plants supplied for 30 min with Fe(III)-citrate in the presence of 1 and 3 mM $KHCO_3$ (pH values 7.0 and 7.5, respectively) (Fig. 5, B and C; Table 2), show similar Fe components to those found in the spectra of the roots supplied for 30 min with Fe(III)-citrate without $KHCO_3$ at pH 6.5-7.5 (Fig. 1, Table 1). However, the relative Fe(II) concentration (15-17%) (Table 2) was higher than those measured at pH values 6.5 and 7.5 set by MES and HEPES buffers (Table 1). Moreover, these values are similar to those measured at pH 4.5-5.5 (12-16%) (Table 1). The Mössbauer parameters calculated for the Fe(II) component in the presence of bicarbonate (Table 2) are slightly different from those of Fe(II) found after Fe supply without bicarbonate at pH 6.5-7.5 (Table 1) since the quadrupole splitting (Δ) values are higher.

The FCR activity was altered by the pH of the assay solution but it was not further influenced by the pretreatment with Fe(III)-citrate in bicarbonate containing solution (Fig. 6, see also Fig. 3). When it was measured at pH 5.5 (MES) after a 30 min pretreatment at pH 7.0 ($KHCO_3$), the FCR activity was similar to that found in other roots pretreated without Fe at pH

4.5-5.5 (MES), but when the assay was carried out in the presence of bicarbonate at pH 7.0-7.5 it was reduced by 50% (Fig. 6).

The Perls/DAB staining of roots treated with bicarbonate during the 30 min incubation with Fe(III)-citrate shows that the Fe incorporation into the root apoplast involved only the outer cortical cell layers just as in the roots treated at pH 7.5 (HEPES) (Fig. 4, C).

When the plants were grown in Fe(III)-citrate containing nutrient solution with 1 mM bicarbonate the inhibited movement of Fe into the root cortex resulted in its precipitation as granules and diffusely scattered grains at the root epidermis surface (Fig. 4, D). These granules may correspond to an Fe plaque. This sort of precipitation was not observed in the control Fe-sufficient plants grown without bicarbonate (Fig. 4, F) or in any other samples.

Discussion

The effect of pH in the bulk solution on the rhizospheric and biochemical processes in connection with Fe uptake has been extensively investigated by several authors (Toulon et al., 1992, Susín et al., 1996, Chang et al, 2003). The main conclusions of these studies were that pH is important in terms of (i) influencing the FCR activity, (ii) interacting with H^+ excretion by root cells and, (iii) determining the charge balance of Fe(III)-chelates modifying their movement in the negatively charged apoplastic spaces.

The FCR activity values measured in the Fe-deficient cucumber roots (Fig. 3) were in the same range as those found in previous studies (Alcantara et al, 1991, Kovács et al, 2009). The decrease in the relative Fe(II) concentration of the Fe-deficient roots supplied with Fe(III)-citrate

for 30 min at high pH (Fig. 1, Table 1) found by Mössbauer spectroscopy is in good agreement with the decreased FCR activity observed at pH 7.5 set by buffers (Fig. 3). On the basis of the relative spectral areas of the Fe(II) doublet and the ICP-MS total Fe concentration data, the total amount of Fe(II) produced (S_r) by the ferric chelate reduction in 30 min could be assessed (Fig. 2). The significantly lower Fe(II) concentration at pH 6.5 and 7.5 compared to those found at pH 4.5 and 5.5 can be explained by the lower FCR activity at high pH. In parallel, at pH 7.5, a significant immobilization of Fe can be observed in the rhizodermal and outer cortical layers in the subapical root regions (Fig. 4, A and B). This immobilization cannot be seen at pH 4.5, since Fe penetrated the root tissue radially until it reached the central cylinder. It can be hypothesized that immobilization may come from the effect of high pH, because Fe solubility is much lower and mineralization of the original Fe(III)-citrate may occur. Immobilization observed as increased accumulation of Fe at the root surface may have lead to an increased total Fe concentration in these roots. However, this is not supported by the total Fe concentrations measured by ICP-MS since the opposite was found (after 30 min Fe supply). (The concentration of soluble iron in the solutions also remained constant during the the experiments – data not shown.) This finding is in agreement with previously published data (Zhao and Ling, 2007). Therefore it is concluded that the high pH (7.5) maintained in the nutrient solution by the buffer, does not necessarily cause an immediate Fe precipitation from Fe(III)-citrate at the root surface. The decrease in FCR activity leading to lower accumulation of Fe(II), the more mobile form of Fe, may partly explain the immobilization. This means that the FCR activity together with the H^+ extrusion is a prerequisite to the accumulation of Fe by creating conditions that increase its mobility in the apoplastic micropore system (Sattelmacher, 2001).

