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Dependency of Iron Reduction on Development of a Unique Root Morphology in *Ficus benjamina* L.[1]

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ABSTRACT

The activity of the Fe^{3+} reductase of excised adventitious roots of *Ficus benjamina* L., grown in hydroponic culture without iron, was determined by a colorometric assay simplified by the use of a microplate reader. Reductase activity remained the same from pH 4.5 to 6.5 and decreased sharply above pH 6.5. Acetate buffer inhibited reduction. During early stages of root growth, excised roots did not exhibit Fe^{3+} reductase activity. After several weeks and extensive root system development, Fe^{3+} reduction still was not detectable in primary roots, but intermediate and high rates of reduction occurred in lateral and newly formed root clusters, respectively. Clustered roots only developed on plants grown at 0 or very low (<1 micromolar) iron. Microscopic examination revealed the root cluster to be composed of up to 30 lateral roots, usually less than 1 millimeter in diameter and 1 centimeter in length, that were completely covered with root hairs.

Iron deficiency triggers some plants to induce biochemical reactions in their root systems that assist the mobilization of insoluble ferric (Fe^{3+}) iron from alkaline soils; these plants are termed Fe-efficient. Dicots and nongraminaceous monocots typically exhibit enhanced root surface reduction of Fe^{3+} to Fe^{2+} by a plasmalemma-bound reductase, proton secretion, and/or release of soluble reducing or chelating compounds (strategy I), whereas, graminaceous species secrete phytosiderophores (strategy II) (16). *Ficus benjamina* L. greatly acidified the rhizosphere (8), and iron reduction at the root surface was enhanced, while Fe^{3+} reduction from released soluble reductants was minimal (9). Thus, *F. benjamina* showed characteristics of a strategy I Fe-efficient species. It was observed that *F. benjamina* only exhibited these characteristic iron deficiency responses after reaching a certain age and developing a unique morphology of terminal root clusters. The objective of these studies was to investigate Fe^{3+} reductase activity related to the appearance of the unique root morphology of *F. benjamina*.

MATERIALS AND METHODS

Plant Material and Culture

Terminal stem cuttings, 10 to 15 cm long, of *Ficus benjamina* L. were rooted under intermittent mist in washed, coarse sand. Adventitious root formation occurred in about 28 d. Rooted cuttings were washed free of sand, rinsed in 2.0 mM Na_2EDTA to remove any adsorbed Fe, and then inserted into Styrofoam trays and floated on aerated modified Hoagland solution 1 (pH 6.3) without Fe, and containing 1 mM KH_2PO_4, 5 mM KNO_3, 5 mM $CA(NO_3)_2$, 2 mM $MgSO_4$, 11 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.16 μM $(NH4)_6Mo_7O_{24}$, and 46 μM H_3BO_3. All materials coming into contact with plants or nutrient solutions were rinsed with 0.1 N HCl followed by 2.0 mM Na_2EDTA to remove iron contamination. Nutrient solutions were changed weekly. The plants were grown under greenhouse conditions of 30°C day/25°C night (±5°C) and 45 to 85% RH.

Excised Root Fe^{3+} Reductase Assay

Roots, about 2.5 cm long, were excised, rinsed briefly in water, then collected and held for 5 to 10 min in 5 mM Mes (pH 5.5) and 0.5 mM $CaSO_4$. Roots were recut to 2 cm and immersed in 3 to 4.5 mL of Fe^{3+} reductase assay solution containing 5 mM Mes (pH 5.5), 0.5 mM $CaSO_4$, 0.1 mM $Fe^{3+}EDTA$, and 0.3 mM BPDS.[2] For the pH experiment, 5 mM Na acetate at pH 4.3 to 5.5, Na succinate at pH 4.5 to 5.5, Mes at pH 5.0 to 7.0, and Hepes at pH 6.8 to 7.6 were used as buffers. Each assay mixture contained 15 to 40 mg fresh weight of excised roots, usually 5 or 10 root tips or 1 or 2 clusters, depending on size and morphology. Samples were incubated in the dark in a shaking water bath at 23°C. At timed intervals, 0.25 mL of assay solution was removed and measured for appearance of Fe^{3+} BPDS in a MR650 microplate reader (Dynatech Labs Inc, Alexandria, VA) at 490 nm. After the last timed sample, roots were removed and blotted dry, and fresh weight was determined. Two or three replicate samples were used for each treatment, and each experiment was repeated at least twice.

