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Iron Uptake by Plants from Microbial Siderophores[1]

A Study with 7-Nitrobenz-2 Oxa-1,3-Diazole-Desferrioxamine as Fluorescent Ferrioxamine B Analog

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ABSTRACT

The synthetically produced fluorescent siderophore NBD-desferrioxamine B (NBD-DFO), an analog of the natural siderophore ferrioxamine B, was used to study iron uptake by plants. Short-term (10-hour) ^{55}Fe uptake rates by cotton (*Gossypium* spp.) and maize (*Zea mays* L.) plants from the modified siderophore were similar to those of the natural one. In longer-term uptake experiments (3 weeks), both siderophore treatments resulted in similar leaf chlorophyll concentration and dry matter yield. These results suggest that the synthetic derivative acts similarly to the natural siderophore. The NBD-DFO is fluorescent only when unferrated and can thus be used as a probe to follow iron removal from the siderophore. Monitoring of the fluorescence increase in a nutrient solution containing Fe^{3+}-NBD-DFO showed that iron uptake by plants occurs at the cell membrane. The rate of iron uptake was significantly lower in both plant species in the presence of antibiotic agent, thus providing evidence for iron uptake by rhizosphere microbes that otherwise could have been attributed to plant uptake. Confocal fluorescence microscopy revealed that iron was taken up from the complex by cotton plants, and to a much lesser extent by maize plants. The active cotton root sites were located at the main and lateral root tips. Significant variations in the location and the intensity of the uptake were noticed under nonaxenic conditions, which suggested that rhizosphere microorganisms play an important role in NBD-DFO-mediated iron uptake.

Studies on Fe nutrition of plants have resulted in the division of higher plants into two groups (strategies I and II) in relation to their Fe uptake mechanisms (18). Strategy I plants (dicots and nongrass monocots) rely mainly on membrane reductase activity and on chemical modifications of the rhizosphere (*e.g.* excretion of reductants and protons) and a subsequent Fe^{3+} reduction. Strategy II plants (grasses) possess a unique system for Fe acquisition based on phytosiderophore excretion and a specific membrane-bound receptor for the ferrated phytosiderophores (18). The abundance of microbial siderophores in many soils, along with their outstanding Fe binding capacity, has raised the question of whether these siderophores may be used by plants as an Fe source (14).

A number of researchers investigated the possibility of plant utilization of various microbial siderophores including ferrichrome A (19), agrobactin (3), pseudobactin (1, 2, 12, 13), and ferrioxamine B (9, 10). A variety of plant species were shown to acquire Fe from ferrioxamine B, such as tomato (23), oat (9, 20, 21), sunflower and sorghum (8), and peanut and cotton (1).

The mechanism by which plants utilize microbial siderophores has not yet been elaborated to a satisfying extent and it is a topic of controversy. Although some investigators proposed a specific Fe uptake mechanism (9, 11), others did not support this hypothesis (1, 5, 22).

In this study, Fe uptake from ferrioxamine B by maize (*Zea mays* L.) and cotton (*Gossypium* spp.) plants was investigated using the synthetic fluorescent ferrioxamine B analog NBD-DFO[2] as a probe. NBD-DFO has been designed to be nonfluorescent when saturated with Fe^{3+}, but to gain fluorescence upon Fe^{3+} release. NBD-DFO, therefore, allows one to monitor the location and the dynamics of cellular Fe uptake. This research focused on the uptake mechanism in maize and cotton (monocot and dicot, respectively) and on the possibility of microbial involvement in the Fe uptake process.

MATERIALS AND METHODS

Siderophores

Desferrioxamine B methanesulfonic acid was obtained as "Desferal" from Ciba-Geigy. The synthesis of NBD-DFO was performed as described by Lytton *et al.* (16). Briefly, 200 mg (1 mM) of NBD-Cl dissolved in 7.5 mL of methanol were added to 656 mg (1 mM) of desferrioxamine B methylsulfonate (Desferal), dissolved in 10 mL of methanol and 20 mL of 0.1 N $NaHCO_3$. The mixture was heated for 1 h in an oil bath of 65°C. The crude mixture was concentrated *in vacuo* and chromatographed through a silica gel column (Woelm 63–100) using chloroform:methanol (9:1 v/v) as an eluent,

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[2] Abbreviations: NBD-DFO, 7-nitrobenz-2 Oxa-1,3-diazole-desferrioxamine B; Ferrozine: 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4 triazine.

and 140 mg of NBD-DFO were collected and purified by crystallization from a mixture of methanol and acetonitrile.

