Acta Bot. Neerl. 38(2), June 1989, p. 155–163

Kinetics of ferric chelate reduction by roots of iron-deficient peanut (*Arachis hypogea*)

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SUMMARY

Experiments were conducted to evaluate the effect of Fe^{3+}-chelate concentration and chemical properties on the rate of reduction by roots of Fe-deficient peanut plants (*Arachis hypogea* L. cv. Florigiant). Reduction studies with the Fe^{3+}-chelates of ethylenediaminetetraacetate (EDTA), hydroxyethylethylenediaminetriacetate (HEDTA), *N,N*-ethylenediaminediacetate, and diethylenetriaminepentaacetate (DTPA) each showed saturation kinetics characteristic of active processes by plants. K_m values varied from 23 to 85 μM, and V_{max} from 0·55 to 5·5 μmol h^{-1} (g fresh roots)$^{-1}$. V_{max} values declined as the Fe^{3+}-chelate formation constant increased. The removal of plant shoots reduced initial (< 6 h) reduction rates by 22%. The K_m' for Fe^{3+}-chelates was much higher than Fe^{3+}-chelate concentrations in soil solutions of problem calcareous soils.

Key-words: chlorosis; Fe-deficient; Hofstee; peanut.

INTRODUCTION

During the last decade, many aspects of iron nutrition in higher plants have become clearer (Chaney & Bell 1987; Romheld & Marschner 1986, Bienfait 1988, 1989). The total soluble iron in calcareous soils is 10^4 times higher than soluble inorganic iron species (O'Connor *et al.* 1971), which indicates that essentially all the iron in the soil solution is chelated by natural organic compounds. These natural chelating materials greatly increase Fe^{3+} diffusion to the root (Lindsay 1974; Hodgson 1968).

Plants respond to iron-deficiency by increasing (derepressing) the ability of their roots to obtain iron from calcareous soil solutions. This regulatory control system has been called the iron-stress-response (Brown 1978). Recently *Poaceae* species have been shown to differ from other plant species in their mechanism of iron mobilization. *Poaceae* species secrete amino acids with moderate Fe^{3+} chelating ability (called phytosiderophores) into the rhizosphere, and the roots are believed to absorb intact Fe-phytosiderophores rather than reducing Fe^{3+}-chelates at the plasma membrane, as found for other plant families (Romheld & Marschner 1986; Bienfait 1988, 1989). In non-*Poaceae* species, iron-stress-response occurs in epidermal cells of subapical portions of the main and lateral roots

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Abbreviations: BPDS, bathophenanthrolinedisulphonate; EDTA, ethylenediaminetetraacetate; DTPA, diethylenetriamine-pentaacetate; HEDTA, hydroxyethylethylenediaminetriacetate; PDTS 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulphonate.

formed after initiation of iron-stress, and on associated root hairs (Ambler *et al.* 1971; Brown & Ambler 1974; Romheld & Marschner 1981, 1983, 1986; Bell *et al.* 1988, Wergin *et al.* 1988). These roots have greatly increased iron uptake-translocation to shoots (as much as 500 times faster than fully iron-sufficient plants) (Chaney & Bell 1987), including increased reduction of Fe^{3+} chelates to Fe^{2+}, absorption of Fe^{2+} iron, translocation of iron from roots to shoots, net release of protons, and (for some plants) the release of reductants such as caffeic acid into the rhizosphere (Bienfait 1985, 1988; Romheld & Marschner 1986; Chaney & Bell 1987). Peanut plants display strong regulatory control of iron reduction and uptake (Chaney & Coulombe 1982).

One of the basic steps in plants obtaining iron from the soil solution is the reduction of Fe^{3+}-chelates (Chaney *et al.* 1972; Schwab 1981). A kinetic analysis of the reduction of different Fe^{3+}-chelates at the root surface would aid not only an understanding of the basic mechanism of reduction, but might also help to explain the differences in availability among Fe^{3+}-chelates. In the present study the K_m and V_{max} for reduction of four Fe^{3+}-chelates by iron-deficient peanut roots were determined. Peanut plants were used because peanut roots release only minute amount of 'reductants' (Brown & Ambler 1973; Romheld & Marschner 1983; Chaney & Bell 1987) into the nutrient solution compared to soybean and tomato plants. The use of PDTS (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulphonate; (also called FerroZine [FZ]) to measure the Fe^{3+}-chelate reduction rate was tested because the reagent normally used, bathophenanthrolinedisulphonate (BPDS) (Chaney *et al.* 1972), is so expensive that the cost limits research.

