



Iron nutrition of plants and interactions with vascular wilt disease and light
by Richard Eugene Macur

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Soils
Montana State University

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Abstract:

The relationship between iron nutritional status and Verticillium Wilt disease in tomato possessing single gene resistance to Race 1 of Verticillium dahliae was investigated using hydroponic culture media. Iron limiting conditions increased the sensitivity of resistant tomatoes to the pathogen as expressed by wilting and chlorosis. Distance of fungal vascular invasion was approximately the same in both iron replete and iron limited treatments. Comparison of near-isolines revealed that the magnitude of disease expressed in Fe deficient Pixie II (resistant) was considerably less than that expressed by the susceptible Pixie variety. Infection of tomato did not enhance iron stress severity as quantified by root peroxidase activity and chlorophyll content of young leaves.

The release of iron from horse spleen ferritin through photochemical reduction of Fe(III) to Fe(II) was studied in vitro. Spectrophotometric measurement of the Fe(ferrozine) 3^{2+} complex (specific for Fe(II)) was used to quantify rates of Fe mobilization: Cool white fluorescent plus incandescent light effectively promoted the rate of Fe release. Compounds known to be present in plants may provide further regulation of photorelease. Reductive removal from ferritin was inhibited by phosphate, and hydroxide, whereas citrate, oxalate, tartrate, and caffeate enhanced the release. Of the organic acids studied, caffeate was the only compound which induced detectable Fe release in the absence of irradiation. Rate constants ranged from $2.7 \times 10^{-3} \text{ sec}^{-1}$ (pH = 4.6) to $2.1 \times 10^{-3} \text{ sec}^{-1}$ (pH = 7.1) at 26.5°C. Synthesis of the photosynthetic apparatus is dependent on both light and iron. Thus, the findings provide one possible mechanism coupling chloroplast iron demand with iron release from ferritin.

Treatments known to alter either phenolic metabolism or overall enzyme activity were utilized to examine the Fe reductive mechanisms involved in iron stress response at the roots. Although specific compounds caused elevation of internal o-dihydroxyphenol content, the overall root reduction capacity of Fe stressed plants was significantly suppressed. However, plant roots retained significant capacity to reduce Fe after tissues were subjected to severe protein denaturizing treatments. Thus, indications for both secreted reductant and enzymatic reduction mechanisms were observed.

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The relationship between iron nutritional status and *Verticillium* Wilt disease in tomato possessing single gene resistance to Race 1 of *Verticillium dahliae* was investigated using hydroponic culture media. Iron limiting conditions increased the sensitivity of resistant tomatoes to the pathogen as expressed by wilting and chlorosis. Distance of fungal vascular invasion was approximately the same in both iron replete and iron limited treatments. Comparison of near-isolines revealed that the magnitude of disease expressed in Fe deficient Pixie II (resistant) was considerably less than that expressed by the susceptible Pixie variety. Infection of tomato did not enhance iron stress severity as quantified by root peroxidase activity and chlorophyll content of young leaves.

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Treatments known to alter either phenolic metabolism or overall enzyme activity were utilized to examine the Fe reductive mechanisms involved in iron stress response at the roots. Although specific compounds caused elevation of internal o-dihydroxyphenol content, the overall root reduction capacity of Fe stressed plants was significantly suppressed. However, plant roots retained significant capacity to reduce Fe after tissues were subjected to severe protein denaturizing treatments. Thus, indications for both secreted reductant and enzymatic reduction mechanisms were observed.

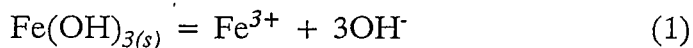
CHAPTER 1

INTRODUCTION

Iron is an element required for the survival of nearly all living organisms. Its electronic structure allows for two stable oxidation states: Fe^{2+} and Fe^{3+} . This property enables iron to play a key role in many of the oxidation-reduction reactions in cells. The cytochromes, ferredoxin, iron-sulfur proteins, leghemoglobin, nitrogenase and the peroxidases are important iron containing molecules utilized in electron transfer reactions. Most of the iron taken up by plants is utilized in photosynthetic and respiratory reactions. In the chloroplasts, it is also required for synthesis of aminolevulinic acid (ALA), a precursor to chlorophyll (Pushnik et al., 1984). Iron limitation in plants results in a dramatic decrease in ALA synthesis and thus leads to the initial most obvious expression of iron stress, interveinal chlorosis of the younger leaves. Prolonged stress leads to leaf necrosis and eventual death of the plant.

Although iron is relatively abundant in most soils, its phytoavailability is often very low. This phenomenon is largely a result of the low solubility of iron hydroxides which frequently control free-iron activity in soils. Amorphous ferric hydroxide, ferrihydrite and soil-Fe are often targeted as being the major solid phases which control iron solubility (Lindsay and Schwab, 1982). Free iron

availability is strongly influenced by pH, an effect which can be explained by examining the dissolution reaction of ferric hydroxide shown in equation 1 below.



As the activity of the hydroxyl ion increases, the reaction shifts to the left with a subsequent decrease of free Fe^{3+} in solution. Theoretically, a tenfold increase in OH^- activity (one pH unit) will cause a one-thousand fold decrease in Fe^{3+} activity. Thus, alkaline conditions are more apt to be iron limiting.

Other factors can also significantly influence iron phytoavailability. Breakdown products of organic matter yield organic acids and other anions which form soluble complexes with iron. These complexes raise the total soluble iron concentration and augment the transport of iron to plant roots (Inskeep and Comfort, 1986). The redox potential (E_h) is another important parameter which can strongly influence availability of iron. (E_h is defined as the millivolt difference in potential between a platinum electrode and the standard hydrogen electrode). The E_h controls the activity of Fe(III) with respect to Fe(II) as shown by the Nernst equation.

$$E_h = E^\circ + 59 \log (\text{Fe}^{3+})/(\text{Fe}^{2+}) \quad (2)$$

Ferrous iron is much more soluble than ferric and hence, soils with lower E_h 's are less likely to be iron limiting. The partial pressure of O_2 is an important determinant of E_h . Poorly aerated soils generally exhibit lower redox potentials due to microbial utilization of O_2 . In fact, the effect in flooded soils can be

great enough to cause iron toxicity in the vegetation. Based on the physical and chemical parameters presented, well aerated, buffered alkaline soils with low organic matter contents are most likely to be iron limiting. Highly calcareous soils in many areas of the world often meet these criteria and consequently are frequently associated with problematic iron deficiency (Clark, 1982).

Limitation of this essential micronutrient in microorganisms and plants results in a series of responses aimed at alleviating the deficiency. Most bacteria, virtually all fungi with the possible exception of Saccharomyces cerevisiae and many plant species of the gramineaceae family synthesize chelates as a means for elevating iron uptake (Neilands and Leong, 1986). These low molecular weight ligands (less than 1 kDa), called siderophores, are exuded into the surrounding environment where they scavenge Fe^{3+} . Siderophores exhibit extremely high affinity for Fe(III). For example, the trihydroxamate type siderophores, often produced by fungi, have stability constants of about 10^{30} . The ferrated siderophores are thought to be taken up by organisms via specific receptor and transport systems (Winkelmann et al., 1988). Molecular genetic studies on these inducible systems are in their initial stages. One of the best known genetically regulated systems was first discovered in Salmonella typhimurium and designated as the fur gene (Fe uptake regulation; Neilands and Nakamura, 1985). Upon binding with iron, the fur protein negatively regulates iron uptake by repressing multiple operons responsible for synthesis of ferrated siderophore transport. Essentially nothing is known about these types of

processes in plants.

In soils, competition for iron between microbial species may be a determinant factor in their function, population and even survival. The relative efficiency of siderophore production and utilization has been cited as one of the important mechanisms in disease suppressive soils (Schroth and Hancock, 1982; Neilands and Leong, 1986). The fluorescent siderophores produced by Pseudomonas spp., have been implicated in suppression of wilt caused by Fusarium oxysporum and root disease caused by Gaeumannomyces graminis. The mechanism is thought to occur via control of growth and/or metabolism of the harmful organism through iron deprivation. Competitive interactions for iron are also thought to occur between plants and microbes. A pseudobactin siderophore produced by Pseudomonas B10 was found to inhibit iron uptake by higher plants (Becker et al., 1985). Verticillium dahliae was also found to be capable of depriving peanut plants of iron when the fungus was present in the rhizosphere (Barash et al., 1988). However, the role of siderophores in this system was not verified. This observation evoked additional studies concerning the interaction between V. dahliae pathogenicity and iron status.

Verticillium dahliae is a soil borne vascular wilt pathogen and a member of the Deuteromycetes class of fungi. It is a pathogen of major economic importance, with worldwide distribution on many vegetables, ornamentals and fruit trees (Pegg, 1974). As a vascular wilt pathogen, V. dahliae's primary method of infection involves direct penetration of the epidermis, intra-and

intercellular growth toward the xylem, penetration of the endodermis and subsequent colonization of the vascular tissue. Disease symptoms are caused by toxins (Nachmias et al., 1982), hydrolytic enzymes, hormone imbalance and vascular plugging (Pegg, 1981). Coprogen B and dimerum acid are the predominant siderophores produced by V. dahliae (Barash et al., 1988). Mechanisms of defense against this pathogen are regulated by a single gene in tomato (Ve gene; Beckman, 1986). These defense mechanisms include: 1) formation of suberized apposition layers (Robb et al., 1987), and 2) formation of tyloses aimed at localizing the pathogen (Sinha and Wood, 1958), 3) hypersensitivity, and 4) rapid accumulation of phytoalexins (Tjamos and Smith, 1975).

It is reasonable to speculate that iron deficiency of resistant plants may result in perturbation of critical defense responses and thus enhanced susceptibility to disease. In 1975, Krikun and Frank observed this phenomena in peanut plants which, when subjected to iron limitation, lost their tolerance to Verticillium Wilt. Tolerance was regained by addition of Fe-amendment to the plants. A similar study by Barash and his coworkers (1988) revealed comparable results with eggplant. In a grant proposal submitted to BARD in 1986, Barash and his coworkers suggested that the differential ability for iron sequestration by the fungal pathogen V. dahliae and its hosts can be a significant virulence factor in Verticillium Wilt diseases. The current study described in Chapter 2, was undertaken to test this hypothesis.

Production of siderophores is not the only mechanism used by organisms to enhance iron uptake. Dicotyledonous and non-gramineous monocotyledonous plants utilize a completely different strategy to elevate iron uptake (Olsen et al., 1981). Their inducible system exhibits at least two components: 1) acidification of the rhizosphere and, 2) a decrease of the oxidation-reduction potential at the rhizoplane and free-space of the root. Acidification of the rhizosphere is thought to occur via exudation of phenolic acids, primarily caffeic and chlorogenic (Olsen et al., 1981; Hether et al., 1984), exuded organic acids (De Vos et al., 1986) and/or a hydrogen ion pumping ATPase. A decrease in pH enhances the solubility of iron by shifting reaction #1 (page 2) forward. Another important aspect of pH is that optimal reduction activity of the roots occurs in the acidic pH range. It has been suggested that reduction of iron is required since the ferrous form, Fe(II), is thought to be the primary form of iron taken up by roots (Chaney et al., 1972). A decreased redox potential also enhances the activity of iron for reasons previously described (page 2).

The primary mechanism of ferric reduction has been the subject of some controversy. Phenolic acid excretion by roots is greatly enhanced when a plant becomes iron stressed. The process is thought to involve variable activity of iron containing peroxidases which polymerize phenolics to form components of suberin (Sijmons et al., 1985). Root peroxidases are sensitive to iron stress. Their inactivation under iron deficient conditions inhibits suberin synthesis and thus allows for accumulation of phenolics which are subsequently exuded from

the roots. The exuded phenolic acids can act as electron donors by forming a redox couple with reduction of ferric Fe (Olsen et al., 1981). The phenolics and their oxidized products (quinones) can also act as Fe ligands which aid in dissolution and transport of Fe(II) or Fe(III). Evidence for this secreted phenolic mechanism of reduction is convincingly supported by studies utilizing an inefficient iron uptake mutant of tomato, T3238 fer (Olsen et al., 1981). This variety does not exhibit the typical acidification and redox responses associated with iron stress. Nutrient solutions supplemented with 100 μ M p-coumaric acid, a precursor to phenolics, caused restoration of these functions and alleviated iron chlorosis of deficient plants. However, kinetic studies and studies involving membrane disruption have led researchers to suggest that exuded reductants may not be the primary source of electrons for reduction of Fe(III). Romheld and Marshner (1983) observed that reduction rates of various ferric chelates by phenolic acids were difficult to reconcile with reduction rates exhibited by roots. Chelates with higher stability constants caused significantly reduced rates of iron reduction by phenolic acids. However, root reduction rates of the various iron chelates were not dependent on chelate binding strength. Thus, enzymatic reduction by a plasma membrane bound reductase was proposed as the main source of electrons in "iron stress response". Later studies by Sijmons and his coworkers (1984) have led them to suggest that concentration of cytosolic NADPH is the rate determining factor in enzymatic reduction. Chapter 4 of this thesis attempts to further clarify the relative significance of these two inducible

mechanisms.

Since micromolar levels of iron within cells can be toxic, an excessive supply is undesirable. Iron overloads commonly occur on flooded soils where a reducing environment leads to high levels of ferrous iron in the vicinity of roots. In addition, activation of "iron stress response" mechanisms may lead to a "flush" of iron throughout the plant whereby excess iron may accumulate and be harmful. To combat these undesirable occurrences, plants utilize the ferritin iron-protein complex to buffer intercellular iron levels (Seckback, 1982). Ferritin is found exclusively in the plastids, primarily chloroplasts, where up to 80% of the total plant iron is located (Seckback, 1968). The ferritin complex consists of a ferric oxyhydroxide core covalently bound to a protein shell composed of 24 subunits. The core may contain up to 4,500 iron atoms in a crystalline structure similar to ferrihydrite. Molecular genetic studies in plant systems have shown that protein subunit synthesis is iron induced at the transcriptional level (Van Der Mark et al., 1983). At least two types of protein subunits are known to exist. In plant leaves, a high iron content has been correlated with a high proportion of 26,500 Da subunits while ferritin in low iron leaves has a considerably higher proportion of lower molecular weight subunits (Van Der Mark and Van Den Briel, 1985).

The specific mechanisms of iron deposition and mobilization from ferritin are still largely undefined. Reductive removal has been shown to be considerably more effective than any other mechanism. Numerous reducing

agents such as caffeic acid (Boyer et al., 1988), superoxide ion (Boyer and McCleary, 1987), thiols, dyhydroflavins (Funk et al., 1985; Sirivech et al, 1974), and ascorbate (Bienfait and Van Den Briel, 1980) have proven to effectively promote iron release from the complex.

