INTERACTION OF *Rhizobium* sp. strain IRBG74 WITH A LEGUME (*Sesbania cannabina*) AND A CEREAL (*Oryza sativa*)

by

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ABSTRACT

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Under the Supervision of Dr. Gyaneshwar Prasad

Rhizobium sp. IRBG74 (IRBG74) develops a classical nitrogen-fixing symbiosis with the legume *Sesbania cannabina* and also promotes the growth of rice (*Oryza sativa*), but not much is known about the rhizobial determinants important for these interactions. We hypothesize that *Rhizobium* sp. IRBG74 utilizes similar mechanisms to endophytically colonize both legume and cereal hosts. In this study, we analyzed the colonization of rice and *S. cannabina* using a strain of IRBG74 marked with β-glucuronidase (GUS) and Green Fluorescent Protein (GFP). IRBG74 infected both of the host plants through crack entry under submerged conditions, but showed root hair mediated infection under aerobic conditions. In rice, IRBG74 was localized within intercellular spaces in the cortex, in the xylem of roots and stems, and intracellularly within epidermal and hypodermal cell layers. We have also shown that the colonization of rice by *Rhizobium* sp. IRBG74 requires the nod factor. A transposon insertion mutant of IRBG74 in *rffB,* which encodes dTDP-glucose dehydratase, exhibited significant defects not only in rice colonization but also in nodulation of *S. cannabina*. IRBG74 was found to synthesize a rhamnose-rich

LPS and the *rffB* mutant produced a truncated version of LPS lacking rhamnose. Colonization of both rice and *S. cannabina* roots by the *rffB* mutant was restored by supplementation with purified LPS from wild type IRBG74, but not by LPS from the *rffB* mutant. Another transposon insertion mutant of IRBG74, *thiQ,* was identified as a biofilm defective mutant. It exhibited significant defects in rice colonization and showed no nodulation phenotype in *S. cannabina*. Preliminary results on the *thiQ* mutant suggest that it is likely involved in flavonoid transport. Taken together, these results indicate that IRBG74 is an effective rice endophyte that utilizes a similar physical mode of entry and a common signaling mechanism to invade rice and its legume host, *Sesbania*. Understanding this rhizobial-cereal interaction may provide novel avenues for engineering a symbiotic nitrogen-fixing system in these important crops.

I dedicate my thesis to my beloved wife, Seema, for her immense support, faith and constant encouragement. This journey would not have been possible without her.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter III

LIST OF TABLES

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Chapter I - Introduction

Significance of nitrogen and the importance of nitrogen fixation in cereals

Atmospheric dinitrogen (N_2), the major gas in earth's atmosphere (~80%) is inert in nature and is disinclined to associate with other molecules. As a result, plants cannot use or assimilate N_2 into biologically active molecules including amino acids or other nitrogen rich compounds such as chlorophyll, nucleic acids, ATP, etc. Nitrogen is the major plant growth-limiting factor in soil (Gruber and Galloway, 2008). As a result, the thriving human population is dependent on synthetic nitrogen fertilizers for food production. Cereal crops, a critical source of food, feed and biofuel, are the most nitrogen hungry plants due to their fast growth rate. To cope up with this nitrogen demand, current agricultural systems are heavily dependent upon nitrogen fertilizers (~60 million tons used worldwide per year). The nitrogen fertilizers are made out of natural gas using the Haber-Bosch (1909 -1913) process. In this process, the N_2 is fixed to ammonia in the presence of hydrogen (obtained from natural gas) and an iron catalyst under 200 atm at 450˚C. The ammonia obtained in this process is utilized to make most of the nitrogenous fertilizers. The major concerns associated with industrial nitrogen production are:

1. Industrial production of nitrogen fertilizer uses hydrogen from fossil fuel, thus the cost of these fertilizers are increased with the increasing cost of natural gas. The production and use of these fertilizers represents a major use of fossil fuels and a significant expense for farmers. In addition, these fertilizers are a major source of environmental pollution.

2. Only 30 - 50 % of the fertilizers used for agricultural purpose is actually taken up by plants and the majority is lost from the field as agricultural runoff to surface water, causing eutrophication of streams and coastal regions as seen in the formation of a massive dead zone in the Gulf of Mexico (Diaz and Rosenberg, 2008; Gutiérrez-Zamora and Martínez-Romero, 2001). A significant fraction of these nitrogen fertilizers is also released to the atmosphere as reactive nitrogen gases, adding more burden to the already existing problem of ozone depletion and global warming (Fig 1) (Sutton *et al.*, 2011).

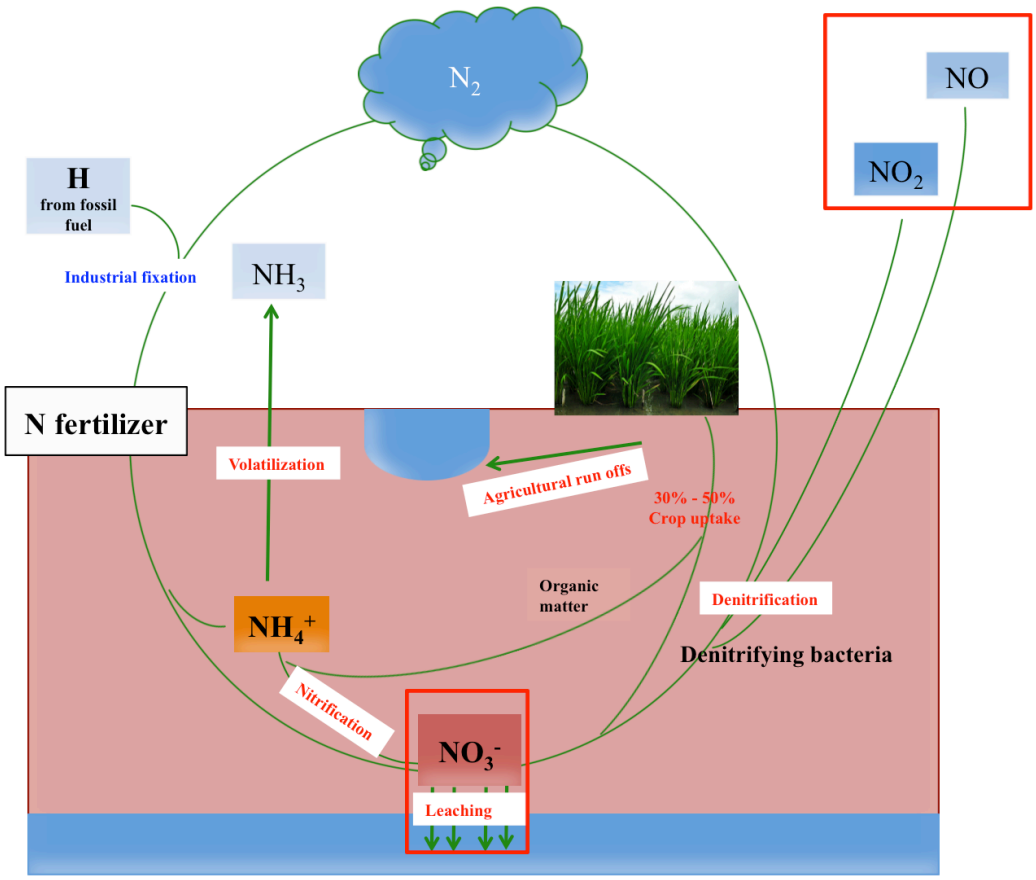


Fig. 1 The Nitrogen cycle, focusing on the environmental impact of nitrogen fertilizers. Only half or less than half of the fertilizers used in agricultural lands are actually taken up by the plants. A significant amount is lost from the field during the process of ammonia volatilization or is converted to nitrate, which is readily soluble in water and leaches into ground water causing serious health hazards in children. Nitrate is also converted to nitric and nitrous oxide (potent green house gases) during the process of denitrification and lost into the atmosphere. But the major problem associated with the excess use of nitrogen fertilizers is eutrophication of surface water bodies from agricultural runoff, which destroys the delicate environmental balance.

