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Translocation of Iron Citrate and Phosphorus in Xylem Exudate of Soybean

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ABSTRACT

Soybean plants, *Glycine max* (L.) Merrill, in standard solution received 2.5 μM ferric ethylenediamine di(*o*-hydroxyphenylacetate (FeEDDHA) and 0 to 128 μM phosphorus. Their stem exudates contained: 32 to 52 μM Fe, 120 to 5000 μM P, and 120 to 165 μM citrate. Electrophoresis of exudates with high P caused Fe trailing that precluded identification of any major form of Fe. Exudate with low P gave an anodic band of Fe citrate as the major Fe compound. Phosphate added to exudate *in vitro* depressed the Fe citrate peak and cause Fe trailing. EDDHA added to exudate *in vitro* pulled Fe from Fe citrate; citrate then migrated as a slower form and Fe migrated as FeEDDHA. A modified preculture system, involving 2-day renewals of 0.2 μM FeEDDHA with 3.2, 9.6, or 16 μM P and low levels of other ions, controlled pH depression and produced considerable change in citrate and P levels. The exudates contained: 45 to 57 μM Fe, 200 to 925 μM P, and 340 to 1025 μM citrate. The high citrate was from plants grown with low P. The major form of Fe in the exudates was Fe citrate. This is probably the form translocated in the plants.

The roles attributed to critic acid relate mostly to cellular metabolism. But released from metabolic sites and moving in the xylem, this acid would perform other functions. Among those reasonably supposed are the binding and translocation of Fe.

Citric acid usually occurs in molar excess of Fe in plant xylem exudates (7, 8). Electrophoresis of sunflower exudate (7) indicated that Fe was present as Fe citrate. Electrophoresis of soybean exudate has usually resulted in heavy streaking of Fe from the origin to the Fe citrate position, with relatively little Fe reaching that position.

Because phosphate can have decided effects on Fe relationships of plants and various inorganic systems, it seemed reasonable that this anion might cause the streaking of Fe in electrophoretic separations. If this were true, electrophoresis of exudates containing low P should give clean separations of Fe. Experiments reported here were designed to answer this question and to identify the principal Fe compound in the exudate.

MATERIALS AND METHODS

Plants and Solution Culture. Hawkeye soybean, *Glycine max* (L.) Merrill, was the experimental plant. Standard nutrient solution (9) and exudate collection (7) have been described. The Fe source was ferric ethylenediamine di(*o*-hydroxyphenylacetate). Two treatment schedules were used. In the first, seeds were germinated 2 days, then transferred to standard nutrient solution and grown under partial shading. At 6 days, seedlings were bound in groups of 15 and transferred into full light, two groups in each 8 liters of nutrient. At 18 days, plants were placed in experimental nutrient solutions (30 plants/2 liters) which were adjusted to pH 6 after addition of ^{59}FeEDDHA[1] and P. After plants were detopped, stem exudate was collected for 10 hr.

A second schedule was identical except that on day 2 and every 2 days thereafter the groups were transferred to 8 liters of new solution in which macroelements were approximately one-fifth and microelements one-half the levels of standard nutrient. Element concentrations (μM) were 250 Ca, 50 Mg, 200 K, 850 N, 30 S, 3 B, 1.2 Mn, 0.25 Zn, 0.10 Cu, 0.05 Mo, 0.2 Fe, and 35 Cl (as NaCl). Table II gives the P levels used for preculture and harvest. Preliminary experiments had shown that concentrations of standard nutrients (1270 μM Ca, 1125 μM K, 270 μM Mg, etc.) could not be used on a 2-day renewal basis without serious plant injury. The roots released enough H^+ to drive pH levels below 4, resulting in stubby roots and generally inhibiting plant growth. In contrast, the dilute nutrient produced excellent growth, and the solution pH was never below 4.4.