On the basis of the Mössbauer spectra, the relationships between Fe immobilization and the Fe(III)-complexes formed in the roots can be studied (Fig. 1, Table 1). At slightly acidic pH, the Mössbauer parameters of the Fe(III) species (δ=0.46(1) mms^{-1} and Δ=0.60(1) mms^{-1}) are in good agreement with those of Fe(III)-carboxylates (e. g. Fe(III)-citrate) found in plant roots (Kovács et al., 2009). At pH values 6.5 and 7.5, the change of the Mössbauer parameters of the Fe(III) component (δ=0.46(1) mms^{-1} and Δ=0.70(1) mms^{-1}) may suggest the formation of a new, immobile Fe-rich phase such as amorphous ferrihydrite or $Fe_xO_y(OH)_z$-type polymers, as it was already shown in several inorganic Fe(III) containing solutions (Cornell and Schwertmann, 2003). Since the direct precipitation from the original Fe(III)-citrate solution is not expected to take place (as judged from the ICP measurements), this is only possible if the formation of this ferrihydrite-like component, which is favoured at the higher pH values, occurs only after the reduction of the original Fe(III)-citrate complex. The resulting Fe(II) is either transported to the cytoplasm or reoxidized to Fe(III) in the apoplast in the presence of molecular oxygen (Davidson and Seed, 1983) or nitrate (Lucena, 2000). In earlier studies it was shown that the transport of Fe(II) through the cell membrane is much slower than the reduction which can lead to Fe(II) accumulation in the apoplast (Lucena and Chaney, 2007, Kovács et al., 2009). At low pH, Fe(II) was shown to accumulate in free hexaaqua complex form in the apoplast (Kovács et al., 2009) while at neutral and alkaline pH the slightly smaller quadrupole splitting of the Fe(II) component suggests its coordination with anionic components (e.g. sulphate or carboxylate) besides water (Vértes, 1979). In the latter case, the oxidation rate in air to different Fe(III) species is much higher, and depending on the anion components of the nutrient solution (e.g. citrate, phosphate), different Fe(III)-oxides and hydroxides of low crystallinity can be formed (Cornell and Schwertmann, 2003). The formation of such hydrous ferric-oxide phases can also be influenced

by the coordination of deprotonated carboxylate moieties of the apoplast to Fe(III). Moreover, the Fe(II) produced by FCR at alkaline pH may also be attached to this mineralized phase, which can result in the decrease of the Fe(II) transport, so that Fe uptake into the cytoplasm may be restricted. This is also supported by the results of Lucena and Chaney (2007) who found that the Fe uptake in similar conditions at pH 7.5 is lower than at pH 6.0. This may explain the low total Fe concentration in the roots and that Fe is localized only in the outer cortical cells of the roots as shown by the Perls / DAB staining.

Bicarbonate is a strong buffer that can maintain neutral or alkaline pH depending on its concentration in the nutrient solution. Moreover, it occurs naturally in calcareous soils. When $KHCO_3$ treatments were applied to the Fe-deficient roots together with Fe(III)-citrate for 30 min, the relative Fe(II) concentration found in the tissues was higher than expected according to the results mentioned above. The pH of the nutrient solution was adjusted to 7.0 (1 mM $KHCO_3$) and 7.5 (3 mM $KHCO_3$), but the relative amount of Fe(II) was similar to that found in roots supplied with Fe(III)-citrate at pH 4.5-5.5 (without bicarbonate). This difference suggests that the presence of bicarbonate in the nutrient solution supports the accumulation of Fe(II) forms in the apoplast. According to the Mössbauer parameters of the Fe(II) component, the coordination sphere of Fe(II) was very similar to that at pH 4.5. On the other hand, FCR activity was found to be lower in plants treated and assayed in bicarbonate containing medium compared to those treated in bicarbonate but assayed at pH 5.5 (Fig. 6) which is in agreement with the previous findings. Indeed, Fe uptake was shown to be inhibited by bicarbonate (Venkatraju and Marschner, 1981, Nikolic et al., 2000). This means that the presence of bicarbonate in the rhizosphere results in a slower reoxidation of Fe(II). As the reoxidation may occur both outside and inside the cells either

the uptake or the apoplastic mineralization (oxidative conversion to ferrihydrite) may be inhibited.

