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Ferric chelate reduction by suspension culture cells and roots of soybean: A kinetic comparison

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Abstract

The abilities of suspension cultures and intact roots of soybean (*Glycine max* L. cv. Hawkeye) to reduce ferric chelate were compared. Ferric chelate was supplied as ferric hydroxyethylethylenediaminetri-acetic acid (FeHEDTA) and reduction was measured spectrophotometrically using bathophenan-throlinedisulfonic acid (BPDS) as the ferrous scavenger. Ferric chelate reduction by cell suspension cultures showed typical saturation kinetics; however, no difference was observed between cells that had been continuously grown with Fe (+Fe) and those that had been grown for four days without added Fe (−Fe). Values for K_m and V_{max}, determined from a Lineweaver–Burk plot, were 57 μM and 28 nmoles mg^{-1} dry weight for the +Fe cells and 50 μM and 22 nmoles mg^{-1} dry weight for the −Fe cells, respectively. Ferric chelate reduction by Fe-deficient roots also exhibited saturation kinetics, while roots grown with adequate Fe did not reduce ferric chelate. The K_m and V_{max} values for Fe-deficient roots were 45 μM and 20 nmoles mg^{-1} dry weight, respectively, and did not differ from values obtained for cells in culture. This study offers strong evidence that the mechanism responsible for the reduction of ferric chelate is the same for cultured cells and roots and that the process is controlled at the cellular level. We propose that suspension cultures can be used as an alternative to intact roots in the study of ferric chelate reduction.

Introduction

The importance of Fe^{3+}-chelate reduction as a prerequisite for Fe uptake by plant roots was demonstrated by Chaney *et al.* (1972). While roots release substances capable of reducing Fe^{3+}-chelates (Brown, 1978; Hether *et al.*, 1984), recent models of Fe uptake emphasize the role of an inducible Fe^{3+}-chelate reducing system in the plasmalemma (Bienfait, 1985; Römheld and Marschner, 1983). Bienfait (1988) compared the inducible 'Turbo' system that reduces Fe^{3+}-chelates to provide Fe^{2+} for the uptake system in dicotyledonous roots with the 'Standard' transmembrane electron transfer system which appears to be present in most plant cells. The latter system reduces ferricyanide, however; it does not reduce Fe^{3+}-chelates, and it is proposed to have a role in regulation of cell growth (Morré *et al.*, 1988).

Sain and Johnson (1986) proposed the use of cell suspension culture in the investigation of Fe uptake by plants and have demonstrated differences in the uptake of Fe by cell cultures from the relatively Fe-efficient Hawkeye and Fe-inefficient T203 soybean (*Glycine max* L.) cultivars. This report provides a kinetic comparison of the Fe^{3+}-chelate reducing system of cultured cells and intact root systems of the Fe-efficient Hawkeye cultivar.

Materials and methods

Cell suspension cultures

Suspension cultures were derived from callus from cotyledons of aseptically grown Hawkeye soybean seedlings. Cells were grown on a modified Gamborg's B5C liquid medium (Gamborg and Wetter, 1975; Sain and Johnson, 1986) supplemented with 86 μM Fe as FeEDTA (ferric ethylenediaminetetraacetic acid). Cells were incubated aseptically on a gyrorotory shaker at 120 rpm in a dimly illuminated (0.1 μE m^{-2} sec^{-1} PAR) chamber at 30°C. A 10 mL volume of cell suspension was transferred to 40 mL of B5C medium in a 250 mL Erlenmeyer flask every fourth day. Iron-deprived cells were grown by transferring Fe-sufficient cells into B5C medium prepared without addition of Fe. Cells were used for Fe^{3+}-chelate reduction assays after four days of growth. The cells were harvested by centrifugation at $300 \times g$ for 5 min followed by three rinses in wash medium (B5C medium prepared without FeEDTA, amino acids, and vitamins). Cells were resuspended in wash medium for use in experiments.

Plant culture

Hawkeye soybean seeds were germinated in vermiculite in the greenhouse and when cotyledons emerged, seedlings were transferred to a 0.1 strength nutrient solution consisting of the macronutrients (mM): KNO_3, 0.5; $Ca(NO_3)_2$ 0.5; $MgSO_4$, 0.2; KH_2PO_4, 0.017 and K_2HPO_4, 0.008. Micronutrient concentrations were (μM): KCl, 2.5; H_3BO_3, 1.25; $MnSO_4$, 0.1; $ZnSO_4$, 0.1; $CuSO_4$, 0.025; and Na_2MoO_4, 0.025. Iron, when supplied, was 10 μM FeEDTA. Forty plants were grown in 10-L containers with continuous aeration. The plants were grown in a growth chamber with a 15/9 h light/dark period at 23°/20°C with 206 μE m^{-2} sec^{-1} PAR supplied by cool white fluorescent and incandescent lamps. When plants without Fe developed a pronounced chlorosis on the second or third trifoliate leaves, they were used for experiments.

