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Accumulation of Apoplastic Iron in Plant Roots[1]

A Factor in the Resistance of Soybeans to Iron-Deficiency Induced Chlorosis?

Nancy Longnecker*[2] and Ross M. Welch

Agronomy Department, Cornell University and U.S. Department of Agriculture-Agricultural Research Service, Plant, Soil and Nutrition Laboratory, Ithaca, New York 14853

ABSTRACT

We hypothesized that the resistance of Hawkeye (HA) soybean (*Glycine max* L.) to iron-deficiency induced chlorosis (IDC) is correlated to an ability to accumulate a large pool of extracellular-root iron which can be mobilized to shoots as the plants become iron deficient. Iron in the root apoplast was assayed after efflux from the roots of intact plants in nutrient solution treated with sodium dithionite added under anaerobic conditions. Young seedlings of HA soybean accumulated a significantly larger amount of extracellular iron in their roots than did either IDC-susceptible PI-54619 (PI) soybean or IDC-resistant IS-8001 (IS) sunflower (*Helianthus annus* L.). Concurrently, HA soybean had much higher concentrations of iron in their shoots than either PI soybean or IS sunflower. The concentration of iron in the root apoplast and in shoots of HA soybean decreased sharply within days after the first measurements of extracellular root iron were made, in both +Fe and −Fe treatments. The accumulation of short-term iron reserves in the root apoplast and translocation of iron in large quantities to the shoot may be important characteristics of IDC resistance in soybeans.

Researchers have attempted to solve the problem of IDC[3] in crop plants for over a century. Currently, it is thought that the best long-term solution to this problem is to breed cultivars that are resistant to iron deficiency (25). Although much has been learned about the physiology of iron uptake in recent years (19), the lack of a clear understanding of the physiology of resistance to iron deficiency has hampered breeding programs.

Graminaceous species respond to iron-deficiency stress by producing phytosiderophores (19). Many other plants, including both soybeans (6) and sunflowers (17), respond to iron-deficiency stress with an increased capacity for root Fe-III reduction. This iron-stress response is a factor in the IDC resistance or 'iron efficiency' of plants (3, 4) and is important because it is thought that iron is absorbed across the root plasmalemma of nongraminaceous plants in the reduced Fe-II form (6). However, Tipton and Thowsen (22) have reported that an increased reducing capacity of roots of soybean cultivars in response to iron-deficiency stress is not quantitatively correlated with their IDC resistance.

In previous experiments, we observed a greening of newly forming leaves of IDC-resistant Hawkeye (HA) soybeans after development of iron-deficiency symptoms, even when no iron had been added externally to the nutrient solution. Thus, the iron must have been mobilized internally. This greening did not occur in IDC-susceptible PI-54619 (PI) soybeans (14). We hypothesized that IDC-resistant HA soybeans can accumulate iron in the root apoplast and that this pool of apoplastic iron can be mobilized as the plants become iron deficient.

There is support in the literature for the importance of the apoplast in iron nutrition in soybeans (15). As previously mentioned, Fe-III reduction is thought to be a prerequisite for iron absorption by nongraminaceous plants. While there is evidence that ferric-iron is reduced at the plasmalemma (1, 5, 6, 18) and that this reduction occurs via an electron transport system operating across the plasmalemma (20, 21), there are also reports that Fe-III reduction occurs in the root apoplast (22) or at the root surface (23).

We studied the accumulation of iron in the root-cell apoplast of IDC-resistant HA soybean, IDC-susceptible PI soybean, and IDC-resistant IS-8001 (IS) sunflower. We present data demonstrating that young HA soybeans accumulated much larger amounts of iron in their root apoplast than either PI soybean or IS sunflower. Young HA soybean seedlings concurrently accumulated very high iron concentrations in their shoots while PI soybean and IS sunflower seedlings did not. These HA soybean traits may be important characteristics of IDC resistance in soybean genotypes.