MATERIALS AND METHODS

Growth

Peanut seeds (*Arachis hypogea* L. cv. Florigiant) were germinated in standard seed germination test papers. The base of rolled papers was placed in 250 μM $CaSO_4$ in the dark at 22°C for 9 days. Seed coats were removed. The stem of each individual seedling was wrapped with polyurethane foam, and placed in a hole in a Plexiglas lid over a 10 litre polyethylene pan (48 plants/pan) in a growth chamber. The pans contained 8 litres of aerated nutrient solution of the following composition: 0·8 mM $Ca(NO_3)_2$, 1·2 mM KNO_3, 0·2 mM $MgSO_4$, 100 μM $NH_4H_2PO_4$, 100 μM $(NH_4)_2HPO_4$, 35 μM NaCl, 3 μM H_3BO_3, 1·0 μM $MnCl_2$, 0·5 μM $ZnCl_2$; 0·10 μM $CuCl_2$, and 0·05 μM $NaMoO_4$. The initial solution pH was 6·5; solutions were completely replaced every 2 days. After 6 days, uniform seedlings were selected and their cotyledons and axial shoots were removed. These were rewrapped in polyurethane foam in bundles of two, and four bundles transferred to each polyethylene bucket containing 8 litres of aerated nutrient solution. The solutions contained 0·2 μM FeEDDHA (ethylene-diamine dihydroxyphenyl acetate) for the first 2 days, and no iron for 6 further days (solutions renewed every 2 days). At the end of this conditioning period, the plants were mildly chlorotic. FeEDDHA was prepared at 1:1 molar ratio according to Chaney & Bell (1987). Other Fe-chelates were prepared with 5% excess ligand.

The plants were grown in a growth chamber with cool-white fluorescent plus incandescent lamps. The lights provided about 200 $\mu E/m^2s$ at plant height during the 16-h day. The air temperature was 25°C.

Reduction assays

One-litre polyethylene beakers (wrapped with black polyethylene, and with a black Plexiglas lid), containing 1 litre of nutrient solution plus Fe^{3+}-chelates and Fe^{2+} colour

reagents, were placed in the growth chamber. The lid had a hole for a glass gas dispersion tube, and a slot and hole for the two peanut plants held by polyurethane foam. The test solutions were set up the day before the reduction experiments to reach temperature equilibrium. Each was continuously aerated. Initial 5-ml samples were obtained from each beaker. Plants were transferred to the test solutions starting 6 h into the daylight period. Five millilitre samples were withdrawn at 30- or 60-min intervals for 6 h depending on the rate of reduction observed. Deionized water was added to replace sample removal and evapotranspired water 5 min before each sample was taken. The fresh weight of blotted roots was obtained at the end of the reduction trial. For each Fe^{3+}-chelate, beakers without plants were included in order to correct reduction rates for slow photoreduction.

Because linear regression was to be used to estimate the kinetic constants based on the whole Fe^{3+}-chelate concentration range, individual concentrations were not replicated in most of the research. For direct comparisons of the effect of PDTS concentration, or of excising the shoots, three replications were used.

The Fe^{2+} produced was measured spectrophotometrically at the appropriate wavelength. Initial rates of reduction (δAh^{-1}) were obtained by linear regression of absorbance (y) versus time (x); δAh^{-1} was converted to $\mu molh^{-1}$ by the appropriate molar absorption coefficient (22 100 at 535 nm for BPDS, and 25 200 at 562 nm for PDTS).

