1.1. Importance of iron and its availability in the soil

The importance of iron in our civilization is perhaps best summarized by the quotation from Rudyard Kipling cited by Philip Aisen in his article on physico-chemical aspects of iron metabolism.

"Gold is for the mistress – silver for the maid - Copper for the craftsman cunning at his trade!
'Good!' said the Baron, sitting in his hall, 'But iron' - Cold Iron - is master of them all"

Certainly, it is due to its biological abundance in the earth’s crust that the practical value of this element was first recognized, particularly compared with elements such as gold, silver, and copper. Iron (Fe) is the fourth most abundant element on Earth’s crust, following oxygen, silicon, and aluminum, mostly found in the form of ferro-magnesium silicates or iron oxides and hydroxides, forms that are not readily utilizable by microorganisms and plants. It has the ability to exist in two forms, the reduced Fe^{2+} (ferrous form) and the oxidized Fe^{3+} (ferric form), but under aerobic conditions, it exists only in the Fe^{3+} form which is not directly available to life forms. In fact, owing to its redox potential ranging from -300 to 1700 mV (Andrews et al., 2003), iron is an all-round prosthetic component for incorporation into proteins that act as biocatalysts or electron carriers.

In aerobic environments, iron generally forms insoluble precipitates of ferric hydroxide [Fe(OH)_3], the solubility of which in the soil is calculated to be 10^{-38} M (Guerinot, 1994) due to which the availability of free Fe^{3+} is as less as 10^{-17} M, which decreases further as follows with rise in pH.

\[
\text{Fe(OH)}_3 = \text{Fe}^{3+} + 3\text{OH}^-
\]

\[
K_{sp} [\text{Fe(OH)}_3] = [\text{Fe}^{3+}] [\text{OH}^-]^3
\]

\[
K_{sp} [\text{Fe(OH)}_3] = 10^{-38} \text{ M}
\]

\[
[\text{Fe}^{3+}] = 10^{-38} / [\text{OH}^-]^3 = 10^{-38} / (10^{-7})^3 = 10^{-38} / 10^{-21} = 10^{-17} \text{ M}
\]

(Because at pH 7.0, the solubility constant of free OH^- ions will be 10^{-7} M). It can be calculated here that pH increase by one unit, decreases the solubility of free Fe^{3+}, thousand fold i.e., at pH 8.0, solubility constant of Fe^{3+} will be 10^{-20} M and so on.
Iron is a vital element required by virtually all living organisms, including bacteria, with the exception of only a few, including *Streptococcus sanguis*, some *Lactobacillus* species, and *Borrelia burgdorferi* (Guiseppi and Fridovich 1982, Archibald 1983, Posey and Gherardini 2000). It is important in many cellular processes including the electron transport chain and in deoxyribonucleotide synthesis and acts as a cofactor for many enzymes, such as ribotide reductase, nitrogenase, peroxidase, catalase, and succinic dehydrogenase (Litwin and Calderwood 1993). In humans, it is a critical component of hemoglobin and myoglobin. Iron deficiency is also associated with decreased immune function, shortened attention span and reduced ability to learn. Iron needs are greatest during periods of rapid growth such as childhood, adolescence, pregnancy and child-bearing years for women. For plants, iron is a catalyst to chlorophyll formation, and a component in several molecules eg., leghemoglobin and those involved in plant respiration and photosynthesis.

### 1.2. Iron requirement by various organisms

Iron requirements of life forms vary, eg., $10^{-9}$ M is required for optimal growth of plants while that required for the optimal growth of microbes is in the range of $10^{-7}$ to $10^{-5}$ M (Raymond *et al.* 2003), both of which are far greater than the biological availability which is $10^{-17}$ M, at physiological pH 7.0. Therefore there is always an “iron stressed” condition prevalent in the soil. Microorganisms has developed various mechanisms in order to overcome the low bioavailability of iron, generally involving synthesis and secretion of low molecular weight, iron specific chelators known as “siderophores”, which are iron carriers with a very high affinity, $K_{afr} > 10^{30}$ M$^{-1}$, for ferric iron and whose biosynthesis is carefully regulated by ferric iron (Byers & Arceneaux, 1998) The first siderophore to be isolated was ferrichrome (Neilands, 1979) from the culture filtrate of the smut fungus *Ustilago sphaerogena* (Neilands, 1979).

### 1.3. Acquisition of iron by life forms:

#### 1.3.1. In humans:

Iron is termed a “micronutrient” because the mineral is essential for life in very small amounts. Humans and other higher animals acquire iron through their diet. In humans iron is stored as bound to iron binding proteins- transferrin and lactoferrin, and thus its availability is restricted to infecting pathogens.
1.3.2. **In higher plants:** Iron uptake in plants is also equally important because plant material is the major source of iron for most human beings. Dicotyledonous plants when subjected to Fe-deficiency, decrease pH in the rhizosphere by proton excretion and reduce ferric iron by an activated reduction system in the plasma membranes of the root or by reductants released from the roots. The efficiency by which these plants take up Fe may strongly depend on their cation-anion balance (Zaharieva and Romheld, 2004). Other mechanisms found in all dicots and monocots with an exception of graminaceous plants, include induction of plasma-membrane bound reductase as well as release of both “reductants” and chelating root exudates (Romheld, 1987). In graminaceous plants, iron limited conditions induce synthesis of phytosiderophores to gather with specific uptake systems for transport of the ferri-siderophore complex into the cell (Marschner et al., 1986).

1.3.3. **In microbial systems:** Iron acquisition through siderophore production is the most common mechanism. The iron transport systems in microorganisms will be discussed under two subheadings: (a) iron acquisition by pathogens in human host (b) iron acquisition amongst rhizospheric microorganisms.

