

Siderophores Production by Some Microorganisms and Their Effect on *Bradyrhizobium*-Mung Bean Symbiosis

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ABSTRACT

Eighty-four microbial isolates were tested for their ability to produce siderophores using four chemical assays. From these isolates, 42 were strongly or moderately positive on chrome-azurool S assay (CAS). However, they showed no reaction with phenolates assay. All bacterial isolates failed in citrate production except *Bradyrhizobium* strains, while *Pseudomonas (Ps.) aeruginosa* showed strong reaction, among positive bacterial isolates, with hydroxamate assay. *Aspergillus* isolates and *Penicillium (P.) chrysogenum* produced moderate and strong reactions with the hydroxamate and citrate assays, respectively. Hydroxamate siderophore production was proportional with iron concentration up to 20 and 40 μM with *Bradyrhizobium* and *Ps. aeruginosa*, respectively. However, maximum siderophore production peak was achieved by *Aspergillus (A.) nidulans* and *P. chrysogenum* at concentration of 80 μM iron. Glucose proved to be the most suitable carbon source for *Ps. aeruginosa*, *A. nidulans* and *P. chrysogenum*; whereas, *B. japonicum* gave its highest yield when grown on mannitol. Siderophore synthesis was inhibited by 4-chloromercuribenzenesulfonic acid, 2,4-dinitrophenol, sodium azide and MgCl_2 suggesting that an energized membrane and a hydroxy group are essential and required for hydroxamate synthesis. The siderophore-producing *P. chrysogenum* and *Ps. aeruginosa* significantly enhanced nodulation and nitrogen fixation of mung bean compared to plants infected with *Bradyrhizobium* strain alone. The ecological advantages in the synthesis of microbial siderophore encourage the use of such microbes as inoculants with root nodule bacteria.

Key Words: Siderophores; Symbiosis; Microorganism; Mung bean

INTRODUCTION

The acquisition of iron by microorganisms in aerobic environments presents a difficult problem since the solubility products constant for ferric hydroxide is about 10^{-38} (Lindsay & Schwab, 1982). Thus at pH 7, the free available iron is at a concentration of no more than 10^{-17} M, which is far below that required for microbial and plant growth. Iron in an aerated environment exists in the ferric form and so is highly insoluble in neutral or alkaline soil (Shenker *et al.*, 1995a). To solve this problem, microorganisms are generally observed to utilize a high-affinity iron transport system. The synthesis and secretion of a low-molecular weight ferric-specific chelation agent to solubilize iron is termed as siderophore (Neilands, 1981; Abd-Alla, 1998).

Microbial siderophore may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake system (Marek-Kozaczuk *et al.*, 1996). In soil, plant roots normally coexist with bacteria and fungi which may produce siderophores capable of sequestering the available soluble iron and so interfere with plant growth and function. Alternatively, plant roots might be capable of taking up ferric complexes of siderophores and using these as sources of iron. Precedents are known for the exploitation by one organism of the synthesis of an iron chelator by another (Neilands, 1982). Thus, ferrichrome is produced by fungi (Neilands, 1977), but *Escherichia coli* can take up its ferritrihydroxamate derivative and use this as a source of iron (Hartmann & Braun, 1980). Ferrichrome derivatives

can also be used as source of iron by higher plants (Orlando & Neilands, 1982; Powell *et al.*, 1982).

Prior research on plant iron nutrition has examined synthetic chelates that donate iron to plant roots by extracellular dissociation (Lindsay & Schwab, 1982). Dissociation occurs when plants alter the redox and pH conditions of the rhizosphere to increase solubility of inorganic iron. Also, iron efficient dicotyledonous plants may remove ferric iron from synthetic chelates with a cell surface reductase that releases inorganic ferrous iron into the root apoplast (Bienfait *et al.*, 1985). In contrast, plants appear to utilize microbial siderophores differently since siderophores do not release iron into the apoplast of dicotyledonous plants nor readily dissociate above pH 4. In studies postulating siderophore transport, Smarrelli & Castigenti (1986) have shown that inorganic iron may be released from hydroxamate siderophore by plant cytosolic NADH: nitrate reductase. Alternatively, Rhomheld and Marschner (1983) have suggested a model that involves a plasmalemma chelate binding site with a reductase-carrier protein for inorganic iron.