It is therefore imperative to engineer nitrogen fixation in cereals for sustainable food, feed and biofuel production (Beatty and Good, 2011).

While some plants, like legumes, are able to obtain their nitrogen directly from the air through efficient associations with nitrogen-fixing bacteria (commonly known as rhizobia), most cereals unfortunately are unable to develop such associations. Though the bacteria from genera *Azospirillum, Herbaspirillum*, *Azoarcus* and *Acetobacter* are reported to form association with economically important crops like wheat, maize and rice, the amount of nitrogen fixation was often reported to be too low to sustain plant growth (Santi *et al.*, 2013).

Rhizobium-Legume symbiosis

By definition, 'rhizobia' are a group of nitrogen fixing soil bacteria capable of forming and inhabiting in a specific plant structure known as root-nodule. Rhizobia, also called the root nodule bacteria (Zakhia *et al.*, 2004), require a huge amount of energy to fix N_2 to ammonia, which they get by respiring on photosynthate provided to it by the plant hosts (O'gara and Shanmugam, 1976). The process of nitrogen fixation by rhizobia in plant root nodules is a symbiotic process, in which the plant provides a safe niche (van Rhijn and Vanderleyden, 1995) and nutrients (Lodwig *et al.*, 2003) to rhizobia for its growth and maintenance inside the plant cell and, in return, receives nitrogen, the major growth-limiting factor in nutrient poor soil.

The beneficial interaction between nitrogen fixing rhizobia and legumes, commonly known as (RLS), is one of the well-studied and most effective nitrogen fixing system known. As described earlier, RLS involves formation of root nodules in legume. Formation of root nodule in the presence of a Rhizobium was first established in late 19th century by Beyerinck, where he induced nodules in faba beans growing in sterile soil, using a pure bacterial culture (Perret *et al.*, 2000).

Mechanism of Rhizobium-Legume symbiosis

The development of RLS starts with a molecular crosstalk between the rhizobia and the host legume to establish an effective symbiotic relationship. Legumes secrete a large amount of organic matter into soil (Perret *et al.*, 2000), which serve as the nutrient source for thriving microbial populations in the rhizosphere. The rhizobia in the host rhizosphere sense and recognize the host root due to the presence of particular plant secondary metabolites known as flavonoids, and initiate the process of root nodule formation. Flavonoids activate rhizobial NodD protein, which is a LysR type of transcriptional regulator, and induce the expression of nodulation specific genes (*nod*, *nol* and *noe*). NodD binds to a highly conserved 47 bp DNA motif (*nod box*), commonly found in promoter regions of the nodulation specific genes, and regulates the expression of these genes. Various nod genes encode approximately 25 proteins involved in synthesis and secretion of nodulation factors in the rhizosphere (Perret *et al.*, 2000).

The Nod factor is a lipochitooligosaccharide (LCO)-like rhizobial signaling molecule, which upon recognition by the host plant receptor, initiates a number of developmental changes in the plant (Jones *et al.*, 2007). The rhizobial LCOs are usually made of a chitin core (4 to 5 molecules of β-1, 4 linked *N*-acetyl glucosamine) with a

lipid moiety attached to the non-reducing end and host specific modification on the backbone, which imparts the specificity of the interaction with the target receptor (Fig 2). Rhizobia carry nodulation specific genes like *nod, noe* and *nol* genes, and mutation in theses genes hinders nodule organogenesis. Some of these genes, such as *nodABCD*, are found in all rhizobia and others may not be present in some rhizobial species (Debellé *et al.*, 2001; Mergaert *et al.*, 1997). Nod factors from different rhizobia differ in their side chain substitution of the chitooligosaccharide backbone and these substitutions confer the specificity of a Nod factor towards its cognate receptor. Some *nod* genes,like *nodABCD*, are intrinsically involved in synthesis of the Nod factor core and strictly required for nodulation, whereas host specificity genes like *nodL* or *nodH* are involved in side chain substitution and determines the host range of those rhizobia (Debellé *et al.*, 2001). Therefore *nod* genes and nod factors are crucial for nodulation and host range determination.

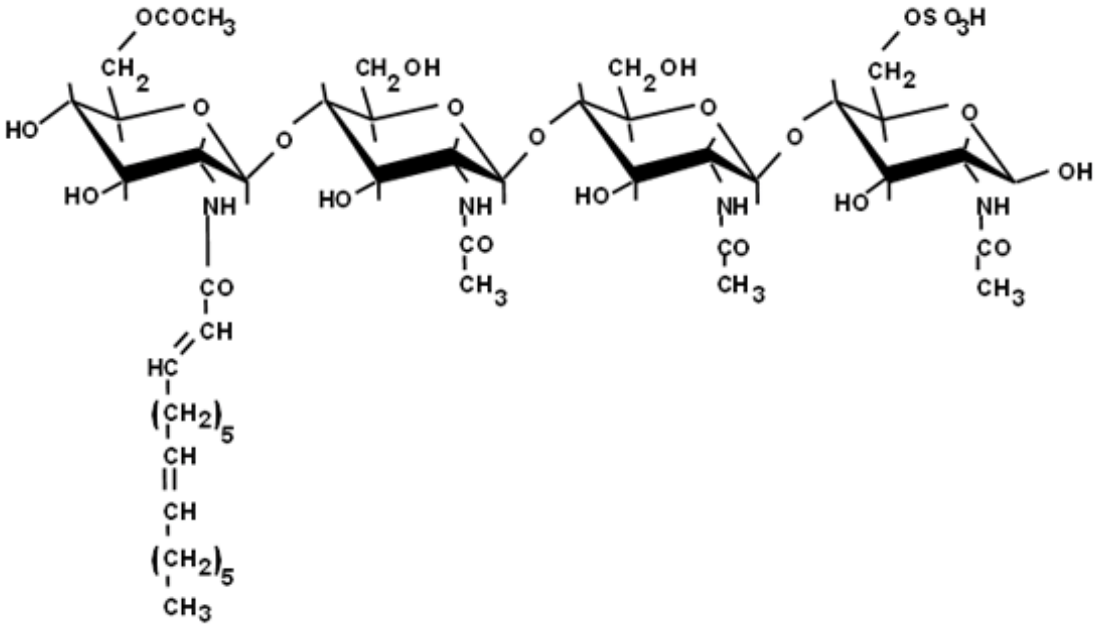


Fig. 2 The structure of Nod factor from *Sinorhizobium meliloti* (Oldroyd, 2013)

Recognition of Nod factor by its cognate plant receptor initiates many developmental changes in the host plant during early stages of the nodulation process. The major events during this process include membrane depolarization, intracellular calcium oscillations and alteration of root hair cytoskeleton that results in root hair deformation and curling. The recognition of LCOs by their cognate receptors also initiate cell division in the root cortex and establish a meristem and nodule primordium (Gage, 2004).