Solution Analysis. The electrophoresis apparatus has been described (7). For electrophoretic separations, 1 ml of exudate was loaded along 20 cm of origin near the center of a 20- × 57-cm strip of Whatman No. 3 paper. After electrophoresis the papers were dried and radiographed with x-ray film. Sections of the papers (0.5 × 20 cm) were taken through the ^{59}Fe-containing areas for analysis. The short vertical lines, *e.g.*, in Figure 2, indicate the 0.5-cm sectioning pattern. The sections were assayed in a gamma scintillation spectrometer. Citric acid in water extracts of the sections and in raw exudate was determined as pentabromoacetone (2). Phosphorus was determined by vanadate-molybdate reagent (3).

RESULTS

Phosphorus Levels and Fe Citrate Stability. Plants that did not receive P in the exudation period (experiment 1, Table I) had 120 μM P in the xylem exudate. The various treatments resulted in a wide range in P concentration and fairly constant levels of Fe and citrate in the exudates. This provided an opportunity to test the effect of P on the stability of Fe citrate.

The Fe distributions (Fig. 1) indicate that increases of P in the exudate suppressed the migration of Fe citrate. After electrophoresis of exudate 2, 57% of the Fe was in sections 32, 33, and 34, a band 1.5 cm wide. For exudate 3, the percentage in these sections was 40; for exudate 4, it was only 12.

Exudates of experiment 2 gave Fe distribution curves (not shown) that were similar to those given by exudates of experiment

[1] Abbreviation: FeEDDHA: ferric ethylenediamine di(*o*-hydroxyphenylacetate).

Table I. *Effect of Phosphorus in Nutrient Solution on Citrate, Iron, and Phosphorus in Soybean Exudate*

Each 30 plants received 64 μM P and 1 μM FeEDDHA in 8 liters of standard nutrient on day 2 from germination and again on day 6. On day 18, 30 plants were placed in 2 liters of experimental nutrient with 2.5 μM FeEDDHA and the designated P treatment. Exudate was collected 10 hr from detopped root systems of 30 plants.

Experiment and Exudate No	Experimental P	Exudate Volume	Fe Absorbed (FeA)	Fe Released (FeR)	FeR/FeA	Exudate Component		
						Fe	Citrate	P
	μM	ml	mμmoles		%	μM		
Experiment 1								
1	0.0	15.2	2910	684	23.5	45	147	120
2	6.4	15.7	2855	691	24.2	44	165	210
3	32	16.6	3010	697	23.2	42	135	1560
4	64	23.8	2980	809	27.1	34	120	4690
Experiment 2								
1	16	22.0	2756	1122	40.7	51	142	1000
2	32	23.2	2642	1206	45.6	52	128	2250
3	64	26.3	2543	973	38.3	37	133	3500
4	128	27.7	2434	942	38.7	34	141	5000
Experiment 3	64	31				32	142	3370

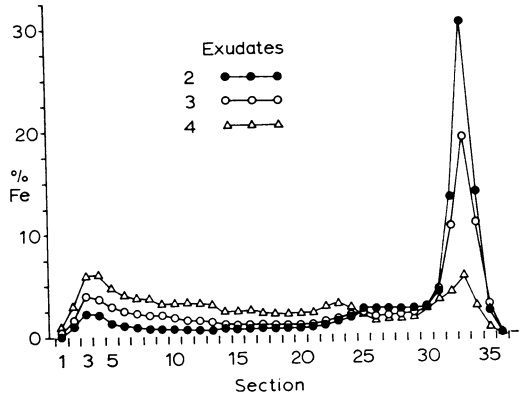

FIG. 1. Effect of phosphorus in soybean exudate on the electrophoresis of Fe. Seven milliliters each of exudates 2, 3, and 4 (experiment 1, Table I) were separated on paper and radiographed. The papers were cut into 36 (0.5-cm) strips and assayed for ^{59}Fe. Electrophoretic conditions: Whatman No. 3 paper, 20 mM sodium acetate at pH 5.4, 18 ma, 100 min, room temperature.