Jurkevitch *et al.* (1986, 1988) reported that peanut plants grown on a calcareous soil amended with Fe-pseudobactin act as Fe source to plants and decreased the symptoms of Fe deficiency. Shenker group demonstrated that microbial siderophore have potential to play a role in Fe nutrition of plants grown on aerated neutral or basic soils (Shenker *et al.*, 1992, 1995a). Utilization of Fe-siderophores by microorganisms is a generally receptor-dependent process (Matzanke, 1991; Jurkevitch *et al.*, 1992). Hence, siderophores may serve as Fe sources or as Fe competitors for organisms, depending on the ability of the organisms to

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acquire Fe from the stable Fe-siderophore complex. Thus, siderophores may play an important role in the competition between microorganisms (Shenker *et al.*, 1995b; Omar & Abd-Alla, 1998) and may act as growth promoters. Since the rhizosphere is very heavily populated with siderophore-producing microorganisms, plants may also encounter similar interaction.

Iron deficiency in nodulated legumes is very common on alkaline soils, and affects such common agricultural crops as chick pea (Rai *et al.*, 1982), French bean (Hemantaranjan & Garg, 1986) and peanut (O'Hara *et al.*, 1988). Although nodule initiation may occur normally in peanut (O'Hara *et al.*, 1988), it is drastically curtailed in lupines under iron deficiency (Tang *et al.*, 1990). There is, however, little evidence that iron deficiency in soil actually decreases the number of root nodule bacteria, implying either that these organisms have lesser demands for iron during normal growth and survival than the plant, or that they have other mechanisms for acquiring iron under deficiency conditions (Abd-Alla, 1999).

The aim of the present study was to investigate the ability of different rhizosphere microorganisms to produce siderophores and their effects on growth, nodulation and nitrogen fixation of mung bean.

MATERIALS AND METHODS

Organisms. Eighty-one isolates of rhizosphere microflora were tested for their capability to produce siderophores. These were *Aspergillus (A.) flavus*, *A. fumigatus*, *A. nidulans*, *Trichoderma koningii*, *Penicillium (P.) chrysogenum*, *Rhizopus arrhizus*, *Mucor hiemalis*, *Cunninghamella elegans*, *Azotobacter Chroococcum*, *Bacillus cereus*, *Pseudomonas (Ps.) aeruginosa*, and *Serratia marscescens*. In addition, three Bradyrhizobial strains (cowpea) CB 1015, USDA 3447 and USDA 3456 were also tested.

Glassware preparation. All glassware used was cleaned in 20% HCl to remove iron and rinsed in deionised water.

Media and culture. Fungal isolates were grown on medium containing (g/L): glucose 10, Na₂HPO₄ 7, KH₂PO₄ 3, NH₄Cl 1, NaCl 0.5, MgSO₄.7H₂O 0.25, CaCl₂.2H₂O 0.015, ZnCl₂ 0.015 and thiamine 0.005. *Azotobacter* strains were grown in a nitrogen-free medium containing (g/L): K₂HPO₄ 1, MgSO₄ 0.2, CaCl₂.2H₂O 0.1, Na₂MoO₄.2H₂O 0.001 and mannitol 10. *Bacillus cereus*, *Ps. aeruginosa*, and *Serratia marscescens* were maintained on the mineral salt medium contained (g/L): K₂HPO₄ 1, KH₂PO₄ 3.4, NaCl 0.45, MgSO₄.7H₂O 0.5, MnSO₄.7H₂O 0.1, CuSO₄.5H₂O, 0.0005, ZnSO₄.7H₂O 0.0005, CaCl₂ 0.1 and glucose 10. *Bradyrhizobium* strains were grown on the medium contained (g/L): K₂HPO₄ 0.5, MgSO₄ 0.2, NaCl 0.1, NH₄Cl 1 and mannitol 10. The pH was adjusted to 7 before autoclaving. Iron was added as 0 or 10 µmol/L FeCl₃. Flasks containing 50 mL of the appropriate media supplemented with or without iron were inoculated with the

tested microbes separately and incubated on orbital shaker at 1.8 Hz and 28°C. Cultures were harvested after 6 d and centrifuged at 67 Hz for 10 min. The experiments repeated twice and five replicates were used for each organism.

Siderophore assay. Catechol-type phenolates were measured on acetyl acetate extracts of the culture supernatant of tested organism using modification of the ferric chloride-ferricyanide reagent of Hathway (Reeves *et al.*, 1983). Ethyl acetate extracts were prepared by extracting 20 mL of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent was prepared by adding 1 mL of 100 mmol/L ferric chloride in 0.1 M HCl to 100 mL of distilled water, and to this, 1 mL 100 mmol/L potassium ferricyanide was then added (Reeves *et al.*, 1983). For the assay, one volume of the reagent was added to one volume of sample, and absorbance was determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2,3-dihydroxybenzoic acid as a standard.