It is becoming increasingly evident that UV and near-UV radiation plays an important role in influencing the bioavailability of iron in plants (Olsen and Brown, 1981; Bennett et al., 1982; Jolley et al., 1987; Pushnik et al., 1987; Krizek et al., 1982). The capability of UV and near-UV radiation to promote reduction of Fe(III) to Fe(II) has been demonstrated in vitro. Evidence has been obtained which suggests the process also occurs in intact leaves. In addition, the studies by Olsen and Brown (1981) and Bennett et al. (1982) indicate that organic acids such as citrate and oxalate can enhance light activated reduction while inorganics such as phosphate and copper have an inhibitory effect. Work by Pushnik, et al. (1987) suggests that the redox state of foliar iron as influenced by UV and near-UV radiation may in turn affect chlorophyll synthesis and ultimately photosynthetic function. Their work with cotton indicates that a similar chloroplastic response results from either exposure to low quantities of UV radiation or iron deficiency.

Chapter 3 of this thesis provides evidence which suggests that light can effectively activate significant mobilization of iron from ferritin with a minimum of metabolic demand on the plant. A mechanism which couples chloroplast iron demand with iron release from ferritin is also proposed.

CHAPTER 2

THE RELATIONSHIP BETWEEN IRON NUTRITION AND
VERTICILLIUM RESISTANCE IN TOMATO

Nearly all living cells require iron for their growth and survival. Though relatively abundant in nature, the low solubility of iron under aerobic conditions limits its availability to organisms. Thus, under iron limiting conditions, plants and microbes utilize iron solubilizing mechanisms to maintain a well regulated supply of this essential element.

Acquisition of iron has been reported to be a critical virulence factor in a number of plant-pathogen interactions (Kloepper et al., 1980; Expert and Toussaint, 1985). The influence of low-iron nutritional status on severity of Verticillium Wilt has been observed in peanut and eggplant (Krikun and Frank, 1975; Barash et al., 1988). Krikun and Frank (1975) reported that peanut varieties which were highly tolerant to Verticillium Wilt in noncalcareous clay soils, succumbed to the disease when grown in iron limiting, calcareous loess soil. The addition of an iron amendment to the calcareous soil allowed the plants to retain tolerance to the disease. A similar study by Barash et al. (1988) utilizing eggplant revealed comparable results. Enhancement of disease expression through iron limitation has also been observed during infection of bean by

Fusarium solani (Guerra and Anderson, 1985) and Botrytis cinerea (Brown and Swinburne, 1982). Reduction in lignin formation and decreased phytoalexin production were cited as reasons for the aggravation of disease symptoms under iron deficient conditions. The mechanisms involved in iron's effect on sensitivity of peanut and eggplant to Verticillium dahliae are not yet clear.

Plants exhibit complex physiological and anatomical responses to various forms of stress induced by different biotic and abiotic factors. A considerable amount of information is known about iron stress response mechanisms in tomatoes (Bienfait, 1985) and tomato response to infection by Verticillium spp. (Pegg, 1981). The present study was undertaken to examine the interaction and combined effect of these two stress-inducing conditions in tomato.

Materials and Methods

Plants, Pathogen, and Inoculation

Pixie II-resistant and Pixie-susceptible tomato varieties were used as near-isolines differing in the presence of the Ve gene for resistance to race 1 of V. dahliae. Ace VF (resistant) and Marglobe (susceptible) were also utilized in experiments. Seeds purchased from W. Atlee Burpee Co. (Warminster, PA) were surface sterilized for three minutes in 0.5% sodium hypochlorite and germinated on stainless steel screens covered with moist cheesecloth. The tomato seedlings were transferred to opaque 10 L polyethylene tubs (24 seedlings per tub) containing 8L of standard nutrient solution plus 36.0 μM

FeEDTA. The standard nutrient solution was composed of 1.90 mM $\text{Ca}(\text{NO}_3)_2$, 0.47 mM $\text{Mg}(\text{NO}_3)_2$, 0.24 mM KCl, 0.61 mM KNO_3 , 0.60 mM K_2HPO_4 , 0.32 mM $(\text{NH}_4)_2\text{SO}_4$, 7.4 μM MnCl_2 , 41.3 μM H_3BO_4 , 1.9 μM ZnSO_4 , 0.5 μM CuSO_4 , and 0.4 μM Na_2MoO_4 with a pH of approximately 7.0. All nutrient solutions in the experiments were continuously aerated and were changed weekly. Inoculum was prepared by incubation of a virulent isolate of V. dahliae (race 1) for two weeks in Czapeks media shake cultures supplemented with 100 ppm kanamycin monosulfate. The race 1 isolate was obtained from K. Kimble, Harris Moran Seed Co., Davis, Calif. At the late 3-leaf stage, plants were inoculated by submergence of the roots into an aerated conidial suspension (1.0×10^6 conidia/ml) of V. dahliae for 24 hours. After inoculation, individual seedlings were placed into opaque polyethylene bottles containing 1L of standard nutrient solution. To allow induction of iron deficiency, 5.0 mM NaHCO_3 , 0.25 g CaCO_3 , and 54.0 μM EDTA were added to all treatments. Iron replete treatments also contained 54.0 μM FeCl_3 . The growth environment consisted of 16 hours light with an approximate energy level of $310 \mu\text{E m}^{-2} \text{s}^{-1}$ followed by 8 hours of darkness. The day and night temperatures were $22^\circ\text{C} \pm 0.7$ and $21^\circ\text{C} \pm 0.7$, respectively. Average relative humidity was 70 %. Analysis of specific physical and biochemical parameters was initiated 20 days after inoculation. Disease, fungal progression, iron stress and peroxidase activity were evaluated in an experiment utilizing the Pixie and Pixie II near-isolines. A $2 \times 2 \times 2$ factorial design in six randomized blocks was implemented with infected vs noninfected

treatments, iron replete vs iron deficient treatments and resistant vs susceptible varieties. Disease was evaluated in two additional experiments with the resistant variety Ace VF. These additional experiments also utilized a randomized block design (6 blocks/experiment).

Evaluation of Disease

Wilt symptoms were quantified by measurement of leaf stomatal resistance with a MK3 automatic porometer (Delta - T devices LTD; Visser and Hattingh, 1980). Measurements were taken on terminal leaflets of lower leaves, 20 and 21 days after inoculation. Visual disease ratings were based on both wilting and chlorosis/necrosis of lower leaves.

Fungal Progression

Upward fungal progression through the vascular system was measured 23 days after inoculation. Leaves and lateral roots were removed and the main axis was dipped into 95% ethanol for 5 seconds followed by submergence in 0.5% sodium hypochlorite for 3 minutes. Segments 0.5 cm in length were cut and placed on Czapeks agar containing 100 ppm kanamycin monosulfate. After 7 days of incubation, the segments were visually inspected for outgrowth of V. dahliae.

Evaluation of Iron Deficiency

Iron deficiency was quantified by visual chlorosis rating (1 = no chlorosis, 5 = severe chlorosis) and determination of chlorophyll content. Two sets of three leaf disks (0.5 cm diameter) were excised from the youngest leaves.

Chlorophyll content of these disks was measured using the technique described by Inskeep and Bloom (1985).

Peroxidase Activity

Root samples (two samples/plant) of Pixie and Pixie II were assayed for peroxidase activity using the technique modified from Reuveni and Ferreira (1985). Immediately following excision, 0.3 g root samples were placed in dry ice until the frozen tissues could be homogenized in a chilled mortar and pestle. The pulverized material was added to 3.0 ml of cold 15.0 mM sodium phosphate buffer, pH 6.0, and the homogenate was centrifuged at 10,000 g for 10 min at 4°C.

Aliquots of the enzyme extract (50 μ L) were added to 3.0 ml of the assay solution consisting of 15 mM sodium phosphate buffer, pH 6.0, 1.0 mM H₂O₂, and 0.1 mM O-methoxyphenol (guaiacol). Enzyme activity was expressed as change in absorbance (470 nm) min⁻¹ g⁻¹ fresh weight.

Total Iron

Total iron analysis of leaf and petiolar tissue was conducted on the susceptible Marglobe variety (4 plants/treatment were sampled). Perchloric/nitric acid tissue digests were analyzed for total iron using atomic absorption spectrophotometry (Perkin-Elmer Model 560 atomic absorption spectrophotometer).

O-Dihydroxyphenols

The method utilized for analysis of total O-dihydroxyphenols was modified from Collier (1979). Root material (0.25 g) was added to 3.0 ml 95% ethanol and homogenized for 10 minutes (Virtis 23 homogenizer). The homogenate was filtered (0.45 μ m) and loaded on 4.0 x 0.8 cm acid alumina columns (activity grade 1). The columns were rinsed with two bed volumes of 95% ethanol followed by diazotization of the O-dihydroxyphenols with two bed volumes of a 5% NaNO₂ plus 5% acetic acid solution. The diazotized compounds were eluted with two bed volumes of 5.0 N NaOH and assayed spectrophotometrically at 520 nm. Caffeic acid was used as the standard.

Siderophore Assay

Siderophore assay agar media was prepared according to the method outlined by Schwyn and Neilands (1987). This assay is non-specific and is very sensitive to molecules possessing iron chelating properties. The assay was conducted as a preliminary test for the presence of iron chelates in the vascular system. Vascular fluid was squeezed out of stem cuttings and placed on the assay media. After 24 hours the media were visually inspected for color change.

Results

Disease Symptoms

Measurements of stomatal resistance provided evidence indicating that iron deficiency of tomato plants bearing major gene resistance to V. dahliae increases

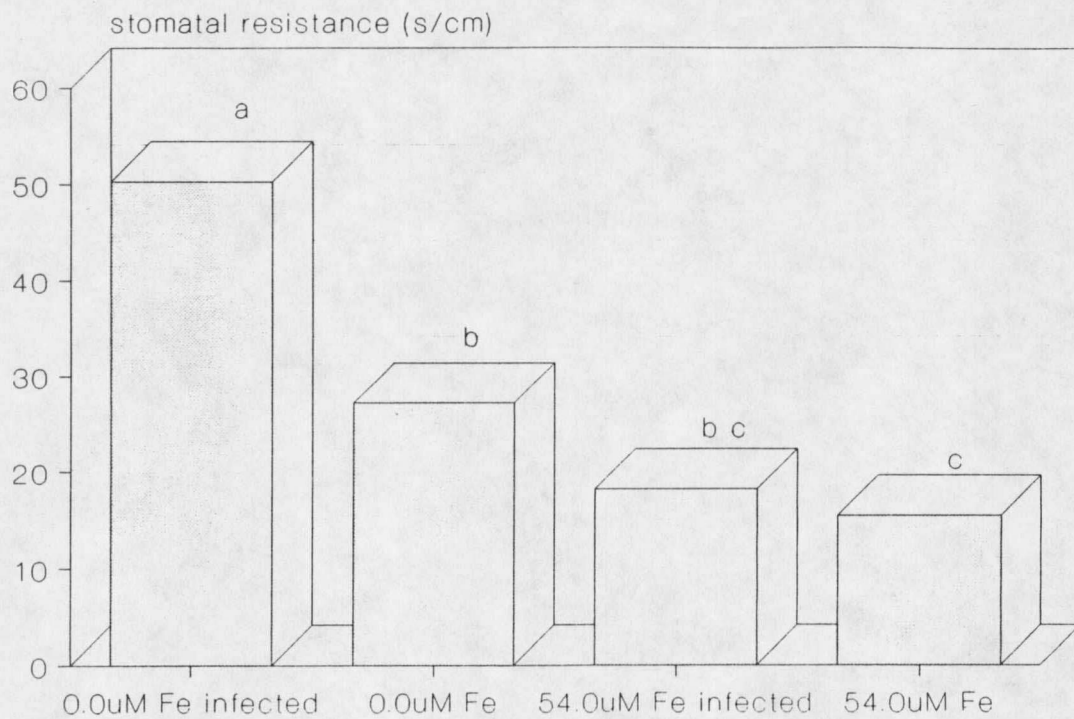


Figure 1. Disease symptoms of the tomato variety Ace VF measured by stomatal resistance (1/transpiration) 20 and 21 days after inoculation with race 1 of *V. dahliae*. Letters are LSD significant differences at the $p = 0.05$ level.

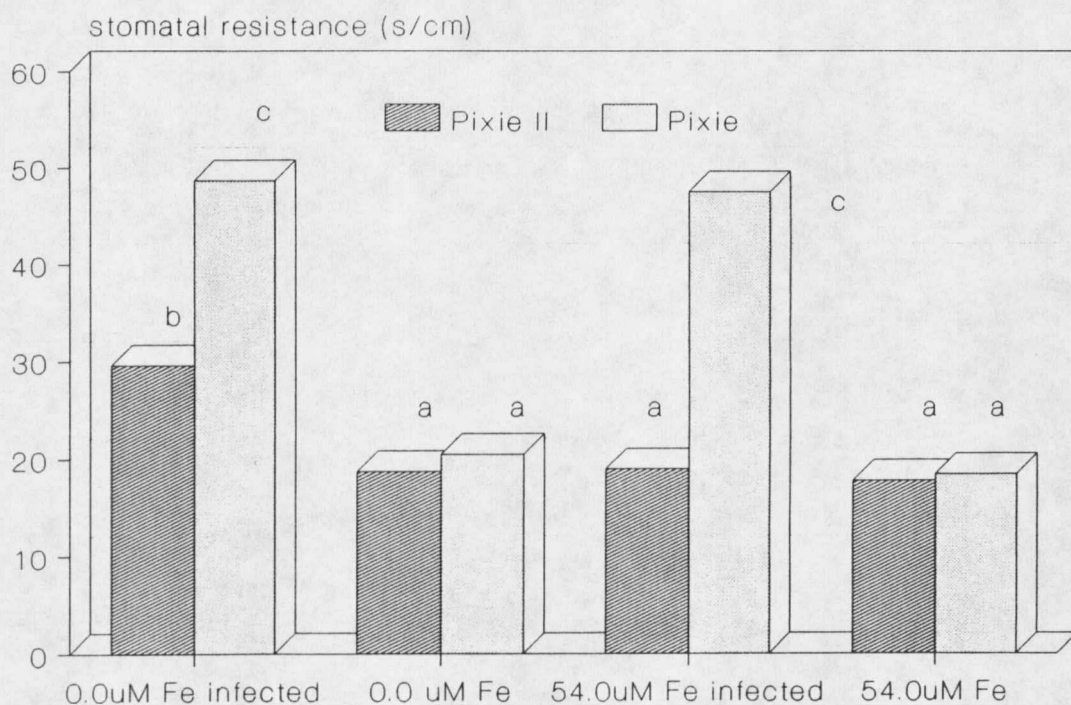


Figure 2. Disease symptoms of tomato near-isolines Pixie (sus) and Pixie II (res) measured by stomatal resistance (1/transpiration) 20 and 21 days after inoculation with race 1 of *V. dahliae*. Letters are LSD significant differences at the $p = 0.05$ level.