1 (Fig. 1), namely, depressed Fe citrate peaks and increased Fe trailing related to increases in P. Continuous electrophoresis (data not shown) of exudates containing low and high P gave similar results. Low P exudate showed a relatively clean migration of Fe citrate.

Effect of Phosphate Added *in Vitro*. Figure 2 shows clearly that phosphate added to exudate caused Fe streaking. Exudate 1, which contained 120 μM P, had 58% of the total Fe in sections 32, 33, and 34. Exudate 1P had 27% of the Fe in those sections. This shows an effect of extraneous inorganic ligand on the natural Fe-binding system of exudate.

EDDHA Extraction of Fe from Fe Citrate. The chelating agent EDDHA has a great affinity for Fe. The stability constant of the Fe chelate is 33.86 (5). Adding the reagent to exudate that contains Fe immediately produces a red solution, indicating formation of FeEDDHA. This suggested that electrophoresis of an exudate treated with EDDHA would produce a decided change in Fe migration. The electrophoretic pattern in Figure 3 (lower path) shows that EDDHA not only eliminated most of the fast Fe band of the control but also prevented Fe streaking. It is obvious that the extraneous agent EDDHA captured Fe and became its principal carrier.

Figure 4 gives quantitative distributions of citrate and Fe after electrophoresis of untreated exudate. Results show enough citrate to bind all the Fe in the heavy Fe band (sections 30 to 35). The excess acid migrated as a slower band with a peak in section 26.

The hump in the leading edge of the citrate curve (Fig. 4) is in the area of concentrated Fe. This suggests that Fe had caused that fraction of the total citrate to migrate at a faster rate. If the faster migration of citrate depends on chelation with Fe, then electrophoresis of EDDHA-treated exudate should result in a loss of the fast citrate band.

Data in Figure 5 confirm the strong chelation of Fe by EDDHA. About 97% of the Fe migrated as a single band of FeEDDHA (sections 10 to 16). Most important, however, is the absence of the hump in the citrate curve. This confirms that the fast citrate band does not exist if citrate is not associated with Fe.

Consistent with this are the quantities of citrate involved in the migration shift. There were 160 mμmoles less citrate in sections 28 to 34 of Figure 5 than were in the same sections of Figure 4. And there were 134 mμmoles more citrate in sections 24 to 26 of Figure 5 than were in those sections of Figure 4. This accounts for 84% of the citrate involved in the shift.

Plant Preculture, Comparative Response. In the first three experiments, plants received standard nutrient on days 2 and 6, and

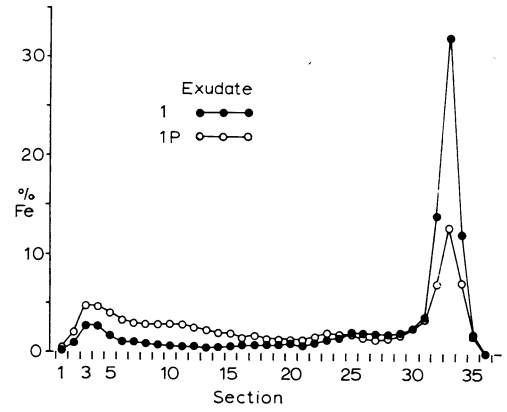

FIG. 2. Effect of phosphorus added *in vitro* on the electrophoresis of Fe in soybean exudate. Exudate 1P is the same as exudate 1 (experiment 1, Table I), except that it was made 4900 μM in K_2HPO_4. Electrophoretic conditions as for Figure 1.