Plant Culture

Seeds of maize (*Zea mays* L. Jubilee) or cotton (*Gossypium* spp. cv Akala) were germinated on either filter paper or vermiculite presoaked with saturated $CaSO_4$ solution. After 4 d the seedlings were transferred to a continuously aerated nutrient solution either without (−Fe plants) or with (+Fe plants) 100 μM FeEDTA. The nutrient solution had the following composition (μM): $Ca(NO_3)_2$, 2000; K_2SO_4, 750; $MgSO_4$, 650; KH_2PO_4, 500; KCl, 100; H_3BO_3, 1; $MnSO_4$, 0.1; $ZnSO_4$, 0.05; $CuSO_4$, 0.05; $(NH_4)_6Mo_7O_{24}$, 0.005. The plants were grown in the nutrient solution for 2 weeks under controlled climatic conditions: 16/8 h day/night cycle; light intensity (PAR) 40 Wm^{-2} (Sylvania cool-white FR 96T 12 fluorescent tubes); day/night temperature 25/23 ± 1°C; RH 70 to 80%.

Axenic Hydroponic Culture

Seeds were surface sterilized in 32% H_2O_2 solution for 30 min, and then rinsed 10 times with sterilized water and germinated on nutrient agar at 28°C for 3 d. Sterile seedlings were transferred to 500-mL Erlenmeyer flasks containing sterilized, threefold-concentrated, nutrient solution. The flask openings were covered with glass beakers connected to the flasks by a silicon rubber compound (Lastic 55, Kettenbach, Eshenburg, Germany). The flasks were continuously aerated through 0.2-μm filters. When the plants reached a height of about 5 cm, the beakers were removed to enable further growth and the flask openings were sealed with 45°C melted paraffin to avoid microbial contamination. Nutrient solution samples were taken after each step for sterility check by incubation for 48 h in 28°C on nutrient agar medium. Contaminated flasks were discarded.

For other treatments, a reduction of bacterial contaminationc was achieved by an addition of 25 mg L^{-1} tetracycline (Sigma) and 25 mg L^{-1} rifampicin (Serva). The microbial population was counted by the dilution method on nutrient agar medium (Difco).

Preparation of ^{55}Fe-Labeled Chelates

An aliquot of Fe-free ligand was mixed with ^{55}Fe-labeled $FeCl_3$ up to 90% of the ligand concentration. After an addition of about 100 mL of nutrient solution and adjustment of the pH to 6.5, the chelate was equilibrated overnight by stirring in the presence of filter paper pulp to adsorb Fe colloids and precipitates. The chelate solution was then filtered through a series of membranes in descending order of pore size, from 100 to 0.1 μm. The specific activity was 40 to 50 TBq/mol Fe.

Short-Term ^{55}Fe Uptake Experiments

Plants were transferred to beakers containing 100 mL of freshly prepared, continuously aerated, Fe-free nutrient solution buffered to pH 6.0 with 0.01 M Mes or to pH 7.3 with solid-phase $CaCO_3$ (primarily adjusted by NaOH). The beakers were placed in 500-mL black plastic pots. ^{55}Fe-labeled chelates were added 30 min afterwards. Ten hours later, the plants were transferred to a fresh, Fe-free nutrient solution for 30 min of washing. Extracellular ^{55}Fe was removed according to the method described by Bienfait *et al.* (6). Plants were then separated into roots and shoot, oven dried at 105°C, weighed, ashed, and resuspended in diluted HCl for ^{55}Fe determination by liquid scintillation.