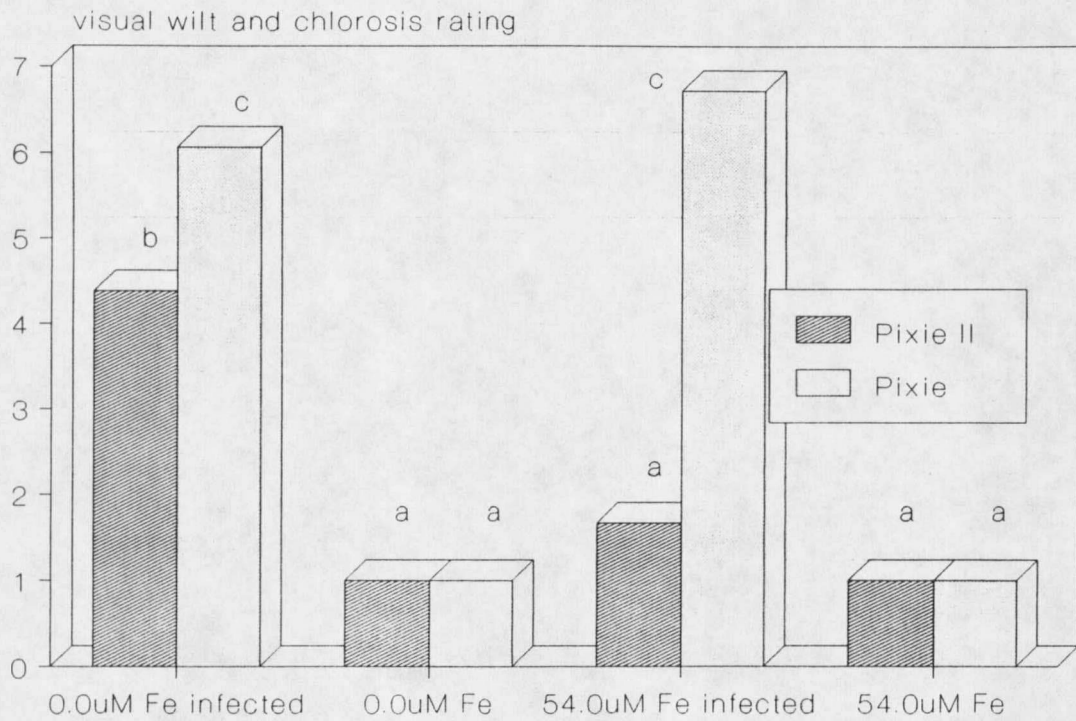


Figure 3. Disease symptoms of tomato near-isolines Pixie (sus) and Pixie II (res) quantified by visual rating of wilt and chlorosis of lower leaves (1.0 = no symptoms, 3.0 = two leaves exhibiting mild symptoms, 3.9 = two leaves exhibiting severe symptoms, 5.0 = four leaves exhibiting mild symptoms). Letters indicate LSD significant differences at the $p = 0.05$ level.

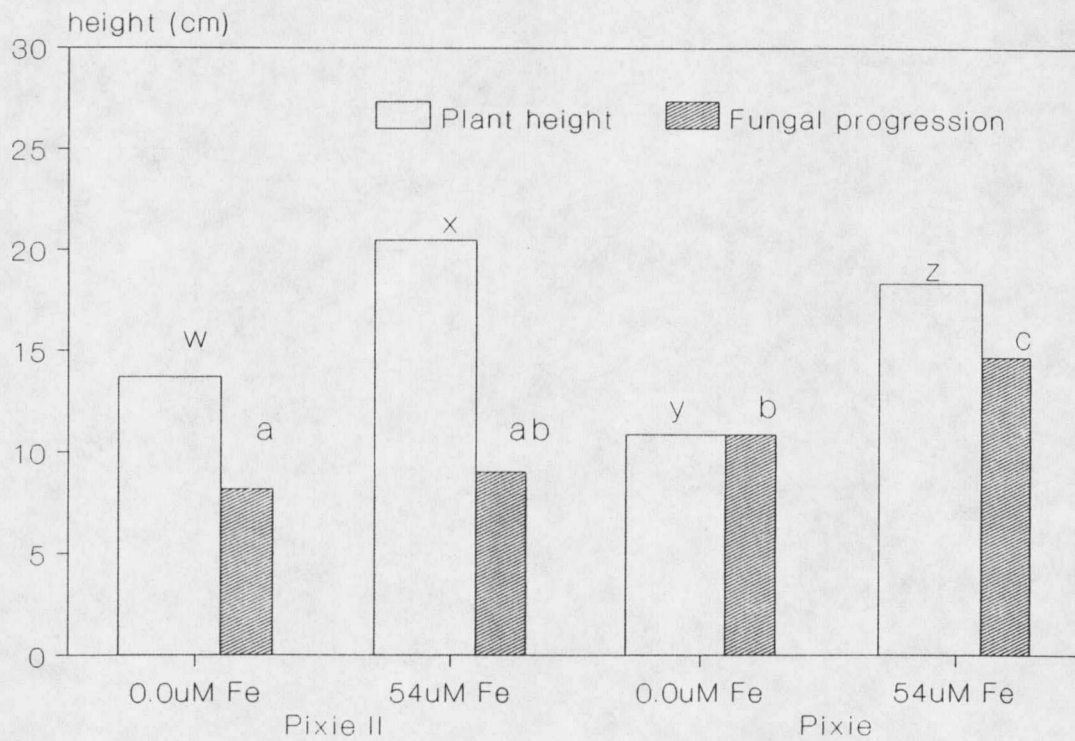


Figure 4. Plant height and distance of upward fungal progression in stems of tomato near-isolines Pixie (sus) and Pixie II (res). Letters w,x,y and z indicate LSD significant differences ($p = 0.05$) between plant height measurements and letters a,b,c and d indicate significant differences in height of fungal progression.

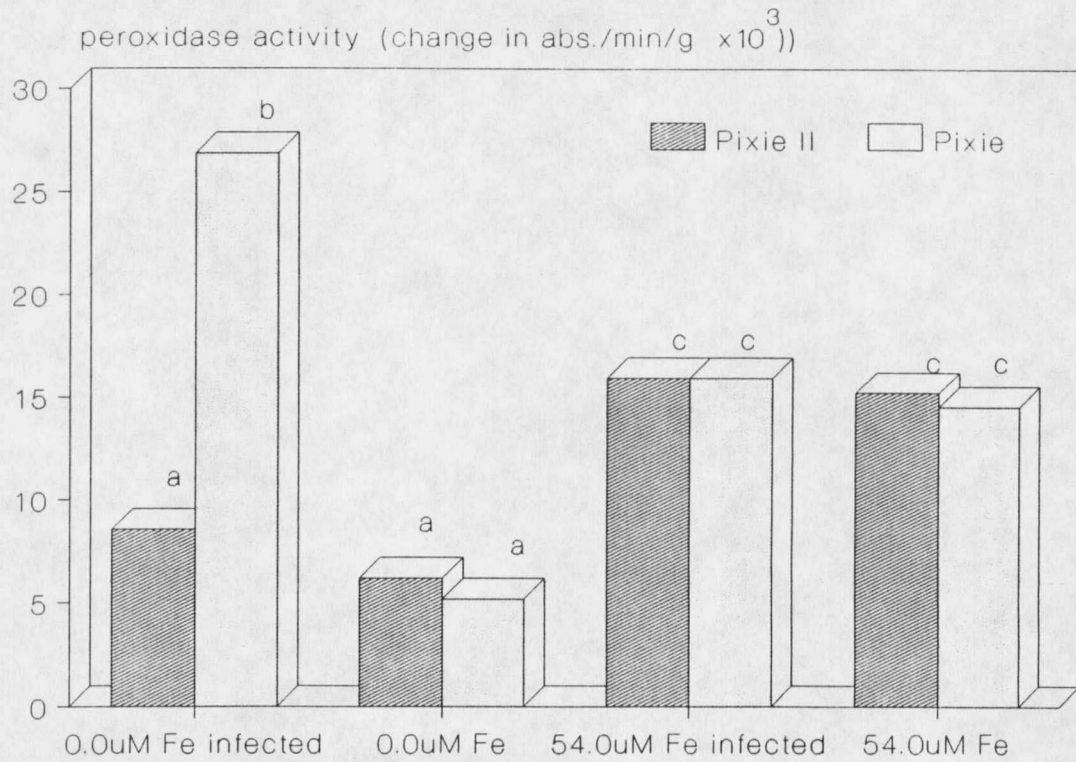


Figure 5. Peroxidase activity of tomato near-isolines. Infection had no significant effect (LSD significance level $p = 0.05$) on peroxidase activity with exception of the iron deficient Pixie (sus) treatment.

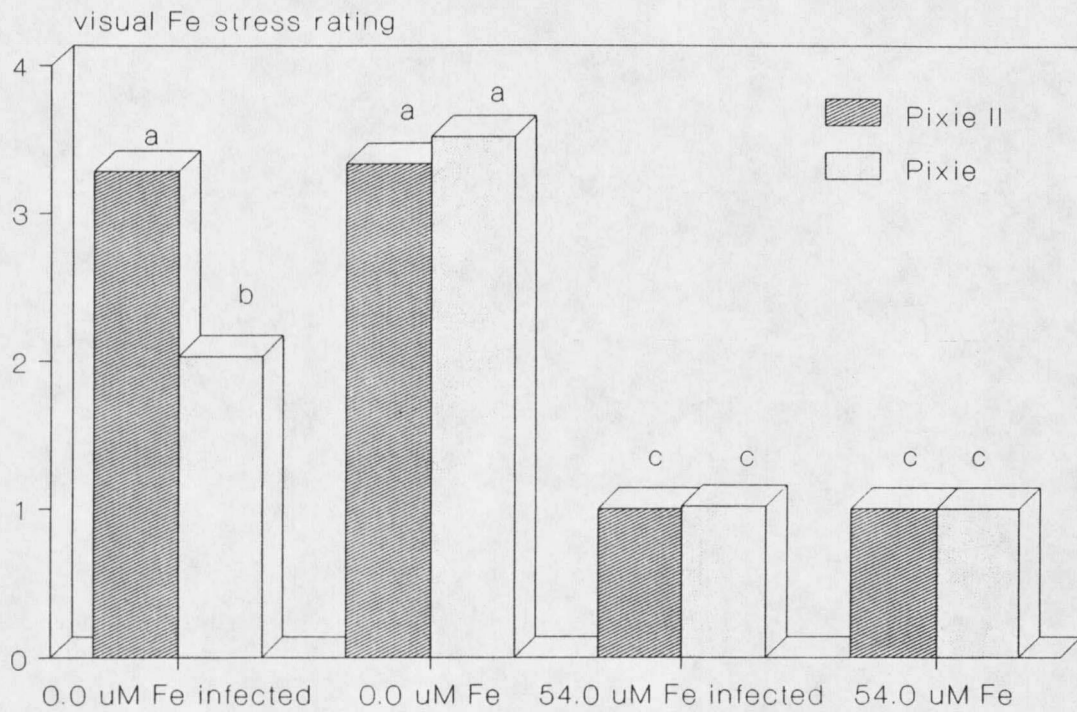


Figure 6. Visual iron chlorosis ratings of tomato near-isolines Pixie (sus) and Pixie II (res) (1.0 = no chlorosis, 5.0 = severe chlorosis and necrosis of younger leaves). Letters indicate LSD significant differences at the $p = 0.05$ level.

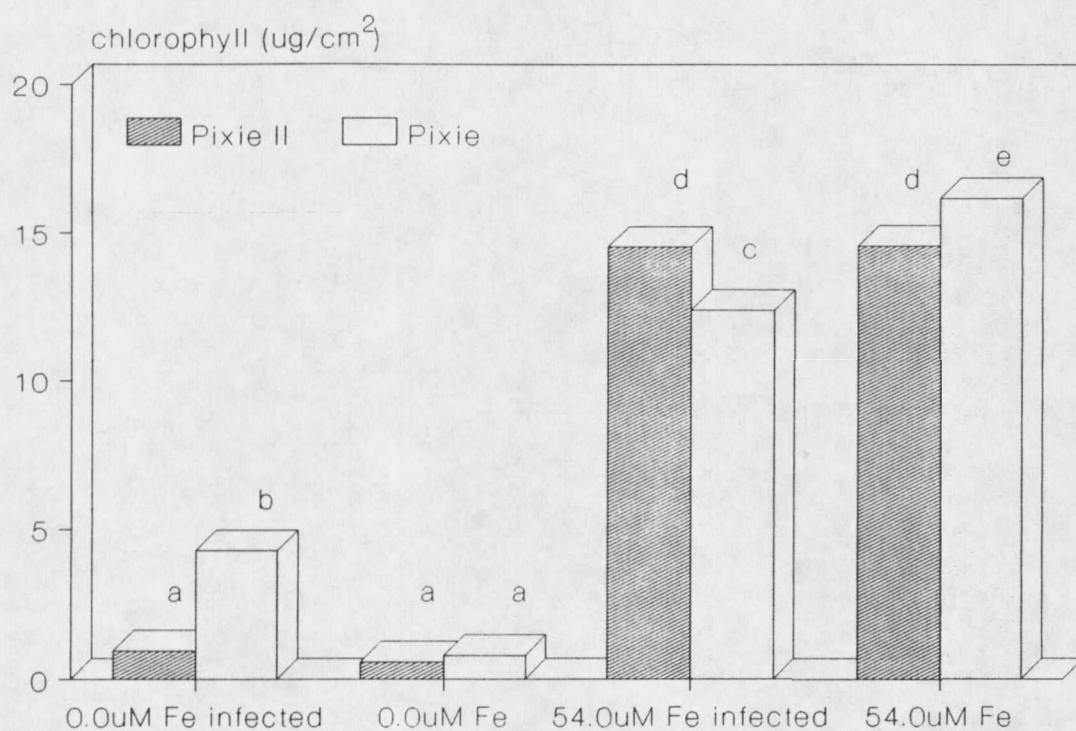


Figure 7. Chlorophyll content in youngest leaves of Pixie (sus) and Pixie II (res) tomato near-isolines. Letters indicate LSD significant differences at the $p = 0.05$ level.

their sensitivity to the pathogen (Figs. 1, 2 and 3). Iron deficient resistant plants exhibited significant wilting while iron replete plants showed little wilting. However, visual observation revealed that slight stunting occurred in lower leaves of iron replete Pixie II. Though sensitivity of resistant varieties was significantly increased by iron deficiency, the magnitude of wilting was still considerably less than that expressed by the susceptible Pixie variety. The iron status of infected Pixie had no significant effect on stomatal resistance.

Fungal Progression

The assay used to determine extent of vascular invasion indicated the pathogen was able to advance a significantly greater distance in Pixie versus Pixie II (Fig. 4). The distance invaded in the resistant varieties was approximately the same for both iron replete and iron limited treatments. Nearly all petiole segments excised from lower leaves of inoculated treatments tested positive for presence of the pathogen.