Short term exposition of Fe-deficient cucumber roots to bicarbonate (discussed above) and high concentration of Fe may create a condition for the so called Fe plaque formation (Hansel et al., 2001, Liu et al., 2007). It has been reported that Fe may be accumulated at the outer surface of the rhizodermis in particulate forms (Longnecker and Welch, 1990, Strasser et al., 1999). The authors questioned the role of this Fe accumulation in the Fe nutrition of the plants. When cucumber plants were grown for 16-18 days in nutrient solution containing sufficient concentration of Fe(III)-citrate, no Fe accumulation was observed in particulate form (Fig. 4, F). However, when 1 mM $KHCO_3$ was also added to the nutrient solution (for the whole growth period) a clear accumulation of Fe was observed as grains at the rhizoplane that is suggested to be Fe plaque.

The Mössbauer parameters of the Fe(III)-components, found in the spectra of the roots grown in the presence of 1 or 3 mM bicarbonate (Table 2) support the presence of hydrated ferric oxides discussed at pH=6.5-7.5. According to the Mössbauer parameters no evidence of basic Fe(III)-carbonate or carbonate containing Fe(II-III)-hydroxy salts (Music et al., 2004, Genin and Ruby, 2008) – contributing to the Fe plaque – could be found.

The above described processes characterised by Mössbauer spectroscopy have been summarized in Fig. 7 giving a schematic representation of Fe microenvironments formed during the short term Fe-citrate treatments of Fe deficient cucumber plants at low and high pH. The apoplastic processes are coupled to the membrane transport machinery associated with Fe uptake (Hell and Stephan, 2003). The Fe(III) and Fe(II) compounds and complexes may be formed in the apoplastic side of the membrane but Fe assimilation leads to the formation of other ones

constituting similar microenvironments. These are not differentiated by Mössbauer spectroscopy but their location may be identified by additional fractionation and analytical techniques. This is in the focus of our future research.

In conclusion, it is suggested that the "pH effect" modifies not only the FCR activity but, through the decreased H^+ excretion, the reoxidation of the enzymatically reduced Fe species may also be enhanced resulting in new immobile Fe(III) species present in the apoplast. The "bicarbonate effect" may involve (i) a pH component decreasing both the FCR activity and the acidification of the apoplast and (ii) a mineralization effect leading to the slow accumulation of extraplasmatic Fe particles forming the Fe plaque trapping Fe and other minerals in biologically unavailable form. It should be noted that these Fe particles are only present in the roots of Fe-sufficient plants.

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Table 1. Mössbauer parameters of the Fe components found in the roots of iron deficient cucumber supplied with 500 μM ^{57}Fe(III)-citrate for 30 min at different pH values. Errors in the last digits are given in parenthesis.

sample	Fe(II) component			Fe(III) component		
	δ mm s^{-1}	Δ mm s^{-1}	S_r %	δ mm s^{-1}	Δ mm s^{-1}	S_r %
pH 4.5	1.34±0.02	3.04±0.04	16±2	0.46±0.01	0.60±0.01	84
pH 5.5	1.30±0.02	2.92±0.04	12±2	0.47±0.01	0.61±0.01	88
pH 6.5	1.25±0.04	2.85±0.07	5±1	0.45±0.01	0.68±0.01	95
pH 7.5	1.36±0.06	2.80±0.10	5±1	0.46±0.01	0.70±0.01	95

Line width are constrained all to be the same.
δ: isomer shift relative to α–Fe
Δ :quadrupole splitting
S_r: relative spectral area

Table 2. Mössbauer parameters of the iron components found in the roots of cucumber treated with bicarbonate. Iron deficient plants (-Fe) were supplied with 500 μM ^{57}Fe(III)-citrate (FeCit) and 1 or 3 mM $KHCO_3$ for 30 min. Iron sufficient plants were grown with 0.01 mM ^{57}Fe(III)-citrate and 1 mM $KHCO_3$ for 14 days. Errors in the last digits are given in parenthesis.