Excised Root Fe^{3+} Reductase Localization

To visualize the discrete areas of excised roots active in Fe^{3+} reduction, excised root sections were embedded in the Fe^{3+} reductase assay solution solidified by the addition of 0.75% (w/v) type II agarose (Sigma Chemical Co., St. Louis, MO). Roots were incubated in the dark to allow for characteristic red color development.

[1] Texas Agricultural Experiment Station article TA25949.

[2] Abbreviation: BPDS, 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid.

Electron Microscopy

Excised roots were fixed in 40% FAA, critical point dried, gold coated, and viewed at 5 or 15 kV with a JSM T330A scanning electron microscope (Jeol Inc., Peabody, MA).

RESULTS

Root Morphology

F. benjamina has been shown to possess the ability to acidify nutrient solutions and exhibit high rates of Fe^{3+} reduction when placed under Fe stress (8, 9). However, newly rooted *F. benjamina* cuttings possessed very low reducing ability. Enhanced reducing capacity of whole root systems appeared only after extensive root growth and when the roots had developed a matted appearance (data not shown). The root system developed at least three morphologies:

Primary Roots

Thick (approximately 1.5 mm diameter) with a distinctive root cap and root hairs several centimeters below that root cap, light in color, and rapidly elongating (Fig. 1A).

Lateral Roots

Thinner roots, usually less than 1 mm in diameter, often arising in close proximity along the primary axis and with well developed root hairs arising within 0.5 cm from the root tip (Fig. 1B).

Clustered Roots

Multibranched roots composed of many (up to 30) short root tips (Fig. 1C), usually less than 1 mm in diameter, arising at the terminal end of a root axis (Fig. 1D) and showing very heavy root hair development (Fig. 1, C and E).

Primary and lateral roots were present at all iron concentrations, while the clustered roots only developed after several weeks in culture under low iron conditions, and after a rather extensive matted root system had developed.

Excised Root Fe^{3+} Reductase Activity

Preliminary studies using an excised root assay for Fe^{3+} reductase activity demonstrated that the primary roots possessed minimal Fe^{3+} reducing ability, the lateral roots were intermediate, and the clustered roots possessed the highest Fe^{3+} reducing ability. This was demonstrable by both qualitative (Fig. 1F) and quantitative techniques (Fig. 2).

To investigate this phenomenon more fully, a time course growth study was conducted to correlate root morphological development with Fe^{3+} reducing ability. For the first 28 d of hydroponic culture of newly rooted *F. benjamina* cuttings, only primary and lateral roots were present and minimal Fe^{3+} reduction was observed (Fig. 3). Between 28 and 35 d, clustered roots appeared, exhibiting very high rates of Fe^{3+} reduction. Lateral roots began reducing at an intermediate rate, while primary roots still exhibited minimal reducing ability. This trend held for 64 d in culture.

Clustered roots developed most extensively at 0 Fe, with some appearance in very low Fe concentrations (<1 μM). Above 2 μM Fe, only primary and lateral roots developed. *F. benjamina* root systems have been shown to exhibit minimal Fe^{3+} reduction above 2 μM Fe (9). Thus, iron stress stimulated the development of the clustered root morphology, which, in turn, enhanced iron reducing ability.

Excised Root Fe^{3+} Reductase Localization

Since the Fe^{3+} reducing capacity of excised root samples varied according to root morphology, samples were embedded in agarose-solidified assay solution in an effort to identify possible regional differences in reducing capacity within root types. Those areas showing reducing capacity were associated with regions of root hair proliferation in both lateral and cluster-type roots (Fig. 1F). Root hairs were conspicuously absent from primary root apices, and these root apices exhibited minimal Fe^{3+} reduction.