Iron Stress Severity

Infection of either resistant or susceptible tomato did not aggravate iron stress in the host as quantified by root peroxidase activity (Fig. 5), visual iron chlorosis rating (Fig. 6) and determination of chlorophyll content (Fig. 7). However, iron stress of diseased Pixie was considerably less severe than any other iron limited treatment. In addition, peroxidase activity in the infected, iron deficient Pixie treatment was significantly higher than other treatments. All other iron limited treatments demonstrated reduced activity. Root peroxidase

is a sensitive indicator of iron stress and an increase in iron stress is correlated with a decrease in enzyme activity (Sijmons, 1985). Infection by vascular wilt pathogens on the other hand, is correlated with elevation of peroxidase activity (Pegg, 1981). Under the present conditions, elevation of peroxidase activity due to infection was only observed in conjunction with iron stress. Reduced activity, which would be expected under iron limitation, was apparently delayed or masked by the additional influence of infection.

In a similar experiment conducted with the susceptible Marglobe variety, total iron analysis of leaf and petiole tissue indicated that infected plants subjected to iron limitation contained 31% more iron than non-infected plants. No difference was observed in the iron replete treatment.

O-Dihydroxyphenols and Siderophores

Analysis of total internal O-dihydroxyphenols yielded results too variable for discernment of significant differences between treatments. All iron deficient treatments tested positive for the presence of siderophores and/or chelates in the xylem fluid. The iron replete treatments tested negative. These results may be attributed to high concentration of iron free EDTA in the nutrient solution of iron deficient treatments.

Discussion

The use of hydroponic culture media permitted study of the interaction between iron deficiency and pathogenicity of V. dahliae in tomato.

Incorporation of near-isolines Pixie (susceptible) and Pixie II (resistant) into the experiments allowed for a viable comparison between resistant and susceptible cultivars. While iron nutritional status of susceptible tomatoes showed no influence on symptom expression, infected tomatoes possessing major gene resistance expressed an increase in wilting upon iron limitation. However, the level of disease expressed by iron stressed Pixie II was still considerably less than that expressed by the susceptible Pixie variety. One possible explanation for this observation is that only a small portion of the multiple resistance factors regulated by the Ve gene (Beckman, 1987) was impaired by iron deficiency.

Progression of the pathogen in stems of Pixie II and Ace VF was not significantly different between iron replete and iron deficient treatments. These results suggest that enhanced expression of disease upon iron limitation is due to an increase in sensitivity of resistant plants to the pathogen rather than restriction of fungal invasion. Since iron is directly involved in many enzymatic and electron transport reactions in cells, its limitation can severely impact plant metabolism and subsequently increase sensitivity to virulence factors such as toxins, hydrolytic enzymes, and/or hormones. Although many of the Ve gene regulated factors are associated with isolation and destruction of the fungus, host sensitivity to virulence factors has been cited as being critical to resistance (Dixon and Pegg, 1969). These specific Ve gene regulated physiological mechanisms are currently unknown.

Guerra and Anderson (1985) have suggested that peroxidases directly or indirectly involved in disease resistance may be negatively impacted by iron deficiency. The peroxidases are iron containing enzymes which catalyze oxidation-reduction reactions with hydrogen peroxide and phenolics. Reduced peroxidase activity resulting from iron stress allows for accumulation of phenolics in the vascular system. These phenolics are thought to play an important role in alleviation of iron stress. In addition, phenolics are known to play a complex role in the pathogenesis of wilt diseases e.g., metabolism of IAA and other growth substances and regulation of enzyme activity including polygalacturonase activity (Pegg, 1981). Thus, phenolic metabolism, as coupled to peroxidase activity, may be one important factor linking disease resistance with iron nutritional status.

Another factor which may be responsible for enhancement of wilt symptoms in resistant varieties is stunting induced by iron limitation. A reduction in total plant volume (quantified by height; Fig. 4) may have allowed for a more concentrated build-up of symptom inducing compounds as compared with non-stunted plants.

Research conducted by Barash and his coworkers (1988) indicated that V. dahliae was able to induce iron deficiency in peanut plants. Other studies with peanuts and eggplants (Krikun and Frank, 1975; Barash et al., 1988) showed that iron deficiency predisposed the plants to the disease. Thus, they proposed that sequestration of iron may be a significant virulence factor in *Verticillium*

diseases. In the current study, the pathogen demonstrated no significant ability to withhold iron from plants when present in planta. However, the use of peroxidase activity and chlorophyll content as parameters for quantification of iron nutritional status may not be appropriate in this plant-pathogen interaction. Nevertheless, the results imply that in vivo iron deprivation by V.dahliae may not be a significant virulence factor in this system.

CHAPTER 3

PHOTOCHEMICAL MOBILIZATION OF FERRITIN IRON

Ferritin is a protein complex utilized for safe, non-toxic storage of iron in a wide variety of organisms (Granick, 1946; Hyde et al., 1963; Steifel and Watt, 1979; Peat and Banbury, 1968). Its structure and function are remarkably similar in many species (Crichton and Charlotiaux-Wauters, 1987). Ferritin consists of a ferric oxyhydroxide core with up to 4,500 Fe atoms per molecule complexed to a proteinaceous shell composed of 24 subunits. The complex is generally viewed as a buffer for iron under conditions in which iron influx is not closely coupled with its use. Iron release from the ferritin reserve occurs most readily by reduction of Fe(III) to Fe(II) followed by passage through micro-pores in the protein shell. Numerous reducing agents such as caffeic acid (Boyer et al., 1988), superoxide ion (Boyer and McCleary, 1987), thiols, dihydroflavins (Funk et al., 1985; Sirivech et al, 1974), and ascorbate (Bienfait and Van Den Briel, 1980) have proven to effectively promote iron release from the complex.

The plant form of ferritin, referred to as phytoferritin, is compartmentalized within plastids of higher plants and is most commonly associated with iron replete tissues exhibiting impaired photosynthetic function (Seckback, 1982; Van der Mark et al., 1981; Craig and Williamson, 1969). In general, actively

photosynthesizing tissues of mature, healthy plants contain little phytoferritin. Iron plays an essential role in many of the electron transfer reactions associated with both photosystems I and II and is utilized in the formation of chlorophyll (Miller et al., 1982; Pushnik et al., 1984). Synthesis and proper function of the photosynthetic apparatus in actively photosynthesizing chloroplasts requires significant amounts of iron which in turn may deplete levels of stored ferritin iron.

It is becoming increasingly evident that UV radiation plays an important role in influencing the bioavailability of iron in plants (Olsen and Brown, 1981; Bennett et al., 1982; Jolley et al., 1987; Pushnik et al., 1987). The capability of UV and near UV radiation to promote reduction of Fe(III) to Fe(II) has been demonstrated in vitro (Olsen and Brown, 1981; Bennett et al., 1982; Krizek et al., 1982). These studies also suggest that Fe reduction occurs in vivo. Studies by Olsen and Brown (1981) and Bennett et al. (1982) indicate that organic acids such as citrate and oxalate can enhance UV activated reduction while inorganics such as phosphate and copper have an inhibitory effect. Work by Pushnik, et al. (1987) suggests that the redox state of foliar iron as influenced by UV radiation may in turn affect chlorophyll synthesis and ultimately photosynthetic function. Their work with cotton indicates that a similar chloroplastic response results from either exposure to low quantities of UV radiation or low iron levels. This current paper provides evidence which suggests that light can effectively induce mobilization of iron from ferritin with a minimum of metabolic demand

on the plant. A mechanism which couples chloroplast iron demand with iron release from ferritin is proposed.

Materials and Methods

Protein Preparation

Several different preparations of horse spleen ferritin were purchased from Sigma Chemical Company. The stock ferritin was further purified by dialysis across 50,000 MW tubing, against a solution containing 1.0 mM EDTA and 0.15 M sodium acetate (pH = 5.5). This was followed by extensive dialysis against 0.15 M sodium acetate and finally, 0.15 M sodium chloride. The final concentration of purified protein was determined by using the Bradford dye-binding procedure with bovine serum albumin as standards (Bradford, 1976). Total Fe was assayed using the Ferrozine method (Stookey, 1970). Iron content was found to vary between 2,500 and 3,000 atoms per molecule, depending on the preparation.

Iron Mobilization Assays

The standard assay for Fe mobilization consisted of 1.0 mM ferrozine (3-(pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine), 0.07 M sodium chloride, and 0.74 μ M ferritin; all adjusted to a pH of 6.0. The chromophoric chelator, ferrozine, was chosen as an indicator of reductive Fe mobilization for reasons described by Boyer et al. (1988). Ferrozine has a strong affinity for Fe(II) while the binding constant for Fe(III) is low. Its large size and negative charge inhibit

its passage through the 3-4 Å pores of the proteinaceous ferritin shell. These micropores are lined with several aspartate and glutamate residues which impart a negative charge in the range of physiological pH. Thus, the properties of ferrozine make it a desirable indicator for studies of ferritin Fe release. The removal of Fe by ferrozine in the dark was undetectable in our assays.

Two mL of standard assay solution was added to 22 x 0.7 cm Bausch and Lomb cuvettes randomly placed in a modified test tube rack. Experiments were conducted at $26.5^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in growth chambers equipped with cool white fluorescent and incandescent bulbs which provided approximately $300 \mu\text{E m}^{-2} \text{ s}^{-1}$ to the assay solutions. After irradiation for various time intervals, the cuvettes were protected from light and immediately assayed for Fe released. The amount of Fe mobilized from ferritin was quantified by spectrophotometric measurement of the $\text{Fe (ferrozine)}_3^{+2}$ complex formed. Concentration of Fe was calculated by use of the reported molar extinction coefficient, $27,900 \text{ cm}^{-1}$ at 562 nm (Stookey, 1970). Measurements were obtained on a Beckman DU-50 spectrophotometer equipped with a kinetics package. All samples were prepared under illumination from either a red safelight or in a darkened room. Each treatment was replicated at least three times.

Effect of Various Factors on Fe Release From Ferritin by Light

pH. To test the effect of pH on Fe mobilization, solutions were adjusted to the desired pH with NaOH prior to illumination.

Temperature. Solutions were placed in a growth chamber adjusted to the desired temperature and allowed to equilibrate for at least 20 minutes prior to an irradiation period of 10 minutes.

Organics and Inorganics. Succinate, fumarate, citrate, oxalate, tartarate, caffeate, cinnamate, phosphate, copper, manganese, and EDTA were added to standard ferritin solutions at various concentrations and their influence on rate of Fe mobilization was determined at pH = 6.0.

Ferritin Fe Release Induced by Light Transmitted Through Leaves

Windows (1.0 cm²) were fashioned with opaque tape and placed over 10 ml beakers. Excised Coleus x hybrid leaves were fastened to the windows in a manner which did not allow light to enter the beakers except by passage through leaves. The amount of Fe mobilized from 10 ml of standard assay solution per beaker was measured after 10 hours of irradiation.

Results

Effect of Light on Ferritin Fe Release

Illuminated standard ferritin solutions released approximately 26 μ M Fe(II) per hour while Fe released from unilluminated solutions was negligible. With the exception of caffeate, none of the organic or inorganic ions tested in this study mobilized ferritin Fe under dark conditions.

Effect of Various Factors on Fe Release from Ferritin by Light

pH. Figure 8 demonstrates the effect of pH on Fe release from ferritin from 0 to 45 minutes. Photorelease of Fe was inversely related to pH. An increase in pH from 4.6 to 7.1 decreased the amount of Fe released by about 20%. A pH change from 6.0 to 7.1 exerted a greater inhibiting influence than a pH change from 4.5 to 6.0. Consequently, release rate does not appear to be linearly related to pH. As with all the light activated reactions in the experiments, the observed rates of Fe release decreased over time.

Iron release from ferritin was assumed to be a first order reaction with respect to ferritin-Fe (Boyer and McCleary, 1987). The rate constant (k) for Fe mobilization with respect to ferritin-Fe was calculated from the integral form of the rate equation $d[\text{ferritin-Fe}/dt] = k [\text{ferritin-Fe}_0 - \text{Fe released}]$. Plots of $-\ln(\text{ferritin-Fe}_0 - \text{Fe released})$ vs time resulted in rate constants ranging from $2.7 \times 10^{-3} \text{ sec}^{-1}$ (pH = 4.6) to $2.1 \times 10^{-3} \text{ sec}^{-1}$ (pH = 7.1).

Temperature. A 28°C increase in temperature (9°C to 37°C) resulted in a 50% increase in the amount of Fe mobilized from ferritin (Fig. 9). The response to temperature was linear ($r^2 = .998$). The Arrhenius activation energy required for Fe release was calculated from a plot of $\ln \text{rate}$ vs $1/T$. The slope of this line was equated to $-E_a/R$ where E_a equals activation energy and R is the gas constant ($r^2 = .995$). The activation energy was calculated and found to equal 10.2 kJ mol^{-1} .

Effects of Organic and Inorganic Ions. Oxalate added to the reaction mixture at 10.0 mM tripled the amount of Fe released after a 30 minute illumination period (Fig. 10). Succinate and fumarate had no significant effect at $p = .05$. The relative order of organic acid enhancement of photoreduction at pH 6.0 was: oxalic > tartaric > citric > fumaric = succinic = control. The presence of cinnamic acid at 1.0 mM did not significantly influence the reaction rate but 1.0 mM caffeic acid increased photorelease by approximately 130% over the control (Fig. 11). About 60% of this increase may be attributed to the reducing capacity of caffeic acid in darkness (Fig. 12), where the amount of Fe released was found to be constant over a time interval of 30 minutes.

Phosphate was found to significantly inhibit the photochemical reaction at concentrations above 10.0 μM (Fig. 13). Citrate concentrations above 25.0 μM were able to alleviate the inhibitory effect of 1.0 mM phosphate. Copper and manganese (25.0 μM) did not exhibit any observable influences on the reaction rate (Fig. 14), while MES buffer (2-[N-morpholino]ethanesulfonic acid) significantly elevated the mobilization rate.

Iron Mobilization by Light Transmitted Through Leaves

The assay used to demonstrate the ability of light transmitted through leaves to activate Fe release from ferritin gave positive results. The results confirmed those obtained by Olsen and Brown (1981) which indicated that light transmitted through leaves was able to induce significant reduction of Fe. Under the current conditions, light was able to induce mobilization of 75.3 $\mu\text{mol Fe(II)}$

$\text{hr}^{-1} \text{ m}^{-2}$ after passage through a Coleus x hybrid leaf. This value indicates a 50% reduction in the Fe mobilizing activity of light when compared to a no-leaf control.