MATERIALS AND METHODS

Plant Culture

Seeds of the IDC-resistant soybean (*Glycine max* L.) variety, Hawkeye, IDC-susceptible PI-54619 soybean, and the IDC-resistant sunflower (*Helianthus annuus* L.) line, IS-8001 contained 73, 72, and 36 mg kg^{-1} iron, respectively, on a dry weight basis. The seeds were surface-sterilized by soaking for 5 min in a solution of 10% sodium hypochlorite containing 0.1% SDS and 10 mM $CaSO_4$ and then imbibed overnight in aerated 0.5 mM $CaSO_4$. The seeds were germinated on paper

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[2] Present address: Soil Science and Plant Nutrition, University of Western Australia, Nedlands, WA 6009, Australia.

[3] Abbreviations: IDC, iron-deficiency induced chlorosis; MDH, NAD^+-dependent L-malate dehydrogenase.

towels set in aerated 0.5 mM $CaSO_4$. After 6 d, seedlings were transferred to nutrient solution containing concentrations one-quarter of that described below with 25 μM Fe (III)-EDTA in a controlled environment growth chamber with 27°C, 16 h d and 18°C nights. The growth chamber contained fluorescent tubes and incandescent bulbs which emitted 300 μmol m^{-2} s^{-1} at plant level. After 2 d (on d 1), the nutrient solution was changed to full-concentration nutrient solution having 50 μM Fe(III)-EDTA and the following composition: 2 mM $Ca(NO_3)_2$, 1 mM KNO_3, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 25 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, and 0.25 μM H_2MoO_4.

Four plants were grown in each 800 mL black plastic pot. There were five replicate pots for each iron treatment. The nutrient solution was changed every 3 to 4 d. Four replicates of each genotype were harvested on d 8. Then, the following iron treatments were imposed: no additional iron (−Fe) and 50 μM Fe(III)-EDTA (+Fe). Subsequent harvests of four replicates of each treatment were taken on d 10, 14, 24, and 28.

Throughout the experiment, the young developing leaves were visually scored for iron-deficiency symptoms as follows: 1, healthy, green leaves; 2, slight chlorosis or interveinal yellowing; 3, marked chlorosis; 4, severe chlorosis with some necrosis or brown spotting; and 5, curled, severely necrotic tissue.

^{59}Fe Uptake Study

HA soybean and IS sunflower were germinated as above and transferred to full-concentration nutrient solution with no added Fe or with 50 μM Fe(III)-EDTA at d 1. On d 17, plants were transferred from the growth chamber to 800 mL pots, one plant per pot, containing full-concentration nutrient solution and the appropriate iron treatment. The pots were placed in the laboratory in a 28°C water bath, under lights with the same photoperiod schedule as in the growth chamber. On d 17, the plants receiving the −Fe treatment were chlorotic, with an average chlorosis score of 3.75. All plants used in the short-term ^{59}Fe uptake study on d 18 were treated as follows: the roots were rinsed for 15 min in full-concentration nutrient solution with no added iron and transferred to black plastic pots containing full-concentration nutrient solution and 45 μM Fe(III)-EDTA labeled with 0.37 TBq of ^{59}Fe. After 1 h, the plants were removed from labeled absorption solutions. Their roots were rinsed in nonradioactive nutrient solution containing 45 μM Fe(III)-EDTA at 4°C for 15 min. The plants were divided into roots and shoots and placed in tared glass digestion tubes. The fresh weight was recorded, and the plant parts were dried at 70°C in an oven for at least 24 h, weighed again, and then digested in concentrated HNO_3-$HClO_4$ (10:1). The resulting digestates were made to 25 mL with water and ^{59}Fe was assayed using an auto-gamma spectrophotometer.