The Fe^{3+}-chelates studied included Fe^{3+}-diethylenetriamine-pentaacetate (FeDTPA), Fe^{3+}-ethylenediaminetetraacetate (FeEDTA), Fe^{3+}-hydroxyethylethylenediaminetriacetate (FeHEDTA), Fe^{3+}-*N,N*-ethylenediaminediacetate (FeEDDA), Fe^{3+}-cyclohexane-1,-2-diamine-tetraacetate (FeCDTA), Fe^{3+}-triethylenetetraaminehexaacetate (FeTTHA), and Fe^{3+}-dihydroxyethylglycine (FeDHEG). Formation constants for Fe^{2+} and Fe^{3+} chelates are shown in Table 1.

The Fe^{3+} reduction data were subjected to kinetic analysis. K_m and V_{max} were calculated by linear regression of rate (y) versus [rate/Fe chelate concentration] (x) (Hofstee plot). The reduction rate versus Fe-chelate concentration curves were calculated by substituting into the Michaelis–Menten equation using the K_m and V_{max} obtained from the Hofstee plot. These curves were superimposed on a plot of the actual experimental results.

RESULTS AND DISCUSSION

Although BPDS has been used in earlier reduction assays (Chaney *et al.* 1972; Bienfait *et al.* 1982, 1983; Romheld & Marschner 1983), the use of PDTS for kinetic analysis research was studied because BPDS is much more expensive (1988 costs were $771/mole for PDTS [Reagent 2304-26, Hach Chemical Co., Ames, IA]* versus $9441/mole for BPDS [Reagent 286, G.F. Smith Chemical Co., Columbus, OH]). The reduction rate for 20 μm FeDTPA by peanut plants was $0{\cdot}160 \pm 0{\cdot}022$ and $0{\cdot}167 \pm 0{\cdot}016$ $\mu mol\ h^{-1}$ (g fresh roots)$^{-1}$ (mean ± sd) for 200 μM PDTS and BPDS, respectively. Based on the similarity of rates of reduction of FeDTPA, measured by PDTS and BPDS, PDTS was used to trap Fe^{2+} in the rest of this study.

The solution pH was not buffered during these experiments. As noted by Chaney & Bell (1987) and others, the nutrient solution pH is rapidly lowered by iron-deficient peanut roots, often reaching pH 3·8 after 6 h of reduction rate or iron uptake assays. However, reduction rates were linear with time, and pH lowering appears to have had little effect on

*The mention of a vendor or product does not imply that they are endorsed or recommended by the US Department of Agriculture over vendors of similar products not mentioned.

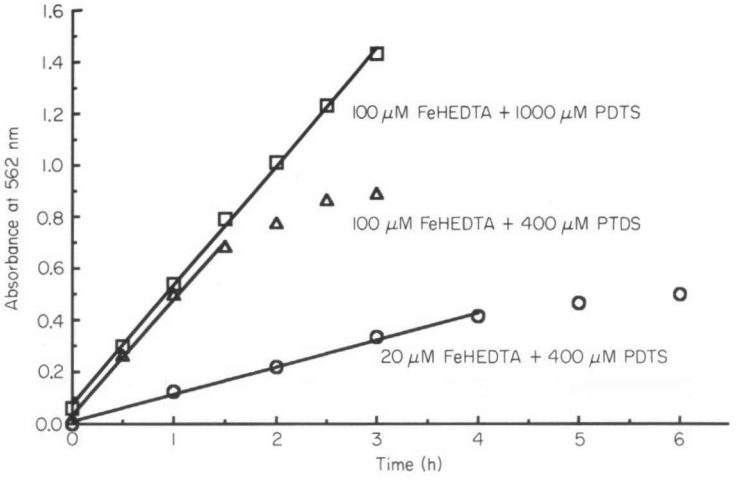

Fig. 1. The effect of FeHEDTA and PDTS concentration on the time course of reduction of FeHEDTA by roots of iron-deficient peanut plants.