Sample	Fe^{II} component			Fe^{III} component		
	δ mm s^{-1}	Δ mm s^{-1}	S_r %	δ mm s^{-1}	Δ mm s^{-1}	S_r %
-Fe/+ FeCit 1 mM $KHCO_3$	1.30±0.01	3.11±0.07	17±2	0.53±0.01	0.69±0.01	83
-Fe/+ FeCit 3 mM $KHCO_3$	1.30±0.04	3.04±0.07	15±2	0.52±0.01	0.67±0.01	85
+FeCit 1 mM $KHCO_3$	-			0.49±0.01	0.68±0.01	100

Line widths are constrained all to be the same.
δ: isomer shift relative to α–Fe
Δ :quadrupole splitting
S_r: relative spectral area

Figure legends:

Fig.1 Mössbauer spectra (T=80 K) of Fe deficient cucumber roots supplied with 500 μM ^{57}Fe(III)-citrate at different pH values for 30 min. Shaded regions represent the contribution of Fe(II).

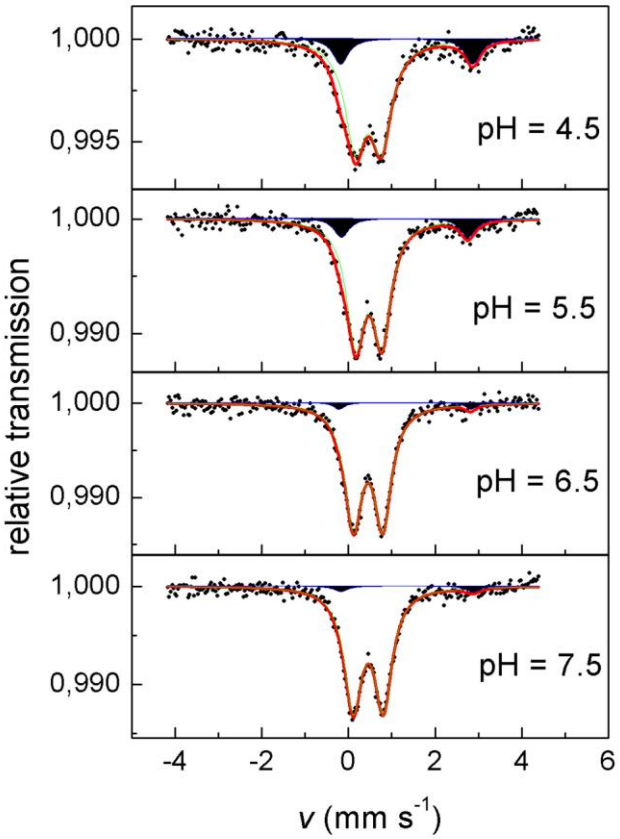


Fig. 2 Iron concentration in the roots of iron deficient cucumber supplied with 500 μM ^{57}Fe(III)-citrate at different pH values for 30 min. Total Fe concentration was obtained by ICP-MS whereas Fe(II) concentrations were estimated from the total Fe using the Fe(II):Fe(III) ratio found in the Mössbauer spectra (data are presented as means±SD, n=3). Statistically different columns are indicated by different letters ($P=0.05$).