Discussion

Phytoferritin is considered a storage form of Fe located within the plastids of plants. It is frequently found in Fe replete tissues with an impaired or non-functioning photosynthetic apparatus (Seckback, 1982). The bulk of the active Fe within plants is concentrated in the chloroplast where it is utilized in important reactions and functional roles associated with photosynthesis. Current research suggests that formation and proper function of the photosynthetic apparatus is dependent on light mediated bioavailability of Fe (Olsen and Brown, 1981; Bennett et al., 1982; Jolley et al., 1987; Pushnik et al., 1987). The regulation of Fe availability within cells by light may include its ability to mobilize ferritin Fe. In this study, attention was focused on the light mediated release of Fe from ferritin and the influence of organic and inorganic ions on this release.

Experimental results indicate that artificial light at about 10% the energy of full sunlight has the capacity to induce significant mobilization of ferritin Fe. In general, results obtained here were similar to those obtained in other in vitro studies utilizing non-ferritin bound Fe (Olsen and Brown, 1981; Bennett et al.,

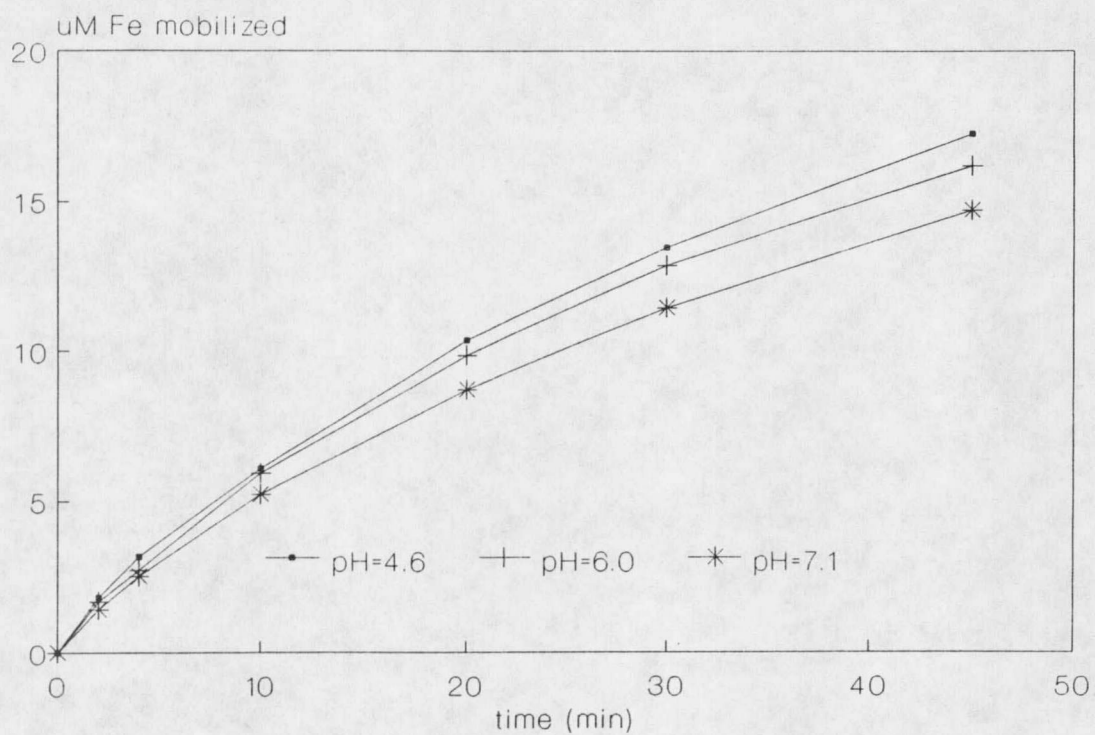


Figure 8. The effect of pH on light activated Fe release from ferritin. All treatments were significantly different at the $p = 0.05$ significance level.

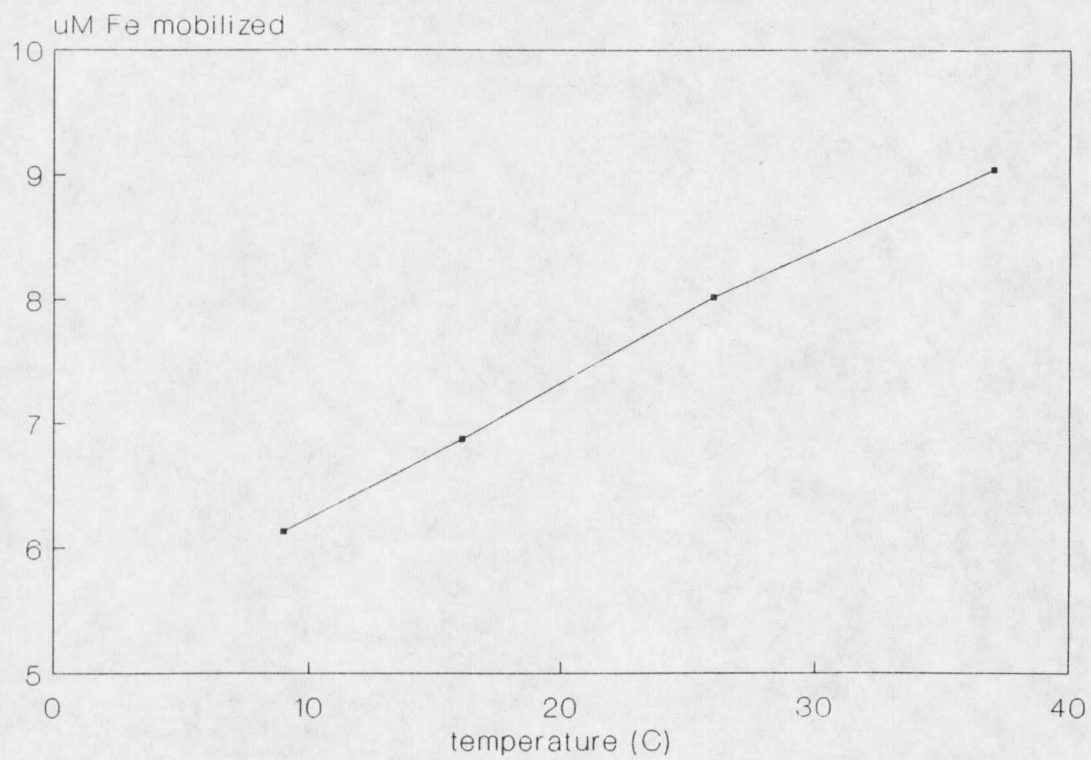


Figure 9. The effect of temperature on photo-induced release of Fe from ferritin. The response to temperature was linear ($r^2 = .99$).

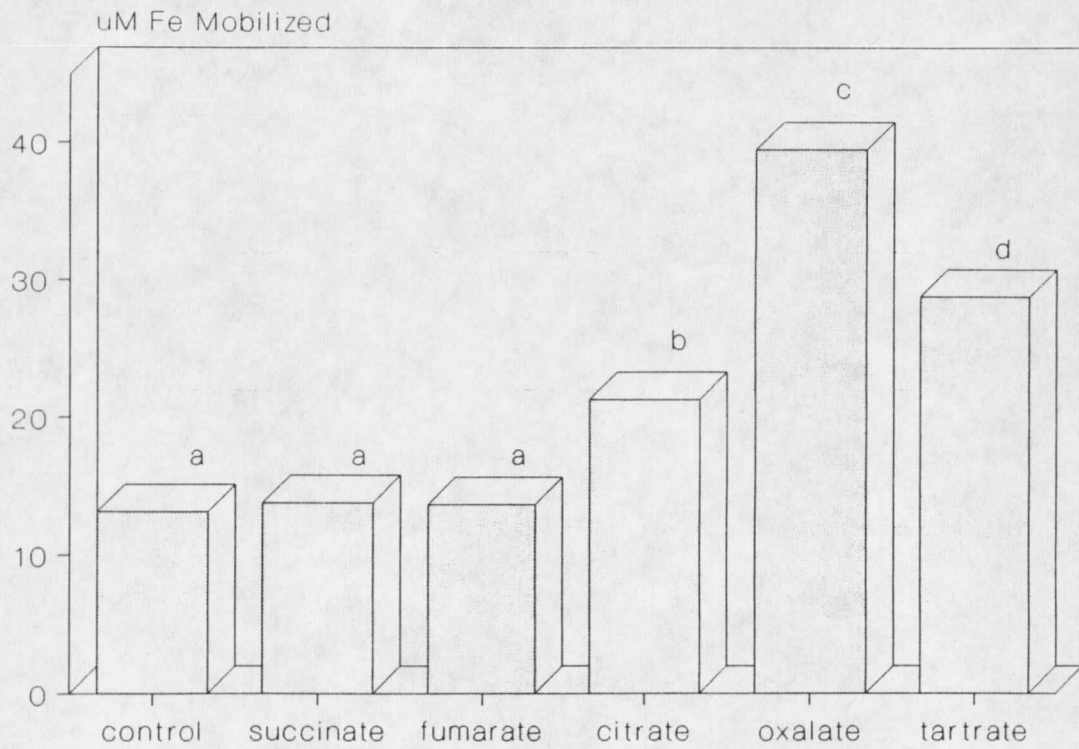


Figure 10. The effects of various organic acids (10.0 mM) on light activated Fe mobilization from ferritin. Letters indicate LSD significant differences at the $p = 0.01$ level.

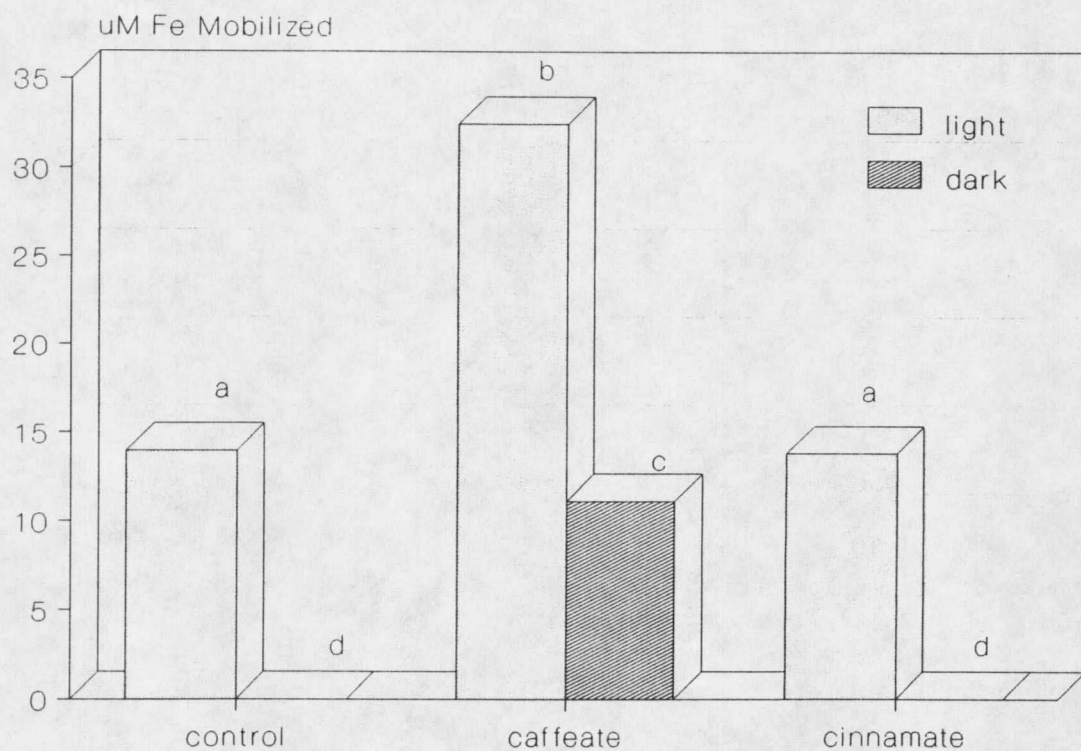


Figure 11. The effect of caffeate and cinnamate on Fe mobilization from ferritin. No Fe release was detected in dark control or dark cinnamate treatments. Letters indicate LSD significant differences at the $p = 0.01$ level.

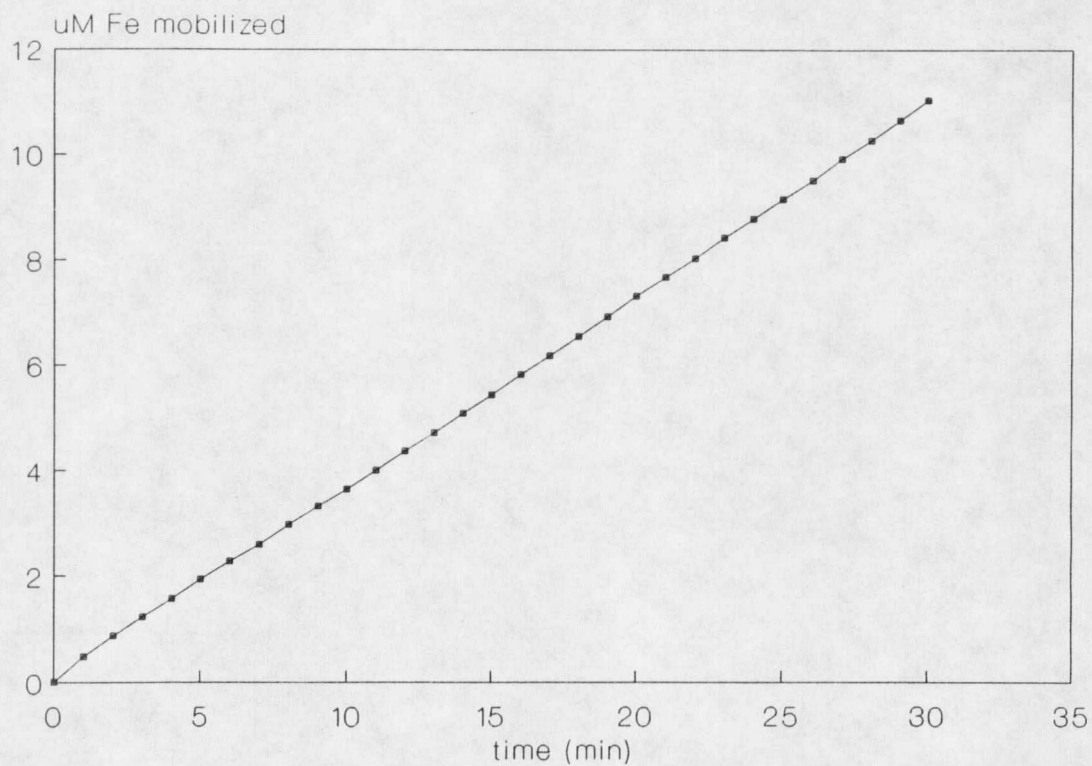


Figure 12. The ability of caffeate to mobilize ferritin Fe in the dark was constant over a time interval of 30 minutes ($r^2 = .99$).