Due to root hair curling the rhizobia, present on the root hair surface, get entrapped between cell walls of a single root hair. Then the entrapped rhizobia undergo several cycles of cell division to form a micro-colony within the curled root hair structure (Jones *et al.*, 2007), degrade the cell wall and induce a progressive ingrowth of the root hair cell membrane and form a tubular structure known as the infection thread. The infection thread grows in through the root hair cell to the basal part of the epidermal cells

and delivers bacteria to the nodule primordium. Once inside, bacteria differentiate to form bacteroids and start fixing atmospheric nitrogen (Fig 3).

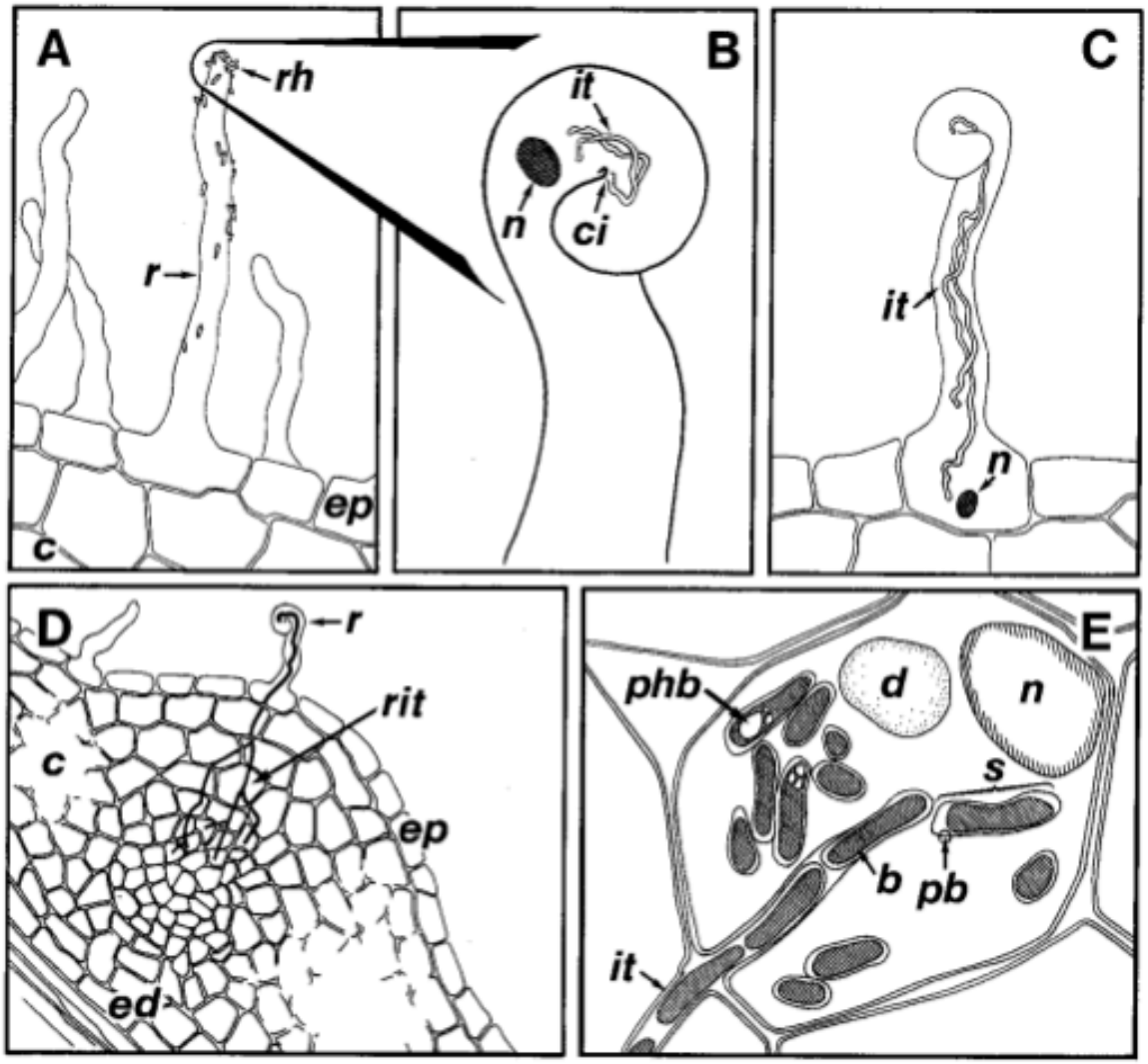


Fig. 3 Root hair mediated rhizobial invasion in legumes (Perret *et al.*, 2000). (A) Rhizobial (rh) attachment to the root hair (r). (B) Nod factor induced root hair curling; formation of center of infection (ci) and initiation of the infection thread (it). (C) Elongating infection thread (it) reaches at the base of the root hair cells. (D) A developing ramified infection thread (rit) reaches to the nodule primordium. (E) Bacteroids (b), enveloped in a peribacteroid membrane (pb) are released from infection thread (it) and

form symbiosome (s).

Abbreviations: phb, poly-β-hydroxybutarate; c, cortex; d, digestive vacuole; ep, epidermis; ed, endodermis (Perret *et al.*, 2000).

Evolution of Rhizobium-Legume symbiosis and the role of common symbiotic genes

It was postulated that, during the evolution of rhizobia legume symbiosis, rhizobia hijacked the plant genetic machinery required for fungal endosymbiosis in legumes. The non-legume *Parasponia* uses a single receptor for lipochitooligosaccharides like signaling molecule to interact with both rhizobia and arbuscular mycorrhizal (AM) fungi (Op den Camp *et al.*, 2011). Genetic and molecular analyses of the rhizobia-legume and the AM fungi-legume interaction have revealed the transduction of these rhizobial and fungal lipochitooligosaccharidic signals in legumes is mediated by a common symbiotic pathway (CSP) that is required for the initiation of both nodulation and mycorrhization (Fig. 4**)** (Mitra *et al.*, 2004). At least eight genes belong to this CSP: a receptor-like kinase with leucine rich repeats - DMI2/NORK/SYMRK (Endre *et al.*, 2002; Stracke *et al.*, 2002); two ion channels localized to the nuclear envelope - DMI1/POLLUX and CASTOR (Ané *et al.*, 2004; Peiter *et al.*, 2007; Riely *et al.*, 2007); three nucleopore-associated proteins - NUP85, NUP133 and NENA (Groth *et al.*, 2010; Saito *et al*., 2007) a calcium and calmodulin dependent kinase - DMI3/CCaMK and a nuclear protein with coiled-coil domains -IPD3/CYCLOPS which interacts with DMI3 (Messinese *et al.*, 2007). The CSP genes are highly conserved throughout land plants and their distribution mirrors the ability of plants to form arbuscular mycorrhizal symbiosis (Zhu *et al.*, 2006). In particular, CSP genes have been shown to be required for arbuscular mycorrhizal

symbiosis in rice through the use of different rice knock out mutants (Chen *et al.*, 2008; Chen *et al.*, 2009; Chen *et al.*, 2007; Gutjahr *et al.*, 2008).