Growth of Plants: Long-Term Experiments

Seeds of maize or cotton were germinated as described above. After germination the seedlings were transferred to a continuously aerated nutrient solution in 1-L plastic pots (30 plants/pot) for 2 to 3 d. Seven to 8 d after germination the plants were transferred to 500-mL pots containing a nutrient solution buffered to pH 7.3 with solid-phase $CaCO_3$ (primarily adjusted by NaOH). The pH was monitored daily and corrected when necessary to give 7.3 ± 0.1 pH units. Fe was supplied at concentrations of $1.5 \cdot 10^{-5}$ and $5 \cdot 10^{-6}$ M for cotton and maize plants, respectively, as ferrioxamine B or NBD-DFO. The nutrient solution was replaced every 7 d. After 3 weeks the plants were harvested, and fresh root weight, fresh shoot weight, and Chl concentration (17) were determined.

Fluorimetry

Fluorescence was measured using an SLM 4800 fluorimeter (SLM Instruments, Inc., Urbana, IL) equipped with an SMC 210 monochromator controller (SLM Instruments). Excitation and emission wavelengths were 480 and 540 nm, respectively.

Microscopy

Roots of 2-week-old −Fe and +Fe precultured plants were cut from various parts of the root system, placed on a microscope slide, and immediately covered with 2% agar-nutrient solution containing 5 μM NBD-DFO, 10 μM Mes buffer (pH 6). When sterile roots were used, 25 mg L^{-1} rifampicin (Serva) and tetracycline (Sigma) were added. Samples were incubated at 25°C in the dark for 6 to 10 h and taken for observation in a fluorescent microscope (Wild Leitz confocal laser scanning microscope; Wild Leitz Instruments, Heidelberg, Germany) operated with a single wavelength excitation laser beam at 480 nm. In most of the observations, roots were incubated as described above in nutrient solution containing the chelate, without an agar support. Small root fragments were placed on a microscope slide and covered with an immersion oil.

RESULTS

Spectral Properties

The molecular structure of NBD-DFO is shown in Figure 1. NBD-DFO has some light absorption peak in the unferrated state near 480 nm. This band is slightly affected by Fe^{3+} binding. In addition, NBD-DFO shows strong fluorescence in the free state, which is quenched upon binding of Fe^{3+} (Figs. 2 and 3). However, the fluorescence is regained when

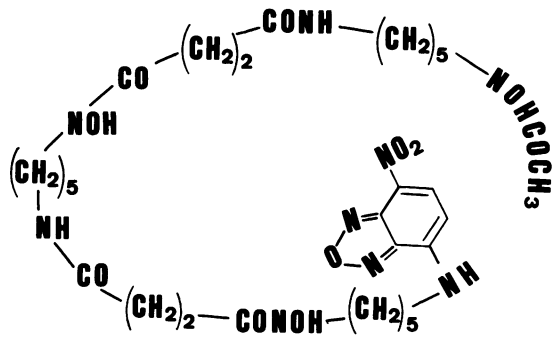


Figure 1. Molecular structure of NBD-DFO.

Fe^{3+} is removed from the NBD-DFO complex by a competing ligand, as shown qualitatively in Figure 2. Ferrioxamine B has an absorbance peak at 430 nm but no fluorescence. These properties render the synthetic ferrioxamine B analog a useful probe to monitor biological Fe uptake processes at the root surface, where Fe removal manifests itself by increased fluorescence intensity of the ligand. This probe can be used quantitatively in nutrient solution studies and qualitatively in fluorescence microscopy studies. Results based on studies of this nature will be shown later.

Uptake Experiments

^{55}Fe uptake rates from NBD-DFO and ferrioxamine B by maize and cotton plants were tested to compare the performance of the two compounds as an Fe source for plants. Cotton and maize plants exhibited similar uptake rate (Fe in shoot and root per root weight per time unit) and translocation rate (Fe in shoot per root dry weight per time unit) from ^{55}Fe-labeled ferrioxamine B and NBD-DFO under short-term (10 h) experimental conditions (Table I). For both plant species