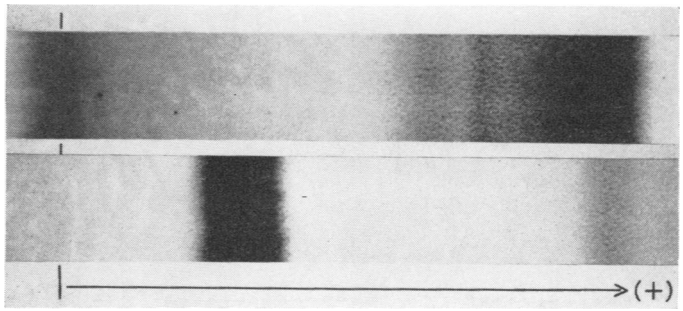

FIG. 3. Radiographs of electrophoretically distributed ^{59}Fe in soybean stem exudate: top: raw exudate (experiment 3, Table I); bottom: the same exudate made 100 μM in EDDHA. Six milliliters of each exudate were analyzed as described in Figure 1. Citrate and Fe distributions are in Figures 4 and 5.

again on day 18 for the absorption and exudation period. One observation invariably made in using this nutrient schedule is that plants lower the pH drastically in the 10-hr absorption period. The solution pH, initially around 6, usually drops below 4 and sometimes reaches 3.5. These plants absorb considerable Fe, and the amounts are fairly constant.

In contrast, the plants in the final experiment (subjected to frequent nutrient renewal at low ion levels) caused little or no change in solution pH, and the quantities of Fe absorbed (FeA, Table II) compared favorably with amounts absorbed in other experiments. The point emphasized here is that lowering of the external pH by roots is not necessarily associated with Fe absorption.

Increased P treatments in the preculture (Table II) did not produce significant changes in root dry weight. And the tops produced on 16 μM P were only 5.7% heavier than those produced on the lowest P treatment. The 3.2 μM P was therefore adequate for plant growth.

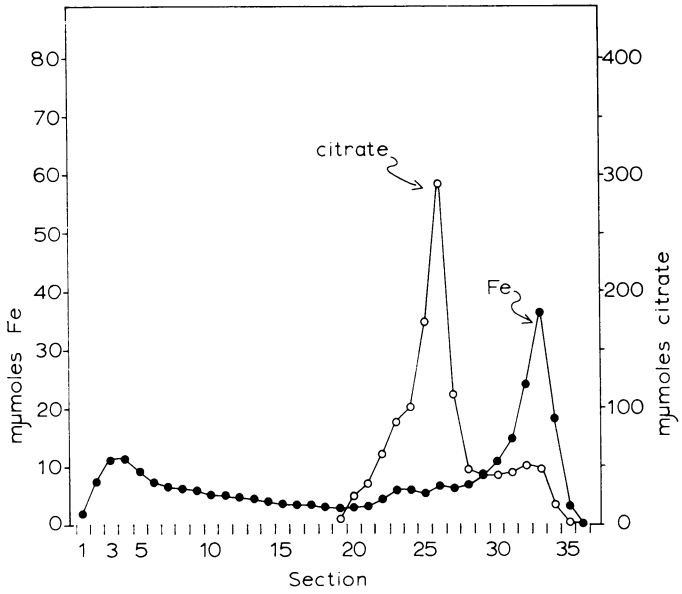

FIG. 4. Electrophoretic distribution of citrate and Fe in soybean exudate. The Fe curve corresponds to the radiograph in Figure 3 (top).