Hydroxamate-type siderophores were determined by the method of Gibson and Magrath (1969). To 0.5 mL of culture supernatant, 0.5 mL 6 mol/L H₂SO₄ was added and the mixture was autoclaved in a glass tube. 1 mL of 1% sulfanilic acid (W/V) in 30% acetic acid (V/V) and 0.5 mL of 1.3% iodine in 30% acetic acid (V/V) were added. The excess of iodine was destroyed by the addition of 1 mL of 2% (W/V) Na₃AsO₄ solution. Then, 1 mL 1-naphthylamine (0.3% in 30% acetic acid) was then added and the total volume made up to 10 ml with distilled water. After 30 min, the absorbance at 526 nm was measured. Hydroxylamine hydrochloride was used as standard and 1.0 µmol/L of compound gave an absorbance of 0.1.

Chrome azurol S (CAS) agar medium was prepared by method of Alexander and Zuberer (1991). CAS agar was prepared from four solutions, which were sterilized separately before mixing. The Fe-CAS indicator solution (solution 1) was prepared by mixing 10 mL of 1 mmol/L FeCl₃.6H₂O (in 10 mmol/L HCl) with 50 mL of an aqueous solution of CAS (1.21 g/L). The resulting dark purple mixture was added slowly, with constant stirring, to 40 mL of an aqueous solution of hexadecyltrimethylammonium bromide (1.821 g/L). This yielded a dark blue solution, which was autoclaved, then cooled to 50°C. All of the reagents in the indicator solution were freshly prepared for each batch CAS agar.

The buffer solution (solution 2) was prepared by dissolving 30.24 g of piperazine-N, N-bis (2-ethanesulfonic acid) (PIPES) in 750 mL of salt solution containing 0.3 g K₂HPO₄, 0.5 g NaCl and 1.0 g NH₄Cl. The pH was adjusted to 6.8 with 50% (W/V) KOH, and water was added to bring the volume to 800 mL. The solution was autoclaved after adding 15 g of agar, then cooled to 50°C. Solution 3 contained the following (in 70 mL water): 2 g glucose, 2 g mannitol, 493 mg MgSO₄.7H₂O, 11 mg CaCl₂, 1.17 mg MnSO₄.2H₂O, 1.4 mg H₃BO₃, 0.04 mg CuSO₄.5H₂O, 1.2 mg ZnSO₄.7H₂O, and 1.0 mg NaMoO₄.2H₂O. Solution 3 was

autoclaved, cooled to 50°C, then added to the buffer solution along with 30 mL filter-sterilized 10% (W/V) casamino acids (solution 4). The indicator solution was added last, with sufficient stirring to mix the ingredients without forming bubbles. A yellow halo surrounding a bacterial colony indicate of a positive CAS reaction. Free citric acid was determined enzymically using the citrate lyase-malate dehydrogenase assay and following the disappearance of NADH at 340 nm (Moellering & Gruber, 1966). The most siderophore-producing organisms (*Ps. aeurginosa*, *Bradyrhizobium* strain USDA 3447, *A. nidulans*, *P. chrysogenum*) were chosen for further investigation.

Iron concentration. Optimum iron concentration was determined by growing the tested organism for 8 d in appropriate medium with different levels (0-400 µM) of iron in the form of FeCl₃.

Carbon source. The effect of different carbon sources (mannitol, glucose, succinate, citrate and fumarate) on siderophore production was investigated.

Metabolic inhibitor agents. The effect some inhibitors (4-chloromercuribenzenesulfonic acid (PCMBs), 2,4-dinitrophenol, (2,4-D carbonyl cyanide 3-chlorophenylhydrazine (CCCP), sodium azide and HgCl₂) on siderophore production were tested.

Plant nodulation test. Clay soil from farmer's fields near Assiut city was used to carry out the experiments in pots. The main physical and chemical characteristics of the experimental soil are shown in Table I. The experiment was performed in a greenhouse using plastic pots of 5 kg capacity, filled with sterilized clay soil. The sterilization was carried out by autoclaving the soil in metal buckets at 121°C for 3 h.