Assay for Extracellular Iron

Extracellular root iron was assayed on d 8, 10, 14, 24, and 28 using the method of Bienfait *et al.* (2). The roots of intact plants were rinsed in 0.5 mM $CaCl_2$ for 5 min, then were placed in large, glass digestion tubes containing 50 mL nutrient solution without iron additions and with 1.5 mM bipyridine (a complexing agent which forms a pink color when complexed with Fe-II). The nutrient solution was purged for 5 min with N_2 gas to displace dissolved O_2, then sodium dithionite (a reducing agent capable of reducing Fe-III to Fe-II) was added to the solutions. Immediately prior to use, 0.25 g of sodium dithionite was dissolved in 5 mL deoxygenated, distilled, deionized water. This was pulled into a syringe, air was removed from the syringe, and 1 mL sodium dithionite was injected into the solution at time 0. Aliquots (5 mL) were taken during a 2 h period, and absorbance was measured at 520 nm.

In the development of this method, Bienfait *et al.* (2) showed that there were was a fast phase of release of iron from roots, followed by a slower phase. During the fast phase, there was no significant decrease in ferritin content of the roots (an indicator of cellular iron which is readily reduced by dithionite) or release of K^+ from roots. During the slow phase, the content of ferritin decreased and K^+ was released from roots. In contrast to ferritin in roots, isolated ferritin released its iron completely in these conditions with a half-time of 1 to 2 min. Bienfait *et al.* (2) concluded that the iron released in the first phase was released from the root free space. Additional evidence for this conclusion is that crude extracts of cell walls from bean plants released iron in a similar length of time as did intact roots, within 5 min of the addition of dithionite (2). In our system, Fe-II efflux curves (representative examples shown in Fig. 1) established that 10 min after the addition of sodium dithionite was an appropriate duration for determining extracellular iron in roots in this system.

After determining the amount of extracellular iron in the roots, the plants were divided into roots and shoots, dried in a forced air oven at 70°C for at least 24 h, and weighed. Shoots were milled with a Udy mill (U.D. Corporation, Boulder, CO) to pass a 0.5 mm mesh screen, digested with concentrated HNO_3-$HClO_4$ (10:1), and analyzed for iron using inductively coupled plasma emission spectrometry (ICP). Statistical analysis of data was performed using SAS (Statistical Analysis System, SAS Institute, Inc., Box 8000, Cary, NC).

RESULTS

The root dry weights (dry weight data not shown) did not differ significantly between the −Fe-treated HA and PI soybean plants and IS sunflower plants at any harvest (14). Similarly, the shoot dry weights of the −Fe-treated HA and PI soybean plants did not differ significantly at any harvest. The shoot dry weights of -Fe-treated IS sunflower plants were significantly greater ($P < 0.01$) than both HA and PI soybean plants at harvests on d 24 and 28, but not at harvest on d 8, 10, or 14. As expected, in all plants studied the +Fe-treated plants produced significantly more root and shoot dry matter than did the −Fe-treted plants by the final two harvest (d 24 and 28).

IDC-resistant HA soybeans developed less severe chlorosis symptoms than either IS sunflower or PI soybean (Fig. 2). The chlorosis symptoms occurred later in HA soybeans than in the other plants, corresponding in time to a drop in iron