1.3.3.1. **Pathogenic microorganisms:** In human systems, iron is bound to heme and heme compounds like hemoglobin, haptoglobin, and hemopexin, and iron storage proteins lactoferrin and transferrin, which the pathogen counteract by producing siderophores. *Vibrio cholerae* synthesizes and secretes catecholate siderophore vibriobactin, the ferri-vibriobactin complex is transported inside the cell by the OM receptor ViuA (Wyckoff et al., 2001). *Pseudomonas aeruginosa*, an important human opportunistic pathogen, produces two siderophores, pyoverdine and pyochelin (Poole and McKay, 2003). Iron is central to pathogenesis to the extent that mutants deficient in the uptake ferri-siderophores complex fail to show pathogenicity. The success of the highly adapted obligate human pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae* is attributed to the expression of multiple high-affinity iron transporters including a family of two-component TonB-dependent receptors as well as multiple single-component TonB-dependent Fe transporters (Schryvers and Stojiljkovic, 1999). In *E. coli*, ChuA is the receptor for heme (ShuA in *Shigella*) and these receptors show some
sequence identity with siderophore receptors (Wandersman and Stojiljkovic 2000). Similar systems have been described, including HemR in *Yersinia enterocolitica* and HmbR and HpuA in *Neisseria meningitidis*, but are not well defined (Wandersman and Stojiljkovic 2000).

**1.3.3.2. Soil microorganisms:** As discussed above the availability of iron in soil is far less than that required for the growth of microorganisms. The most common mechanism by which soil bacteria (endophytes, epiphytes, rhizospheric microorganisms) and fungi acquire iron is through siderophore production. *Information under this heading is available throughout the thesis.*

**1.3.3.3. Saprophytic organisms:** They are also reported to produce siderophores. The independent identification of the *des* and *cch* gene clusters that direct production of the tris-hydroxamate ferric iron-chelators desferrioxamine E and coelichelin, respectively, which could potentially act as siderophores in the saprophyte *Streptomyces coelicolor* A3(2), has recently been reported (Barona-Gomez *et al.*, 2006).

**1.3.3.4. Marine microorganisms:** The iron availability in marine environments is very limited, but siderophore production was not observed to be a regular feature. More than 60% of species examined from a total of 421 strains of heterotrophic marine bacteria which were isolated from marine sponges and seawater were observed to have no detectable siderophore production even when Fe$^{43}$ was present in the culture medium at a concentration of 1.0 pM but growth of siderophore non-producing organisms was stimulated upon addition of exogenous siderophores, pointing towards the existence of siderophore uptake systems in marine bacteria (Guan *et al.*, 2001).

**1.4. Siderophore production: the most common mechanism for iron acquisition by microorganisms – in detail**

In aerobic environments, as most of the iron is present in the oxidized ferric form, and hence is in its unavailable form, most of the soil dwelling organisms produce siderophores. Siderophores are low-molecular-weight iron chelators, secreted by the organisms in conditions of iron deficiency and bind ferric (Fe$^{43}$) ion with high affinity, but with respect to the structure they possess, a less accurate definition which can be given to them is that they are small peptidic molecules, readily
assembled by short, dedicated metabolic pathways and which contain side chains and functional
groups which make them high-affinity ligands for coordination (Crosa, 1989; Neilands, 1981;
Neilands, 1995).}

1.5. Types of siderophores
Different organisms produce different types of siderophores, and they are categorized on the basis
of the iron chelating functional group they possess. Fig. 1.1 shows the structural formulas for siderophores from various bacteria. There are four main types of iron-coordinating functional
groups in siderophores.

1.5.1. Hydroxamate type: Siderophores which possess hydroxamic acids as the functional group. Eg., anguibactin, ferrichrome (Fig. 1.1), rhodotorulic acid.

1.5.2. Catecholate type: Hydroxyls of catechol rings bind to iron, Eg., enterobactin (Fig. 1.2) anguibactin, and acinetobactin.
1.5.3. Ferrioxamine type: represented by ferrioxamine B the mesylate salt of which is marketed by Ciba-Geigy as Desferal, the major drug used in chelation therapy of secondary iron overload in man (Fig. 1.3).

Fig. 1.1: (A) Ferrichrome, the prototype of the hydroxamate type of siderophores (Neilands, 1981) and (B) Desferrichrome (Byers and Arceneaux, 1977)

Fig. 1.2: Spatial structures of enterobactin without and with Fe$^{3+}$. The groups coordinating with iron are six oxygens from the three diphenolic groups. The complex requires a single molecule of enterobactin.
1.5.4. **Nitrogen atoms of five membered thiazoline and oxazoline rings**: They result from enzymatic cyclization of cysteinyl, seryl or threonyl side chains, respectively, and can also coordinate Fe$^{3+}$. This type of coordination is a common feature in the pyochelin, yersiniabactin, vibriobactin, anguibactin, and acinetobactin siderophores.

1.5.5. **Citrate type**: Carboxylate ion participates in iron binding, the example of which is citrate siderophore produced by *Bradyrhizobium japonicum* strains (Guerinot et al. 1990).

1.5.6. **Complex siderophores**: When different iron-chelating functional groups are combined in the same siderophore, such as mycobactin and anguibactin.

1.6. **Pyoverdines**: Several *Pseudomonas* sp. produce and excrete fluorescent yellow-green siderophores, named pyoverdines or pseudobactins (Budzikiewicz, 1993; Meyer, 2000), which are composed of a conserved dihydroxyquinoline chromophore and a variable peptide chain. The diversity in the peptide chain is so large that pyoverdines of different strains can be easily differentiated by isoelectric focusing and this approach termed “siderotyping” can be used to distinguish different *Pseudomonas* sp.
Apart from these, there are also a few exceptional siderophores which cannot be classified into any of the above groups, eg., **rhizobactin** produced by *S. meliloti* DM4, which possess ethylenediamine as the chelating ligand (Smith et al., 1985). **Mugineic acid** is a phytosiderophore excreted by roots of graminaceous plants for Fe³⁺ up take by plants. Mugineic acid is closely related to its biochemical precursor, nicotinamine, and to a number of other compounds that also have been identified as phytosiderophores in graminaceous plants: 3-hydroxymugineic acid, 2'-deoxymugineic acid, avenic acid and distichonic acid.

**Rhizobactin 1021** produced by *Sinorhizobium meliloti* 2011, is a citrate-based dihydroxamate siderophore that has a core structure identical to that of sehizokinin from *Bacillus megatarium* (Persmark et al., 1993).