Five surface-sterilized seeds of mung bean (*Vigna radiata*) cv. Berkum were planted per pot. Five mL of *Bradyrhizobium* USDA 3447 (approximately 10⁶ mL⁻¹) grown on yeast extract mannitol medium for 7 d was added for each pot along with the seeds. After germination, the number of plants was reduced to three plants per pot. The plants were irrigated with water daily or when required. The temperature during the experiment averaged 33/22 °C

Table I. Physical and chemical characteristic of the experimental site

Property	Value (%)
Clay	53.000
Silt	26.000
Sand	14.000
Electrical conductivity (S m ⁻¹)	0.132
pH (1:2 soil:water)	7.900
Organic matter%	0.250
Total N%	0.020
Fe%	0.320

(day/night). These pots were divided into three groups. The first group inoculated with *Bradyrhizobium* strain USDA 3447 and used as control. The second group was inoculated with *Bradyrhizobium* strain USDA 3447 and *Ps. aeurginosa*. The third group inoculated with *Bradyrhizobium* strain USDA 3447 and *P. chrysogenum*. The treatments were arranged in a randomized block design with three replicates for each treatment.

Analysis. The nitrogenase activity was determined on detached root systems using the spectrophotometric method of LaRue and Kurze (1973). Nodules of each individual root were counted and the total fresh mass was recorded. Plant tops and root were dried at 80°C for 48 h and dry mass was determined. Total nitrogen content of the shoots and roots were determined calorimetrically using Nessler's reagent (Vogel, 1968).

RESULTS AND DISCUSSION

Screening for siderophore-producing ability. When 84 microbial isolates were tested, using four different siderophore assays, for their ability to produce siderophore under iron-limiting conditions, 42 isolates were positive (Table II). It was obvious that, all positive isolates produced strong or moderate reaction using the CAS assay, while these isolates showed no reaction with the phenolates assay. From fungal isolates, all positive *Aspergillus* isolates as well as *P. chrysogenum* produced moderate and strong reactions with the hydroxamate and citrate assays, respectively. All positive bacterial isolates produced moderate reaction with

Table II. Siderophore production by different microbial species grown under iron deficiency

Microorganisms	No. of tested isolates	No. of positive isolates	Activity in different siderophore assays on medium			
			CAS	Hydroxamate	Phenolates	Citrate
<i>Aspergillus flavus</i>	9	3	++	++	-	+++
<i>A. fumigatus</i>	8	4	++	++	-	+++
<i>A. nidulans</i>	9	3	+++	++	-	+++
<i>Cunninghamella elegans</i>	7	4	+++	-	-	-
<i>Mucor hiemalis</i>	10	2	++	-	-	-
<i>P. chrysogenum</i>	6	3	+++	++	-	+++
<i>Rhizopus arrhizus</i>	10	7	+++	-	-	-
<i>Trichoderma koningii</i>	9	3	++	-	-	-
<i>Azotobacter Chroococcum</i>	3	3	++	++	-	-
<i>Bacillus cereus</i>	4	2	++	++	-	-
<i>Bradyrhizobium sp.</i>	3	3	++	++	-	++
<i>Pseudomonas aeruginosa</i>	3	3	+++	+++	-	-
<i>Serratia marscescens</i>	3	2	++	++	-	-

Assay scoring: strong reaction (+++), moderate (++), no reaction (-)

the hydroxamate assay except *Ps. aeruginosa*, which showed a strong reaction. All the tested bacterial isolates failed in citrate production except *Bradyrhizobium* strains. When several fungi and bacteria were screened for their siderophore-producing ability, Adriane *et al.* (1999) recorded that fungal and bacterial strains reacted in different manners to the CAS assay. *Aspergillus* strains produced the fastest colour-change reactions in the CAS-blue agar. Moreover, Alexander and Zuberer (1991) found that a large proportion of rhizosphere bacteria that grew on a non-selective medium failed to grow on CAS agar, and several isolates that showed no sign of siderophore production on CAS agar produced siderophore in liquid culture.