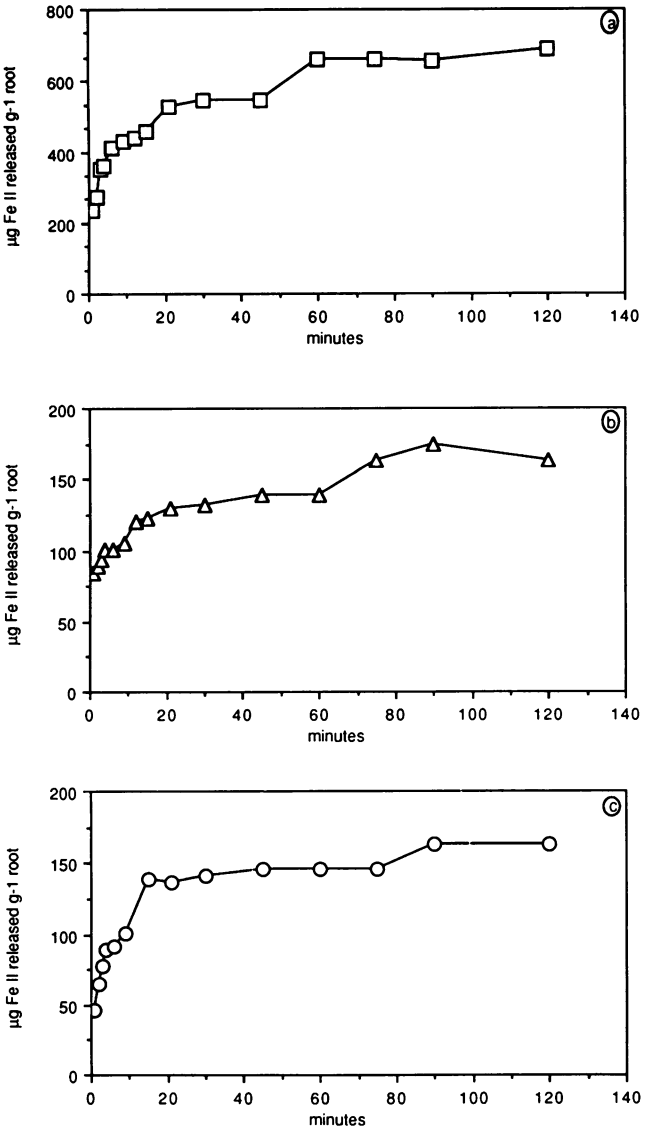

Figure 1. Representative examples of Fe-II efflux from roots of intact (a) HA soybean, (b) PI soybean, and (c) IS sunflower plants treated with addition of sodium hydrosulfite to nutrient solution.

concentration after an early accumulation of iron in the shoots (Figs. 4 and 5). The IDC-resistant IS sunflowers were intermediate in the development of chlorosis. IDC-susceptible PI soybeans became chlorotic first and developed the most severe chlorosis.

The concentration (mg kg^{-1} root dry weight) of iron accumulated in the root apoplast of HA and PI soybean and IS sunflower plants at various harvest dates is shown in Figure 3, a (−Fe) and b (+Fe). Roots had considerably higher concentrations of extracellular iron at the first harvest (d 8) than at subsequent harvests. Young HA soybean seedlings contained a significantly larger pool of apoplastic root-iron early in their development (d 8) than either PI soybean or IS sunflower seedlings receiving the same treatments. By d 14, there were no significant differences among HA and PI soybean and IS sunflower in apoplastic root-iron concentrations

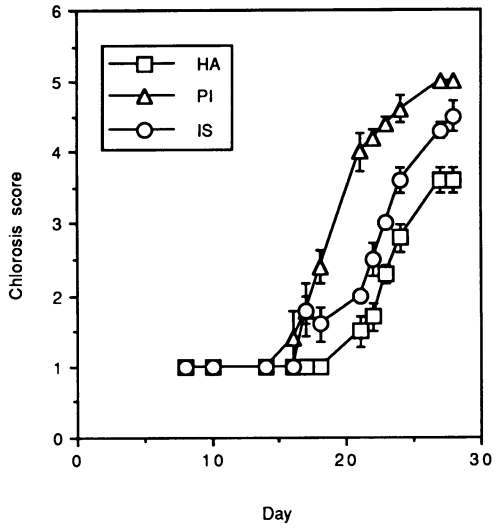

Figure 2. Chlorosis development in HA soybean, PI soybean, IS sunflower not supplied with iron after d 8 (−Fe treatment). Chlorosis scores of leaves are 1, healthy and green; 2, slightly chlorotic; 3, markedly chlorotic; 4, severely chlorotic; and 5, stunted and necrotic. Error bars represent standard errors of the mean of four replicates.

(Fig. 3). Additionally, there were no differences in apoplastic iron between the +Fe and −Fe treatments.

The change between the first and final harvest in total iron in the root apoplast of the −Fe treated plants was −45 µg for HA soybean, −6 µg for PI soybean, and +4 µg for IS sunflower.