Ferric chelate reduction

Ferric EDTA was prepared from Na_2EDTA according to Steiner and van Winden (1970) and Fe^{3+}HEDTA (ferric hydroxyethylethylenediaminetriacetic acid) was prepared as described by Chaney (1988). Bathophenanthrolinedisulfonic acid (BPDS) was used as the Fe^{2+} scavenger; absorbency of the $Fe^{2+}(BPDS)_3$ complex was measued at 535 nm and a molar extinction coefficient of 22,140 was used (Chaney *et al.*, 1972).

The reduction of Fe^{3+}-chelate by washed suspension culture cells was assayed in the wash medium containing 350 μM BPDS and, unless otherwise noted, 86 μM Fe^{3+}-chelate. The cells were incubated under normal growth conditions in foil-covered flasks. Cells were separated from the assay medium by vacuum filtration using a Filtrator (Fisher Scientific, Pittsburg, PA 15219) and pre-weighed Shark Skin analytical filter paper (Schleicher and Schuell, Inc., Keene, NH 03431). The absorbency of the filtrate was then measured. The cells were washed on the filter paper with redistilled water, dried at 60–70°C, and weighed. Ferric chelate reduction activity in nmoles Fe^{2+} mg^{-1} dry weight cells h^{-1} was computed as the difference in the absorbency of an initial sample filtrate and filtrates obtained after an incubation period of 1 h.

Root systems of intact soybean seedlings were rinsed with distilled water and individually placed in 200-mL of 1.0-strength nutrient solution containing 55 μM BPDS in a 250-mL flask. The plants were maintained at 26°C with illumination at 50 μE m^{-2} sec^{-1} and the nutrient solution was continually aerated. Reduction was initiated by the addition of FeHEDTA. Solution samples were taken initially and after 2 h to measure $Fe^{2+}(BPDS)_3$. The remaining volume of the nutrient solution was measured and the weight of the root system determined after drying at 60°C. Ferric chelate reduction activity was computed as nmoles Fe^{2+} mg^{-1} dry weight roots h^{-1}.

Ferric chelate reducing substances released by roots were measured by a modification of the method of Ao *et al.* (1985). Roots of intact plants were incubated in fresh 1.0-strength nutrient solution without Fe^{3+}-chelate, as described

for the root system assay of Fe-chelate reduction. After 24 h the nutrient solution was centrifuged at 12,000 × g for 15 min. A 50-mL sample of the supernatant was incubated with 219 μM BPDS and 45 μM FeHEDTA at 26°C in the dark for 20 h prior to measurement of the $Fe^{2+}(BPDS)_3$ complex. Ferric chelate reduction in the medium that previously contained roots was corrected for reduction occurring in nutrient solution that had not contained roots.

Results and discussion

Ferric chelate reduction by cell suspensions

Non-biological reduction of FeEDTA in both the cell culture and wash media was evaluated in the dark and light in preliminary experiments (Fig. 1). The rate of non-biological Fe-chelate reduction did not differ with the type of medium when incubated in the dark. When incubated under fluorescent lamps, an increase in Fe-chelate reduction was observed in both media. Ferric EDTA prepared from $FeSO_4$ resulted in higher values for non-biological reduction than when FeEDTA was prepared from $FeCl_3$. Based on these results, cell mediated Fe-chelate reduction experiments were incubated in the dark using the wash medium and FeEDTA was prepared from $FeCl_3$.

Ferric EDTA reduction by cell suspension cultures was essentially linear for at least 2 h and a standard incubation time of 1 h was used for experiments (data not reported). Reduction of FeEDTA by suspension cultures did not vary between pH 4 and 8, although activity tended to be highest in the range of pH 5 to 7. In subsequent reduction experiments, a wash medium pH of 5.5 was used.