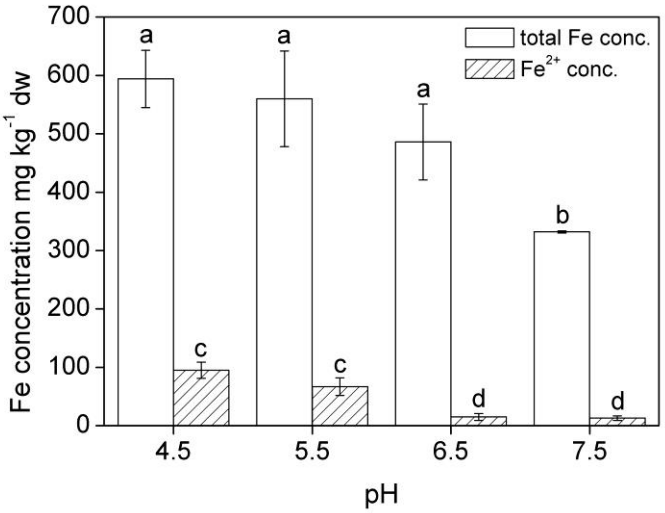


Fig. 3 Ferric chelate reduction of Fe deficient cucumber roots pretreated with MES/HEPES buffers at different pH values (indicated inside the columns) with (+Fe) or without (-Fe) 500 μM ^{57}Fe(III)-citrate for 30 min. The FCR assay was conducted afterwards at the same pH as that used in the pretreatment (data are presented as means±SD, *n*=5). Statistically different columns are indicated by different letters (*P*=0.05).

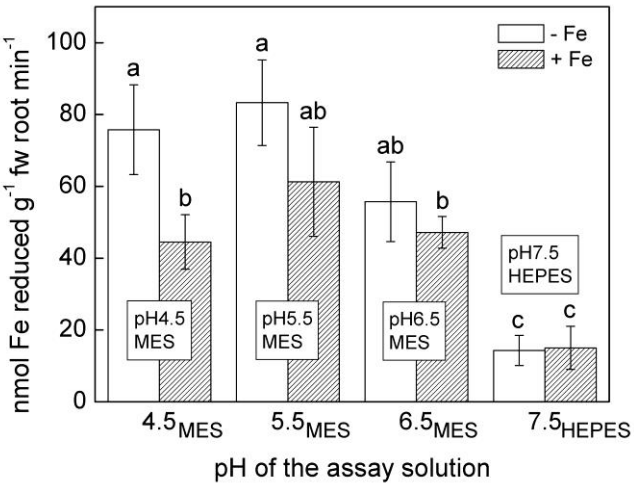


Fig. 4 Microscopic analysis of Fe distribution in cross sections of young roots of cucumber (about 3-5 mm behind the tip). Iron has a brownish colour due to Perls stain and DAB

intensification. A. Iron deficient root supplied with 500 μM Fe(III)-citrate at pH 4.5 (MES) for 30 min; B. Iron deficient root supplied with 500 μM Fe(III)-citrate at pH 7.5 (HEPES) for 30 min; C. Iron deficient root supplied with 500 μM Fe(III)-citrate at pH 7.5 ($KHCO_3$) for 30 min; D. Iron sufficient root grown with 10 μM Fe(III)-citrate and 1 mM $KHCO_3$ for 14 days; E. Iron deficient root without any Fe supply (control for A, B, C); F. Iron sufficient root grown with 10 μM Fe(III)-citrate (unbuffered) for 14 days (control for D). The scale bar represents 100 μm.