### 1.7. Mechanisms other than siderophore production to acquire iron

In addition to producing one or multiple siderophore acquisition systems, bacteria have evolved other mechanisms for obtaining scarce iron from a wide range of environments. Many Gram-negative bacteria possess a system for transporting ferrous iron, termed the Feo system, which is typically only expressed under anaerobic conditions (Kammler et al. 1993). In some bacteria this system is essential, particularly in organisms that colonize the stomach or intestine, such as *Helicobacter pylori* and *Salmonella enterica* (Wandersman and Delepelaire 2004). Another putative mechanism for iron acquisition is the SitABCD system found in *Salmonella* and *Shigella* species and in enteroinvasive *E. coli*. Depending on the organism, this system is thought to transport ferric or ferrous iron (Runyan-Janeccky et al. 2003). It is not certain that this system transports iron, but studies have shown it is partially regulated by Fur and available iron in the environment (Janakiraman and Slauch 2000). Additionally, it has been shown that induction of the *sit* genes can improve growth of mutants deficient in iron transport (Runyan-Janeccky et al. 2003).

### 1.8. What is iron siderophore affinity?

The formation constants of the ferric siderophore complexes is of particular interest, since these constants define the ability of the siderophore to extract iron from other biological ferric complexes like lactoferrin, transferrin (animal host), phytoferritins (plant host) and other
siderophore complexes produced by other organisms in the soil. It is due to these affinity differences that complex interactions occur in the rhizosphere with respect to iron nutrition. The affinity of the siderophore mainly is a result of its structure, the aminoacid composition and the way they are arranged with respect to the iron chelating group present.

Given below are the affinity constants or the formation constants (at pH 7.0) of ferric-siderophores commonly found in soil (Guerinot, 1994):

- Ferrichrome, made by numerous fungi - $10^{-25}$
- Desferrioxamine B, made by actinomycetes - $10^{-27}$
- Enterobactin – $10^{-52}$
- Pseudobactin (pyoverdin), made by pseudomonads - $10^{-25}$
- Mugineic acid, a phytosiderophore - $10^{17}$

The three catecholate side chains in *E. coli* enterobactin makes it a complete hexadentate ligand which can bind to ferric ion, accounting for the estimated $K_d$ (Dissociation constant) of $10^{-52}$ M that makes enterobactin such a good scavenger for iron (Neilands, 1981; Neilands, 1995) (Fig. 1.2).

1.8.1. Significance of iron-siderophore affinity in a nich colonization

Plant deleterious organisms possessing siderophores with comparatively high affinity, succeeds in colonizing plant rhizosphere and hence cause plant disease. Similarly animal pathogens having siderophores with high affinity for iron can successfully sequester iron from host iron storage proteins- transferrin and lactoferrin and establish pathogenesis. Based on this concept it is obvious that if plant growth promoting (PGP) organisms (free-living of symbiotic) used as bioinocula produce siderophore of significant high affinity, it will not only succeed to establish itself, but will also be able to inhibit the growth of pathogenic organisms and hence can act as a biocontrol agent (Chakraborty and Purkayastha, 1984; Ehteshamul-Haque *et al.*, 1992).
1.9. Significance of biological nitrogen fixation (BNF) and its dependence on iron

Symbiotic bacteria, are plant-interacting microorganisms of major agronomic importance. *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium* are some of the well-known genera of rhizobia, nitrogen-fixing bacteria, which live in soil and the rhizosphere and may enter into symbioses with leguminous plants such as peas, alfalfa, clover and soybeans. They induce the formation of root nodules in which they persist as nitrogen fixing bacteroids. They produce nitrogenase enzyme complex and nod factors, which are important in symbiosis with their host plant. They provide the major biological source of fixed nitrogen in the soil, estimated at over $2 \times 10^{13}$ grams per year (Falkowski 1997), they are therefore being used commercially as bio-inoculants to increase legume plant productivity (Carson *et al.* 2000). About 65% of the biosphere’s nitrogen is produced via the biological reduction of atmospheric nitrogen to ammonium, which is mediated to a large extent by legume–rhizobia symbioses (Lodwig *et al.*, 2003). This symbiosis is highly dependent on iron due to several proteins involved in the process containing iron. The nitrogenase enzyme complex which reduces molecular nitrogen to ammonium constitutes up to 10–12% of the total soluble bacteroid protein (Verma & Long, 1983). Leghemoglobin, which represents about 25–30% of the total soluble protein in infected plant cells; is crucial for symbiosis as it serves in binding and transport of oxygen, thereby maintaining a low concentration of free oxygen and avoiding the inactivation of the oxygen-labile nitrogenase (Ott *et al.*, 2005). The importance of iron in the symbiosis has been demonstrated by O’Hara *et al.* (1988), who showed that iron deficiency limits nodule development in peanuts inoculated with *Bradyrhizobium* species.

1.10. Siderophore production and utilization in rhizobiales (nodulating group of bacteria) and its implications in Rhizobium -legume symbiosis

In addition to the iron requirement during the nitrogen fixation process, rhizobia must be also able to survive and grow in the soil and be able to colonize the host plant rhizosphere. Siderophore production by rhizobia is of particular interest due to the prominent role of iron in the nitrogen fixation and assimilation process. As in other organisms, siderophore production in rhizobia is strain specific. For eg., *Rhizobium meliloti* DM4 produces rhizobactin in which...
ethylenediamine is the iron chelator (Smith et al., 1985), whereas a dihydroxamate siderophore rhizobactin 1021 is produced by Sinorhizobium meliloti under iron stress, rhhABCDEF genes are responsible for its biosynthesis (Lynch et al., 2001). Rhizobium leguminosarum bv. viciae, synthesizes a cyclic trihydroxamate type siderophore called vicibactin which is taken up by cells via outer and inner membrane machinery encoded by fhuABCD operon (Dilworth et al., 1998). Catecholate siderophores are known to be produced by rhizobia from the cowpea group (Jadhav and Desai, 1992; Modi et al., 1985) and catecholates viz., salicylic acid and dihydroxybenzoic acid are produced by Rhizobium ciceri isolates from chickpea nodules (Berraho et al., 1997). Citrate as a siderophore is released by Bradyrhizobium japonicum under iron-limited conditions (Guerinot et al., 1990) but it also capable of utilizing ferrichrome and rhodotorulic acid, siderophores from fungal origin (Plessner et al., 1993). Siderophore production and cross-utilization has good implications for free-living rhizobia of being able to survive better in the soil as compared to siderophore non-producing strains (Raaijmakers et al., 1995). There is a high demand of iron in the nitrogen fixation symbiosis, although the mechanism by which it is supplied to the root nodule is not yet understood. It is reported that iron nutrition of the bacteroids in the nodules is through ferrous form of iron which become available to the bacteroids through the reductases present on the peribacteroid membrane (Moreau et al., 1998). It is for this reason that in certain cases biosynthesis of siderophore and its up take system in rhizobia is not in direct concurrence with the nitrogen fixation efficiency. A fhuB mutant of Rhizobium leguminosarum, defective in the up take of siderophore vicibactin, produced apparently normal nitrogen fixing nodules on peas (Stevens et al., 1999). By contrast there are reports in R. meliloti 1021, that the ability to produce and take up siderophore may significantly increase the ability of the differentiated bacterium to fix nitrogen leading to an increase in plant growth (Gill et al., 1991). It is also reported that iron deficiency in peanuts results in arrested nodule development with fewer bacteroids in nodules, decreased amounts of leghemoglobin and decreased nitrogenase activity (O’Hara et al., 1988a). An adequate supply of iron is therefore essential for the establishment of a functional symbiosis. It has been suggested that differences in nodule development under iron deficient conditions may be due to varying abilities of different strains of root nodule bacteria to acquire iron for nodule initiation and development (O’Hara et al., 1988b).
1.11. Mechanisms of transport of ferri-siderophores from outside the cell to its interior