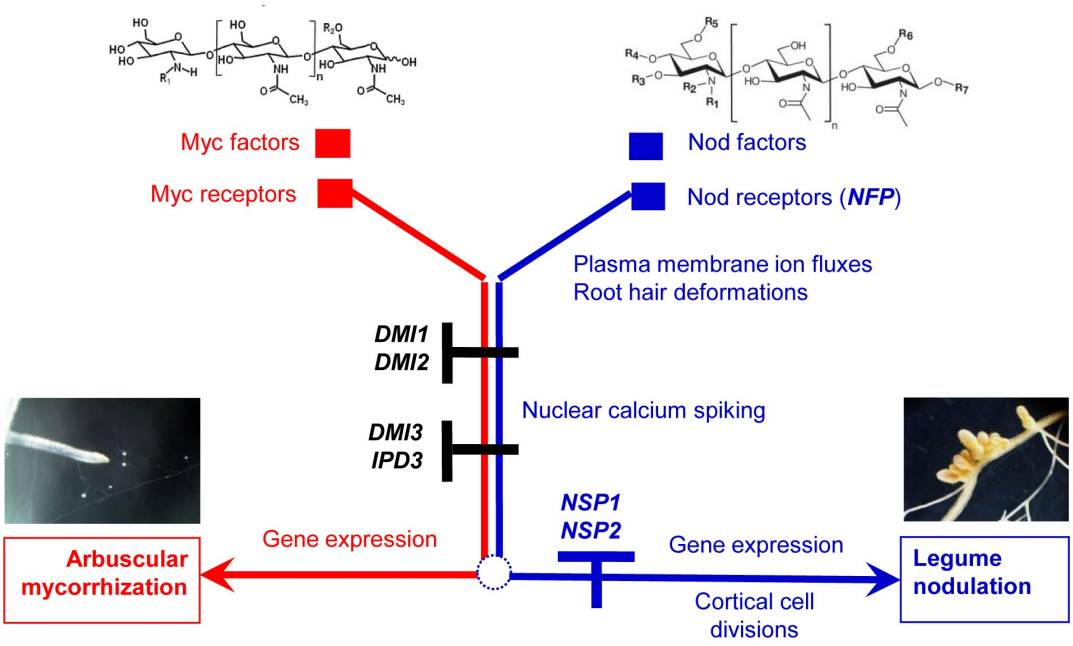


Fig. 4 Signaling pathway controlling mycorrhizal and rhizobial symbioses in the model legume *Medicago truncatula*. The recognition of microbial lipochitooligosaccharides (Myc and Nod factors) triggers nuclear calcium spiking, regulates gene expression and allows the colonization of plant roots. These processes are controlled by common symbiotic pathway (CSP) genes like *DMI1*, *DMI2*, *DMI3* and *IPD3* (Courtesy Jean M Ané).

Rice as a model system for paving the way to engineering nitrogen fixation in cereals

Recent studies have revealed that rice has a natural ability to form an association with rhizobia, the bacteria that forms N fixing symbiosis with legumes (Biswas *et al*., 2000a,b; Yanni and Dazzo, 2010; Chaintreuil *et al.*, 2000; Chi *et al.*, 2005; Peng *et al.*, 2008). These observations have renewed interest in exploring the possibility of extending N-fixation to rice. Rice requires the homologs of legume CSP pathway genes for mycorrhizal infection (Chen *et al.*, 2008; Chen *et al.*, 2009; Chen *et al.*, 2007; Gutjahr *et al.*, 2008), indicating that the mechanism of mycorrhizal symbiosis in rice is similar to that in legumes. Additionally, many strains of rhizobia form growth-promoting associations with cereals (Chaintreuil *et al.*, 2000; Yanni and Dazzo, 2010). 16S rDNA and *nifH* sequences of rhizobia were found in rice (Sessitsch *et al.*, 2012) and rhizobial sequences were significantly decreased in a rice mutant lacking *Os*CCaMK (Ikeda *et al.*, 2011). Rice, one of the most important cereal crops for humans, has emerged as an excellent model for genomics and systems biology of cereals because of its small genome size, sequenced genome, established genetic modification techniques and resources for various "omics" studies (Devos, 2010; Kellogg, 2001; Wood *et al.*, 2001). In addition, rice has also been a main target crop to characterize nitrogen fixation with cereals. Studies over the last few decades have revealed that many features of the rhizobial-legume interactions are also involved in rhizobial-rice interactions. Rhizobial *nod* genes are induced, though at a lower level, by rice root exudates. Transgenic rice containing promoter-reporter fusions of legume nodulin genes could express these genes in a way similar to their expression in legumes, and homologues of legume CSP genes are well conserved in rice (Zhu *et al.*, 2006). These results suggest that rice contains many of the

genetic determinants required for forming a symbiosis with rhizobia. However, very little is known about the mechanisms that rhizobia and rice use for mutual recognition and the subsequent formation of beneficial associations. The molecular mechanisms underlying the rhizobial legume symbiosis, and the apparent evolution of RLS using legume-mycorrhizal symbiosis suggest that development of rhizobial symbiosis with cereals is feasible in the long term. However, in order to realize this potential it is necessary to identify the genetic determinants and the key events involved in rice-rhizobial interactions.

***Rhizobium* sp. IRBG74**

Rhizobium sp. strain IRBG74 was originally isolated in the Philippines from the root nodules of the aquatic legume *Sesbania cannabina*. *Sesbania* is a genus of 60 species of tropical legume, out of which 40 plant species were reported to form nodule with a variety of rhizobia. Due to its nitrogen fixing ability with different rhizobia, it is widely used as a green manure in wetland rice production in different Asiatic countries.

Nodulation studies with different *Sesbania* sp. showed that, *Rhizobium* sp. IRBG74 forms nitrogen-fixing root nodules with at least eight different *Sesbania* species. Additionally, the rice growth promotion ability of *Rhizobium* sp. IRBG74 (Biswas *et al.,* 2000 a,b), makes it a good model system for determining the mechanisms of *Rhizobium*-cereal interactions. 16SrDNA, *rpoB* and *fusA* sequence analysis identified *Rhizobium* sp. strain IRBG74 as a close relative to the plant pathogen *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*), but DNA: DNA hybridization with other *Agrobacterium* (*R. radiobacter*, *R. rubi*, *R. vitis* and *R. huautlense*) suggested that IRBG74 is potentially a new species in the *Rhizobium*/*Agrobacterium* clade (Cummings *et al.*, 2009). Unlike

pathogenic *Agrobacterium* species, *Rhizobium* sp. strain IRBG74 doesn't have virulence genes (*virABCDEFG*) and as a result it lacks tumor-forming ability in plants. But it has a large plasmid, containing a number of genes (*nod*, *nif*, and *fix*), which helps it to associate with legume plants for symbiotic nitrogen fixation.