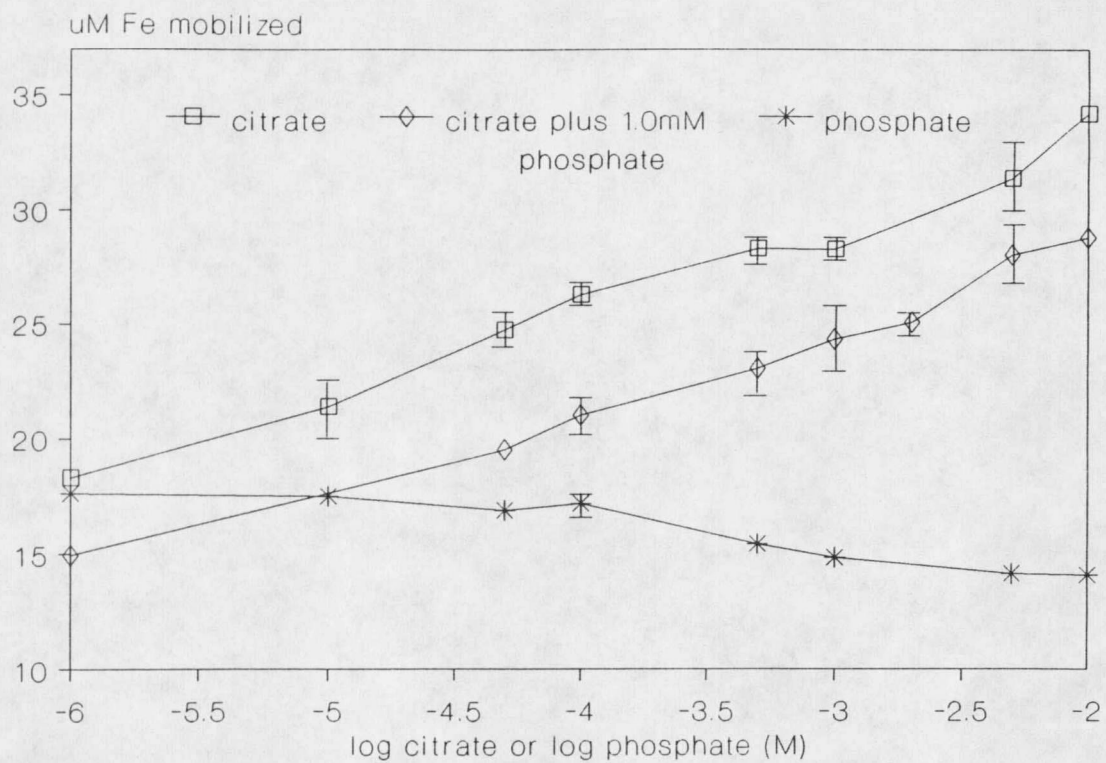


Figure 13. The effects of various concentrations of citrate and phosphate on light activated mobilization of Fe from ferritin after an illumination interval of 45 minutes (mean $\pm$ standard deviation).

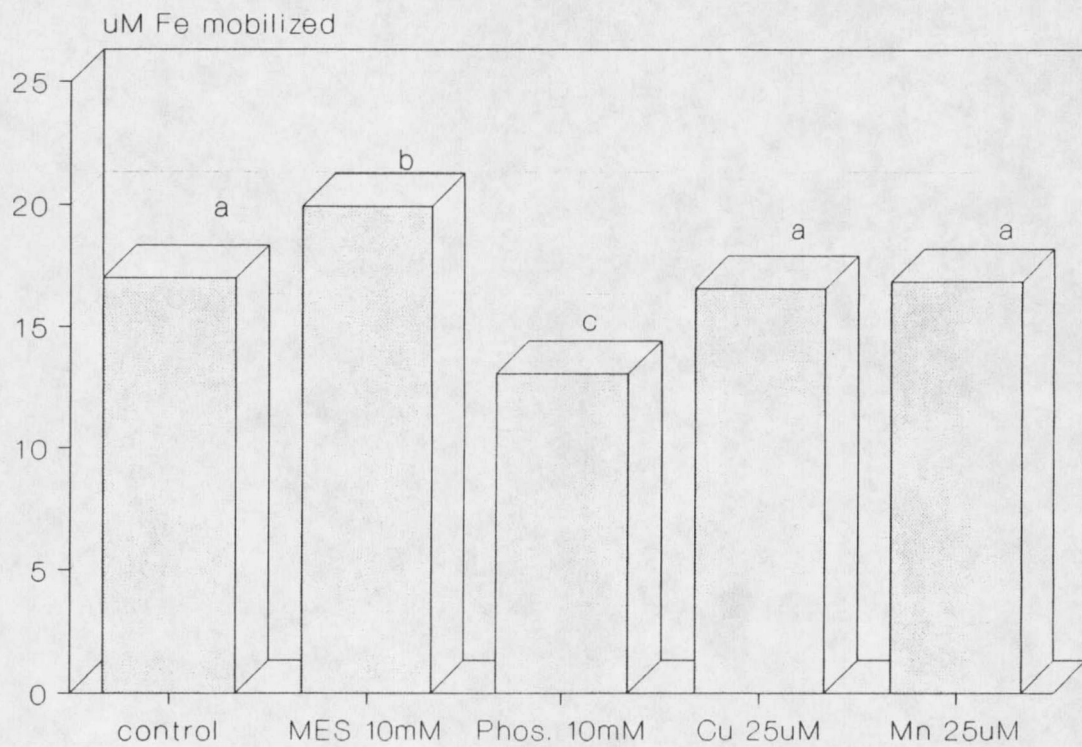
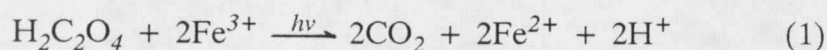


Figure 14. The comparative effects of MES buffer and various inorganics on light activated Fe mobilization from ferritin. Letters indicate LSD significant differences at the $p = 0.01$ level.

1982; Waite and Morel, 1984). These studies involved the effect of UV radiation and other factors on reduction of dissolved and mineral bound Fe(III). The most notable differences between the ferritin and non-ferritin Fe systems is related to the magnitude of inhibition caused by inorganic ions. Orthophosphate and copper ions proved to be potent inhibitors of Fe(III) reduction in the non-ferritin systems. In the current work, ferritin with 25 μ M copper showed no apparent inhibitory effect while 10.0 mM orthophosphate decreased photoreduction rate to about 80% of the control. The proteinaceous ferritin shell may be responsible for the observed differences of inhibitor effectiveness through: 1) its catalytic role of regulating Fe transport to and from the central core (Bryce and Crichton, 1973) and 2) photooxidative degradation of the subunits coupled to reduction of Fe in the mineral core.

Organic acid enhancement of UV induced Fe reduction has been substantiated in many studies (Olsen and Brown, 1981; Bennett et al., 1982; Krizek et al, 1982; Waite and Morel, 1984). The mechanism is thought to proceed in two steps: 1) chelation of Fe(III) by the organic acid and 2) Fe(III) reduction to Fe(II) through coupling with oxidative decarboxylation of the organic acid in the presence of light (Frahn, 1958). The relative amount of Fe^{2+} and Fe^{3+} complexed by each organic acid at pH = 6 was calculated with the equilibrium computer program GEOCHEM (Sposito and Mattigod, 1979) as modified by Parker et al. (1987). Citrate demonstrated the highest affinity for

both Fe^{2+} and Fe^{3+} ions. In contrast, the experimental results indicated that oxalate was most effective in increasing the rate of light-induced Fe mobilization. This suggests that a mechanism other than complexation was the rate limiting step for the reaction. An alternative mechanism would be the Fe-organic acid redox coupled reaction (Frahn, 1958). For example, the reaction with Fe(III) and oxalate:



would yield Fe(II).

The positive effect of caffeic acid on ferritin Fe release with and without irradiation is shown in Figure 11. The mechanism of Fe reduction by caffeate in the dark proceeds by oxidation of the hydroxyl substituents on the benzene ring forming caffeoyl-o-quinone as summarized in the reaction sequence shown in Figure 15 (Olsen et al., 1982).

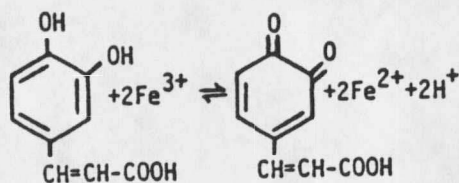


Figure 15. Oxidation of caffeic acid to caffeoyl-o-quinone coupled to reduction of Fe(III) to Fe(II).

Cinnamate, in contrast with caffeate, has no hydroxyl substituents on the benzene ring and thus, demonstrates no ability to mobilize Fe from ferritin in the dark. Also, the presence of 1.0 mM cinnamate did not influence the rate of Fe mobilization by light. Caffeate's ability to enhance light induced Fe mobilization above the control was greater in magnitude than its ability to mobilize Fe in the dark. Since cinnamate exhibited no enhancement, the additional amount of Fe released by caffeate was attributed to an increase in the rate of reaction 2 in the presence of light.

Bienfait and Van Den Briel (1980) have suggested that ascorbate, which may be present at micromolar levels in the chloroplasts, is the primary reductant responsible for Fe removal from phytoferritin. This mechanism of Fe mobilization is now considered to occur via formation of the superoxide ion (Boyer and McCleary, 1987). However, the high levels of superoxide dismutase present within cells may deem this process to be nonsignificant.

This study provides evidence that light itself can directly mediate significant release of iron from ferritin. It is known that light plays an essential role in formation of the photosynthetic apparatus (Miller et al., 1982). Thus, the ability of light to mobilize significant amounts of iron from ferritin would provide a mechanism for coupling chloroplast iron demand with Fe release from ferritin. This hypothesis is supported by evidence of low ferritin Fe levels in leaves of healthy plants grown under illumination. The observations in this paper also

suggest that compounds known to be present in plants may either enhance or inhibit the rate of release and thereby contribute to the control of bioavailable Fe.

CHAPTER 4

CHARACTERIZATION OF REGULATED REDOX PROCESSES
IN TOMATO ROOTS

Specific details of the regulatory mechanisms involved in Fe uptake by plants are still largely unknown. To date, two types of inducible uptake systems have been proposed. The two systems, named Strategy I and Strategy II, are activated by Fe deficiency and cause significant elevation in the rate of Fe uptake by roots. (Marshner et al., 1986). The Strategy II system, utilized by graminaceous species, involves production, excretion and uptake of siderophore type compounds (Mino et al., 1983). These phytosiderophores possess high stability constants for ferric Fe and can greatly elevate plant available Fe in the rhizosphere.

Plants possessing the Strategy I system include all dicotyledonous and monocotyledonous species with the exception of the grasses. The Strategy I system involves at least two physiological responses: 1) enhanced rhizosphere acidification and 2) a decreased redox potential at the rhizoplane. The responses are correlated to transfer cell formation in a swollen subapical zone of the roots (Landsburg, 1986). Acidification of the rhizosphere is thought to involve activation of a membrane bound ATPase and enhanced excretion of

organic and phenolic acids (Marshner et al., 1986; De Vos et al., 1986; Olsen et al., 1981).

The mechanism of ferric reduction has been subject to some controversy. Excreted phenolic acids can act as electron donors by forming a redox couple with reduction of ferric Fe (Olsen et al., 1981; Landsburg, 1986). However, kinetic studies and studies involving membrane disruption suggest that exuded reductants are not the primary source of electrons for reduction of Fe(III) (Romheld and Marshner, 1983; Barrett-Lennard et al., 1983). Those studies provide evidence for another iron stress induced redox mechanism, namely a plasma membrane (P.M.)-bound reductase. The relative significance of the excreted reductant versus the P.M. bound reductase in "iron stress response" has yet to be determined.

In this investigation, nonspecific enzyme denaturizing treatments and compounds known to disrupt phenolic acid biosynthesis were used to further elucidate the significance of the two inducible reductive mechanisms.

Materials and Methods

Plant Growth

Seeds of Marglobe and Ace VF tomato varieties were purchased from W. Atlee Burpee Co. (Warminster, PA). The seeds were surface sterilized for three minutes in 0.5% sodium hypochlorite and germinated on stainless steel screens covered with moist cheesecloth. The tomato seedlings were transferred to

opaque 10L polyethylene tubs (24 seedlings per tub) containing 8L of standard nutrient solution plus 36.0 μM FeEDTA. The standard nutrient solution was composed of 1.90 mM $\text{Ca}(\text{NO}_3)_2$, 0.47 mM $\text{Mg}(\text{NO}_3)_2$, 0.24 mM KCl, 0.61 mM KNO_3 , 0.60 mM K_2HPO_4 , 0.32 mM $(\text{NH}_4)_2\text{SO}_4$, 7.4 μM MnCl_2 , 41.3 μM H_3BO_4 , 1.9 μM ZnSO_4 , 0.5 μM CuSO_4 , and 0.4 μM Na_2MoO_4 with a pH of approximately 7.0. All nutrient solutions in the experiments were continuously aerated and were changed weekly. After 21 additional days of growth, the plants were subjected to Fe limitation by ceasing supplementation of Fe to nutrient solutions.

Alteration of Phenolic Metabolism

One week after initiation of Fe limitation, individual plants were transferred to 1L bottles containing the standard nutrient solution plus various compounds aimed at altering phenolic metabolism. The potent inhibitor of phenylalanine ammonia lyase (PAL), p-fluorophenylalanine (Kudakasseril and Minocha, 1984), was utilized (0.2 mM) in conjunction with cinnamic acid (0.3 mM), a product of the PAL catalyzed reaction. Glyphosate, which is known to perturb phenolic metabolism (Duke et al., 1980) was also incorporated as a treatment (0.3 mM). After 36 hours, root tissues were assayed for content of o-dihydroxyphenols and reduction activity.

O-Dihydroxyphenols

Quantitative analysis of exuded phenolic compounds is hindered by rapid oxidation and polymerization in the free space and at the root surface.

Therefore, the internal concentration of phenolic compounds was assayed. The method utilized for analysis of total O-dihydroxyphenols was modified from Collier (1979). Root material (0.4g fresh weight) was added to 3.0 ml 95% ethanol and homogenized for 10 minutes (Virtis 23 homogenizer). The homogenate was filtered (0.45 μm) and loaded on 4.0 x 0.8 cm acid alumina columns (activity grade 1). The columns were rinsed with two bed volumes of 95% ethanol followed by diazotization of the O-dihydroxyphenols with two bed volumes of a 5% NaNO_2 plus 5% acetic acid solution. The diazotized compounds were eluted with two bed volumes of 5.0 N NaOH and assayed spectrophotometrically at 520 nm. Caffeic acid was used as the standard. The presence of cinnamate did not demonstrate any ability to affect measurements when using this method.

Fe Reducing Activity

Root Fe reducing activity was assayed in a manner modified from Olsen and Brown (1980). Excised terminal roots (0.4 g fresh weight) were placed in 50.0 ml of reduction assay solution. The solution consisted of 100 μM FeCl_3 and 400 μM ferrozine (3-(pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) adjusted to a pH of 4.0 with HCl. After 21 hours, the absorbance of the filtered assay solution was measured at 562 nm.