The chelates FeEDTA and FeHEDTA at a concentration of 86 μM were compared as substrates for reduction by suspended cells. The relationship of cell density to reduction activity is illustrated in Figure 2. The reduction activity mg^{-1} cells for both chelates was a maximum at low cell densities and decreased as cell density exceeded 1 to 2 mg dry cells mL^{-1}. Equations were developed to describe the relationship between cell density and Fe-chelate reduction activity (Fig. 2). The basis for the decrease in reduction activity with FeEDTA or FeHEDTA at higher cell densities is uncertain. At higher

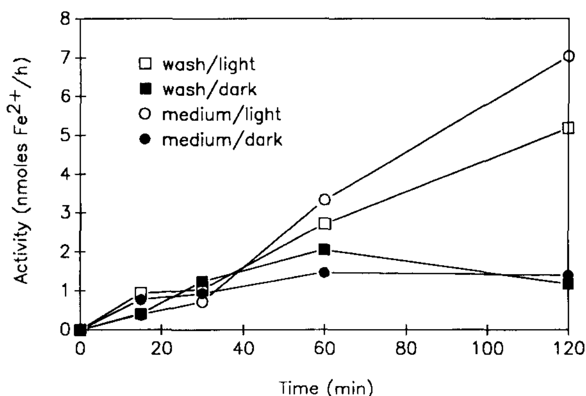

Fig. 1. Non-biological reduction (nmoles Fe^{2+} h^{-1}) of FeEDTA in wash medium (wash) and soybean cell culture medium (medium) in light and dark. Data are means of two to five replicate determinations.

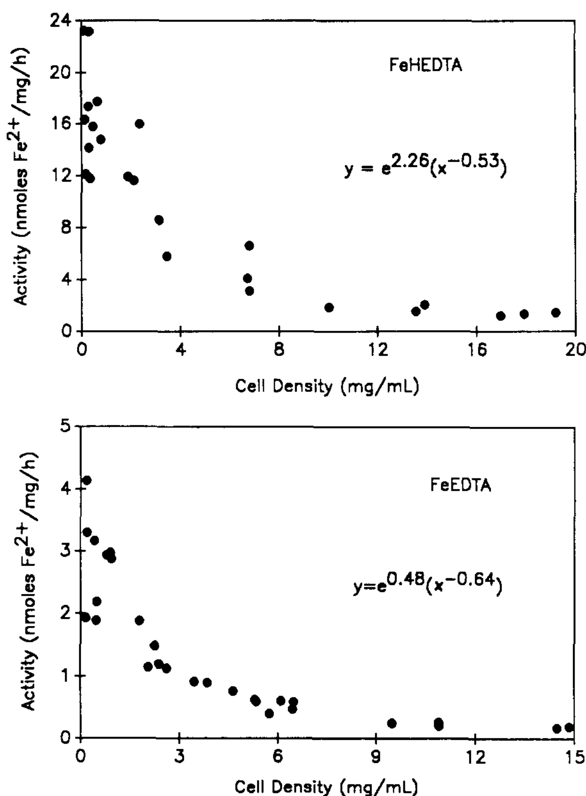

Fig. 2. Reduction activity (nmoles Fe^{2+} mg^{-1} h^{-1}) at various cell densities for Fe-sufficient soybean suspension culture cells with 86 μM FeHEDTA or FeEDTA. Each point represents one flask of cells incubated with Fe-chelate.

cell densities, increased binding of BPDS and $Fe^{2+}(BPDS)_3$ by the cell walls may have lowered the apparent reduction rates. Additionally, high cell densities may have adversely effected reduction activity. At low cell densities (<1 to 2 mg mL^{-1}), reduction activity for FeHEDTA was approximately 5-fold greater than for FeEDTA (Fig. 2).

A kinetic study of FeHEDTA reduction was conducted using low cell densities (<1 mg mL^{-1}) in the reaction medium (Fig. 3). The reaction closely followed Michaelis-Menten kinetics, and a Lineweaver–Burk double reciprocal transformation compared to a linear model resulted in R^2 values exceeding 0.90. Based on Lineweaver–Burk transformations, a maximum velocity (V_{max}) for the reduction reaction of 28 nmoles mg^{-1} h^{-1} and a concentration at half-maximum velocity (K_m) of 57 μM FeHEDTA was obtained (Table 1).