Chapter II

Rhizobium sp. IRBG74, a classic nitrogen fixing symbiont of *Sesbania* sp. and an endophytic colonizer of rice, requires rhamnose-rich LPS for endophytic colonization of rice and for nodulation of *Sesbania cannabina*

Introduction

Legume root- and stem-nodulating bacteria belonging to the Alpha- and Betaproteobacteria, collectively called rhizobia (Graham, 2008; Gyaneshwar *et al*., 2011), are among the most effective plant growth-promoting rhizobacteria. Rhizobia form intimate symbiotic associations with legumes that result in the formation of nitrogen fixing root nodules, which can supply all of the hosts N-requirements (Graham and Vance 2003, Peoples *et al*., 2009). The legume-rhizobia symbiosis has been studied extensively, and it requires a complex signal exchange between host plants and their microbial partners. A combination of microbial genetics and biochemistry has elucidated bacterial and plant factors that mediate the often-high level of host specificity observed in the association (Oldroyd and Downie, 2006; Stacey *et al*., 2006; Jones *et al.*, 2007).
In addition to forming nodules on legumes, many strains of rhizobia develop non-nodulating and often endophytic associations with cereal crops, including maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (Biswas *et al*., 2000a, b; Gutierrez-Zamora and Martinez-Romero 2001; Chaintreuil *et al*., 2000; Chi *et al*., 2005; 2008; Peng *et al*., 2008; Sun *et al*., 2008; Yanni and Dazzo, 2010). This indicates that rhizobial-cereal associations, both endophytic and epiphytic/rhizospheric, are widely prevalent in nature,

especially in agricultural systems. These associations can lead to a significant increase in plant biomass and improved nutrient uptake (Biswas *et al*., 2000a, b; Yanni and Dazzo, 2010), but the growth promotion is not thought to involve N-fixation, and instead has been attributed to the ability of some rhizobia to secrete plant growth-promoting hormones, such as indole acetic acid (IAA) (Biswas *et al*., 2000; Misra *et al*., 2008). While rhizobial strains have consistently been reported to colonize and stimulate rice growth, very little is known about the signals and genetic mechanisms that regulate rice-rhizobial interactions and growth promotion. In order to elucidate the mechanisms involved in the establishment of rhizobial-cereal associations, we have chosen as a model *Rhizobium* sp. IRBG74, a symbiont of some species in the aquatic legume genus *Sesbania* (Cummings *et al*., 2009), and for which the genome sequence is available (Crook *et al*., 2013). *Rhizobium* sp. IRBG74 can enhance the growth of many rice cultivars (by approx.18-20%) under both gnotobiotic and greenhouse conditions (Biswas *et al*., 2000a, b; Peng *et al*., 2002). Based on phylogenetic analyses using both 16S rRNA and other housekeeping genes, *Rhizobium* sp. IRBG74 was found to belong to a novel lineage within the *Rhizobium-Agrobacterium* cluster (Tan *et al*., 2001; Cummings *et al*., 2009), which is supported by its genome sequence (Crook *et al*., 2013). As *Rhizobium* sp. IRBG74 nodulates *S. cannabina*, a semi-aquatic legume that grows in conditions similar to rice paddies, we hypothesized that it may possess mechanisms to interact with and colonize rice, and that these mechanisms might have commonalities with its interaction with its natural legume host, *S. cannabina*. As a first step towards a systematic dissection of these mechanisms, we analyzed the colonization of rice and *S. cannabina* by *Rhizobium* sp. IRBG74. In this report we show that *Rhizobium* sp. IRBG74 infects both *S.*

cannabina and rice through crack entry under submerged conditions. Under aerobic conditions, however, rhizobia commonly enter *S. cannabina* through root hairs via the formation of infection threads. Similarly, in rice grown under aerobic conditions, *Rhizobium* sp. IRBG74 can colonize root hairs internally, albeit at a low frequency, by an as yet unknown infection process. Our studies have demonstrated that *Rhizobium* sp. IRBG74 forms extensive cell aggregates on the surface of rice roots, including root hairs, and then penetrates into the interior of the roots and stems, where it is localized within intercellular spaces and in the xylem.

As *Sesbania* and rice both share the same habitat and similar colonization pattern we hypothesized that the interaction of *Rhizobium* sp. IRBG74 with rice is specific and that IRBG74 Nod factor may play a crucial role in rice colonization. To test this hypothesis, we have generated a Nod factor defective mutant and performed a rice colonization study. The Nod factor defective mutant of *Rhizobium* sp. IRBG74 showed reduced rice colonization, suggesting a role of *Rhizobium* sp. IRBG74 Nod factor in rice. However, further plant studies are required to uncover the specifics of this interaction and to understand the role of Nod factor in rice signaling.

A mutant of *Rhizobium* sp. IRBG74 that produces altered lipopolysaccharide (LPS) displayed a reduced ability not only to colonize rice, but also to colonize and form nodules on *S. cannabina*. These data establish that there are commonalities in the processes involved in the endophytic colonization of rice and in the establishment of symbiotic association in legumes by this rhizobial strain.

Materials and Methods

Bacterial strains and growth conditions

The strains, plasmids and DNA primers used in this study are listed in Table 1. Rhizobial strains were grown at 28°C and maintained on Yeast Mannitol (YM) (Vincent, 1970).

E. coli strains were grown at 37°C and maintained on LB agar. For inoculation studies, rhizobia were grown on YM medium and washed with Phosphate buffered saline (PBS) at least 2 to 3 times before plant inoculations.

Table 1: Strains, plasmids and primers used in this study

Strains/Plasmids/ Primers	Description	Reference
Strains		
IRBG74-GUS	Wild type IRBG74 marked with pCAM120 (*papH-gusA*) Spec^r	Cummings et al. (2009)
IRBG74-GUS-GFP	*Rhizobium* sp. IRBG74-GUS containing pHC60 Tet^r	This study
IRBG74-Δ*nodABC*	*Rhizobium* sp. IRBG74-GUS *nodABC* deletion mutant Gen^r	This study
IRBG74-GUS-*rffB::Himar*	*Rhizobium* sp. IRBG74-GUS *rffB::Himar* mutant Kan^r	This study
IRBG74-GUS-GFP *rffB::Himar*	*Rhizobium* sp. IRBG74-GUS-GFP *rffB::Himar* mutant Kan^r	This study