pH Response

As a refinement of the excised root assay and to more fully investigate the reducing capacity of the clustered roots, the effect of pH was investigated. Highest rates of reduction occurred at pH 5.5 to 6.7 with Mes buffer, reduction being inhibited below pH 5.5 with acetate buffer, and above pH 6.8 with Hepes buffer (Fig. 4). This is similar to the pH response of soybean cell wall reductase using acetate buffer at low pH (18) and barley root reductase using Mes buffer at low pH (2). However, *F. benjamina* has been shown to depress the pH of nutrient solutions to pH 4 when placed under iron stress (8) and exhibited its highest rate of whole root system Fe^{3+} reduction under these conditions (9). Thus, it seemed inconsistent that the Fe^{3+} reduction mechanism would exhibit sensitivity at acid pH. The experiment was repeated with other buffers at low pH and with Mes buffering extended to a lower pH range. In the presence of succinate or Mes buffer, high rates of reduction were observed in the pH range 4.5 to 6.5 (Fig. 5). Therefore, decreased reduction at low pH in the previous experiment (Fig. 4) was due to acetate inhibition. The decreased reduction at high pH is probably real, because reduction decreased at pH 7 with both Mes and Hepes buffer (Fig. 5). A broad range of high Fe^{3+} reducing ability, extending into the acid pH range, has been demonstrated for peanut roots (15) and bean roots (1). Reduction of rhizosphere pH is common under conditions of Fe deficiency for most non-graminaceous plant species (16). In *Lupinus albus* L., the greatest Fe^{3+} reduction occurred in proteoid root zones, where pH was reduced to 4.5 (10).

DISCUSSION

Most studies on Fe^{3+} reduction utilize young (1–4 weeks old), actively growing herbaceous annuals, where a relatively large portion of the root system is functional. Agar-embedded primary and lateral roots of *Helianthus annuus* seedlings exhibited enhanced Fe^{3+} reduction around the apical root zones (11), and enhanced reduction of excised lateral roots has been demonstrated (17). However, with the woody *F. benjamina*, newly developed root systems of adventitious