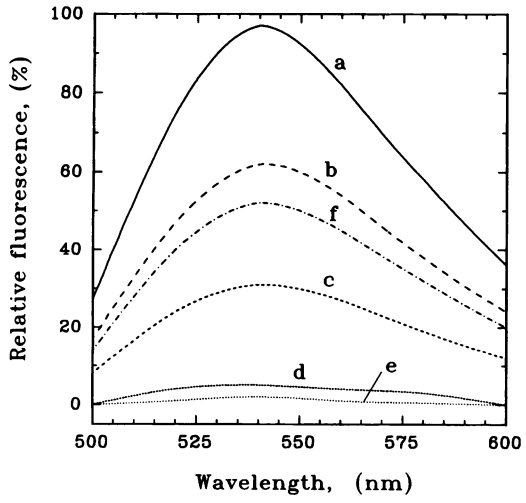


Figure 2. Fluorescence emission spectra of NBD-DFO as affected by the addition of $FeCl_3$. a, 0.30 mM ligand; b, +0.06 mM $FeCl_3$; c, +0.12 mM $FeCl_3$; d, +0.24 mM $FeCl_3$; e, +0.30 mM $FeCl_3$; f, +0.30 mM $FeCl_3$ + 0.30 mM EDTA.

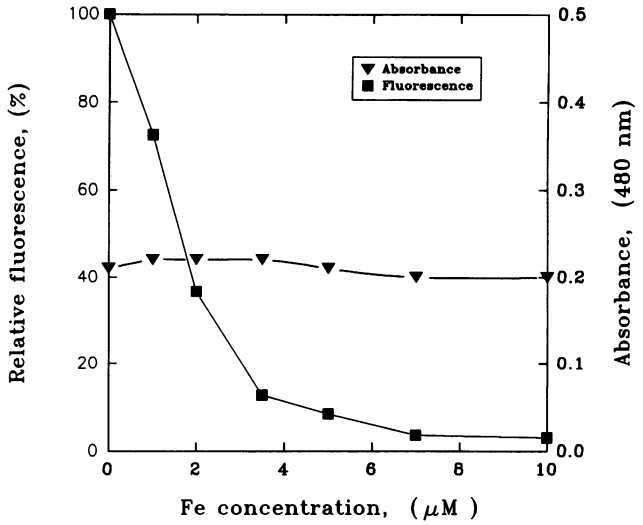


Figure 3. Relative fluorescence (■) and absorbance (▼) of 5 μM NBD-DFO solution as a function of Fe concentration at pH 6.0 (10 mM Mes buffer).

Fe uptake was higher in plants that were precultured without Fe(−Fe), and ^{55}Fe translocation rates were low compared with the uptake rate (0.3–1.0%). These characteristics of ^{55}Fe uptake and translocation rates are typical for Fe uptake from microbial siderophores. Similar values were reported by Barness *et al.* (1) for Fe-pseudobactin and by Bienfait *et al.* (5) and Romheld and Marschner (22) for ferrioxamine B.

Under long-term conditions (3 weeks) both chelators supported normal growth of maize and cotton plants (Table II). The Fe concentration required for normal growth of cotton plants was higher than that needed for maize plants (Table II). These differences are attributed to the different mechanisms of Fe acquisition operating in the two plant species (18).

Similar results were obtained for the two plant species between the plants' response to both chelators in both short-term (^{55}Fe uptake rate, Table I) and long-term (plant growth, Table II) experiments. On the basis of this similarity it was concluded that both chelators are utilized analogously by plants as an Fe source, and that observations obtained for NBD-DFO by fluorescence measurements, including fluorescence microscopy, reflect processes similar to those occurring with ferrioxamine B.

Fluorescence Measurements

The fluorescence properties of NBD-DFO were used to study the rate and pathway of Fe removal from the siderophore by maize and cotton plants. The fluorescence of a nutrient solution containing 5 μM NBD-DFO, in which plants were grown, was measured during a period of 15 h. Because we hypothesized that the chelator may be utilized by rhizosphere microorganisms, a treatment in which antibiotics were added to the nutrient solution was included for comparison in this experiment. The antibiotic treatment had typically reduced the microbial population to 10^3 colony-forming

Table I. *Rates of ^{55}Fe Uptake and Translocation to the Shoot of Maize (Z. mays L. Jubilee) and Cotton (Gossypium spp. cv Akala) Plants from Ferrioxamine B and NBD-DFO*

Plants were incubated for 10 h in aerated nutrient solution containing 1 μM Fe as either ^{55}Fe-NBD-DFO or ^{55}Fe-ferrioxamine B. The pH was maintained at 6.00 by 10 mM Mes buffer.