Protein Denaturation

Several harsh treatments aimed at denaturing root plasma membrane-bound enzymes were performed. Excised roots (0.5 g fresh weight) of Fe stressed

plants were subjected to various treatments and immediately assayed for Fe reducing activity.

Results

Alteration of Phenolic Metabolism

The addition of p-fluorophenylalanine to nutrient cultures decreased root reduction capacity to about one-third the capacity expressed by the control (Fig. 16). Although, the internal concentration of o-dihydroxyphenols was also reduced, the decrease was not significant at the 5% level. The addition of cinnamate, a phenolic precursor, to the p-fluorophenylalanine treatment resulted in elevation of both o-dihydroxyphenol content and root reduction activity. However, reduction activity was still only about 50% that of control. The glyphosate treatment also induced elevation of total o-dihydroxyphenols in root tissue but reduction activity was inhibited to the level of the p-fluorophenylalanine treatment. All of the treatments utilized to manipulate phenolic metabolism resulted in a significant decrease of reduction activity relative to control.

The measured concentration of o-dihydroxyphenols in roots was always greater than the total amount of Fe(II) reduced after the 21 hour reduction assay period. Thus, o-dihydroxyphenols could have theoretically been responsible for all of the reduction observed.

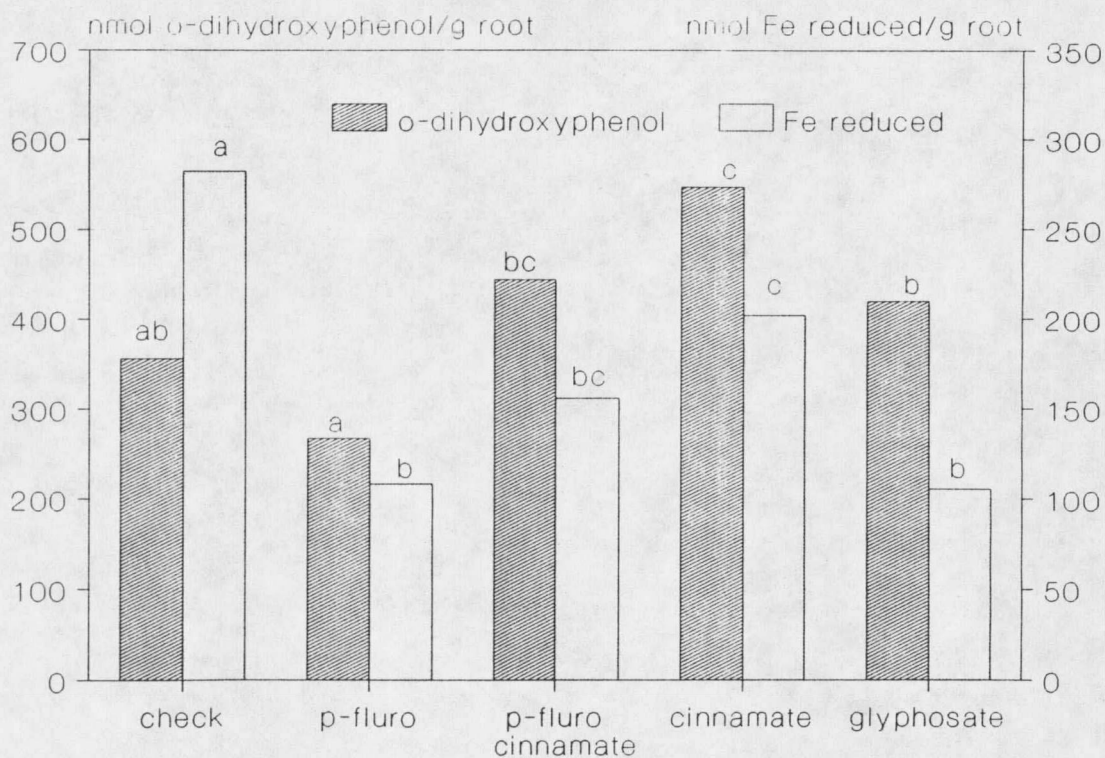


Figure 16. Comparison of total o-dihydroxyphenol content and reducing activity of Fe stressed tomato roots pretreated with various compounds (0.2 mM p-fluorophenylalanine, 0.3 mM cinnamate and 0.3 mM glyphosate) known to influence phenolic metabolism. Letters are LSD significant differences at the $p = 0.05$ level.

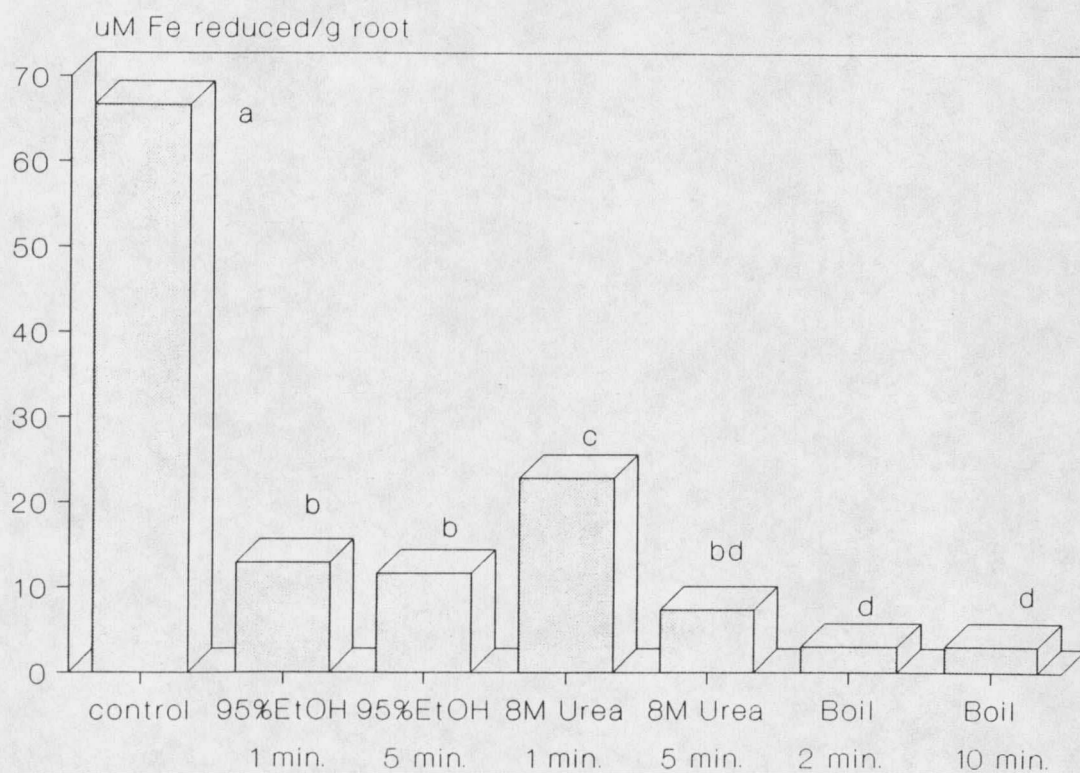


Figure 17. Reducing activity of excised Fe stressed tomato roots subjected to various protein denaturing treatments. Letters are LSD significant differences at the $p = 0.05$ level.

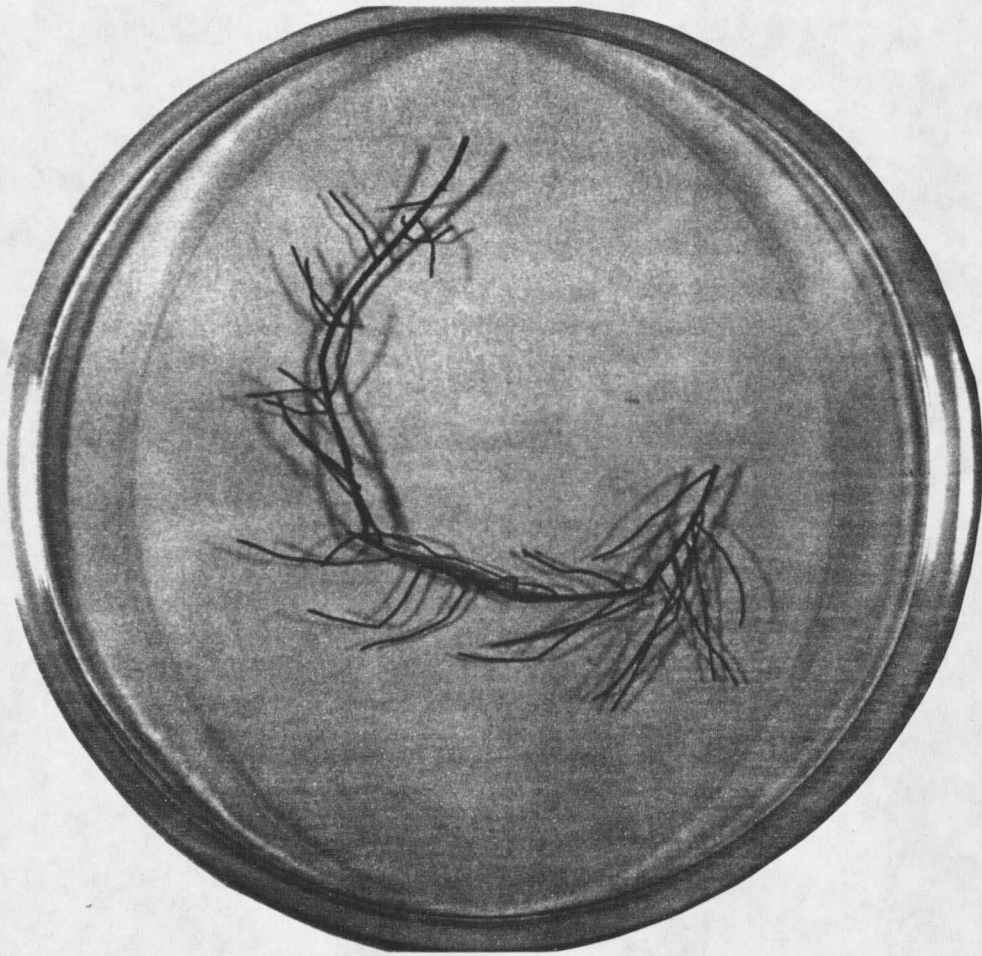


Figure 18. Ferrozine-Fe staining of tomato roots treated with 95% ethanol to denature plasmamembrane and cell wall enzymes. The dark staining demonstrates root reducing activity (unstained roots are white).

Protein Denaturation

The results of the protein denaturation treatments are displayed in Figure 17. Although root tissues were subjected to severe treatments, some reducing activity was retained. It is unlikely that this activity was due to a membrane bound reductase since enzymes at the root surface should have been inactivated by the various treatments. Staining of treated roots by the tris-ferrozine-ferrous complex (indication of Fe reduction) demonstrated that reduction had occurred along the root surface (Fig. 18).

Discussion

The roots of dicotyledonous plants possess an inducible reduction system utilized for promotion of Fe uptake. Two types of mechanisms are thought to exist: 1) exudation of reductants, primarily phenolics (Olsen et al., 1981) and 2) NADPH driven enzymatic reduction (Sijmons et al., 1984). The relative significance of these two systems in "iron stress response" has yet to be clarified.

In this current work, the secreted reductant hypothesis is supported by the observation that cinnamate added to p-fluorophenylalanine treatments caused enhanced Fe reduction. In addition, the presence of root reducing activity after severe protein denaturizing treatments indicated that significant concentrations of compounds capable of reducing Fe(III) were present within plants.

Olsen and his coworkers (1981) have observed that acetic acid treated roots demonstrated increased efflux of compounds capable of reducing Fe(III). More

recent studies by Barrett-Lennard et al. (1983) also showed that the acetic acid treatment caused rapid decreases in rates of Fe(III) reduction by roots of peanuts and sunflowers. Their observations suggest that membrane integrity is an important component in maintaining the high rates of Fe reduction observed in Fe stressed roots.

In the present work, evidence against the secreted reductant hypothesis stems from the observation that treatments which elevated the internal o-dihydroxyphenol content also caused significant inhibition of root reduction activity. In theory, rate of phenolic exudation would be regulated by two factors: 1) internal phenolic concentration and 2) membrane permeability. Since membrane permeability is known to be a function of pH and the reduction assay solution pH was approximately 4.0, root "leakiness" should have been fairly constant between treatments. Therefore, if exuded phenolics alone were of primary importance in reduction, greater internal concentration should have resulted in greater reduction at the root surface. However, it can be argued that internal phenolic concentration does not necessarily correlate with rate of secreted phenolics. It is possible that the phenolic content of vacuoles could be elevated without an increase in the rate of secreted phenolics.

The addition of the various phenolic metabolism altering compounds to nutrient solutions apparently hindered some aspect of Fe reduction. The most likely aspect affected would be enzymatic activity. However, these observations do not rule out the importance of phenolics in reduction. Plasma membrane

bound enzymes or enzymes present in the cell wall may be crucial to phenolic reduction of Fe(III) (Olsen et al., 1981). An enzymatic mediated reaction would help reconcile the observation (Romheld and Marshner, 1983) that various chelate binding strengths significantly affect in vitro reduction rates by phenolics while reduction rates by roots are not affected. After enzyme catalyzed oxidation, the quinone products could still function as ligands for increased dissolution of Fe(III) minerals in the soil and subsequent transport to roots.

Although the results of these experiments do not provide unequivocal evidence to verify either the secreted reductant or the membrane bound reductase mechanisms, they suggest that both processes are involved in reduction at the root.

CHAPTER 5

SUMMARY

Iron is an element required for many critical electron transfer reactions in cells. In plants, iron is concentrated within the chloroplasts where it is primarily used for synthesis and function of the photosynthetic apparatus. Ferritin, a storage form of iron located exclusively in plastids, is an important buffer form of iron employed under conditions where iron uptake is not closely coupled with its utilization. The current study (Chapter 3) provides evidence demonstrating that light can activate significant release of iron from ferritin. It is known that light plays an essential role in formation of the photosynthetic apparatus (Miller et al., 1982). Thus, the ability of light to mobilize significant amounts of iron from ferritin would provide a mechanism for coupling chloroplast iron demand with Fe release from ferritin. This hypothesis is supported by evidence of low ferritin Fe levels in leaves of healthy plants grown under illumination. The observations in this paper also suggest that compounds known to be present in plants may either enhance or inhibit the rate of release and thereby contribute to the control of bioavailable Fe.

Under conditions of iron deficiency, iron uptake from the environment and release from ferritin reserves falls below metabolic demand. Consequently,

important biochemical reactions are disturbed which likely include reactions crucial to defense against pathogens. This condition may result in greater susceptibility to disease. Supplementation of iron may therefore be a practical agronomic tool for combating diseases in crops grown on iron limiting soils.