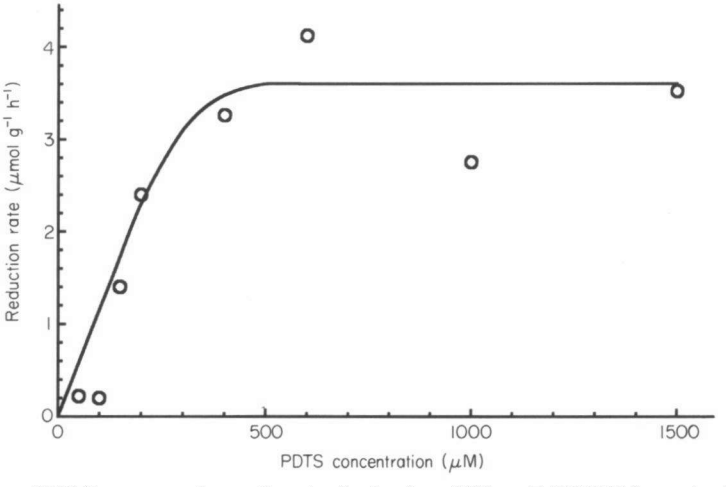

Fig. 2. The effect of PDTS concentration on the rate of reduction of 100 μM FeHEDTA by roots of iron-deficient peanut plants.

the rate of reduction measured. Figure 1shows examples of reduction assay experiments. The lines are calculated by linear regression of the data, including all points before the reduction rate departed from the linear response. If we compare 400 and 1000 μM PDTS as an Fe^{2+} trap, the initial reduction rates are similar, but the linear reduction curve continued for a longer period (over 3 h) with the higher PDTS concentration.

The effect of the PDTS concentration on the apparent rate of reduction of 100 μM FeHEDTA is shown in Fig. 2. Up to 400 μM PDTS, increasing PDTS increased the measured initial rate of reduction although higher PDTS produced linear reduction curves for longer periods (Fig. 1). Thus, 400 μM PDTS were used in subsequent reduction assays.

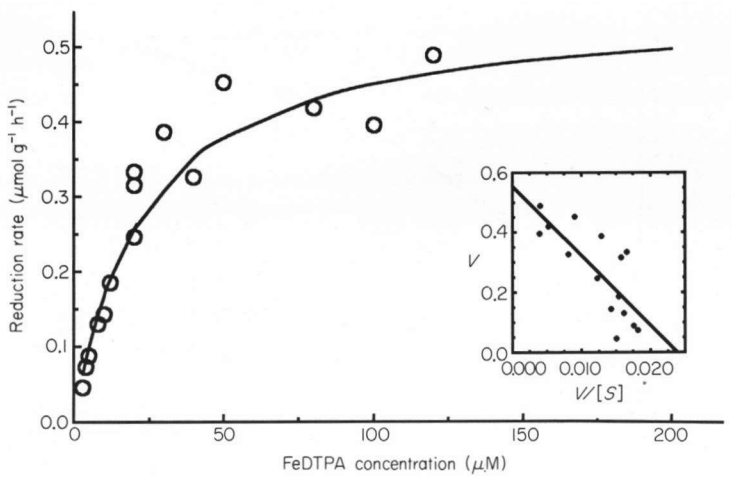

Fig. 3. The effect of FeDTPA concentration on the rate of its reduction by roots of iron-deficient peanut plants. The line is the calculated Michaelis–Menten equation with $K_m = 23{\cdot}2$ and $V_{max} = 0{\cdot}554$. The inset curve is the Hofstee plot of data with a linear regression line used to estimate K_m and V_{max}.