Plant	Pre-culture	^{55}Fe Uptake Rate		^{55}Fe Translocation Rate	
		NBD-DFO	Ferrioxamine B	NBD-DFO	Ferrioxamine B
		nmol/g dry weight/10 h			
Maize	+Fe	240.2*[a]	166.7*	2.44†	1.46†
	−Fe	357.8†	319.6†	1.87†	0.98†
Cotton	+Fe	307.1*	145.6*	2.39†	1.76†
	−Fe	380.2†	245.6†	1.72†	1.69†

[a] Different symbols represent a significant difference at P = 0.05.

units/mL compared to 10^6 colony-forming units/mL in the control.

A significant increase in the fluorescence of the nutrient solution containing no antibiotics was measured during the experiment period (Fig. 4), indicating that Fe was removed from NBD-DFO by both plant species. The fluorescence increased with time for −Fe and +Fe precultured plants, but the slope obtained for the +Fe precultured plants was markedly lower. The fluorescence intensity of the +Fe precultured plants exhibited a lower starting value (80%), probably because of $Fe(OH)_3$ precipitates on the roots of the Fe-sufficient plants (precultured with 100 μM FeEDTA). Some of these precipitates could have been chelated during the first hours of the experiment by the NBD-DFO. In both plant experiments a significant portion of fluorescence increase was eliminated upon the addition of an antibiotic agent, suggesting that NBD-DFO was recognized as an Fe source by microorganisms, that microbial uptake processes were taking place, and that these processes are considerably faster than those of the plant. It should be noted that these processes may interfere with root uptake measurements of microbial siderophores.

Fluorescence Microscopy

In all our experiments, −Fe-cultured plant roots had higher indigenous fluorescence intensity than did +Fe-cultured plant roots. This observation was similar for cotton and maize plants. This phenomenon had been observed earlier by Landsberg (15) and was attributed to the accumulation of phenolic compounds in the rhizodermal cells. Fluorescence was located mainly at the root tips of main and lateral roots, at root hairs, and around emergence sites of young lateral roots.

Axenically grown plant roots exhibited less indigenous fluorescence than did nonaxenically grown ones. Increased fluorescence intensity in maize or cotton plants upon administration of Fe^{3+}-NBD-DFO, when compared with similarly grown plants in the absence of the chelator, was used as an indicator of the Fe removal process. When agar-embedded maize roots were examined, a halo was observed mainly around the main and lateral root tips (Fig. 5). A slightly brighter halo was observed around lateral root emergence sites and at sporadic sites on the root surface. At these spots, an accumulation of root exudates (mucigel) was observed (Fig. 6). Because these sites are heavily loaded with microorganisms (7), we suggest that microbial activity was the main cause for the observed fluorescence. This hypothesis is supported by the fact that axenically grown maize roots did not show any sites of increased fluorescence when supplied with Fe^{3+}-NBD-DFO.

Cotton roots showed significant Fe uptake. The brightest areas of these roots were about 0.4 mm from the root tip (Fig. 7A) and were brighter near the mucigel layer. This fluorescence could have been enhanced by rhizosphere microorganisms because root exudates and mucigel are likely to be heavily populated by microorganisms. Axenically grown cotton roots, on the other hand, showed Fe removal activity at

Table II. *Chl Concentration and Dry Matter Weight of Maize (Z. mays L. Jubilee) and Cotton (Gossypium spp. cv Akala) Plants After 3 Weeks of Growth in Aerated Nutrient Solution with Either Ferrioxamine B or Fe-NBD-DFO as an Fe Source at pH 7.3, Buffered by Solid-Phase $CaCO_3$*

Statistical analysis was performed separately for each parameter and for each plant species.

Plant	Fe conc.	Chlorophyll		Dry Matter Yield	
		NBD-DFO	Ferrioxamine B	NBD-DFO	Ferrioxamine B
	μM	*mg·g dry weight^{-1}*		*g*	
Maize	0	0.38*[a]	0.32*	0.82*	0.84*
	5	1.80†	1.69†	3.67†	3.46†
Cotton	0	0.15*	0.16*	1.23*	1.14*
	15	2.96†	2.92†	4.45†	4.16†

[a] Different symbols represent a significant difference at P = 0.05.

