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REACTION OF FERRIC IRON BY SIDEROPHORE PRODUCED BY A *BRADYRHIZOBIUM* STRAIN

P.T.C. Nambiar, S. Sivaramakrishnan and B. Srinivasa Rao

International Crops Research Institute for the Semi-Arid Tropics Patancheru, A.P. 502 324
India

ABSTRACT: Ferric iron (Fe^{+3}) chelating compounds, including siderophores produced by microorganisms, help in the Fe nutrition of plants. Dicotyledonous plants absorb Fe in the ferrous (Fe^{+2}) form. The ability of siderophores produced by a *Bradyrhizobium* strain, a rhizosphere bacterium of groundnut (*Arachis hypogaea*), to reduce Fe^{+3} was tested. Two Fe^{+3} (^{58}Fe) binding fractions were separated from the culture supernatant of a *Bradyrhizobium* strain grown in an iron deficient medium. One of the fractions isolated reduced Fe^{+3} to Fe^{+2}, unlike the synthetic chelator ethylenediaminetetraacetic acid (EDTA). It has been proposed that Fe-chelators supply Fe^{+3} to groundnut roots, and Fe^{+3} reduction to Fe^{+2} and its uptake occurs at the plasmalemma. Since siderophores can reduce Fe^{+++}, they may help in Fe nutrition of groundnut plants better than chelators like EDTA. There is no evidence to indicated siderophore uptake

by groundnut plants, but we have detected ethyl acetate insoluble Fe^{+3} reducing activity in the xylem sap of the plants.

INTRODUCTION

Although Fe is abundant in soils (1-8%), it is often unavailable to plants because of its insolubility. Most soil Fe exists in the insoluble ferric (Fe^{+3}) form in aerobic soils. Iron availability to plant roots may be modified by pH and organic chelators (1,2). When grown under conditions of Fe deprivation, microorganisms secrete ferric-specific ligands called siderophores, small moleoular weight compounds which exhibit a strong affinity for Fe^{+3}, and have a formation constant in the range of IO^{30} or higher (1). Most of the siderophores can be classified into two types, *i*) the catechol-like compounds found only in bacteria, and *ii*) the hydroxamate-like compounds found in fungi, yeasts and bacteria (1,2).

The importance of siderophores produced by microorganisms to supply Fe to plants has been suggested by many workers (2-6). Jurkevitch et al. (7) reported that bacterial siderophores may help overcome lime-induced chlorosis in groundnut (*Arachis hypogaea*) grown in calcareous soils. In most cases, the role of siderophores has been attributed to the Fe^{+3} binding function of siderophores (1,8,9). In some dicotyledonous plants, separation and absorption of Fe from Fe^{+3} chelates appears to require reduction of Fe^{+3} to Fe^{+2} before the uptake of Fe^{+2} by the plant (10,11). It is important to understand the mechanism of siderophore mediated Fe uptake by plants. We

describe a method for estimating the Fe^{+3} reducing ability of a siderophore produced by a *Bradyrhizobium* strain (NC 92) that nodulates groundnut, and we suggest that this function of siderophores could be important in the Fe uptake by groundnut.

MATERIALS AND METHODS

Strains Culture Conditions and Isolation of Siderophore: Details of the test strain NC 92, and culture conditions and isolation of catechol type siderophores have been described previously (12).

Partial Purification of Siderophore and Estimation of Ferric Iron Binding and Reducing Activity: An ethyl acetate siderophore extract prepared from a 2 L culture strain NC 92 was dissolved in 1 mL ethanol and loaded on a lipophilic Sephadex (LH-20-100, Sigma Chemical Co., St Louis, MO, USA) column (2 cm diameter, 35 cm long, and equilibrated with ethanol). Two mL fractions were collected and assayed for Fe^{+3} binding and reducing activities. Ferric binding activity was assayed using ^{58}Fe. Two µg ^{58}Fe was mixed with 6 µg cold Fe (as ferric chloride) in 0.5 mL dilute HCl ($5x10^{-4}$ N). To this mixture, 0.1 mL of the fraction was added, stirred well and the mixture incubated for 1 h at room temperature ($27\pm2^{0}C$). At the end of the incubation period, the siderophore-Fe complex was extracted in 2 mL ethyl acetate, and the 0.5 mL ethyl acetate layer was added to a vial containing Instagel scintillation fluid and counted in a Beckman (5801) counter (12). Ferric reducing activity was assayed by adding 0.5 mL of the fraction to 0.5 mL freshly prepared ferric chloride (2 mM, pH 2.0) and incubating 2

h at room temperature. Ferrous in the sample was estimated by adding 1 mL 1-10 o-phenanthroline [o-ph, 1.5%, pH 2.0, (12)]. Optical density (O.D.) at 510 nm was recorded 10 min after o-ph addition. To create a blank, siderophore was added to a mixture of o-ph and ferric chloride solution at the above concentrations just before recording O.D. Various concentrations of ferrous ammonium sulphate added to ferric chloride (2 mM) were used as standards. EDTA (ethylenediaminetetraacetic acid, 5 mL, pH 2.0) was also used as a control instead of siderophore.