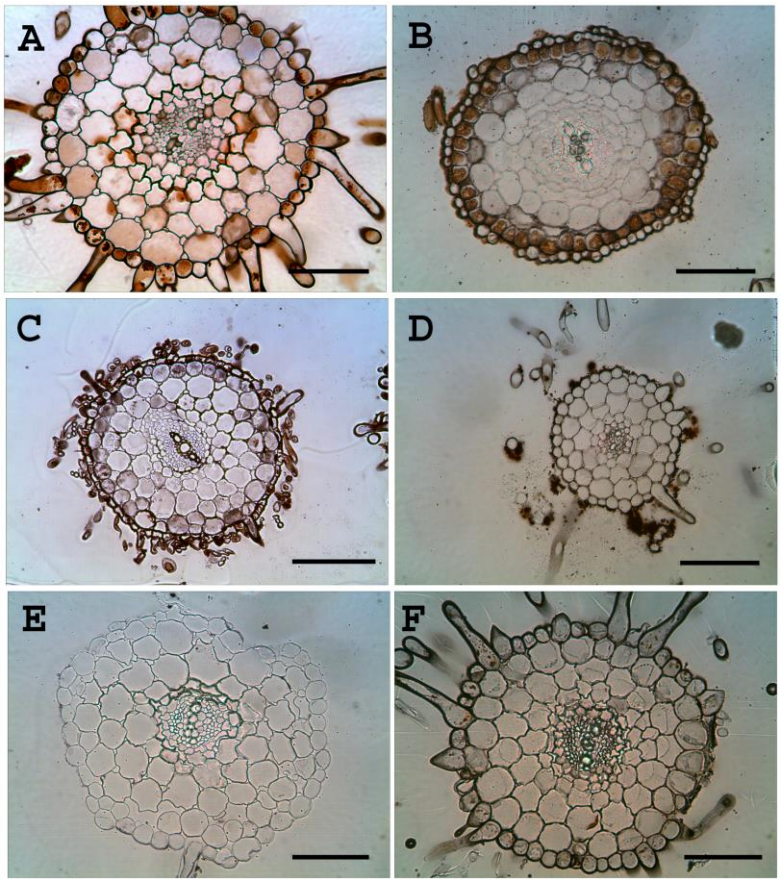


Fig. 5 Mössbauer spectra (T=80 K) of cucumber roots. A. Iron sufficient root grown with 10 μM ^{57}Fe(III)-citrate and 1 mM $KHCO_3$ for 14 days; B, Iron deficient root supplied with 500 μM ^{57}Fe(III)-citrate at pH 7.0 (1 mM $KHCO_3$) for 30 min. C. Iron deficient root supplied with 500

μM ^{57}Fe(III)-citrate at pH 7.5 (3 mM $KHCO_3$) for 30 min. Shaded regions represent the contribution of Fe^{II}.

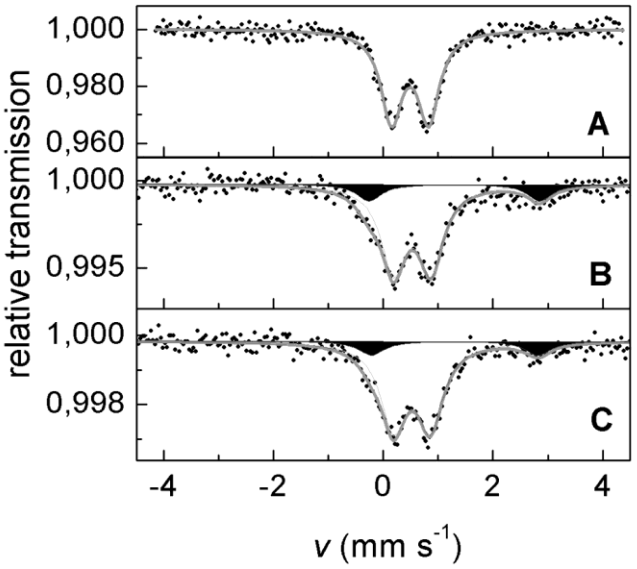


Fig. 6 Ferric chelate reduction of Fe deficient cucumber roots pretreated with $KHCO_3$ at different pH values (indicated inside the columns) and with (+Fe) or without (-Fe) 500 μM ^{57}Fe(III)-citrate for 30 min. The FCR assay was conducted at the same or different pH (MES/$KHCO_3$) as that used in the pretreatment (data are presented as means±SD, n=5). Statistically different columns are indicated by different letters (P=0.05).