1.11.1. Outer Membrane Receptors

Extensive research has been done to study the ferri-siderophore uptake in gram-negative bacteria (Braun et al., 1998; Braun 1998; van der Helm, 1998). Ferri-siderophore complexes are actively transported across the OM of Gram-negative bacteria through a specific OM receptor proteins, whose genes are also induced only under iron-deficient conditions (Earhart and McIntosh 1977). The crystal structures of four outer membrane receptor proteins responsible for transporting ferric-siderophore complexes have recently been solved. These include FepA (Fig.1.4A), which transports ferric enterobactin (Buchanan et al. 1999), FhuA (Fig.1.4B), which transports ferrichrome (Ferguson et al. 1998), FecA (Fig.1.4C), which transports ferric dicitrate (Ferguson et al. 2002), and FpvA, which transports ferric pyoverdine (Cobessi et al. 2005). The crystal structures of the receptors show remarkable similarities; they are each composed of a β-barrel, consisting of 22 anti-parallel β-strands and a globular domain, which is referred to as a “cork” or “plug” because of the way it is oriented into the β-barrel domain (Endriss and Braun 2004). The β-barrel has short periplasmic loops and longer extracellular loops, which allow entrance of the ligand (Chakraborty et al. 2003).

![Fig. 1.4: Crystal structures of (A) FecA (ferric-citrate receptor), (B) FhuA (ferrichrome receptor) and (C) FepA (ferric-enterobactin receptor)](image-url)
Most of the receptors are multifunctional. Thus FhuA also serves as the receptor for the antibiotic albimycin, colicin M and phages T1, F5, and φ80; IutA for cloacin DF13, synthesized by *Enterobacter cloacae*; Fepa for colicins B and D; and Cir for colicin I and V, where the latter is a microcin rather than a colicin (Braun *et al.*, 1994).

1.11.2. Passage of the whole ferri-siderophore complex into the cytoplasm

This is a widespread mechanism in most of the gram negative bacteria. The OM receptors are very specific to the ferri-siderophore it identifies, as shown in table 1, the ferric complexes of ferrichrome, coprogen, aerobactin, enterochelin, salmochelin, dihydroxybenzoic acid and citrate, bind specifically and without any receptor cross-reactivity to their respective outer membrane proteins. The structure of the OM receptor is as shown in Fig. 1.4. The transport of the ferrisiderophore through the specific receptor is an energy dependent process. Translocation of iron through the bacterial outer membrane (Fig. 1.5) as the ferric-siderophore requires the formation of an energy transducing complex with the proteins TonB, ExbB, and ExbD, which couple the electrochemical gradient across the cytoplasmic membrane to a highly specific receptor and so promote transport of the iron complex across the outer membrane. In the periplasm, they probably bind to so-called binding protein in the periplasm (Koster and Braun, 1990). The periplasmic binding proteins loaded with ferric siderophore complex are presumed to activate the ATPases FhuC, FepC and FecE. ATP hydrolysis provides energy for opening the presumptive channels, formed in the cytoplasmic membrane by FhuB, FepDG and FecCD and the ferrisiderophore complex is transported into the cytoplasm.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Outer Membrane protein</th>
<th>Periplasmic protein</th>
<th>Cytoplasmic membrane proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobactin</td>
<td>FepA</td>
<td>FepB</td>
<td>FepD, FepG, FepC</td>
</tr>
<tr>
<td>Salmochelin</td>
<td>IroN</td>
<td>FepB</td>
<td>FepD, FepG, FepC</td>
</tr>
<tr>
<td>Catecholates</td>
<td>Cir</td>
<td>FepB</td>
<td>FepD, FepG, FepC</td>
</tr>
<tr>
<td>Catecholates</td>
<td>Fiu</td>
<td>FepB</td>
<td>FepD, FepG, FepC</td>
</tr>
<tr>
<td>Ferrichrome</td>
<td>FhuA</td>
<td>FhuD</td>
<td>FhuB, FhuC</td>
</tr>
<tr>
<td>Aerobactin</td>
<td>IutA</td>
<td>FhuD</td>
<td>FhuB, FhuC</td>
</tr>
<tr>
<td>Coprogen/</td>
<td>FhuE</td>
<td>FhuD</td>
<td>FhuB, FhuC</td>
</tr>
<tr>
<td>Rhodotorulic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>FecA</td>
<td>FecB</td>
<td>FecC, FecD, FecE</td>
</tr>
<tr>
<td>Heme</td>
<td>ChuA</td>
<td>ChuT</td>
<td>ChuU, ChuV</td>
</tr>
<tr>
<td>Yersiniabactin</td>
<td>FyuA</td>
<td>NI</td>
<td>YbtP, YbtP</td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td></td>
<td></td>
<td>FeoB</td>
</tr>
</tbody>
</table>

(Braun, 2005)

It could be observed in the above table that the outer membrane receptor is highly specific for a particular ferrisiderophore complex, while the cytoplasmic and periplasmic membrane machinery has relatively relaxed specificity.
1.11.3. Siderophore shuttle mechanism of iron transport

Here, the whole ferrisiderophore complex does not enter the cytoplasm, only the iron(III) molecule enters inside, by a siderophore shuttle mechanism. The mechanism can be explained as follows:
Fig. 1.6: Proposed model of the siderophore shuttle iron exchange mechanism for iron transport in gram-negative bacteria.