RESULTS AND DISCUSSION

O-Ph reacts with both Fe^{+2} and Fe^{+3} (14), but Fe^{+2} can be determined spectrophotometrically in the presence of Fe^{+3} under the assay conditions described in materials and methods (13). Fractions 30 to 38 and 72 to 75 from the Sephadex column showed Fe^{+3} binding activity, but Fe^{+3} reducing activity was associated with the fractions 72 to 75 only (Fig. 1). Fractions exhibiting the Fe^{+3} reducing activity were pooled and used for other experiments. The Fe^{+3} reducing activity was linear for about 2 h before leveling off (Fig. 2). No change in the absorption spectrum of the EDTA-Fe^{+3} complexes was noted with the addition of o-ph (Fig. 3).

The importance of siderophore production by strain NC 82 has been suggested earlier (12,15). Inoculation with strain NC 82 increased the yield of a few groundnut cultivars in India, China and Cameroon (16), while inoculation with other strains (which produced lesser amounts of siderophores) did not, de-

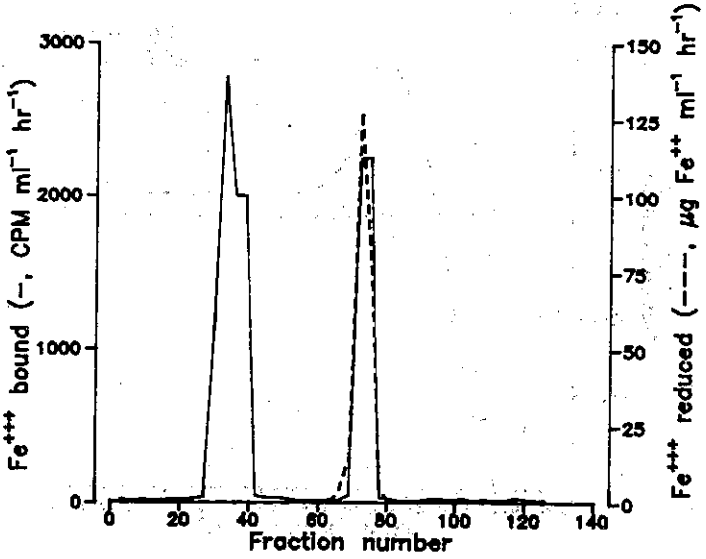

Figure 1. Elution profile of siderophore from Sephadex (LH-20-100) column. Fe^{+3} binding (—, CPM/mL siderophore/h) and Fe^{+3} reduction (---, ug Fe^{+2} formed/mL siderophore/h) was estimated as describe in materials and methods.

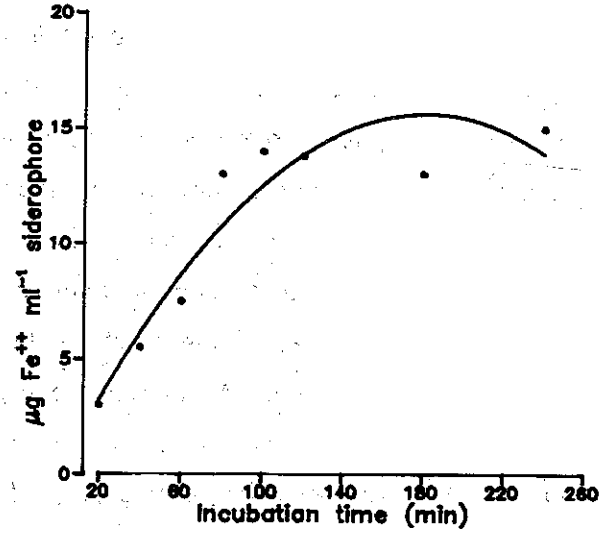

Figure 2. The course of Fe^{+3} reduction by siderophore.