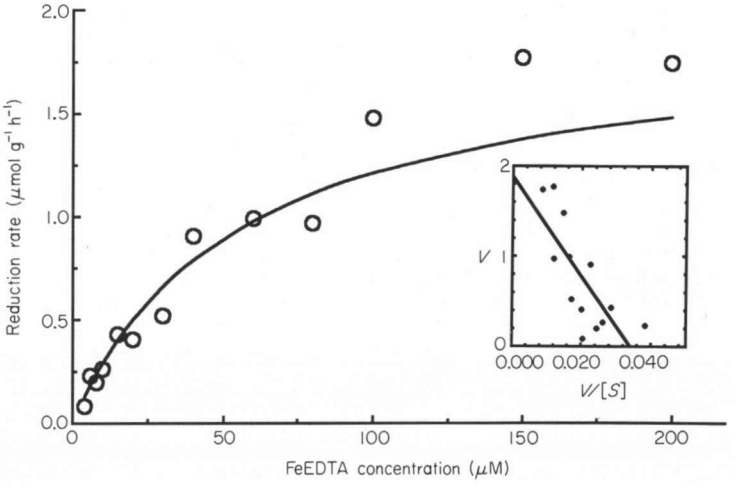

Fig. 4. The effect of FeEDTA concentration on the rate of its reduction by roots of iron-deficient peanut plants. The line is the calculated Michaelis–Menten equation with $K_m = 55{\cdot}3$ and $V_{max} = 1{\cdot}88$. The inset curve is the Hofstee plot of data with a linear regression line used to estimate K_m and V_{max}.

Results for FeDTPA are shown in Fig. 3. The reduction rate aproached a plateau at higher FeDTPA levels. The inset on Fig. 3 shows the linear regression of rate versus rate/[*S*]. The equation for the line is:

$$\text{reduction rate} = 0{\cdot}554 - 23{\cdot}2\ (\text{reduction rate}/[\text{FeDTPA}]),$$

with $r = 0{\cdot}78$. Results for FeEDTA are similarly shown in Fig. 4. The equation for the linear regression is:

$$\text{reduction rate} = 1{\cdot}88 - 55{\cdot}3\ (\text{reduction rate}/[\text{FeEDTA}]),$$

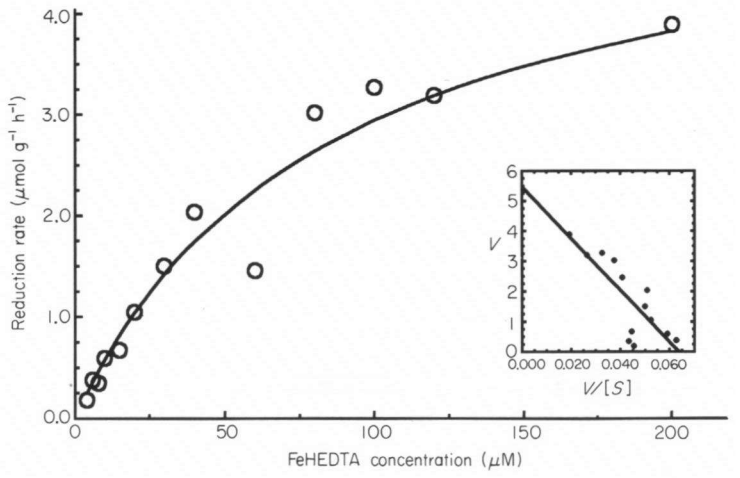

Fig. 5. The effect of FeHEDTA concentration on the rate of its reduction by roots of iron-deficient peanut plants. The line is the calculated Michaelis–Menten equation with $K_m = 85{\cdot}7$ and $V_{max} = 5{\cdot}47$. The inset curve is the Hofstee plot of data with linear regression line used to estimate K_m and V_{max}.

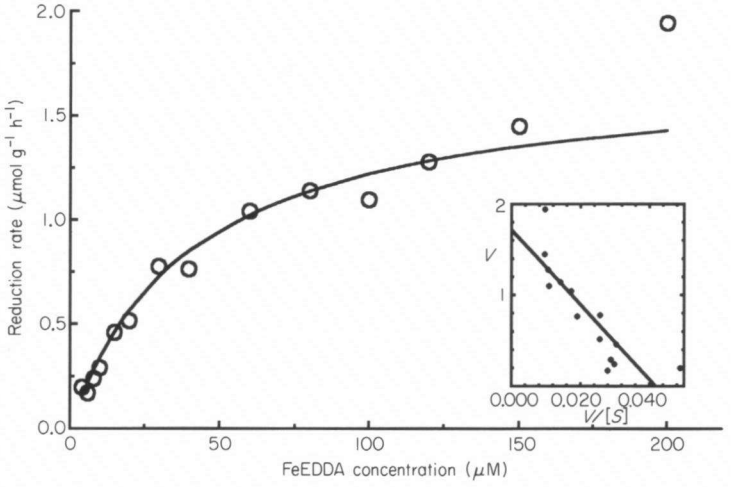

Fig. 6. The effect of FeEDDA concentration on the rate of its reduction by roots of iron-deficient peanut plants. The line is the calculated Michaelis–Menten equation with $K_m = 41{\cdot}0$ and $V_{max} = 1{\cdot}72$. The inset curve is the Hofstee plot of data with linear regression curve used to estimate K_m and V_{max}.