Effect of iron. Siderophore production by the tested microorganisms was affected by iron concentration in the growth medium with different degrees (Fig. 1). Hydroxamate siderophore production increased with increasing in iron concentration up to 20 and 40 μM with *Bradyrhizobium* and *Ps. aeruginosa*, respectively. However, maximum siderophore production peak was achieved by *A. nidulans* and *P. chrysogenum* at concentration of 80 μM iron. It was obvious that, increasing in iron concentration above 80 μM resulted in a significant decrease in siderophore production by the tested organisms. In a similar study, Dave and Dube (2000) tested the regulation of siderophore production by 20 fungi and three rhizobacterial *Pseudomonas* in response to iron concentration in the growth media. They found that organisms showed difference in threshold values at which they stopped siderophore elaboration. In nine fungi (3 *Aspergilli*, 1 *Penicillium*, *N. crassa*, *F. dimerum* and 3 mucors) siderophore production was repressed at 3 μM Fe (III). Siderophore production was repressed at 27 μM of Fe (III) in 3 aspergilli, 2 penicillia and 3 pseudomonads. Rest of the fungi had cut off values at 6, 9, 15 and 21 μM of Fe (III) concentration.

Effect of carbon source. Siderophore production by the tested microorganisms was affected by carbon source (Table III). According to our data, glucose proved to be the most suitable source for *Ps. aeruginosa*, *A. nidulans* and *P. chrysogenum* whereas, *B. japonicum* gave its highest yield when grown on mannitol. On contrary, malate appeared to be a poor substrate for siderophore production by the tested isolates. The amount and the type of siderophore produced by an organism depends on the availability of organic and inorganic nutrients (Neilands, 1982; Abd-Alla, 1998).

Table III. Effect of Carbon source on siderophore production

Carbon source	Hydroxamate production ($\mu\text{M mg}^{-1}$ protein)			
	<i>Ps. aeruginosa</i>	<i>B. japonicum</i>	<i>A. nidulans</i>	<i>P. chrysogenum</i>
Mannitol	14.6	19.8	9.8	10.5
Glucose	16.9	17.1	11.4	13.7
Succinate	4.2	4.4	6.2	5.3
Fumarate	5.8	5.5	5.1	4.8
Citrate	3.2	3.8	10.6	9.4
Malate	4.8	5.1	3.4	3.1

Effect of metabolic inhibitors on siderophore production. All metabolic inhibitor agents used in this study (Table IV) dramatically repressed siderophores production by the tested organisms. These results reveal that an energized membrane and a mercapto group are essential and required for siderophore synthesis.

Effect of siderophore producing-organisms on nodulation and nitrogen fixation of mung bean. Inoculation of mung bean (grown in alkaline soil) with siderophore producing microorganisms significantly enhanced nodulation, nitrogenase activity, dry matter accumulation and nitrogen yield (Table V). Mixed inoculum of *Bradyrhizobium* and *P. chrysogenum* achieved the maximum enhancement in all tested parameters. Furthermore, since, the motivation of this work is related to iron deficiency, which effects nodulation and nitrogen

Fig. 1. Effect of iron concentration on siderophore production by some bacteria and fungi. Vertical bars represent LSD at 0.05 level between treatments within each organism