E. coli β2155	*thrB*1004 *pro thi strA hsdsS lacZ* ΔM15 (F9 *lacZ* ΔM15 *lacI*^{q} *traD*36 *proA*1 *proB*1) Δ*dapA*::*erm* (Erm^{r}) *pir*::RP4 [::*kan* (Km^{r}) from SM10]	Dehio and Meyer (1997)
E. coli S17.1	*thi pro* res^{-} mod^{+} Sm^{r} Tp^{r} *recA1* RP-4-2[Tc::Mu; Km::Tn7]	Simon *et al*. (1983)
Plasmids		
pHC60	IncP Tet^{r}, *gfp* under the control of a constitutive *lac* promoter	Cheng and Walker (1998)
pMiniHiMar	*mini-HimarRB1* transposon, R6K *ori*	Bouhenni *et al*. (2005)
pCAM121	Sm/Sp, Ap; mTn5SS*gusA2l* (P*aph-gusA-trpA* ter) in pUT/mini-Tn5 Sm/Sp	Wilson *et al*. (1995)
pENTR™/D-TOPO® Vector	Entry Vector for TOPO cloning Kan^{r}	Life Technologies
pMBC219	The Gateway destination vector derivative of the suicide plasmid pJQSK200 Gen^{r}	Quandt and Hynes (1993) J.M Ane Lab
Primers		
nodABC upstream_F	GGCGTGCTAAAGTCATGGGAG	This study
nodABC upstream_R	CCATCGGCAGAGATTTAAGCGATTG CAGTTCAGTTTCCCAGC	This study
nodABC downstream_F	GCTGGGAAACTGAACTGCAATCGC TTAAATCTCTGCCGATGG	This study
nodABC downstream_R	CCTTCGCTGAAGACCTGGAAC	This study
*nodA*_RT_FP	TTGCTGGGAAACTGAACTGC	This study

nodA _RT_RP	GCGTGCTATCGGAAAAACGA	This study
*rpoB*_RT_FP	CGAGTTCGACGCCAAGGATA	This study
*rpoB*_RT_RP	ACGCGGTAGATGTCGAACAG	This study
Himar 1	CATTTAATACTAGCGACGCCATCT	This study
Himar 615	TCGGGTATCGCTCTTGAAGGG	This study
rffBC F	AATCCCGGCTCGTTTCGGCG	This study
rffBC R	TGTCGCGTCGATGGCGTAGC	This study

GUS and GFP marking of rhizobial strains

Rhizobium sp. IRBG74 was marked with *gusA* (encodes β-glucuronidase), using *E. coli* harboring pCAM121 containing P*aph- gusA-trpA* ter cassette inserted as a NotI fragment in mini-Tn*5* encoding Sm^r/Sp^r. Conjugants were selected on YMA (Yeast mannitol agar) containing 25 μg ml^{-1} spectinomycin. The GUS marked *Rhizobium* strains were transformed by electroporation with plasmid pHC60 (Cheng and Walker 1998). pHC60 is a broad host range plasmid which expresses GFP under the control of a constitutive *lac* promoter (Cheng and Walker 1998).

Colonization of rice and *S. cannabina* by *Rhizobium* sp. IRBG74

The bacterial inoculation of rice was performed as described earlier (Gyaneshwar *et al.* 2001; James *et al.* 2002). Briefly, de-hulled seeds of *Orzya sativa* cultivar Nipponbare were surface sterilized with 70% ethanol followed by 0.2% mercuric chloride for 30 seconds and were washed three times with sterile water. The seeds were germinated on 0.1% agar plates, and seedlings free of visual bacterial and fungal contamination were used for inoculation with the *gusA*-marked strains. The latter were grown in YM

supplemented with spectinomycin (100 μg ml^{-1}) until they reached an optical density of 0.6. The cells were then harvested by centrifugation, washed twice with normal saline, and resuspended in PBS (10^7 bacteria ml^{-1}) and used for inoculation. Surface-sterilized seedlings were grown in 100 ml glass tubes filled with Fahraeus medium (Fahraeus 1957) without nitrogen covered with Parafilm® such that the roots were submerged into the growth medium. The inoculated plants were incubated in a growth chamber (14 h light/10 h dark cycle) at 27°C (day) and 25°C (night). Uninoculated plants served as controls. The inoculation and nodulation of *S. cannabina* was determined as described by Cummings *et al.* (2009). Seeds of *S. cannabina* were surface sterilized and their dormancy broken by treating them with concentrated sulfuric acid for 20 min. The seedlings were grown in 100 ml tubes containing N-free nutrient solution as for rice. The nodulation was determined at 30 days after inoculation (DAI) and the nodules were stained for GUS activity using X-Gluc as described previously (Cummings *et al*., 2009).

Enumeration of *Rhizobium* sp. IRBG74 colonizing rice roots and stems

The colonization of rice roots and stems was quantified as described earlier (Gyaneshwar *et al.*, 2001). Briefly, rice seedlings were sampled at various times, from 1 to 14 DAI. Loosely attached bacteria were removed by washing the roots in excess sterile water, and the roots were then immersed in sterile water and vortexed for 30s. The resulting solution was serially diluted and placed on LB agar plates containing spectinomycin (100 μg ml^{-1}) and X-Gluc (40 μg ml^{-1}). Bacterial colonies showing blue coloration were then counted, and these counts were assumed to be those bacteria that were closely associated with the root surface. In another set, the roots were surface sterilized by immersion in 95% ethanol for 5 min followed by treatment with 3% calcium hypochlorite containing 0.1% sodium

dodecyl sulfate for 1 min. After three washes with sterile distilled water followed by maceration in saline, the homogenate was serially diluted and plated on LB agar as described above. The saline solution before maceration was plated to determine the efficiency of surface sterilization, and the number of bacteria present in this solution, if any, was subtracted from the total count after maceration.

Microscopic studies of plant infection and colonization by *Rhizobium* sp. IRBG74

At least three rice seedlings from three independent inoculations were collected at 10 DAI and GUS activity was determined using X-Gluc as described earlier (Gyaneshwar *et al.*, 2001). Portions of roots and shoots that were stained blue were cut into small (1 - 2 mm) pieces and fixed in 4% glutaraldehyde in 50 mM phosphate buffer (pH 7.0) containing 0.1% (vol/vol) Triton X-100. The fixed samples were prepared for light microscopy and TEM, and TEM sections were immunogold-labeled with an antibody raised against *Rhizobium* sp. IRBG74 as described earlier (Cummings *et al.*, 2009). Briefly, ultrathin (80 nm) sections were collected on grids, treated with the *Rhizobium* sp. IRBG74-antibody, washed, and incubated in a 15 nm gold-labeled goat anti-rabbit antibody (Amersham International). The sections for light microscopy were viewed under a Zeiss Axiophot 2 optical microscope, and the ultrathin sections were viewed using a JEOL 1200 EX TEM.

Green fluorescent protein (GFP)-marked *Rhizobium* strains showed bright fluorescent signal, which allowed their detection on rice and *S. cannabina* tissues by fluorescence microscopy. The plant tissues were treated with 10 μg ml^{-1} propidium iodide (Sigma-Aldrich) for 5 min to stain the plant cell wall to differentiate between inter or

intra cellular presence of *Rhizobium* sp. IRBG74 in rice and *S. cannabina*. Free hand sectioning was done on stained roots and three-dimensional (3D) laser scanning confocal microscopy performed with a TCS SP2 (Leica Microsystems, Bannockburn, IL) using an excitation wavelength of 488 nm to detect the fluorescence of GFP and propidium iodide in two specific emission windows of 500-550 nm (GFP) and 640-700 nm (propidium iodide) (Marcel *et al.*, 2010).

RNA isolation

To isolate the bacterial RNA in the presence of rice, the strains were grown to mid-logarithmic phase in YM minimal medium. For the isolation of RNA from the rice rhizosphere, bacteria (10^7 cells ml^{-1}) were inoculated in sterile Magenta boxes containing aseptically germinated rice seedlings. The entire setup was then incubated in a growth chamber for 7 days. At 7DAI bacteria from the rice rhizosphere were harvested aseptically and RNA was isolated using TRIzol reagent (Sigma).