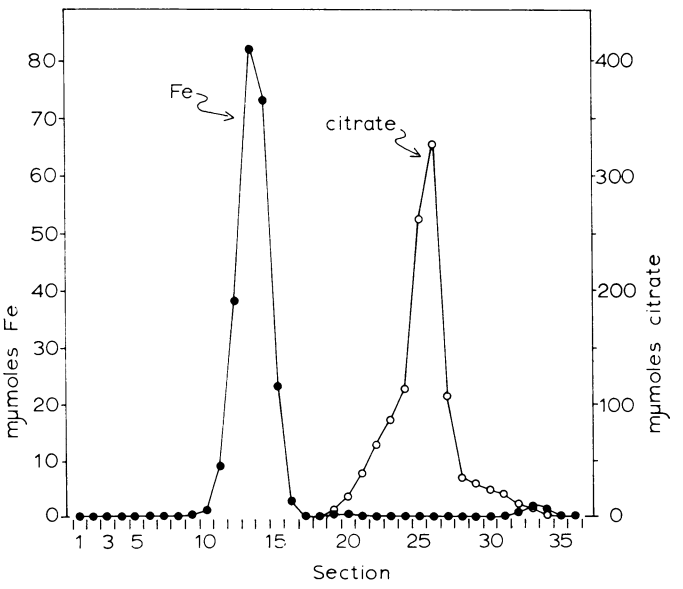

FIG. 5. Effect of EDDHA on the electrophoretic migration of citrate and Fe in soybean exudate. The Fe curve corresponds to the radiograph in Figure 3 (bottom).

Table II. *Effect of Varied Phosphorus in Nutrient Solution on Citrate, Iron, and Phosphorus Concentrations in Soybean Exudate*

Thirty plants received every 2 days (starting day 2 from germination) 8 liters of complete nutrient containing 0.2 μM FeEDDHA with low levels of other ions and the designated P treatment. The experimental nutrients (2 liters given at detopping on day 18) were identical except that three replicates were without P and all solutions contained 2.5 μM FeEDDHA. The pH levels, initially at 6.0, were checked after 10 hr. Exudate volumes are for 10-hr collections from 30 plants.

Exudate No	Preculture P	Experimental P	Final pH	Exudate Volume	Fe Absorbed (FeA)	Fe Released (FeR)	FeR/FeA	Exudate Component		
								Fe	Citrate	P
	μM			ml	mμmoles		%	μM		
1	3.2	0.0	5.1	13.3	2396	718	30.3	54	800	235
2	3.2	3.2	5.1	13.7	2466	735	30.5	55	1025	200
3	9.6	0.0	6.0	20.0	2370	1020	43.0	51	520	315
4	9.6	9.6	6.0	20.5	2480	963	38.3	47	368	665
5	16.0	0.0	6.3	19.8	2500	1128	45.1	57	561	310
6	16.0	16.0	6.4	22.7	2340	1021	43.6	45	340	925

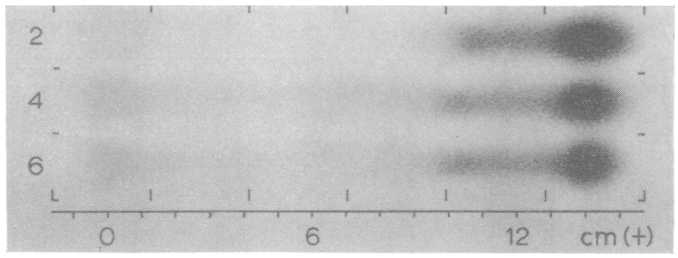

FIG. 6. Radiograph of Fe in soybean exudate. Exudates 2, 4, and 6 (Table II), corresponding to paths 2, 4, and 6, were the samples used (volume, 50 μl). Electrophoretic conditions as for Figure 1. Markings indicate blocks assayed for ^{59}Fe.

The most striking result of this experiment was the high citrate in exudates obtained under low P culture. Higher levels of P lowered citrate, but it was still considerably higher than was observed in previous experiments.

Radiographs after electrophoresis (Fig. 6) show heavy deposits of anodic ^{59}Fe. Assays (not given) revealed 63 to 69% of the Fe in the position of Fe citrate. The high citric acid in these exudates undoubtedly enhanced the stability of Fe citrate.

Relation of Fe Uptake to Release. Although P levels supplied to roots were very different, the quantities of Fe absorbed were similar (FeA, Tables I, II).

The molarity of Fe was usually lower in exudates containing high P concentrations. However, increases in exudate volume associated with increased P resulted in fairly constant recoveries of Fe. In several cases the highest recoveries occurred under conditions of high P transport. The tables present the values as Fe released in total exudate (FeR).