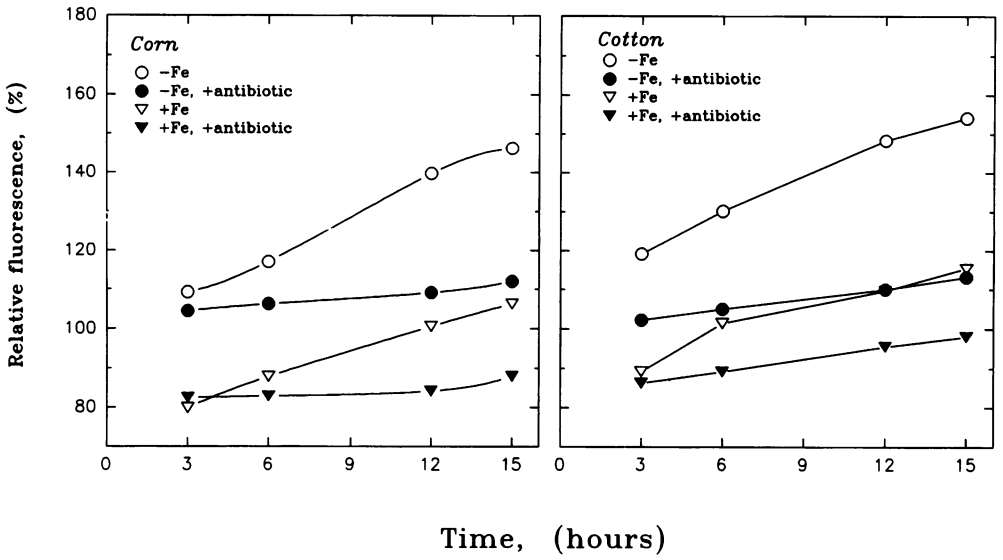


Figure 4. Fluorescence increase of nutrient solution of −Fe and +Fe precultured maize and cotton plants supplied with NBD-DFO. Plants were incubated for 15 h in aerated nutrient solution containing 5 μM Fe as NBD-DFO. Antibiotic treatment included 25 mg L^{-1} rifampicin and 25 mg L^{-1} tetracycline, pH 6.0 (10 mM Mes buffer).

the root tips only (Fig. 7B). Roots of control plants (−Fe, no NBD-DFO added) did not exhibit any fluorescence (Fig. 7C). It should be noted that we had to use a sevenfold higher excitation intensity to observe the fluorescence emitted as a result of the root activity (Fig. 7B), compared with the intensity needed for the observation of fluorescence emitted by nonaxenically grown roots (Fig. 7A).

When Ferrozine was included with Fe^{3+}-NBD-DFO in the agar layer covering cotton roots, a purple stain of the Fe^{2+}-Ferrozine complex was observed under visible light near the root tip (0.1–0.4 mm from the tip, Fig. 8). The location of the Fe^{2+}-Ferrozine complex was also examined in a fluorescence microscope (data not shown). Fluorescence derived from NBD-DFO was observed at this site of the root. This is direct evidence for Fe^{3+} reduction at the root tip of cotton. As shown in Fig. 6 (maize) and in Fig. 7, A and B (cotton), in both plant species studied no NBD-DFO was detected beyond the cell membrane, indicating that the Fe removal processes occur at the plasma membrane.

DISCUSSION

NBD-DFO may be used as an analog of ferrioxamine B because its major properties as an Fe source for strategy I and strategy II plants and the plants' response are similar to those of ferrioxamine B. The use of NBD-DFO has the advantage of facilitating the monitoring of Fe uptake through its fluorescence properties.

