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

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 x 10^-3 sec^-1 (pH = 4.6) to 2.1 x 10^-3 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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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, leghemogloben, 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.

$$Fe(OH)_{3(s)} = Fe^{3+} + 3OH^- \qquad (1)$$

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.

$$E_h = E^o + 59\log (Fe^{3+})/(Fe^{2+}) \qquad (2)$$

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 graminiaceae 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 uM 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 $Ca(NO_3)_2$, 0.47 mM $Mg(NO_3)_2$, 0.24 mM KCl, 0.61 mM KNO_3, 0.60 mM K_2HPO_4, 0.32 mM $(NH_4)_2$ 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 $\mu E\ m^{-2}\ s^{-1}$ followed by 8 hours of darkness. The day and night temperatures were 22°C ± 0.7 and 21°C ± 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*2*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 μL) were added to 3.0 ml of the assay solution consisting of 15 mM sodium phosphate buffer, pH 6.0, 1.0 mM H_2O_2, 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 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.

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