Initially, the iron concentration in the shoots of young, −Fe-treated HA soybean seedlings was much higher (614 mg kg^{-1} dry weight) than in similarly treated PI soybean (100 mg kg^{-1}) or IS sunflower (105 mg kg^{-1}) seedlings (Fig. 4). However, as the treatment period continued, the iron concentration in −Fe-treated HA soybean shoots decreased markedly, so that by d 24 it was similar to those in −Fe-treated PI soybean and IS sunflower shoots, both of which decreased slightly from d 8.

The −Fe-treated HA soybean plants also contained a much greater total amount of iron in their shoots on d 8 (346 µg $shoot^{-1}$) than similarly treated PI soybean (80 µg $shoot^{-1}$) or IS sunflower plants (72 µg $shoot^{-1}$) (Fig. 5). This difference in shoot iron of HA soybean could not be accounted for by seed reserves since the average amount of iron in seeds of HA, PI and IS was 16, 16, and 5 µg, respectively. Thus, when compared to PI soybean or IS sunflower, the HA soybeans absorbed and translocated more iron to their shoots during the early period of growth, when all were supplied 50 µM Fe (III)-EDTA. However, while the total iron content of the −Fe-treated PI soybean and IS sunflower shoots increased markedly from d 8 to d 28, the iron content of HA shoots remained fairly constant during this period, perhaps increasing slightly (Fig. 5). Therefore, growth dilution (*i.e.* new growth without concomitant iron uptake) could account for the subsequent reduction in iron concentrations observed in the HA shoots at later harvests (Fig. 4). The total iron content of all +Fe-treated plants steadily increased during the course of the experiment, from 467 to 1444 µg $shoot^{-1}$ for HA soybean,

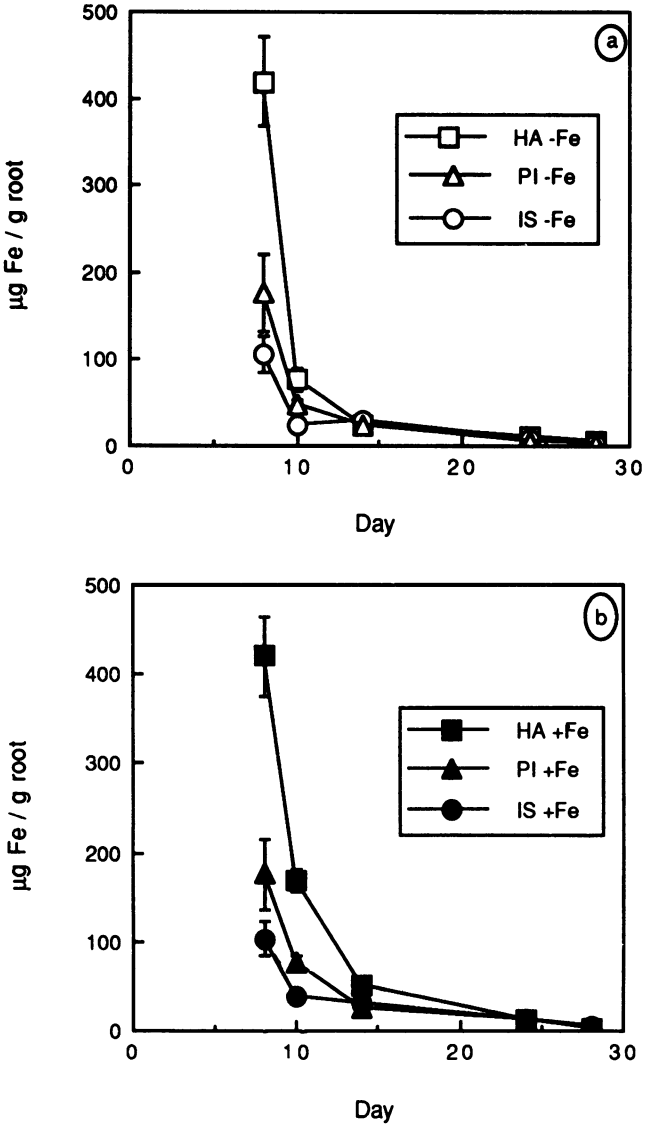

Figure 3. Concentration of iron in the root apoplast of HA soybean, PI soybean, and IS sunflower (a) not supplied with iron after d 8 (−Fe treatment) and (b) supplied 50 µM FeEDTA (+Fe treatment). Error bars represent standard errors of the mean of four replicates.

from 147 to 1150 µg shoot^{-1} for PI soybean, and from 145 to 861 µg shoot^{-1} for IS sunflower.