with $r = 0{\cdot}75$. Results for FeHEDTA are shown in Fig. 5. The equation for the linear regression is:

$$\text{reduction rate} = 5{\cdot}47 - 85{\cdot}7\ (\text{reduction rate}/[\text{Fe/HEDTA}]),$$

with $r = 0{\cdot}81$. Results for FeEDDA are shown in Fig. 6 The equation for the linear regression is:

$$\text{reduction rate} = 1{\cdot}72 - 41{\cdot}0\ (\text{reduction rate}/[\text{FeEDDA}]),$$

with $r = 0{\cdot}85$.

Table 1. Summary of chemical characteristics and kinetic analysis of Fe^{3+}-chelate reduction for iron chelates studied (in order of increasing iron-chelate formation constant). Formation constants (log K_{ML}) at 0 ionic strength are taken from the corrected GEOCHEM-PC database, GEODATA (Chaney 1988). The predominant chemical species at pH 6 was identified by GEOCHEM

Chelate	K_m (μM)	V_{max} (μmol h^{-1} g^{-1})	Rate at 10 μM chelate (μmol h^{-1} g^{-1})	Formation constant		Charge at pH 6
				Fe^{2+}	Fe^{3+}	
FeEDDA	41	1·7	0·34	10·7	18·2	0
FeHEDTA	86	5·5	0·57	13·5	21·8	−1
FeEDTA	55	1·9	0·29	16·0	27·6	−1
FeDTPA	23	0·55	0·17	18·5	31·2	−2

The predominant chemical species at pH 6 were: Fe(OH)EDDA, Fe(OH)HEDTA (ionization of the hydroxyethyl), FeEDTA, and FeDTPA.

Results for FeCDTA and FeTTHA are not shown. PDTS was not able to retain Fe^{2+} produced by peanut roots in the presence of these chelators in aerated nutrient solution, particularly with FeCDTA. In other studies, however, BPDS prevented absorption of iron from FeCDTA by algae (Owens & Chaney, 1971). Thus PDTS chelates Fe^{2+} too weakly for use with strong Fe^{3+} chelators. BPDS could be used in a study of stronger chelators.

The rate of reduction of FeDHEG by roots could not be determined because addition of PDTS to the FeDHEG solution caused a chemical reaction whereby Fe^{2+} was immediately formed. Addition of an Fe^{2+}-trap alters the redox equilibria in these systems (see Romheld & Marschner 1986), and apparently DHEG can be oxidized by Fe^{3+} to produce Fe^{2+}.

The influence of excising the plant shoots on the apparent rate of reduction was tested using 100 μM FeHEDTA plus 400 μM PDTS. The rate for intact plants was 3·16 μmoles h^{-1} (g fresh roots)$^{-1}$, while the rate for topped plants was 2·47, a non-significant 22% reduction. The assays were completed by 3 h, and root energy reserves appear to have prevented a large effect of shoot excision on Fe^{3+}-chelate reduction.

Kinetic analysis of the Fe^{3+}-chelate reduction results was conducted because the reduction rate approached saturation at higher Fe^{3+}-chelate concentrations. This pattern is characteristic of 'active' plant uptake of nutrient elements (Epstein 1972), and other biological reactions. V_{max} and K_m were evaluated in the normal fashion (Epstein 1972) used in the analysis of the kinetics of ion uptake using the methods developed for enzymes. Bienfait *et al.* (1982, 1983) had previously examined the kinetics of FeEDTA reduction. They studied the effect of solution temperature and FeEDTA concentration on the FeEDTA reduction rate by roots of chlorotic beans. They determined that the reduction rate was diffusion-limited at low FeEDTA concentration (20 μM), but that the reduction rate shifted to an activation energy limitation at higher (300 μM) FeEDTA concentration.