The iron-free siderophore (in red) is in large excess over iron-loaded siderophore (in blue) and consequently predominates as bound to its cognate receptor (Fig. 1.6A). Iron-loaded siderophore is brought close enough to the iron-free bound siderophore to promote the exchange of iron. The ligand exchange induces a conformational change of the N-terminal globular domain (Fig. 1.6B, as represented in orange), signaling the iron-loaded status of the bound siderophore to the protein TonB (Fig. 1.6C). Energized TonB then triggers a conformational change of the receptor, allowing translocation of the ferric-siderophore from the cell surface to the periplasmic space. The ferric-siderophore that gave up its iron then binds to the receptor, replacing the transported ferric-siderophore complex (Fig. 1.6D). Finally, the receptor returns to its initial conformation. The net result is a shuttle mechanism in which siderophore ligands pass serially through a channel, with iron exchange between the siderophores a key step in the process. This mechanism suggests an increase of the iron uptake rate, with increasing concentration of iron-free siderophore. The ability to select for Fe$^{3+}$ via ligand exchange enables production of a single receptor with a lower affinity for its cognate siderophore but with a broad range of siderophore recognition, as observed in *A. hydrophila*. In a medium with a low ferric-siderophore concentration, this iron acquisition strategy is more efficient than one in which iron is acquired via a set of receptors (usually inducible), each of which is specific to a single siderophore. This
mechanism is observed in *Aeromonas hydrophila, E coli, and P. aeruginosa.* It is proposed that this mechanism may be widespread in organisms.

### 1.12. Distribution of siderophore receptors among rhizobiales

There are a number of reports of Iron Regulated Outer Membrane Proteins (IROMPs) in rhizobia, correlating with the production and release of specific siderophores (Jadhav and Desai, 1994; Reigh and O’Connell, 1993; Patel *et al.,* 1994). Not many OM siderophore receptors are reported in rhizobia.

1. The rhizobial ferrichrome OM receptor is the hydroxamate-type siderophore receptor **FegA** of *B. japonicum* 61A152 (LeVier and Guerinot, 1996). The *fegA* gene is organized in an operon with *fegB* which probably encodes an inner membrane protein (Benson *et al.,* 2005). Mutant analysis revealed that both genes are required for utilization of the siderophore ferrichrome, and a *fegAB* double mutant, but not a *fegB* mutant, fails to establish a normal symbiosis. Soybean nodules elicited by the *fegAB* mutant lack leghemoglobin and do not fix nitrogen (Benson *et al.,* 2005).

2. The **FhuA** of *Rhizobium leguminosarum* specifies the OM receptor which works in association with FhuCDB (inner membrane proteins) for the uptake of vicibactin. FhuC is the ABC-transporter ATPase, FhuB is the permease and FhuD is the periplasmic siderophore binding protein which brings the ferri-vicibactin complex to the inner membrane machinery for its transport from the periplasm to the cytoplasm.

3. **RhtA** is the OM receptor responsible for rhizobactin uptake in *Sinorhizobium meliloti,* where a specialized single permease RhtX is responsible for its transport from periplasm to cytoplasm (O’Cuiv *et al.,* 2004).

4. **HmuR** is the heme receptor in *Bradyrhizobium japonicum* (Nienaber *et al.,* 2001)

Overall, iron uptake genes are not very widely studied in rhizobia. Using BLASTp tool, homologs of FegA were searched in the whole genome databases and was found that a homolog 42% identical to FegA is present in *S. meliloti* (SMc01611), and that with 84% identity to FegA is present in *B. japonicum* strain 110 (Bll4920) Based on sequence similarity to FegA of *B. japonicum* 61A152, strain 110 encodes additional candidate OM receptor protein Bll7968 (26%
identical to FegA), most probably organized in an operon with bll7967, whose predicted product shows weak similarity to FegB (24% identity).

1.13. The TonB-ExbB-ExbD complex

In many Gram-negative bacteria, the proteins TonB, ExbB and ExbD form a complex that transduces the energy provided by the electrochemical charge gradient of the cytoplasmic membrane in order to transport iron-loaded siderophores through OM receptors (Moeck & Coulton, 1998). The TonB protein is associated with both the inner and the outer membrane, with a large part of the protein occupying the periplasmic space. TonB spans the periplasm and directly contacts OM active transport proteins. TonB mediated uptake of iron-loaded siderophores requires interaction of TonB proteins with the TonB box of receptors. The TonB box which is located at the N-terminus of OM receptors, is a moderately conserved stretch of hydrophobic aminoacid residues (Ferguson & Deisenhofer, 2002). Located on the periplasmic side, it enables physical interaction with the TonB protein. TonB provides the structural link between the proton motive force (pmf) of the inner membrane and high-affinity transporters of siderophores, heme or vitamin B12 through the OM. The importance of the TonB box was shown previously for FhuA and FepA (Braun et al., 2003) and more recently for HasR, the heme uptake receptor of S. marcescens (Wandersman & Delepelaire, 2004). It was reported recently that HasR, lacking the TonB-box-containing plug, is nonetheless inserted into the OM and still binds the hemophore HasA. However, TonB-dependent active transport of the HasA-bound heme is abolished (Letoffe et al., 2005). The three proteins TonB, ExbB and ExbD seem to act as a complex, as ExbB and ExbD interact with each other in vitro (Braun et al., 1996) and TonB can be cross-linked in vivo to ExbB through transmembrane domains and to ExbD through periplasmic domains (Higgs et al., 1998; Postle & Kadner, 2003). Different models for TonB-dependent energy transduction have been proposed. The 'shuttle model' is based on in vivo labeling of the TonB N-terminus with cysteine-specific fluorescent markers. This model postulates that ExbB and ExbD transduce the pmf to TonB whose amino-terminal signal anchor breaks its association with the CM. TonB then shuttles to the OM to deliver conformationally stored potential energy to OM transporters and becomes associated with the OM. Discharged TonB is then recycled to the CM to be re-energized by ExbB and ExbD (Larsen et al., 2003; Postle & Kadner, 2003). The 'propeller model' is derived from crystallographic studies of the C-terminal TonB domain (Chang et al., 2004; Higgs et al., 1998; Postle & Kadner, 2003).
2001). The model suggests that two intertwined TonB monomers interact with OM receptors and undergo a torsional motion that is probably caused by the proton gradient across the CM. Thus, a mechanical force is transduced from the inner to the outer membrane, a mechanism similar to that described for the bacterial flagellar powered by the ExbB/ExbD homologs MotA/MotB (Zhai et al., 2003). For unknown reasons, some bacteria possess only one TonB–ExbB–ExbD system (e.g. E. coli) whereas others such as Vibrio cholerae have two TonB systems, one of which is necessary for hemin utilization (Seliger et al., 2001). A B. japonicum strain lacking exbD–tonB (bll7072–bll7071) is still able to use ferrichrome or ferric citrate as iron source, suggesting an alternative TonB–ExbB–ExbD system. Indeed, a putative second copy of exbBD–tonB is encoded by the genes Bfr3906–3908 in the B. japonicum genome (Nienaber et al., 2001).