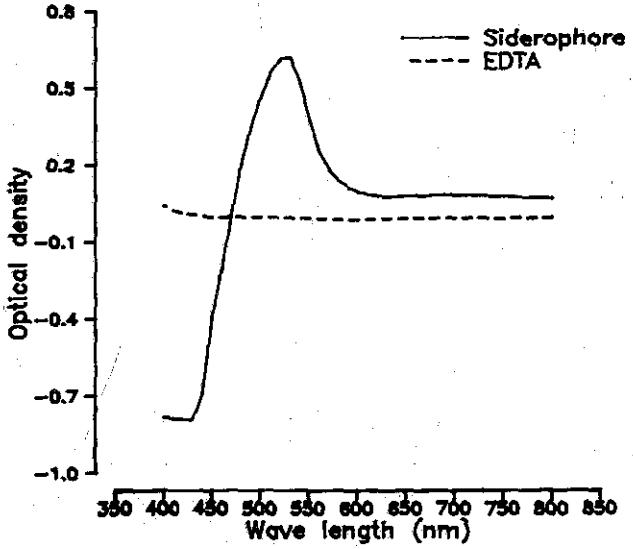

Figure 3. Spectra of reaction products of siderophore-Fe^{+3} (—) or EDTA-Fe^{+3} (- - -) with o-ph.

spite some of the strains being equally efficient in N fixation in pot experiments and competitive in nodule formation in the field (16). However, there is no direct evidence to demonstrate the effect of *Bradyrhizobium* siderophore on groundnut growth and yield in the field. O'Hara et al. (15) suggested that the ability of strain NC 82 to form nodules on groundnut under conditions of Fe stress may be related to the ability of the strain to produce catechol type siderophores. A siderophore produced by a *Pseudomona* strain was reported to correct Fe chlorosis of *Arachis hypogaea* (5,7) and enhance plant growth of *Solanum tuberosum* (4). The effects of siderophores on plant nutrition

and growth have been discussed only from the standpoints of Fe^{+3} binding ability of siderophores and siderophore-mediated Fe^{+3} transport (1,3,4,9). The function of siderophores has been compared to that of synthetic chelators such as EDTA and EDDHA [ethylenediamine-di(o-hydroxyphenylacetic acid] (5,11).

Reduction of Fe^{+3} is an obligatory step for higher uptake rate of Fe in plant species like *Glycine max* and *Arachis hypoaea* grown under conditions of Fe deficiency (10,11). Romheld and Marschner (11) suggested that chelators supply Fe^{+3} to the plant and binding of Fe chelates would occur at the outer surface of the plasmalemma of root cells where Fe^{+3} is reduced by plant enzymes. They further suggested that the reduction of the Fe^{+3} chelate by roots is probably preceded by a chelate binding and weakening of chelate bonds which in turn leads to a facilitated electron transfer in the subsequent reduction process (11).

Chaney et al. (10) suggested that reduction of Fe^{+3} chelates could occur at the plasmalemma, and a cytochrome or flavin on the cell membrane could transfer electrons inside the cell after Fe reduction. Since microbial siderophores are present in soils, we suggest that Fe^{+3} reducing ability of these chelating compounds should be considered in models describing Fe uptake by groundnut (and possibly other plants) roots from soils. Since siderophores can reduce Fe^{+3}, they may help in Fe nutrition of groundnut plants better than chelators like EDTA. Direct uptake of Fe^{+2} by plant cells could occur at the outer surface of the

plasmelemma of root cells. Alternatively, the Fe^{+2} siderophore could be taken up directly. To test this possibility, we assayed the xylem sap of groundnut plants inoculated with *Bradyrhizobium* (strain NC 92) for the presence of siderophore. Although we could detect a Fe^{+3} reducing activity in the xylem sap, we could not detect any Fe^{+3} reducing activity in the ethyl acetate extract of xylem sap (xylem sap adjusted to pH 2.0 with 0.1 N HCl, and then extracted with an equal amount of ethyl acetate). This indicates that perhaps the ethyl acetate soluble siderophore is not taken up by the plants. Romheld and Marschner (11) also observed that increases in reduction and uptake of Fe from Fe-chelates by roots of *Arachis hypogaea* was not associated with a corresponding increase in chelator uptake. The role of ethyl acetate insoluble Fe^{+3} reducing activity in the xylem sap of the plants is not clear.

Howell (17) recently reported that groundnut inoculated with different *Bradyrhizobium* strains contained different concentrations of mineral nutrients, including Fe. Since *Bradyrhizobium* strains differ in siderophore production (12), it may be possible that the differences in metal-ion uptake with different strains are influenced by siderophores. Extraction of the culture filtrate into ethyl acetate is an effective purification step for catechol siderophores (1), and our results indicate that different types of catechol Fe^{+3} binding compounds are produced by *Bradyrhizobium* strains.

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