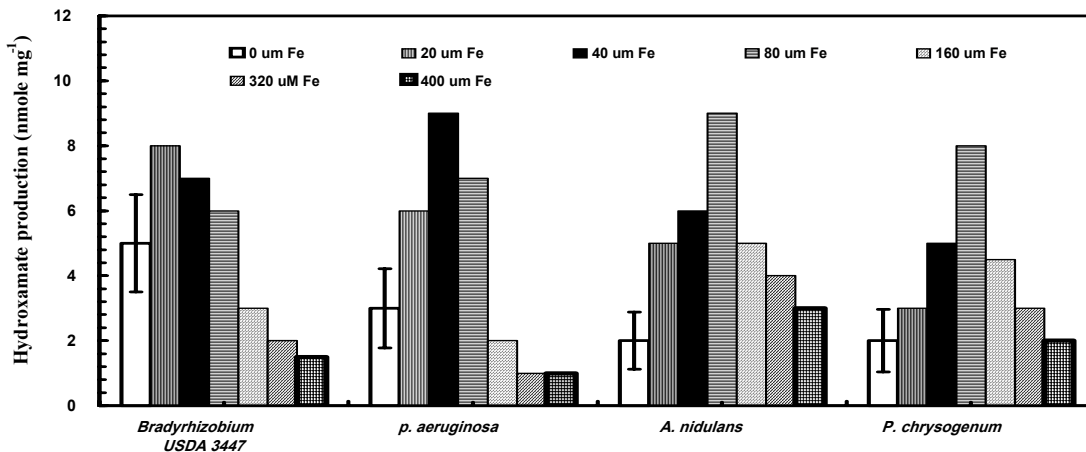


Table IV. Effect of some metabolic inhibitor agents on siderophore production

Compound	Conc. in medium (mmol L ⁻¹)	Hydroxamate production (% of control)			
		<i>Ps. aeruginosa</i>	<i>B. japonicum</i>	<i>A. nidulan</i>	<i>P. chrysogenum</i>
PCMBs	0.1	38	28	24	30
	0.2	20	17	14	18
2,4-D	0.2	47	34	30	26
	0.4	26	21	19	17
CCCP	0.1	32	20	26	21
	0.2	14	11	14	12
HgCl ₂	0.1	35	22	19	23
	0.2	15	13	11	11
Sodium azide	0.2	45	49	28	20
	0.4	19	27	16	9

fixation, the results obtained *in vitro* were confirmed *in vivo*. The trends of our results agree with those of Marek-Kozaczuk *et al.* (1996) and Abd-Alla (1998, 1999).

There are some reports that specific siderophore producing microorganisms stimulated the nodulation, nitrogen fixation and plant growth of leguminous plants (Grimes & Mount, 1987; Omar & Abd-Alla, 1994; Shenker *et al.*, 1999). The authors suggested that, nodulation promoting microorganisms could be included in rhizobial inoculants to improve efficacy of these products. The mechanisms of action under field and laboratory conditions are still under investigation. One of the possible modes of growth promotion of nodulated legumes under field conditions, is production of siderophores which control the proliferation of soil-born pathogens or facilitate the uptake of iron from environment (Schippers *et al.*, 1987; Omar & Abd-Alla, 1998). A nodulated legume has an increased need for iron compared to a non-nodulated plant (Guerinot, 1991; Deryto & Skorupska, 1992) since this metal is a constituent of key proteins such as nitrogenase and leghaemoglobin. Nitrogenase is made up of two proteins, both are rich in iron and essential for activity. Non-heme iron electron transfer proteins such as ferredoxin and flavodoxin are essential in nitrogen fixation (Eady & Postgate, 1974). Moreover, microbial siderophores may be involved in the biosynthesis of leghaemoglobin and iron containing enzymes such as hydrogenase, peroxidase and catalase. High levels of catalase had been reported in the effective nodules of some

Table V. Effect of siderophore-producing organisms on nodulation, nitrogenase activity and plant growth of mung bean (*Vigna radiata*) cv. Berkum (n=5)

Microorganism	Nodules plant ⁻¹		Nitrogenase activity (μmol C ₂ H ₄ plant ⁻¹ h ⁻¹)	Dry weight (mg plant ⁻¹)		Total nitrogen (mg plant ⁻¹)	
	No.	Fresh wt (mg)		Shoots	Roots	Shoots	Roots
Bradyrhizobium (mung bean) USDA 3447	90	480	7.5	1110	512	55	14
Bradyrhizobium (mung bean) USDA 3447 + <i>Pseudomonas aeruginosa</i>	150	810	10.2	1600	780	85	25
Bradyrhizobium (mung bean) USDA 3447 + <i>P. chrysogenum</i>	173	920	12.4	1815	870	102	29
LSD (P=5%)	20	105	1.8	196	95	19	10

legumes (Francis & Alexander, 1972). Furthermore, Shenker *et al.* (1992) and Wolfgang *et al.* (2000) recorded that siderophore mixture from some fungi are an excellent source for Fe nutrition of non-leguminous plants. They found that siderophore mixture from *P. chrysogenum* and *Rhizopus arrhizus* significantly improved the iron status of cucumber, maize and tomatoes plants as measured by chlorophyll concentration to the same degree as high as from FeEDTA supply.

A number of soil-inhabiting fungi have been shown to release both citric and malic acids; release of organic acids in response to iron stress is well documented for *Neurospora crassa* (Holzberg & Artis, 1983). The acids excreted by these fungi are proposed to interact with iron which has been concentrated at the cell surface, solubilizing the iron and making it available for use by the fungi (Winklemann, 1979). Although the surface of rhizobia has been the recognition and attachment of rhizobial cell to host plant cell and its apparent role in nodule formation itself, there are no reports of surface iron deposition per se. It is also possible that the citric acid released by the rhizobial cells in the rhizosphere aids plants (Guerinot *et al.*, 1990). The ecological advantages in the synthesis of microbial siderophores encourage the use of such microbes as inoculants with root nodule bacteria. Therefore, further studies about the use of such mixed inoculants in iron deficient alkaline soil are mandatory.

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