Table I shows the results of the ^{59}Fe-labeled uptake study comparing the short-term iron absorption rates of −Fe- and +Fe-treated HA soybean and IS sunflower seedlings. The iron absorption rates of both the −Fe-treated HA soybean and IS sunflower were much higher than those rates obtained for the +Fe-treated soybean or sunflower plants. The +Fe-treated HA soybean plants had significantly higher short-term iron absorption rates than did the +Fe-treated IS sunflower plants. Of the absorbed iron, 86 to 98% was in the roots.

DISCUSSION

All of the genotypes studied here accumulated relatively more iron in their root apoplast at the early harvest compared

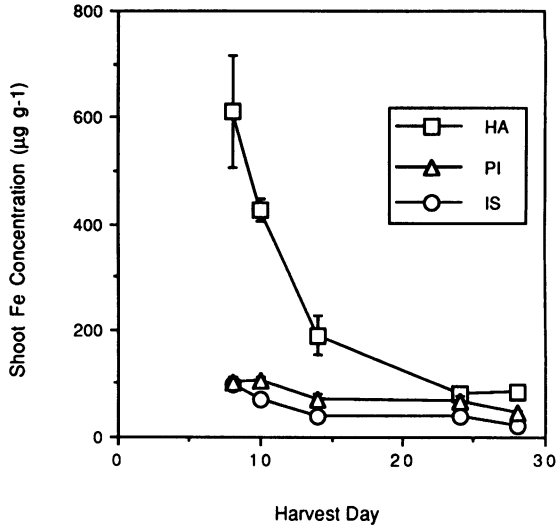

Figure 4. Concentration of iron in shoots (dry weight basis) of HA soybean, PI soybean, and IS sunflower not supplied with iron after d 8 (−Fe treatment). Error bars represent standard errors of the mean of four replicates.

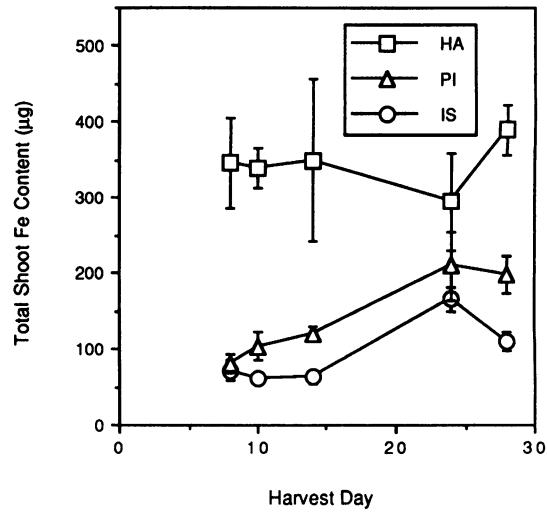

Figure 5. Total amount of iron in shoots of HA soybean, PI soybean, and IS sunflower not supplied with iron after d 8 (−Fe treatment). Error bars represent standard errors of the mean of four replicates.

Table I. *Short-Term (1 h) Uptake Rates of Iron from Nutrient Solutions Containing 45 µM ^{59}Fe-labeled Fe (III)-EDTA by 17 d old HA Soybean and IS Sunflower Plants Grown with 50 µM FeEDTA (+Fe) or No Added Iron (−Fe)*

	Rate of Iron Uptake	
	+Fe	−Fe
	µmol ^{59}Fe·g dry wt root^{-1} h^{-1}	
HA Soybean	31.1 (10)[a]	45.3 (2)
IS Sunflower	11.2 (3)	45.3 (11)

[a] Values in parentheses are standard errors of the mean of four replicates.