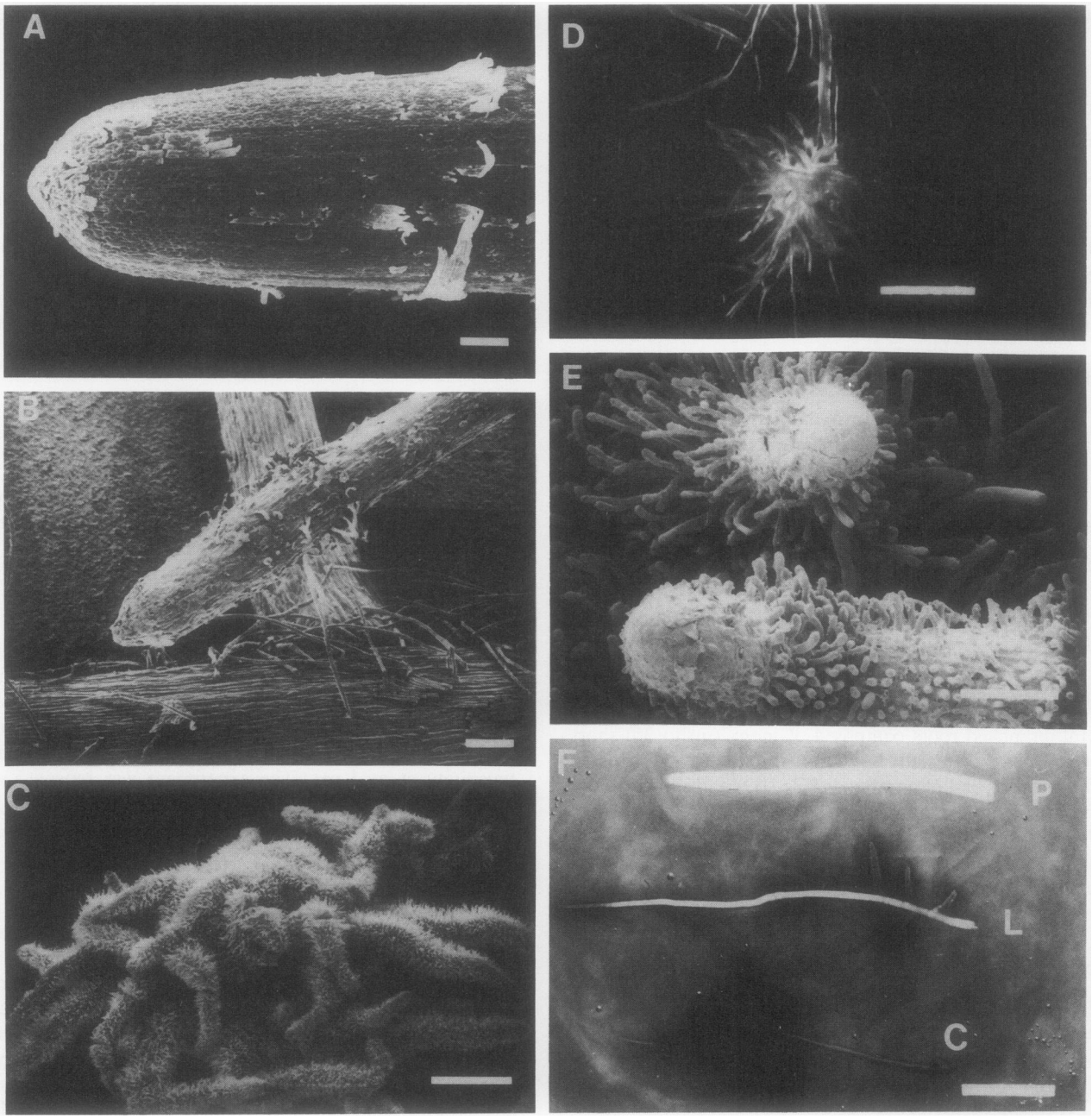

Figure 1. Roots of *F. benjamina* L. grown approximately 6 weeks in 0 Fe aerated Hoagland solution. A, Scanning electron micrograph of primary root with typical root cap; bar = 100 μm. B, Scanning electron micrograph of lateral root showing main axis and tip of a secondary root, with typical root hairs; bar = 100 μm. C, Scanning electron micrograph of root cluster with root hair proliferation; bar = 1 mm. D, Photograph of root cluster formation at terminus of a secondary root; bar = 5 mm. E, Scanning electron micrograph of root hair proliferation on cluster roots; bar = 100 μm. F, Photograph of primary (P), lateral (L), and cluster (C) roots embedded in agarose-solidified reduction assay solution. Dark areas localize Fe^{3+} reduction to Fe^{2+} BPDS (red); bar = 5 mm.

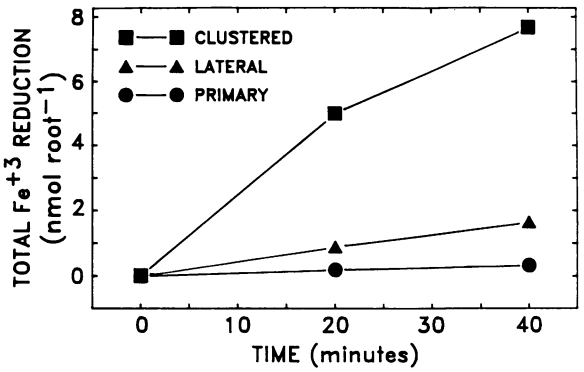

Figure 2. Total Fe^{3+} reduction per root of ten 2 cm excised *F. benjamina* L. root tips from plants grown approximately 6 weeks in 0 Fe aerated Hoagland nutrient solution. Figure compares total reduction of primary roots (13.3 mg $root^{-1}$), lateral roots (0.31 mg $root^{-1}$), and terminal root clusters (0.57 mg $root^{-1}$).

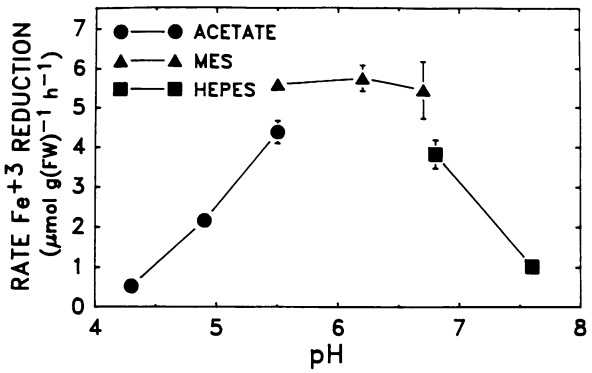

Figure 4. Effect of pH on Fe^{3+} reducing activity of excised root clusters of *F. benjamina* L. grown approximately 42 d in 0 Fe aerated Hoagland nutrient solution. Assay solution was buffered with either 5 mM Na acetate, Mes, or Hepes. Bars equal standard error of the mean.

origin did not exhibit high rates of Fe^{3+} reduction. After several weeks of culture, the plants developed a thick mat of roots composed of highly branched lateral roots and terminal clusters with multiple apices (Fig. 1). These root morphologies exhibited enhanced Fe^{3+} reducing ability (Fig. 3). Often, large, rapid-growing, light-colored primary roots grew from this thick mat, but when assayed they exhibited minimal reducing capacity. At that stage, the older, suberized portions of the root system were nonfunctional. Thus, for *F. benjamina* and possibly other woody plants, expressing reduction on a root system basis (*e.g.* per unit weight or volume) can lead to an underestimation of reducing ability.