Chapter 2 of this thesis shows that Fe deficiency of resistant tomato varieties increases their susceptibility to attack by V. dahliae. However, there is only speculation as to the specific mechanisms involved in this interaction. The role of iron deprivation from tomato by V. dahliae does not seem to be an important aspect of virulence in this system.

Plants respond to iron deficiency in a variety of ways. In an attempt to alleviate the stress, dicots activate mechanisms which decrease the redox potential at the rhizoplane. To date, the specifics of these Fe reduction mechanisms are not well understood. Although the results presented in Chapter 4 do not provide unequivocal evidence to verify either the secreted reductant or the membrane bound reductase hypotheses, they suggest that both processes are involved in reduction at the root.

REFERENCES CITED

1. Barash, I., R. Zion, J. Krijun and A. Nachmeas. 1988. Effect of iron status on Verticillium wilt disease and on *in vitro* production of siderophores by Verticillium dahliae. J. Plant Nut. 11:893-905.
2. Barrett-Lennard, E.G., H. Marshner and V. Romheld. 1983. Mechanism of short term Fe(III) reduction by roots. Plant Physiol. 73:893-898.
3. Becker, J.O., R.W. Hedges and E. Messens. 1985. Inhibitory effect of pseudobactin on the uptake of iron by higher plants. Appl. Envir. Microbio. 49:1090-1093.
4. Beckman, C.H. 1987. Genetic variation in the host and pathogen. The Nature of Wilt Diseases of Plants, Ed. by Beckman, C.H., pp 103-116. American Phytopathological Society Press.
5. Bennett, J.H., E.H. Lee, D.T. Krizek, R.A. Olsen and J.C. Brown. 1982. Photochemical reduction of iron. II. Plant related factors. J. Plant Nutr. 5:335-344.
6. Bienfait, H.F. 1985. Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. J. Bioenerg. Biomem. 17:73-83.
7. Bienfait, H.F. and M. Van Den Briel. 1980. Rapid mobilization of ferritin iron by ascorbate in the presence of oxygen. Biochim. Biol. Acta. 631:507-510.
8. Boyer, R.F. and C.J. McCleary. 1987. Superoxide ion as a primary reductant in ascorbate-mediated ferritin iron release. Free Rad. Biol. Med. 3:389-395.
9. Boyer, R.F., H.M. Clark and A.P. LaRoche. 1988. Reduction and release of ferritin iron by plant phenolics. J. Inorg. Biochem. 32:171-181.
10. Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

11. Brown, A.E. and T.R. Swinburne. 1982. Iron chelating agents and lesion development by Botrytis cinerea on leaves of vicia faba. *Physiol. Pl. Path.* 21:13-21.
12. Bryce, C.F.A. and R.R. Crichton. 1973. The catalytic activity of horse spleen apoferritin. *Biochem. J.* 133:301-309.
13. Chaney, R.L., J.C. Brown and L.O. Tiffin. 1972. Obligatory reduction of ferric chelates in iron uptake by soybeans. *Plant Physiol.* 50:208-213.
14. Clark, R.B. 1982. Iron deficiency in plants grown in the Great Plains of the U.S. *J. Plant Nut.* 5:251-268.
15. Collier, G.F., V.C. Huntington and E.F. Cox. 1979. A possible role for chlorogenic acid in calcium-related disorders of vegetable crops with particular reference to lettuce tipburn. *Commun. Soil Sci. and Plant Anal.* 10:481-490.
16. Craig, A.S. and K.I. Williamson. 1969. Phytoferritin and viral infection. *Virology.* 39:616-617.
17. Crichton, R.R. and M. Charlotheaux-Wauters. 1987. Iron transport and storage. *Eur. J. Biochem.* 164:485-506.
18. De Vos, R.C., H.J. Lubberding and H.F. Bienfait. 1986. Rhizosphere acidification as a response to iron deficiency in bean plants. *Plant Physiol.* 81:842-846.
19. Dixon, G.R. and G.F. Pegg. 1969. Hyphal lysis and tylose formation in tomato cultivars infected by Verticillium albo-atrum. *Trans. Brit. Myco. Soc.* 53:109-118.
20. Duke, S.O., R.E. Hoagland and C.D. Elmore. 1980. Effects of glyphosate on metabolism of phenolic compounds. *Plant Physiol.* 65:17-21.
21. Expert, D. and A. Toussaint. 1985. Bacteriocin-resistant mutants of Erwinia chrysanthemi: Possible involvement of iron acquisition in phytopathogenicity. *J. Bact.* 163:221-227.
22. Frahn, J.L. 1958. The photochemical decomposition of the citrate-ferric-iron complex: A study of reaction products by paper ionophoresis. *Aust. J. Chem.* 11:399-405.

23. Funk, F., J. Lenders, R.R. Crichton and W. Schneider. 1985. Reductive mobilization of ferritin iron. *Eur. J. Biochem.* 152:167-172.
24. Granick, S. 1946. Ferritin: Its properties and significance for iron metabolism. *Chem. Rev.* 38:379-403.
25. Guerra, C. and A.J. Anderson. 1985. The effect of iron and boron amendments on infection of bean by Fusarium solani. *Phytopathology*. 75:989-991.
26. Hether, N.W., R.A. Olsen and L.L. Jackson. 1984. Chemical identification of iron reductants exuded by plant roots. *J. Plant Nut.* 7:667-676.
27. Hyde, B.B., A.J. Hodge, A. Khan and M.L. Birnsteil. 1963. Studies of phytoferritin. I. Identification and characterization. *J. Ultrastr. Res.* 9:248-258.
28. Inskeep, W.P. and P.R. Bloom. 1985. Extinction coefficients of chlorophyll a and b in N,N-Dimethylfomamide and 80% acetone. *Plant Physiol.* 77:483-485.
29. Inskeep, W.P. and S.D. Comfort. 1986. Thermodynamic predictions for the effects of root exudates on metal speciation in the rhizosphere. *J. Plant Nut.* 9:567-586.
30. Jolley, V.D., J.C. Brown, J.C. Rushnik and G.W. Miller. 1987. Influences of ultraviolet-blue light radiation on the growth of cotton. I. Effect on iron nutrition and iron stress response. *J. Plant Nutr.* 10:333-351.
31. Kloepper, J.W., J. Leong, M. Teintze and M.N. Schroth. 1980. *Pseudomonas siderophores*: A mechanism explaining disease-suppressive soils. *Cur. Microbio.* 4:317-320.
32. Krikun, J. and Z.R. Frank. 1975. Effect of sequestrene on the reaction to Verticillium dahliae of peanuts growing in calcareous loess soil. *Phytoparasitica*, 3:77-78.
33. Krizek, D.T., J.H. Bennett, J.C. Brown, T. Zaharieva and K.H. Norris. 1982. Photochemical reduction of iron. I. Light reactions. *J. Plant Nutr.* 5:323-333.

34. Kudakasseril, G.J. and S.C. Minocha. 1984. Regulation of L-Phenylalanine Ammonia-lyase in germinating lettuce seeds: Effect of substrate analogues and phenylpropanoid compounds. *Z. Pflanz. Bd.* 114:164-171.
35. Landsberg, E.C. 1986. Function of rhizodermal transfer cells in the Fe stress response mechanism of Capsicum annuum L. *Plant Physiol.* 82:511-517.
36. Lindsay, W.L. and A.P. Schwab. 1982. The chemistry of iron in soils and its availability to plants. *J. Plant Nut.* 5:812-840.
37. Marshner, H., V. Romheld and M. Kissel. 1986. Different strategies in higher plants in mobilization and uptake of iron. *J. Plant Nut.* 9:695-713.
38. Miller, G.W., A. Denny, J.C. Pushnik and M.H. Yu. 1982. The formation of delta-aminolevulinate, a precursor to chlorophyll, in barley and the role of iron. *J. Plant Nutr.* 5:289-300.
39. Mino, Y., T. Ishida, N. Ota, M. Inoue, K. Nomoto, T. Takemoto, H. Tanaka and Y. Sugiura. 1983. Mugineic acid-iron (III) complex and its structurally analogous cobalt (III) complex: Characterization and implication for absorption and transport of iron in gramineous plants. *J. Am. Chem. Soc.* 105:4671-4676.
40. Nachmias, A., V. Buchner and J. Krikun. 1982. Protein-lipopolysaccharide complexes produced by pathogenic and nonpathogenic strains of Verticillium dahliae Kleb. from potato. *Physiol. Plant Path.* 20:213-221.
41. Neilands, J.B. and S.A. Leong. 1986. Siderophores in relation to plant growth and disease. *Ann. Rev. Plant Physiol.* 37:187-208.
42. Neilands, J.B. and K. Nakamura. 1985. Regulation of iron assimilation in microorganisms. *Nutr. Rev.* 43:193-197.
43. Olsen, R.A. and J.C. Brown. 1980. Factors related to iron uptake by dicotyledonous and monocotyledonous plants. II. The reduction of Fe(III) as influenced by roots and inhibitors. *J. Plant Nut.* 2:647-660.
44. Olsen, R.A. and J.C. Brown. 1981. Light-induced reduction of Fe³⁺ as related to causes of chlorosis in cotton. *J. Plant Nutr.* 3:767-787.

45. Olsen, R.A., J.H. Bennett, D. Blume and J.C. Brown. 1981. Chemical aspects of the Fe stress response mechanism in tomatoes. *J. Plant Nut.* 3:905-921.
46. Olsen, R.A., J.C. Brown, J.H. Bennett and D. Blume. 1982. Reduction of Fe^{3+} as it relates to Fe chlorosis. *J. Plant Nutr.* 5:433-445.
47. Parker, D.R., L.W. Zelazny and T.B. Kinraide. 1987. Improvements to the program GEOCHEM. *Soil Sci. Soc. Am. J.* 51:488-491.
48. Peat, A. and G.H. Banbury. 1968. Occurrence of ferritin-like particles in a fungus. *Planta.* 79:268-270.
49. Pegg, G.F. 1974. *Verticillium* diseases. *Rev. Plant Path.* 53:157-182.
50. Pegg, G.F. 1981. Biochemistry and physiology of pathogenesis. In *Fungal Wilt Disease of Plants*, Ed. by Mace, M.E., A.A. Bell and C.H. Beckman, pp 193-253. Academic Press, New York.
51. Pushnik, J.C., G.W. Miller and J.H. Manwaring. 1984. The role of iron in higher plant chlorophyll biosynthesis, maintenance and chloroplast biogenesis. *J. Plant Nut.* 7:733-758.
52. Pushnik, J.C., G.W. Miller, V.D. Jolley, J.C. Brown, T.D. Davis and A.M. Barnes. 1987. Influence of ultraviolet-blue light radiation on the growth of cotton. II. Photosynthesis, leaf anatomy, and iron reduction. *J. Plant Nutr.* 10:2283-2297.
53. Reuveni, R. and J.F. Ferreira. 1985. The relationship between peroxidase activity and the resistance of tomatoes (*Lycopersicum esculentum*) to *Verticillium dahliae*. *Phytopath. Z.* 112:193-197.
54. Robb, J., D.A. Powell and P.F.S. Street. 1987. Time course of wall-coating secretion in *Verticillium*-infected tomatoes. *Physiol. and Mole. Path.* 31:217-226.
55. Romheld, V. and H. Marshner. 1983. Mechanism of iron uptake by peanut plants. *Plant Physiol.* 71:949-954.
56. Romheld, V. and H. Marshner. 1983. Mechanism of iron uptake by peanut plants. Fe(III) reduction, chelate splitting, and release of phenolics. *Plant Physiol.* 71:949-954.

57. Schroth, M.N. and J.G. Hancock. 1982. Disease-suppressive soil and root-colonizing bacteria. *Science*. 216:1376-1381.
58. Schwyn, C. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160:47-56.
59. Seckbach, J. 1968. Studies on the deposition of plant ferritin as influenced by supply to iron deficient beans. *J. Ultrastr. Res.* 22:413-423.
60. Seckbeck, J. 1982. Ferriting out the secrets of plant ferritin - A review. *J. Plant Nutr.* 5:369-394.
61. Sijmons, P.C., P.E. Kolattukudy and H.F. Bienfait. 1985. Iron deficiency decreases suberization in bean roots through a decrease in suberin specific peroxidase activity. *Plant Physiol.* 78:115-120.
62. Sijmons, P.C., W. Van Den Briel and H.F. Bienfait. 1984. Cytosolic NADPH is the electron donor for extracellular Fe(III) reduction in iron deficient bean roots. *Plant Physiol.* 75:219-221.
63. Sinha, A.K. and R.K.S. Wood. 1968. Studies on the nature of resistance in tomato plants to Verticillium albo-atrum. *Ann. Applied Bio.* 62:319-327.
64. Sirivech, S., E. Frieden and E. Osaki. 1974. The release of iron from horse spleen ferritin by reduced flavins. *Biochem J.* 143:311-315.
65. Sposito, G. and S.V. Mattigod. 1979. GEOCHEM: A computer program for the calculation of chemical equilibria in soil solutions and other natural systems. Univ. Calif., Riverside.
66. Steifel, E.I. and G.D. Watt. 1979. Azotobacter cytochrome $b_{557.5}$ is a bacterial ferritin. *Nature*. 279:81-83.
67. Stookey, L.L. 1970. Ferrozine - A new spectrophotometric reagent for iron. *Anal. Chem.* 42:779-781.
68. Sugiura, Y. and K. Nomoto. 1984. Phytosiderophores. *Struct. Bond.* 58:107-135.
69. Tjamos, E.C. and I.M. Smith. 1975. The expression of resistance to Verticillium albo-atrum in monogenically resistant tomato varieties. *Physiol. Plant Path.* 6:215-225.

70. Van Der Mark, F., T. de Lange, and H.F. Bienfait. 1981. The role of ferritin in developing primary bean leaves under various light conditions. *Planta*. 153:338-342.
71. Van Der Mark, F., H.F. Bienfait and H. Van Den Ende. 1983. Variable amounts of transferable ferritin mRNA in bean leaves with various iron contents. *Biochem. Biophys. Res. Commun.* 115:463-469.
72. Van Der Mark, F. and W. Van Den Briel. 1985. Purification and partial characterization of ferritin from normal and iron-loaded leaves of Phaseolus vulgaris. *Plant Sci.* 39:55-60.
73. Visser, S. and M.J. Hattingh. 1980. Criteria for quantal host response of tomato plants to infection by Verticillium dahliae. *Plant Dis.* 64:207-208.
74. Waite, T.D., and F.M.M. Morel. 1984. Photoreductive dissolution of colloidal iron oxide: Effect of citrate. *J. Coll. Interface Sci.* 102:121-137.
75. Winkelmann, G., I. Berner and H. G. Huschka. 1988. Structure-activity of siderphores in fungi. *J. Plant Nut.* 11:883-892

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