to later harvests. However, 8 d old IDC-resistant HA soybean plants accumulated a much larger pool of iron in their root apoplast than did either IDC-sensitive PI soybean or IDC-resistant IS sunflower plants (per g of root; see Fig. 3). Therefore, the data presented here can be interpreted to support the hypothesis that IDC-resistant HA soybean plants can accumulate iron in their root apoplast. This accumulation of iron in the root apoplast may be a factor in the IDC resistance of HA soybean as there may be a relationship between the higher amount of iron in the root apoplast of HA soybean than the other plants studied and the extremely high concentration of iron in HA shoots at the first harvest. The iron bound in the root apoplast may serve as a short-term iron storage pool which is more readily available for absorption and translocation than iron which has already been absorbed by root cells and incorporated into metabolites and/or accumulated in root-cell organelles (*e.g.* vacuoles, plastids, and mitochondria).

A significant problem with the original hypothesis is that the +Fe-treated HA soybeans did not maintain a large amount of iron in their root-cell apoplast over time as measured in this study (Fig. 3b). While young HA plants obviously accumulated iron in their root apoplast, one cannot ascertain from these data whether HA soybeans maintained a pool of apoplatic iron as a buffer against potential further deficiency. Two possible explanations for the lower amount of iron in the apoplast of HA soybean at the later harvests are discussed below.

It is possible that the original hypothesis is true and HA soybean does accumulate more iron in the root apoplast than the other genotypes, as seen in the first harvest. However, if there are differences in the iron accumulation by various cell types at different stages of development and if the cell types of interest represented a lower proportion of the total at the later harvests, an accumulation of apoplastic iron at the later harvests may not have been large enough to measure because of the low apoplastic iron in the rest of the root system. Thus, the first harvest may have occurred at a point in time when the cells involved in adsorption of iron in the apoplast and absorption into the symplast represented a relatively large proportion of the total root biomass. In contrast, at the later harvests these cell types may have represented a lower proportion of the total because of growth of the roots. For example, if iron is accumulated preferentially in the apoplast of immature root cells which are not actively involved in iron uptake and is absorbed into the symplast once that region of the root matures, the average concentration of iron in the apoplast of root cells (expressed per g of root) would decrease as the roots grow and the proportions of immature cells to mature epidermal and cortical cells are reduced.

There is evidence in the literature to support this explanation. Reports have shown that iron was preferentially accumulated in the apical portions of roots (*i.e.* cell maturation zones) of certain plant species (1, 8, 12, 17). Clarkson and Sanderson (8) reported that iron was readily accumulated in a zone of maturing or recently matured root cells of barley (*Hordeum vulgare* L.). The rates of iron translocation from this root zone to shoots were also higher than from elsewhere along the root (8).

Another possibile explanation of the difference in accumulation at the early and late harvests is that the early accumulation of iron in the root apoplast was a transient iron-deficiency stress response. This is possible if the rapidly growing soybean seedlings exhausted their seed-iron stores before developing adequate capacity for iron uptake. Thus, the newly forming root cells could have been iron-deficiency stressed and 'programmed' to accumulate iron in their cell walls as they developed. Perhaps the IDC-resistant HA soybean accumulated more iron in its apoplast because it had a greater response to iron-deficiency stress. Since the −Fe-treated plants only received iron until d 8, iron-deficiency stressed root cells which would have been programmed after that would not have iron in the growing medium to accumulate. Once the iron uptake capacity of the plants receiving the +Fe treatment was adequate to keep up with growth, the root cells of +Fe treated plants would not be programmed to accumulate more iron. The two hypotheses concerning lower apoplastic iron at the later harvest dates could be tested by (a) supplying older iron-stressed plants with an adequate iron supply and measuring the amount of iron accumulated in the apoplast and (b) measuring apoplastic iron in different regions of the roots.

The possibility that HA soybeans accumulate storage pools of iron in their shoots will be discussed in another paper. The data support the hypothesis that HA soybean accumulate more iron than PI soybean, whether they are iron-deficient or sufficient (N Longnecker, RM Welch, unpublished data).