Ratios of Fe released to that absorbed (FeR/FeA) were similar in the individual experiments, despite very different P concentrations in exudates and nutrients. The FeR/FeA values ranged from 23 to 45%. Over-all, the highest P treatments did not hinder Fe uptake by the root, and the highest levels of P moving in the xylem did not hinder the passage of Fe through the root.

DISCUSSION

Although Fe was supplied as a synthetic chelate, it did not move through the xylem in that form. It is obvious from electrophoretic patterns (upper path, Fig. 3) that FeEDDHA was not present in the exudate. Plants thus separate Fe from FeEDDHA and transport the metal in a different form.

Electrophoretic Analysis of Exudate Iron. The first requirement in using electrophoresis to identify a particular Fe compound in exudate is that the complex migrate intact in sufficient quantity for analysis of its components. Analysis of sunflower exudate by this method (7) revealed two electrophoretic bands of citrate. The slower one contained most of the citrate and was Fe-free. The faster band moved at the same rate as the fast moving Fe and presumably was bound to it.

It has been impossible in most studies of soybean exudates to identify a predominant form of Fe, because such large amounts of Fe trail on the paper. Patterns, for example, resemble curve 4, Figure 1, where little Fe migrates as Fe citrate. Electrophoresis of low P exudates, however, has given discrete bands of Fe, apparently Fe citrate, and considerably less Fe trailing.

The EDDHA competition experiment (Fig. 5) has given more direct evidence that citrate is the natural carrier of Fe. After releasing Fe to EDDHA, citric acid no longer migrated to the Fe citrate position. The opposite of this occurred in an earlier experiment (7) when increasing quantities of Fe were added to citric acid *in vitro*. The result was that increasing amounts of citrate migrated to the Fe citrate position.

Translocation of Iron *in Vivo*. In 1932, Rogers and Shive (4) proposed that if organic acids can maintain soluble Fe in an external solution they should serve the same purpose within the plant. Although this is reasonable, there are no direct observations of Fe being held in solution and transported, for example, in the xylem. Much information, however, has accumulated from studies of Fe solutions *in vitro*.

One important area of study relates to stability constants of organic and inorganic complexes of Fe in simple solution. The stability values (1, 6), compiled from many sources, give indications of Fe-binding mechanisms one might expect in biological solutions.

The presence of soluble Fe and citric acid in xylem fluid suggests a probable association and means for transporting Fe. The separation of discrete bands of electrophoretic Fe, with Fe citrate as the major form, provides further presumptive evidence for that system. For if Fe citrate is the major form of Fe in an exudate, it is probably the form that moves in the plant.

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LITERATURE CITED

1. Chaberek, S. and A. E. Martell. 1959. Organic Sequestering Agents. John Wiley & Sons, New York.
2. Dickman, S. R. and A. A. Cloutier. 1951. Factors affecting the activity of aconitase. J. Biol. Chem. 183: 379–388.
3. Jackson, M. L. 1958. Soil Chemical Analysis. Prentice-Hall, Inc., Englewood Cliffs, N. J. p. 151.
4. Rogers, C. H. and J. W. Shive. 1932. Factors affecting the distribution of iron in plants. Plant Physiol. 7: 227–252.
5. Schroder, K. H. 1964. Complex formation of trivalent iron and N,N'-ethylene bis[2(*o*-hydroxyphenyl)]glycine. Nature 202: 898–899.
6. Sillen, L. G. and A. E. Martell. 1964. Stability Constants of Metal-ion Complexes, Ed. 2. Special Publication No. 17. The Chemical Society, London.
7. Tiffin, L. O. 1966. Iron translocation. I. Plant culture, exudate sampling, iron-citrate analysis. Plant Physiol. 41: 510–514.
8. Tiffin, L. O. 1966. Iron translocation. II. Citrate/iron ratios in plant stem exudates. Plant Physiol. 41: 515–518.
9. Tiffin, L. O. 1967. Translocation of manganese, iron, cobalt, and zinc in tomato. Plant Physiol. 42: 1427–1432.