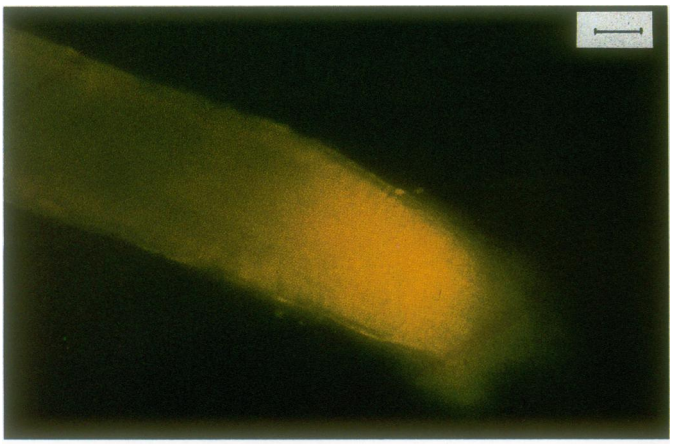
Monitoring fluorescence increase in a nutrient solution containing Fe^{3+}-NBD-DFO led to similar results in terms of trends and intensity for both maize and cotton plants. A critical portion of the fluorescence increase (due to Fe removal from the chelate) was eliminated upon addition of an antibiotic agent. The results obtained in nutrient solution experiments were in good agreement with observations made by microscopy. Root Fe uptake could not be observed unless most of the microbial activity was eliminated. Thus, microbial activity must be considered when studying Fe acquisition by plants from microbial siderophores.

Fluorescence microscopy revealed some important aspects of Fe uptake by both plant species. Maize plants exhibited NBD-DFO-derived fluorescence at the root tips of the main and lateral roots. Root tips are areas in which most of the Fe uptake activity takes place (4). However, this activity was observed only when roots were agar embedded. This fluorescence may have originated from some fluorescent compounds (*e.g.* phenolics) that were diffusing away from the root.

Cotton roots removed Fe from Fe^{3+}-NBD-DFO, as demonstrated by the increased fluorescence in some parts of the root. The observed root activity was biased by microbial activity, as shown in this study by comparing axenically and nonaxenically grown roots. Lower root activity was obtained when the number of microbes was reduced by antibiotics.

The data shown in this study indicate that a major part of Fe removal from NBD-DFO is a result of microbial activity. In the absence of microbial activity, the differences in Fe acquisition from NBD-DFO by strategy I and strategy II plants are significant. These differences are expressed in the ability of strategy I to reduce ferric chelates. The agreement between the location of the fluorescence increase and the location of the Fe^{2+}-Ferrozine complex on the root surface indicates the reductive nature of the Fe uptake process. It should be noted that researchers have proposed that strategy II plants, lacking the ability to reduce ferric chelates, may use their phytosiderophore receptors for Fe acquisition from microbial siderophores (9–11). However, this hypothesis was not supported by Fe uptake rates reported in other studies (1, 5, 22).

By using Fe^{3+}-NBD-DFO as an Fe uptake probe in combination with confocal fluorescence microscopy, areas of root and of predominant microbial activity could be differentiated,



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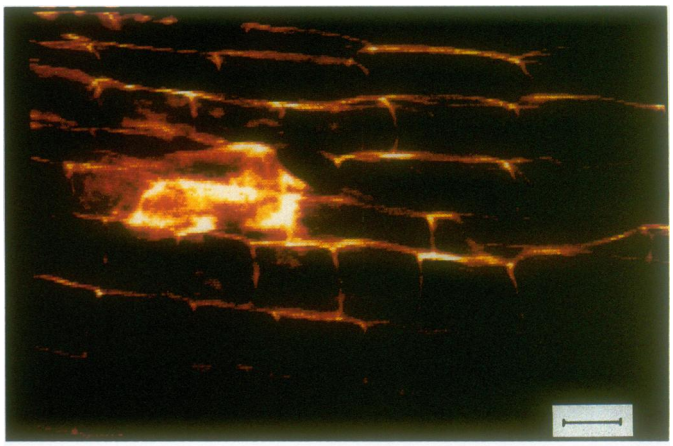

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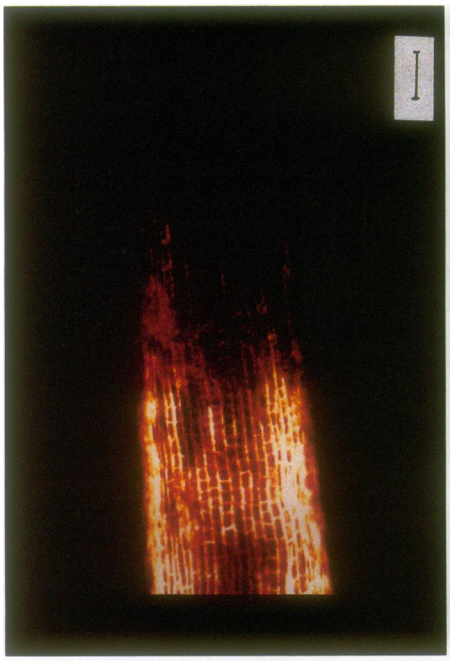