1.14. Periplasmic binding proteins

Once inside the periplasm, specific periplasmic binding proteins (PBP) have been identified that bind the ferric siderophore complexes. These proteins are generally synthesized at a lower level compared with PBPs that bind amino acids or sugars and have a lower affinity for ferrisiderophore complexes as compared with OM receptors (Sprenzel et al. 2000). They have a broad specificity, for eg., FhuD which bind the siderophore ferrichrome, also binds to other hydroxamate type siderophores aerobactin, copragen, and rhodotorulic acid. This can be explained by a wider and shallower binding site than that of other PBPs (Wandersman and Delepelaire 2004). The crystal structure of FhuD has been solved (Clarke et al. 2000), and from this it is thought that human iron-binding proteins, transferrin and lactoferrin, evolved from bacterial PBPs as they resemble the N- and C-terminal lobes of these proteins (Braun and Braun 2002).

The PBP-ferric siderophore complex provides a substrate for the next component of these iron transport systems, the ATP-dependent ABC-type transporter. It is not in all organisms that PBPs are involved in the transport of ferrisiderophore complexes. For eg: In E. coli for the transport of ferrichrome a Fhu-PBT (Fhu- Periplasmic Binding protein dependent Transport) system occur. But in B. japonicum, transport of ferrichrome is through a two-component system (Benson et al., 2005). FegA transports it from outside to the periplasm and a specialized permease FegB transports it from periplasm to the cytoplasm. No periplasmic binding protein is involved to carry the ferrisiderophore complex to the inner membrane protein. A similar system of transport of
rhizobactin occurs in *S. meliloti* (O’Cuiv et al, 2004). We call this transport system as “Periplasmic binding protein independent”.

1.15. **Transport across the periplasm and cytoplasmic membrane**

Once the iron–siderophore complex or heme is inside the periplasm, it is transported across the cytoplasmic membrane by ABC (ATP-binding cassette) transporters. These ABC-type permeases consist of a periplasmic binding protein and an inner membrane complex energized by an ATPase (Köster, 2001). Upon release from the OM receptors, siderophores or heme are shuttled by periplasmic binding proteins (with relaxed specificity for substrate) to the cognate permeases in the inner membrane. In *R. leguminosarum*, the *fhuA* gene and the *fhuBCD* operon are induced under iron-limiting conditions. The genes are homologous to the *fhu* genes of *E. coli* and other bacteria (Yeoman *et al.*, 2000) and encode the vicibactin uptake system of *R. leguminosarum*. The OM receptor is encoded by *fhuA*, while gene products of *fhuD* and *fhuBC* are the periplasmic transporter and the inner membrane ATPase proteins, respectively. The composition of the heme uptake system of *B. japonicum* follows the same principle. The receptor (*HmuR*), the periplasmic transporter (*HmuT*) and the inner membrane ATPase proteins (*HmuUV*) are encoded by the *hmuTUV*, *hmuR* genes, which are located in a cluster together with *exbBD-tonB* genes encoding the Tonsystem (Nienaber *et al.*, 2001). *Pseudomonas aeruginosa* possesses in addition to the *HasA–HasR* system the *Phu* system composed of a heme uptake receptor *PhuR* and a binding-protein-dependent ABC system encoded by the *phuSTUVW* genes (Ochsner *et al.*, 2000).

A siderophore uptake system dissimilar from the iron/heme uptake systems described above was discovered in *S. meliloti*. The rhizobactin biosynthesis genes *rhbABCDEF* are located upstream from *rhtA*, encoding the OM receptor (Lynch *et al.*, 2001). An AraC-like regulator encoded by *rhrA* is located between the *rhbABCDEF* operon and *rhtA* and was shown to regulate positively the production and transport of the siderophore. There is strong evidence that the gene product of *rhtX*, which is located upstream of the *rhbABCDEF* operon, is a novel permease that functions as a single-subunit transporter of rhizobactin 1021 (O’Cuiv *et al.*, 2004). *Escherichia coli* takes up the siderophore aerobactin, which is structurally similar to rhizobactin, via the OM receptor *IutA* and the inner membrane transport system *FhuCDB* (Köster, 2001). O’Cuiv *et al.* (2004)
demonstrated that RhtX alone could substitute for FhuCDB to transport rhizobactin 1021 in *E. coli*.

### 1.16. ABC-type Transporters and the Fate of Complexes upon Entering the Cytoplasm

Ferric siderophores bound to their respective PBP are transported to a specific ATP-dependent ABC-type transporter located in the inner membrane. A typical ABC-type transporter has four subunits. Two are hydrophobic and span the membrane multiple times, and two bind nucleotides and are exposed to the cytoplasm. Interaction between ligand-bound PBP and this transporter stimulates the ATPase activity of the transporter, initiating transport (Locher 2004). In the ferrichrome transport system, FhuB is a hydrophobic cytoplasmic membrane protein, and FhuC is a cytoplasmic ATPase associated with FhuB. FhuD with bound Fe$^{3+}$-siderophore complex appears to insert deeply into the FhuB channel, coming into close proximity of the binding site of FhuC ATPase. This observation suggests that the triggering of ATP hydrolysis occurs via direct contact of FhuD with FhuC, or possibly through a short FhuB linker. This is an interesting mechanism, as the other well-studied mechanisms of transport through an ABC-type transporter depend on transmembrane signaling as demonstrated by the maltose transport system (Braun and Braun 2002).