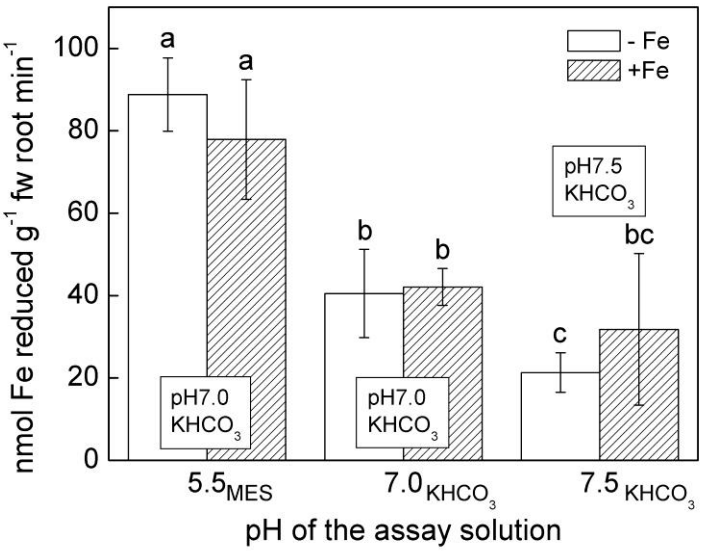
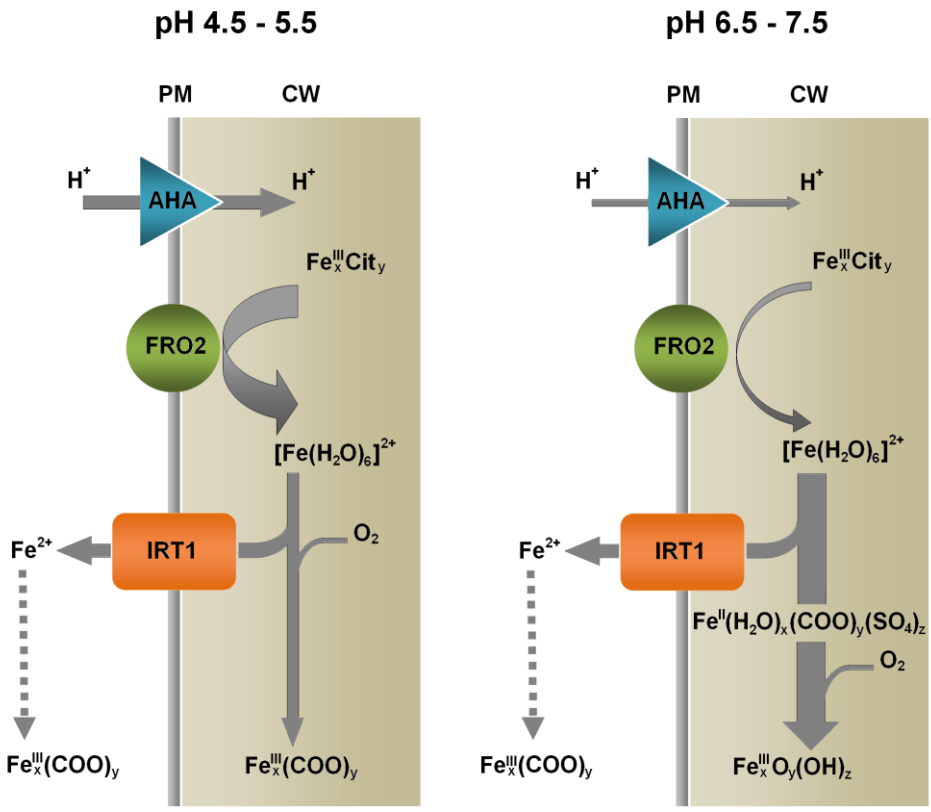


Fig. 7 Schematic representation of the processes during the short term Fe(III)-citrate treatments of Fe-deficient cucumber roots at pH 4.5-5.5 and 6.5-7.5. Fe(III)-citrate complex may be present at different stochiometry in the solution depending on pH ($Fe^{III}_xCit_y$). It is reduced by FRO2 and Fe(II) is released in hexaaqua complex form. Fe(II) is taken up by IRT1 and can be reoxidised and complexed to nicotianamine and other metabolites ($Fe^{III}_xCOO_y$). But Fe(II) can also be reoxidised by O_2 outside the cytoplasm and bind to the apoplastic charges or free citrate residues again ($Fe^{III}_xCOO_y$) at low pH (MES). At alkaline pH maintained by HEPES or $KHCO_3$ Fe(II) may also bind to carboxylic groups or sulphate thereby restricting its mobility ($Fe^{II}(H_2O)(SO_4)_y(COO)_z$). Reoxidation results in further reduction of Fe mobility producing amorphous ferrihydrites or polymers ($Fe_xO_y(OH)_z$). (Line width represents the up or down regulation in the chemical/enzymatic processes. PM, plasma membrane; CW, cell wall)


pH 4.5 - 5.5
PM
CW
H+
AHA
H+
Fe^III_x Cit_y
FRO2
[Fe(H2O)6]2+
Fe2+
IRT1
O2
Fe^III_x(COO)_y
Fe^III_x(COO)_y
pH 6.5 - 7.5
PM
CW
H+
AHA
H+
Fe^III_x Cit_y
FRO2
[Fe(H2O)6]2+
Fe2+
IRT1
Fe^II(H2O)_x(COO)_y(SO4)_z
O2
Fe^III_x(COO)_y
Fe^III_x O_y(OH)_z

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