High Fe^{3+} reducing capacity in *F. benjamina* root systems appeared to be limited to regions of root hair development. While root clusters were entirely covered with root hairs, young, actively growing primary roots were usually devoid of root hairs for several centimeters below the root apex. Primary roots developed at a later time, *i.e.* after conditioning to low Fe status, had a greater propensity for root hair development. The formation of increased numbers of root hairs has been shown to be a morphological iron efficiency response by plants under iron stress (13, 14). Therefore, the proliferation of secondary and tertiary roots with an increased density of root hairs seems to be one strategy for iron scavenging in *F. benjamina*.

Increased capacity for Fe^{3+} reduction at the surface of rhizodermal cells has been demonstrated in proteoid roots of white lupin (3, 10). In many ways the clustered roots observed with Ficus (Fig. 1) resemble the proteoid roots of Proteaceae (12) and white lupin (*Lupinus albus* L.) (5). The proteoid roots of white lupin have been shown to secrete reducing compounds that are involved in the acquisition of P on soils were its availability is limited (4). In acidic soils this may be accomplished primarily through the solubilization of Fe- and Al-phosphates (6, 7). *Lupinus cosentinii* forms clustered roots in response to low Fe levels, and these clustered roots show a 50 to 60% increase in Fe^{3+} reduction over other root morphologies, and under normal Fe status clustered roots are not formed (19).

F. benjamina is unusual in its ability to thrive under

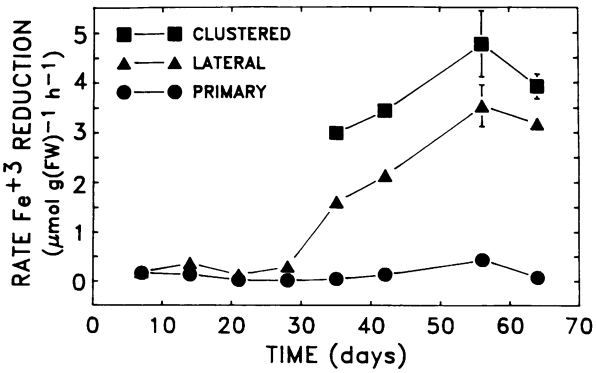

Figure 3. Time course of Fe^{3+} reducing activity of excised primary, lateral, and cluster roots of rooted *F. benjamina* L. cuttings grown in aerated Hoagland nutrient solution containing 0 Fe. Bars equal standard error of the mean.

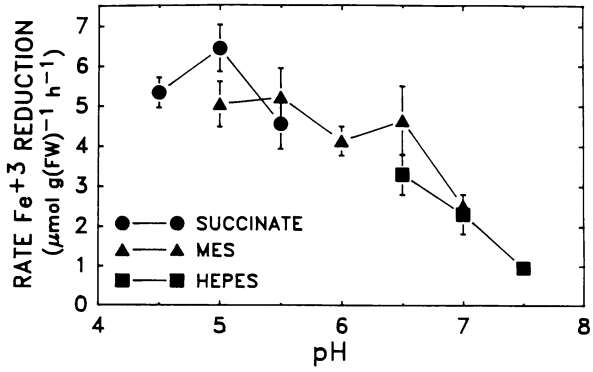

Figure 5. Effect of pH on Fe^{3+} reducing activity of excised root clusters of *F. benjamina* L. grown approximately 42 d in 0 Fe aerated Hoagland nutrient solution. Assay solution was buffered with either 5 mM Na succinate, Mes, or Hepes. Bars equal standard error of the mean.

extremely low iron concentrations. Plants grown without iron in hydroponic culture show minimal chlorosis and continue to grow (8). Contaminate levels of iron, while experimentally undetectable, may be sufficient for *F. benjamina* to continue growth when other species would become chlorotic and die in a short period.

There are several implications of these findings. Iron stress not only turned on the Fe^{3+} reducing system, but also stimulated the development of a unique root morphology as a prerequisite to enhanced reduction. Restricting studies to young actively growing root systems may not demonstrate iron reducing potential. Of course, this may be unique to *F. benjamina*, or possibly other woody species.

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