Once transport into the cytoplasm has occurred, there are two possible ways that iron is released from the siderophore. One of these is that ferric reductases reduce ferric iron to ferrous iron, which is released from the siderophore because of reduced binding affinity in the ferrous state (Wandersman and Delepelaire 2004). The second is that the siderophore is broken down, which releases the iron. Genes have been identified in the enterobactin transport system, which code for ferric enterobactin esterase (*fes*) that are thought to breakdown enterobactin (Brickman and McIntosh 1992). More work needs to be done in order to fully understand these processes. A generalized mechanism for siderophore mediated transport is given in figure 1.7.
1.17. Regulation of ferrisiderophore uptake systems

Even though the reactivity of the iron atom makes it useful in many different biological applications, undesirable side reactions can occur. Through Fenton-type chemistry, iron catalyzes the production of toxic hydroxyl radicals from hydrogen peroxide, which arises from the spontaneous combination of superoxide anions created by oxidative metabolism in cells (Arroyo et al., 1994; Touati, 2000). Oxygen radicals and peroxides are highly destructive, damaging lipids, proteins and nucleic acids in the cell. Radicals induce the formation of unsaturated bonds in lipids, decreasing membrane fluidity and causing cell lysis. They also react with thiol groups in proteins, causing cross-linking and inactivation. Hydroxyl radicals can also extract hydrogen atoms from DNA and RNA, causing mutations or cleavage of the phosphodiester backbone.

A collection of different mechanisms have evolved in order to deal with toxic radicals. A number of enzymes and cofactors function in prokaryotes to detoxify oxygen radicals. However, a simpler
method to reduce radical formation by iron is to limit the availability of the iron atom itself; by sensing adequate iron levels and limit its uptake.

In the majority of Gram-negative organisms, ‘Fur’ (ferric uptake regulator) is considered to be the key regulator for expression of genes involved in iron transport (Hantke 1981). Fur is a transcriptional repressor of more than 90 different genes in for eg., *E. coli* and *Pseudomonas aeruginosa*, many of which are involved in siderophore synthesis and uptake (Wexler *et al.* 2003). In environments where iron is abundant, the Fur protein (in its repressive mode) attached to Fe$^{3+}$ binds fur boxes, blocking transcription of target genes (Masse and Gottesman 2002). In some gram-positive bacteria, Fe-responsive gene regulation is mediated by members of the DtxR family, identified in *Corynebacterium diphtheriae* as a regulator of Fe-dependent diphtheria toxin (Boyd *et al.* 1990). This protein also uses ferrous iron as a corepressor (Qian *et al.* 2002) but shows no sequence homology to Fur (Wexler *et al.* 2003). In addition to Fur, there are at least three other subgroups in the Fur superfamily. PerR is an oxidative-stress-response regulator (Bsat *et al.*, 1998) and Zur regulates genes involved in Zn uptake (Hantke, 2001). The fourth member of the Fur family is Irr (iron response regulator), which is restricted to a few α-proteobacteria including rhizobia, *Agrobacterium*, the animal pathogen *Brucella* and *Rhodopseudomonas palustris*. Regulation of iron responsive genes in *R. leguminosarum* and *S. meliloti* is not mediated by Fur but rather by the dissimilar RirA (rhizobial iron regulator) protein, a member of the Rrf2 family of regulators. The role of Fur-like protein Mur (Manganese uptake regulator) in these species appears to be restricted to the regulation of manganese uptake genes. Comparative models of the iron-regulatory mechanisms in *E. coli*, *Neisseria meningitides* and selected rhizobia are shown in Fig. 1.8.
Fig. 1.8: Comparative models of iron-regulatory mechanisms in *Escherichia coli*, *Neisseria meningitidis* and selected rhizobia. Iron regulators, their predicted effectors, DNA target sites and selected target genes (open horizontal arrows) are indicated for each organism. High- and low-iron conditions are indicated by +Fe and -Fe, respectively. Promoter regions are highlighted by dark gray boxes. Fur box, ICE (iron control element), IRO (iron responsive operator) and MRS (Mur-responsive sequence) are DNA-binding sites for Fur, Irr, RirA and Mur, respectively. Fe2+, Fe-S and Mn2+ denote (putative) effectors of regulatory proteins. *Rhizobium leguminosarum* genes that are under the control of both RirA and IrrA are highlighted on a gray background. Question marks refer to postulated effectors and target genes in (c) and (d). Solid arrows indicate activation of gene expression. Interference with gene expression or protein activity is symbolized by blocked arrows. Open arrowheads indicate gene expression.
1.18. Role of siderophores produced by rhizosphere colonizing and nodulating bacteria in control of plant pathogens

It is not known clearly what advantages are conferred on a free-living Rhizobium strain possessing a siderophore, but it is possible, and even likely, that siderophores in Rhizobium sp. play a role in competition in the rhizosphere, perhaps in a manner similar to that of siderophores in Pseudomonas strains. In the competitive soil environment plant growth promotion and nodulation by rhizobia is promoted by certain rhizobacteria (Bai et al., 2002; Dahsti et al., 1998; Rao and Pal, 2003), which indirectly become beneficial for plant growth. However, other plant growth inhibitory bacteria also exist in the soil, and if they are capable for rhizospheric colonization, they negatively affect plant growth. In addition to the nitrogen fixation activity performed by rhizobia, they are also reported as effective biocontrol agents for the inhibition of these soil-borne plant pathogens (Chakraborty and Purkayastha, 1984; Ehteshamul-Haque et al., 1992), the mechanism involved being production of diverse microbial metabolites like siderophores (Persmark et al., 1993), rhizobitoxin (Chakraborty and Purkayastha, 1984), plant growth enhancement through IAA production, uptake of phosphorus and other minerals etc. Many species of rhizobia promote plant growth and also inhibit the growth of certain pathogenic fungi. There are reports where Paecilomyces lilacinus, Trichoderma harzianum (Ehteshamul-Haque et al., 1992) and Memnoniella ehinata (Dawar et al., 1993) have shown promising results for the control of root infecting fungi. Rhizobia are also reported to significantly inhibit the growth of pathogenic fungi i.e Macrophomina phaseolina (Tassi) Gold, Rhizoctonia solani Kuhn and Fusarium sp., in both leguminous and non-leguminous plants (Ehteshamul-Haque and Ghaffar, 1993). Rhizobium meliloti and Bradyrhizobium japonicum bacterized seeds are known to have reduced Macrophomina phaseolina infection, the mechanism involved being siderophore production which inhibit the growth of Macrophomina phaseolina by starving it for iron (Arora and Kang, 2001; Deshwal et al., 2003). Siderophores produced by several of the fluorescent Pseudomonas sp. play a role in biological control of plant pathogens and in plant growth promotion through competition for Fe$^{3+}$ (Kloeper et al., 1980; Schippers et al., 1987). The excreted pseudobactin chelates Fe$^{3+}$ because it has a higher affinity for Fe than do the siderophores from most microorganisms that are deleterious to plant growth (Scher and Baker, 1982).
1.19. Dependence of rhizospheric stability and colonization of organisms on iron acquisition systems

Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and can influence plant growth positively or negatively. The rhizobial high affinity iron uptake systems may be of use in the competition among soil microbes for access to available iron and may enhance the survival of the free-living forms. Evidence has been presented to indicate that soil competition among rhizosphere pseudomonads may occur at the level of the uptake system of the ferric-complex specific for the individual Pseudomonas sp. siderophore (Buyer and Leong, 1986). A comparable competition may also occur among rhizobial strains. R. meliloti DM4 and R. meliloti 1021 produce different siderophores, each stimulating growth of the source organism, but antagonizes the growth of the other. It could be therefore said that possession of uptake system for the siderophore produced by majority organisms in the soil, and hence predominantly present in the soil can have positive implications for growth and survival of the possessing organism. If this organism is a plant growth promoting organism like rhizobia, or many pseudomonads, the growth and propagation of the organism allows it to successfully colonize the rhizosphere, and hence it becomes capable of benefitting plant growth. Plant growth beneficial effects can be brought about by organisms by various mechanisms (as discussed above), the prime necessity being stable rhizospheric colonization. Competition for iron is one of the major factors which determine the successful maintenance of organisms in the rhizosphere. Utilization of foreign siderophores is considered to be an important mechanism to attain iron sufficiency. Fluorescent pseudomonads are known to efficiently colonize various ecological niches, which is largely attributed to the diverse and sophisticated iron uptake systems they possess. They have an ability to utilize a large number of heterologous siderophores via different TonB dependent siderophore receptors. Earlier studies report 32 putative siderophore receptors in P. aeruginosa (Dean and Poole 1993; Ankenbauer and Quan, 1994), 29 in P. putida, 27 in P. fluorescens and 23 in P. syringae (Cornelis and Matthijs, 2002). Bradyrhizobium japonicum 61A152 also cross-utilizes ferrichrome (FC) and rhodotorulic acid (RA), produced by soil fungi (Plessner et al. 1993). This led us to think that increasing the number of outer membrane siderophore receptors
could make our rhizobial strains more efficient with respect to iron acquisition, and hence colonizing the rhizosphere.

1.20. Occurrence of siderophore ferrichrome and ferrichrome receptors

Majority of soil fungi produce hydroxamate type of siderophores, and hydroxamates are stably present in the soil in large concentrations (Powell et al., 1980). By bioassay method it was proved that a greater portion of the hydroxamates in the soil consists of the FC type siderophores which are present at concentrations as high as 78 nM (Powell et al., 1983).

Field tests performed with Bj61A152, consistently gave high yields of soybean cultivars (Hume and Shelp 1990). These data led us to a speculation that iron-scavenging properties of organisms is the major determinant of stability of the organism in the rhizosphere, in case of rhizobia can lead to better competitive survival and hence efficient nodulation on host roots. It was due to this reason that we selected to clone fegA gene of Bradyrhizobium japonicum 61A152, which encoded for FC receptor. It was thought that by expressing FegA in our rhizobial strains, we will be able to make a large pool of iron available to them, which was otherwise, unavailable, which would definitely increase their survival in the soil. Increased survival would mean increased competition for nodulation sites, which would benefit the plant health as a direct consequence.
Present investigation:

Rhizobia are nodulating bacteria, which are amended into agricultural fields as nitrogen biofertilizer. The successful performance of rhizobial inoculant strains depends upon their capability to outcompete the indigenous soil bacteria, survive and propagate, and enter into effective symbiosis with the host plant. The strains which fail to survive under soil conditions, are most of the times ineffective in enhancing legume productivity because vast majority of nodules formed are not by the inoculated strain, but by indigenous rhizobia in the soil (Miller and May 1991; Streeter 1994).

One of the major factors which contribute to survival competitiveness in the rhizosphere is iron availability. Highly competitive and efficient rhizospheric colonizing ability of *Pseudomonas* species has been attributed to the presence of large number of Ton B dependent outer membrane siderophore receptors in this group of organisms (Cornelis and Matthijs, 2002). This led us to conceptualize that increasing the repertoire of Outer Membrane siderophore receptors in rhizobia would confer upon them better iron acquisition in the soil, and in turn could help for better nodulation and efficient nitrogen fixation.

The present investigation was therefore aimed to begin with the study of siderophore production and cross-utilization among rhizobia and rhizospheric bacteria, and understanding how:

- Possession of a low/broad specificity siderophore receptor enable the organism to utilize different types of siderophores
- Possession of multiple siderophore receptors impart upon the organisms utilization of multiple siderophores
- Production of high concentrations of siderophore by an organism help in establishing effective colonisation
- Production of a siderophore having comparatively higher affinity for iron help in competitive survival
- Possession of a specific receptor enable utilization of siderophore present predominantly in the soil
The above understanding was extrapolated to hypothesize that cloning and expression of ferrichrome receptor in rhizobial isolates would confer upon them better survivability and hence better rhizospheric colonization to the rhizobial strains.

The objectives followed were:

1. Isolation of siderophore producing organisms from nodules and rhizosphere of peanut (Arachis hypogea) plants and their characterization with respect to siderophore production and siderophore cross-utilization efficiency.

2. Understanding the interactions taking place among the rhizospheric bacteria with respect to siderophore production and cross-utilization, by growing the organisms in medium with varying forms of iron available.

3. Cloning of the ferrichrome receptor gene (fegA) from Bradyrhizobium japonicum 61A152 and its expression into commercial rhizobial bioinoculant strains of Arachis hypogea and Cajanus cajan.

4. Check the performance of the rhizobial fegA transconjugants with respect to survivability in the soil as well as nodulation on the respective host plants.