Similar kinetics were obtained for Fe-deprived cells (Fig. 3); and the kinetic parameters did not differ compared to Fe-sufficient cells (Table 1). That cells grown with 86 μM FeEDTA were not Fe limited was demonstrated by growth experiments with higher concentrations of FeEDTA (data not reported). These results suggested that soybean cells in suspension culture had an active 'Turbo' system of Fe^{3+}-chelate reduction regardless of their Fe nutritional status. In contrast, Fe-sufficient roots had very little Fe^{3+}-chelate reducing activity and the 'Turbo' system was

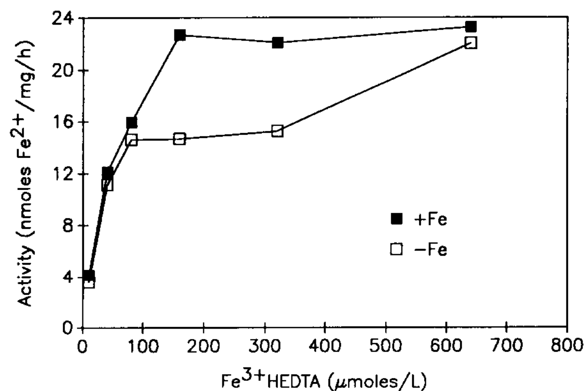

Fig. 3. Reduction activity (nmoles Fe^{2+} mg^{-1} h^{-1}) for Fe-sufficient (+Fe) and Fe-deprived (−Fe) soybean suspension culture cells at various concentrations of FeHEDTA. Data are means of three experiments with +Fe cells and two experiments with −Fe cells.

Table 1. Kinetic values for Fe^{3+} HEDTA reduction by cell suspension cultures and roots. K_m units are μM and V_{max} units are nmoles mg^{-1} dry weight h^{-1}

System	Fe status	K_m [a]	V_{max} [b]
Cells	+	57	28
Cells	−	50	22
Roots	−	45	20

[a] K_m values do not differ significantly ($P = 0.77$).
[b] V_{max} values do not differ significantly ($P = 0.32$).

induced by Fe-deficiency (Brown, 1978; Chaney *et al.*, 1972; Römheld and Marshner, 1983). In experiments with Hawkeye soybean cells in culture, Sain and Johnson (1986) found that while both Fe-sufficient and Fe-deprived cells took up Fe from FeEDTA, uptake was stimulated in cells that were stressed by growth in medium without added Fe. Our results suggest that the mechanism controlling induction of the 'Turbo' system in roots is not functional in cells in culture and that 'Turbo' is always active in cells in suspension culture.

It is of interest to compare the kinetic values for FeHEDTA reduction in Table 1 with those reported for Fe uptake by Hawkeye soybean cells (Sain and Johnson, 1986). The K_m values for Fe uptake from FeEDTA ranged from 0.13 to 1.5 μM while V_{max} values varied from 0.3 to 2.3 nmoles mg^{-1} h^{-1}. While the K_m and V_{max} values for reduction of FeEDTA would be predicted to be lower than for FeHEDTA, the large difference in K_m values for Fe uptake and Fe^{3+}-chelate reduction was surprising.

Ferric chelate reduction by roots

Reduction of FeHEDTA by roots of Fe-deficient soybean plants also followed Michaelis–Menton kinetics (Fig. 4); and the Lineweaver–Burk transformation resulted in a V_{max} of 20 nmoles mg^{-1} h^{-1} and a K_m of 45 μM FeHEDTA (Table 1). Iron-sufficient soybean roots did not reduce FeHEDTA, confirming the inducible nature of the Fe-chelate reducing system (Fig. 4). The kinetic parameters for FeHEDTA reduction did not differ for Fe-deficient roots and cultured cells (Table 1), suggesting that the same reducing system was active both in roots and cells in culture. Furthermore, FeEDTA was reduced by Fe-deficient Hawkeye soybean roots with a V_{max}

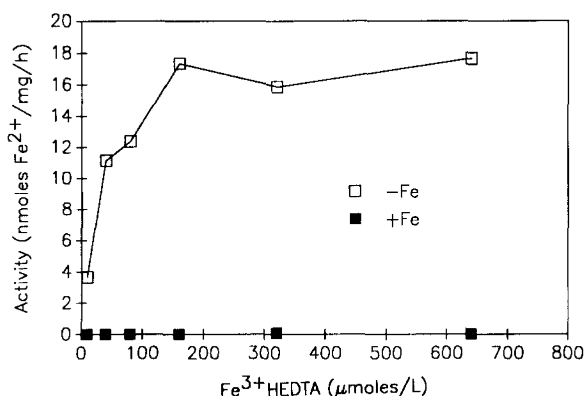

Fig. 4. Reduction activity (nmoles Fe^{2+} mg^{-1} h^{-1}) of roots of Fe-deficient (−Fe) and Fe-sufficient (+Fe) soybean plants at various concentrations of FeHEDTA. Data are means for three replicate plants.

of 6 nmoles mg^{-1} h^{-1} and a K_m of 28 μM (Cornett, 1989). Thus roots as well as cultured cells more readily reduced FeHEDTA than FeEDTA (Fig. 2).