An aspect of iron absorption that requires further evaluation is the importance of cation exchange capacity in the root-cell apoplast (11). Several questions need to be addressed. First, can the accumulation of iron in the root apoplast be accounted for by precipitation of Fe III oxides and hydroxides or is the accumulation a cation exchange phenomenon? Second, are there adsorption sites which are specific for iron binding in the root-cell walls of HA soybean? There is evidence that cell walls from soybean seed coats have iron-specific binding sites which differ from the majority of ion exchange sites in plant cell walls (13). Third, do differences exist in the capacity of cell walls to adsorb polyvalent cations at different stages of root-cell differentiation and maturation? Answers to these questions are germane to understanding the role of cell-wall cation exchange sites in iron absorption by plant roots.

Tipton and Thowsen (22) have proposed a model of iron uptake based on Fe-III reduction in the root apoplast. Iron-deficiency stress is known to induce the release of L-malate from root-cells (5, 7, 9, 24). In their model, the released L-malate increases the activity of NAD^+-dependent L-malate dehydrogenase (MDH) in the root apoplast in a reaction that also reduces NAD^+ in the cell wall. The resulting NADH is presumed to be the electron donor for Fe-III reduction. To support the model, Tipton and Thowsen (22) showed: (a) that L-malate stimulated Fe-III reduction, (b) L-malate increased in roots of iron-stressed soybean seedlings, with a somewhat greater increase in IDC-resistant varieties, and (c) MDH activity can be found in washed root-cell walls.

However, there are problems with this model. For example, the presence of MDH activity in cell walls remains controver-

sial. It has been shown that MDH does not exist in purified cell wall preparations from corn roots (16), whereas cell wall MDH activity has been reported in horseradish (10). Cakmak *et al.* (5) reported that there was MDH activity in cell walls of iron-stressed and iron-adequate bean roots, but that the amount of MDH present was not enough to account for the levels of Fe-III reduction in those roots. Also, the addition of malate by Cakmak *et al.* (5) inhibited ferric reducing activity of Fe-deficient bean roots, in contrast to the stimulation of Fe-III reduction by addition of malate to excised soybean roots in the Tipton and Thowsen report (22).

While some evidence casts doubt on the Tipton and Thowsen model of Fe-III reduction in the cell wall via MDH, the role of the apoplast in the iron nutrition of plants is an area which deserves further study. The data presented here show that HA soybean has the ability to accumulate large amounts of iron in the root apoplast early in its growth (Fig. 3). This ability is greater for IDC-resistant HA soybean than for IDC-susceptible PI soybean or IDC-resistant IS sunflower. Additionally, Cakmak *et al.* (5) did measure Fe-III reduction activity in isolated cell walls. Their data suggest that reduction of Fe-III can occur without the binding of Fe-III at the plasmalemma surface.

Both HA soybean and IS sunflower responded to iron-deficiency stress with increased short-term rates of iron uptake (Table I), thus confirming previous observations for these species (6, 17). Interestingly, the +Fe-treated HA soybean has a threefold higher iron absorption rate than the +Fe-treated IS sunflower. Of this iron taken up, the vast majority was retained in the root systems. Because our calculated rates of iron absorption included both adsorbed and absorbed root-iron, it is uncertain how much of the iron taken up was absorbed across the plasmalemma of root cells, was bound to polyvalent cation cell-wall exchange sites, or was precipitated in the extracellular apoplastic spaces in the roots. Further studies, which determine the partitioning of ^{59}Fe-labeled iron between apoplastic and symplastic pools within the roots over time, are needed to accurately determine the short-term iron absorption rates of soybeans.

In the past, the lack of a physiological characteristic which clearly and quantitatively demonstrates IDC-resistance in soybean genotypes has hindered soybean breeding programs. If the determination of iron in the root apoplast of young soybean seedlings is shown to be positively correlated to the ranking of IDC-resistance in soybean genotypes in the field, this nondestructive assay for root apoplastic iron could be developed as a screening technique for IDC-resistance in soybeans. This remains to be demonstrated, using a wider range of soybean genotypes.

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