The RNA concentrations were determined by UV/Vis spectrophotometry. RNA samples were further treated with RNase free DNase (Promega) to eliminate any DNA contamination.

Reverse Transcription PCR

cDNA synthesis and RT-PCR were performed using the AccessQuick RT-PCR system (Promega) as follows. The DNase treated RNA sample (0.3 µg) was used as template for cDNA synthesis at 45°C for 45 min. PCR amplification was performed using primers listed in Table 1 as follows: 2 min of incubation at 94°C, followed by 25 cycles of amplification with denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, and

extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. RNA samples that had not been subjected to reverse transcription were used as negative controls and *rpoB* was used as the internal control.

Construction of *nodABC* deletion mutant

To construct a deletion mutant of *nodABC*, Gateway® Cloning Technology (Life Technologies) was employed to clone a 2 kb IRBG74 Δ*nodABC* PCR amplicon in the destination vector pMBC219. The deletion vector pMBC219 (pJQ200SK modified with the Gateway® cloning cassette from pDEST24™ [Invitrogen]) was kindly provided by Matthew Crook (University of Wisconsin–Madison). Briefly, PCR primers were designed to amplify approximately 1kb upstream and 1 kb downstream sequence of the IRBG74 *nodABC* operon. Overlap extension PCR (OE-PCR) was used to generate the IRBG74 *nodABC* deleted (Δ*nodABC*) amplicon. The Δ*nodABC* amplicon was then cloned into pENTR™/D-TOPO® Vector (entry vector) using manufacturer's (Life Technologies) protocol. The vector containing the IRBG74 Δ*nodABC* fragment was then chemically transformed into One Shot® TOP10 chemically competent *E. coli* cells using manufacturer's protocol and selected on LB plates containing 50µg ml^{-1} of kanamycin. The kanamycin resistant TOP10 *E. coli* colonies were inoculated in LB broth and the entry vectors containing 2 kb IRBG74 Δ*nodABC* fragment were isolated and confirmed by PCR. Using LR cloning technique the 2 kb IRBG74 Δ*nodABC* PCR fragment was then transferred to the destination vector pMBC219. The destination vector containing IRBG74 Δ*nodABC* was then chemically transformed into β2155 cells and selected on LB gentamycin 50 µg ml^{-1} plates (Dehio and Meyer, 1997). For efficient transfer of destination vector (containing Δ*nodABC* fragment) to IRBG74 *gusA* strain the process of

conjugation was employed. The single recombinants of *Rhizobium* sp. IRBG74 containing the IRBG74 Δ*nodABC* fragment were then selected on LB gentamycin 150 µg- ml^{-1} plates and confirmed with PCR. Sucrose selection was performed on YM minimal media plates, containing 10% sucrose, to obtain the double recombinants. The double recombinants obtained were then tested for their gentamycin sensitivity to confirm loss of the destination vector. Then, sucrose resistant and gentamycin sensitive IRBG74 colonies were screened for Δ*nodABC* using PCR.

Genome sequencing and contig analysis

For whole-genome sequencing, 100-bp paired-end libraries were generated from *Rhizobium* sp. strain IRBG74, which was previously marked with mTn*5*ss*gusA20* (Cummings *et al.*, 2009).

Sequencing was done using Illumina's Phusion-based library kits according to their protocols (Illumina, Hayward, CA) and sequenced on Illumina GAIIx machines at the National Center for Genome Resources (Santa Fe, NM). Base calling was done according to the manufacturer's protocols. The insert sizes averaged 309 nucleotides (nt), and 90,615,496 reads were obtained, comprising approximately 300X coverage of the genome. Read assembly was performed *de novo* using ABySS (http://www.bcgsc.ca/platform/bioinfo/software /abyss/), resulting in 26 contigs. Several *k*-mers were run, and the best resulting assembly was chosen based on assembly contiguity statistics, the placement of a subset of high-quality read pairs in the assembly with correct spacing, and orientation. Potential contig junctions were predicted based on the conservation of synteny with two reference genomes (those of *Agrobacterium tumefaciens* C58 and *Agrobacterium* sp. strain H13-3) and an analysis of contig ends.

These predicted contig junctions were resolved by PCR.

Construction and screening of a *Rhizobium* sp. IRBG74 mutant library

A random insertion mutant library of *Rhizobium* sp. IRBG74 was constructed using a modified mariner transposon miniHimar (pMiniHimarRB1) that encodes kanamycin resistance (Bouhenni *et al.*, 2005) by conjugation and subsequent selection of kanamycin resistant mutants. The mutants were isolated on modified YM medium in which ammonium chloride replaced the yeast extract, thus eliminating rhizobial auxotrophs.

The transconjugates were isolated and purified on YM plates containing kanamycin, and then maintained in YM medium in 96-well plates. The mutants were then screened for defects in biofilm formation ability in 96-well plates using crystal violet staining and also for defects in motility on LB motility plates as described below.

Identifying the transposon insertion sites

The genes disrupted by the transposon were identified by PCR amplification and by sequencing of genomic regions flanking the transposon as described by Bouhenni *et al*. (2005). Genomic DNA from the mutants was isolated, digested with SphI and self-ligated with T4 DNA ligase. The transposon insertion region was amplified from the ligation mixture using inverse PCR with the Himar1 and Himar 615 primers, gel purified and sequenced directly.

Motility assays

The motility of the wild type and mutant strains was assessed using swim plates (0.3% agar) in rice media containing 10 mM mannitol and 5 mM ammonium chloride as carbon and nitrogen sources, respectively. The bacteria were grown in YM media overnight, and 5 µl of each strain was inoculated into the center of the plates. Motility was assessed by measuring the diameter of the swim circle after incubation at 30°C for 24 and 48 hours.

LPS isolation and characterization

LPS was extracted from the wild type and *rffB* mutant strain by hot phenol extraction, essentially as described earlier (Noel *et al.*, 2004). The bacteria were grown in YM, harvested by centrifugation, washed and resuspended in distilled water. The bacterial pellet was heated at 65^0C and mixed with an equal volume of hot phenol. The sample was further incubated at 65^0C for 15 min, and the water and phenol phase was separated by centrifugation at 13,000 X g for 15 min at 10^0C. The water phase was dialyzed against distilled water and lyophilized. The lyophilized sample was resuspended in 0.05 M Tris-HCl, pH 7.0 and treated with RNase A, DNase I and proteinase K. The crude LPS extract was analyzed by SDS-PAGE and silver-stained as described by Noel *et al*. (2004).

LPS composition analysis

Sugar and fatty acid composition of the purified LPS was determined at the Complex Carbohydrate Research Center, University of Georgia. Briefly, 100 µl of each sample was incubated in 4N HCl for 2 hrs at 100 °C followed by drying under nitrogen, and the residue was used for composition analyses. Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-

trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. Methyl glycosides were then prepared from the dry samples by methanolysis in 1 M HCl in methanol at 80°C (16 hours), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 hours). These procedures were carried out as previously described (Merkle and Poppe 1994). GC/MS analysis of the TMS methyl glycosides was performed on an Agilent 7890N GC interfaced to a 5975C MSD (EI-MS), using a Grace EC-1 fused silica capillary column (30m × 0.25 mm ID). Additionally, the TMS methyl glycosides was performed on a Shimadzu QP2010 (GC/CI-MS), using a Grace EC-1 fused silica capillary column (30m × 0.25 mm ID).