Chaney (1989) reported K_m values of 86 and 55 μM for reduction of FeHEDTA and FeEDTA, respectively, in experiments with roots of Fe-deficient peanut (*Arachis hypogae* L.). While the V_{max} values for peanut roots cannot be directly compared to those for soybean roots, the V_{max} value for FeHEDTA was three times the V_{max} for FeEDTA with either peanut or soybean roots. Clearly FeHEDTA was more readily reduced than was FeEDTA by the inducible Fe^{3+}-chelate reducing system ('Turbo') implicated in Fe uptake.

Solutions obtained by incubating roots of intact Fe-deficient plants in fresh nutrient solution for 24 h reduced 1.06 ± 0.28 nmoles FeHEDTA mg^{-1} dry root compared to 0.42 ± 0.09 nmoles mg^{-1} dry root for Fe-sufficient plants. While significantly ($P = 0.02$) more reducing substances were released by Fe-deficient than Fe-sufficient plants, the FeHEDTA reduced was about 0.4% of the reducing capacity of roots of Fe-deficient plants compared on an hourly basis at similar FeHEDTA concentrations (Fig. 3). These results are in agreement with the conclusion of Römheld and Marschner (1983) that released reducing substances make only a minor contribution to total Fe-chelate reduction by peanut roots.

In conclusion, the results of FeHEDTA reduction experiments with suspension culture cells and intact soybean roots demonstrated that the Fe^{3+}-chelate reducing systems exhibited similar behavior. In addition, both systems reduced FeHEDTA at a higher rate than FeEDTA. While Fe-chelate reducing activity was found in Fe-deficient but not Fe-sufficient roots, Fe-chelate was reduced by cultured cells regardless of their Fe nutritional status, indicating that the induction of Fe-chelate reduction may be controlled differently in the two systems. This investigation demonstrates that cell suspension cultures can be used to study Fe-chelate reduction, and may provide insights concerning the induction of Fe-chelate reducing activity compared to intact roots.

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References

Ao T Y, Fan F, Korcak R F and Faust M 1985 Iron reduction by apple roots. J. Plant Nutr. 8, 629–644.

Bienfait H F 1985 Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. J. Bioenerg. Biomembr. 17, 73–83.

Bienfait H F 1988 Mechanisms in Fe-efficiency of higher plants. J. Plant Nutr. 11, 605–629.

Brown J C 1978 Mechanisms of iron uptake by plants. Plant Cell Environ. 1, 249–257.

Chaney R L 1988 Plants can utilize iron from Fe-N, N′-di-(2-hydroxybenzoyl)-ethylenediamine-N,N′-diacetic acid, a ferric chelate with 10^6 greater formation constant than FeEDDHA. J. Plant Nutr. 11, 1033–1050.

Chaney R L 1989 Kinetics of ferric chelate reduction by roots of iron-deficiency peanut (*Arachis hypogea*). Acta Bot. Neerl. 38, 155–163.

Chaney R L, Brown J C and Tiffin L O 1972 Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol. 50, 208–213.

Cornett J D 1989 Iron chelate reduction: a comparison of soybean suspension cultures and roots. M.S. Thesis. University of New Mexico, Albuquerque, NM.

Gamborg O L and Wetter L R 1975 Plant Tissue Culture Methods. National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, 109 p.

Hether N H, Olsen R A and Jackson L L 1984 Chemical identification of iron reductants exuded by plant roots. J. Plant Nutr. 7, 667–676.

Morré D J, Brightman L-Y W, Barr R, Leak B and Crane F L 1988 Role of plasma membrane redox activities in elongation growth in plants. Physiol. Plant. 73, 187–193.

Römheld V and Marschner H 1983 Mechanism of iron uptake by peanut plants. 1. Fe^{III} Reduction, chelate splitting, and release of phenolics. Plant Physiol. 71, 949–954.

Sain S L and Johnson G V 1986 Characterization of iron uptake by iron-efficient and iron-inefficient soybeans in suspension culture. J. Plant Nutr. 9, 729–750.

Steiner A A and van Winden H 1970 Recipe for ferric salts of ethylenediaminetetraacetic acid. Plant Physiol. 46, 862–863.