K_m may be a measure of affinity of these Fe^{3+}-chelates for the site on the root where reduction occurs. Table 1 shows a summary of the kinetic data for the four iron chelates studied in detail. Formation constants for each chelator with Fe^{3+} and Fe^{2+} are the ionic strength 0 constants in the GEOCHEM-PC database GEODATA (Chaney 1988). The charge on the Fe^{3+}-chelate in Table 1 is that for the chemical species which predominates at pH 5–6 according to GEOCHEM-PC with constants for all important species formed

(Fe(OH)EDDA; Fe(OH)HEDTA; FeEDTA; FeDTPA). There is no apparent relationship between K_m and chelate formation constants. However, V_{max} decreased with increased Fe^{3+}-chelate formation constant. FeHEDTA had the lowest affinity (K_m) at 86 μM but the highest reduction rate V_{max} (5·5 μmol h^{-1} (g fresh roots)$^{-1}$).

The calculated rate of reduction for each Fe^{3+}-chelate at 10 μM is shown in Table 1 to provide a typical rate for concentrations ordinarily used in nutrient solution culture by many authors. The higher V_{max} for FeHEDTA allows this iron-chelate to have the highest reduction rate under practical conditions of nutrients solutions (10 μM iron-chelate).

Ionic charge on the Fe^{3+}-chelate might be expected to affect binding to the reduction site on the root. FeDTPA with charge = −2 had a lower K_m value than iron-chelates with −1 or 0 charge.

The K_m values observed (23–86 μM) are much higher than the usual concentration of total iron dissolved in soil solution of calcareous soils (0·1 μM). Although one should not expect Fe^{3+}-chelates, which are not the natural substrate of the reduction system on roots, to have relevant K_m's, these values are very much higher than iron-chelate levels in natural conditions.

As reported by Chaney *et al.* (1972), Romheld & Marschner (1983), and by Kojima & Bates (1981), a reduction of physiologically relevant concentrations of strong Fe^{3+}-chelates (e.g. DTPA, EDTA) by *o*-diphenol 'reductants' released by soybean and sunflower is very slow. Roots of iron-deficient plants of both crops reduce these same Fe^{3+}-chelates very rapidly. Iron-deficient peanut plants release only traces of reductants in contrast to soybean and some other crops (Chaney & Bell 1987; Romheld & Marschner 1983). $Fe^{2+}(PDTS)_3$ accumulated in the nutrient solution rather than inside the root epidermis cells. The reduction process shows the kinetic properties of active uptake and biological enzymatic processes. A reduction of strong Fe^{3+} chelates by the root is an integral step in their obtaining iron from their environment. Although reductants may be important in the release of iron from soil hydrous Fe^{3+} oxide, allowing this inert iron to become chelated and soluble for faster diffusion to roots, reduction is also required for the release of iron from the chelate before transport of Fe^{2+} into root cells. Fe^{2+} has a short half-life in calcareous soil solutions, and natural Fe^{3+}-chelates should carry all soluble iron released from soil particles.

Further studies are required to elucidate the kinetic properties of reduction of other Fe^{3+}-chelates, and reduction of Fe^{3+}-chelates by plants other than the peanut. BPDS should be used in a study of chelators which have higher formation constants with Fe^{2+}. Many chelators catalyse the oxidation of Fe^{2+} (Kurimura *et al.* 1968), particularly at higher solution pH. BPDS competes better for the Fe^{2+} than PDTS, and should be able to trap Fe^{2+} more effectively if this is needed.

Differences among Fe^{3+}-chelates in their ability to correct chlorosis are affected by many soil and plant characteristics. In this study, differences were observed both in the affinity for the reduction site and in the maximial reduction rate. More research is needed with Fe^{3+}-chelates, which have a wider range of chemical properties, to characterize the nature of the reduction site and process. The present work identified constraints for these needed studies.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the technical assistance of Ms L. Muranaka in conducting the experiments, and Ms C. A. Lloyd in preparing the figures.

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