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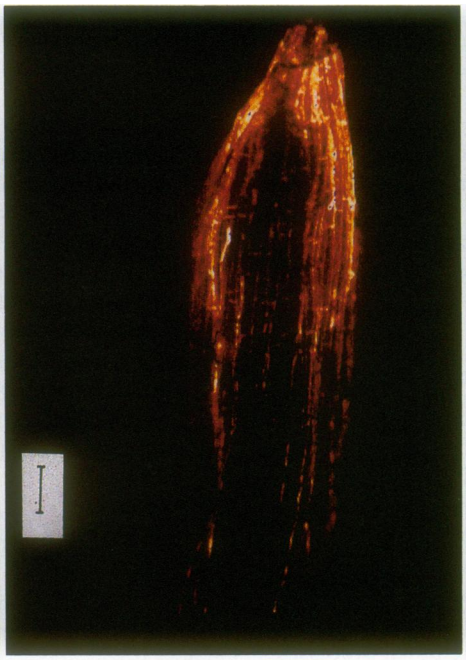

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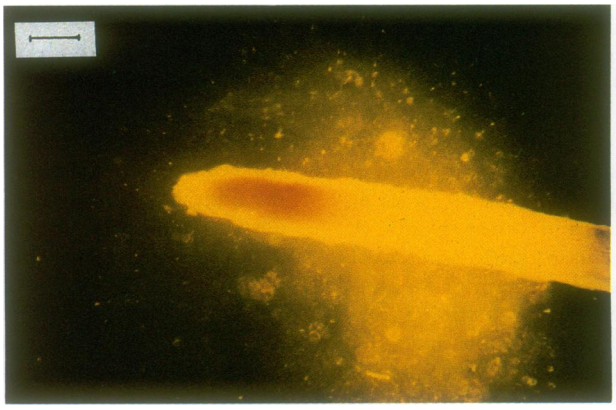

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and the role of rhizosphere microorganisms in siderophore-mediated Fe uptake by plant roots is demonstrated.

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Figures 5 through 8. Fluorescence microscopy studies. **Figure 5**, A halo of fluorescent NBD-DFO around maize (*Z. mays* L.Jubilee) root tip supplied with Fe^{3+}-NBD-DFO. Plants were grown for 2 weeks in nutrient solution without Fe. Root cuts were embedded in agar layer containing 5 μM Fe^{3+}-NBD-DFO and buffered with 10 mM Mes (pH 6). Photography was performed after 6 h of incubation at 25°C in the dark. Bar = 100 μm. **Figure 6**, Fluorescent area at a maize (*Z. mays* L. Jubilee) root surface supplied with Fe^{3+}-NBD-DFO. The fluorescent area represent a site of mucigel accumulation. Plants were grown for 2 weeks in nutrient solution without Fe. Root cuts were incubated in nutrient solution containing 5 μM Fe^{3+}-NBD-DFO and buffered with 10 mM MES (pH 6). Photography was performed after 6 h of incubation at 25°C in the dark. Bar = 10 μm. **Figure 7**, Fluorescence developed by a cotton root tip supplied with Fe^{3+}-NBD-DFO. The plants were grown for 2 weeks in nutrient solution without Fe. Root cuts were incubated in nutrient solution containing 5 μM Fe^{3+}-NBD-DFO and buffered by 100 mM Mes (pH 6). Photos were taken after 6 h of incubation at 25°C in the dark. A, Nonsterile root; B, axenically grown root; C, control (no Fe added) cotton root tip. The fluorescence intensity in (A) is sevenfold higher than that in (B) and 10-fold higher than that in (C). Growth conditions were as described in Figure 6. Bar = 50 μm. **Figure 8**, An Fe^{2+}-Ferrozine stain at the root tip of cotton supplied with 5 μM Fe^{3+}-NBD-DFO. Bar = 200 μm.