Complementation studies with purified LPS

Germinated rice seeds were divided into two sets of 10 seeds each. Seeds from one set were soaked in isolated bacterial LPS overnight, and seeds from the second set were soaked in sterile water. The next day, seeds were transferred into glass tubes containing N-free growth medium and were inoculated with approximately 2×10^7 *Rhizobium* sp. IRBG74 *rffB* mutant. Bacterial colonization was determined at 7 DAI by harvesting the seedlings, and then by performing histochemical staining with X-gluc as well as making total bacterial counts. Similar experiments were performed to study the effect of rhamnose supplementation.

Results

Rhizobium sp. IRBG74 infects *S. cannabina* through root hairs under aerobic condition and via crack entry under flooded conditions

In order to study the plant colonization pattern of *Rhizobium* sp. IRBG74, we utilized a strain chromosomally marked with a constitutively expressed β-glucuronidase (GUS) that was previously shown to form effective nodules with *S. cannabina* similar to the wild type strain (Cummings *et al*., 2009). To facilitate easy determination of the colonization pattern, the GUS strain was also marked with a plasmid containing constitutively expressing green fluorescent protein (GFP) (Cheng and Walker, 2008). *Rhizobium* sp. IRBG74 infection occurred mainly through curled root hairs via infection thread formation when *S. cannabina* was grown under aerobic conditions (Fig. 5A-C) and were found to infect roots through crack entry at the point of lateral root emergence in *S. cannabina* seedlings when grown under submerged conditions (Fig. 5D). Regardless of the infection process it formed a typical N-fixing rhizobial endosymbiosis in the mature nodules (Fig. 5E-F). These results are similar to those from studies on the nodulation of *S. rostrata* by *Azorhizobium caulinodans* ORS571 (Goormachtig *et al*., 2004) and *S. virgata* by *Az. doebereinerae* (Bomfeti *et al*., 2013) i.e., *Rhizobium* sp. IRBG74 can infect *S. cannabina* by two mechanisms: crack-entry under submerged conditions and through root hairs under aerobic conditions.

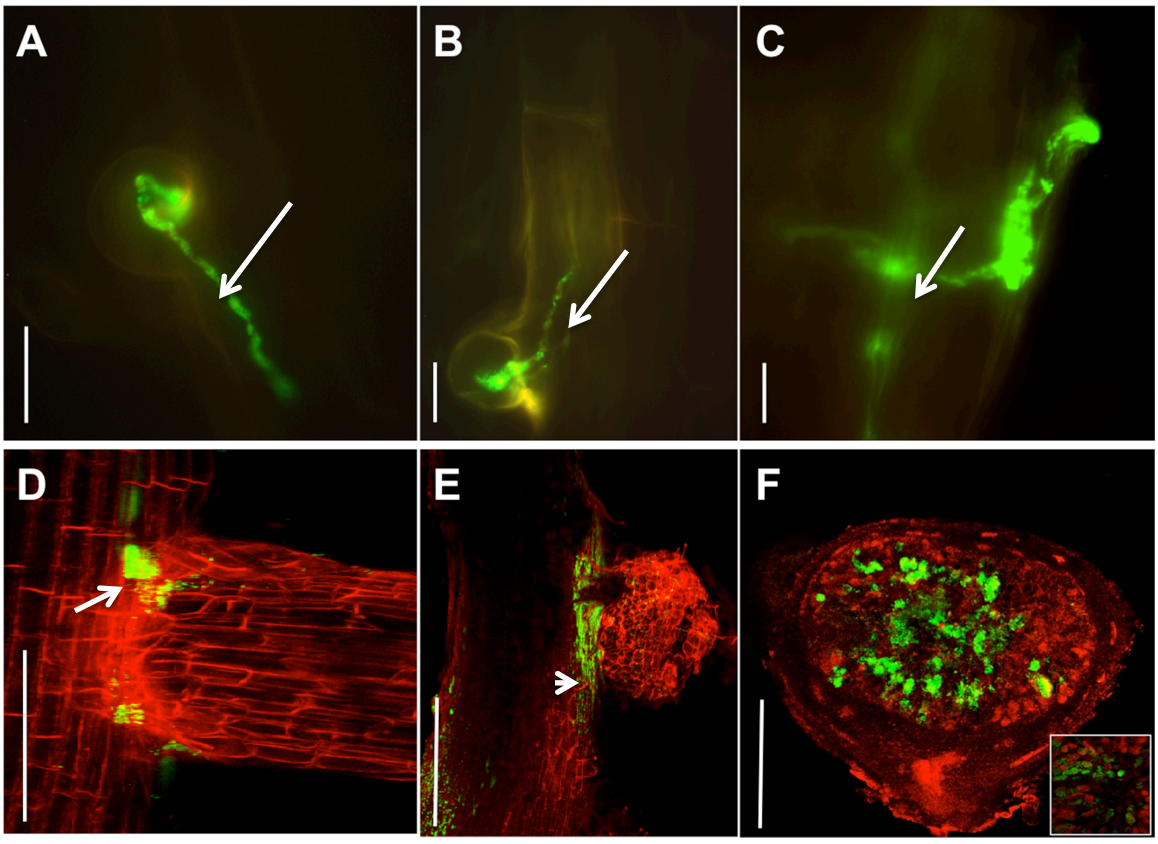


Fig. 5 Colonization of *S. cannabina* by *Rhizobium* sp. IRBG74-GFP. (A-C) Under aerobic conditions, *Rhizobium* sp. IRBG74 colonized *S. cannabina* via root hair curling and infection thread (arrow) formation. Scale bar = 10 µm (D) The GFP marked strain colonized *S. cannabina* roots through crack entry at the point of lateral root emergence (small arrow) under submerged conditions. Scale bar = 150 µm; (E) GFP fluorescing bacteria can be seen on the base of the nodule (arrow head) Scale bar = 600 µm. (F) GFP fluorescing bacteria can be seen inside the infected nodule. Scale bar = 600 µm; inst showing symbiosomes containing GFP fluorescing bacteria.

Rhizobium sp. IRBG74 endophytically colonizes Japonica rice variety nipponbare via cracks in lateral root junctions and through root hairs

To determine how *Rhizobium* sp. IRBG74 enters and colonizes rice, rice seedlings were inoculated and the colonization patterns were determined at 3 and 10 days after inoculation (DAI) by staining the plants for GUS activity (Gyaneshwar *et al.*, 2001). At 3 DAI, GUS activity was primarily localized at the sites of lateral root emergence (Fig. 6A arrows). At 10 DAI, however, intense GUS activity was observed throughout the roots of inoculated plants as well as on the basal portions of stems and leaves (Fig. 6B-arrow). No GUS activity was found on uninoculated seedlings (Fig. 6C). In accordance with the GUS staining, bacteria were found accumulated at the lateral root junctions, as determined by scanning electron microscopy (SEM) (Fig. 6D). When viewed under the confocal laser-scanning microscope (CLSM), GFP-expressing rhizobia were observed on the root surface (Fig. 6E small arrow) as well as at the points of lateral root emergence (Fig. 6E large arrow).

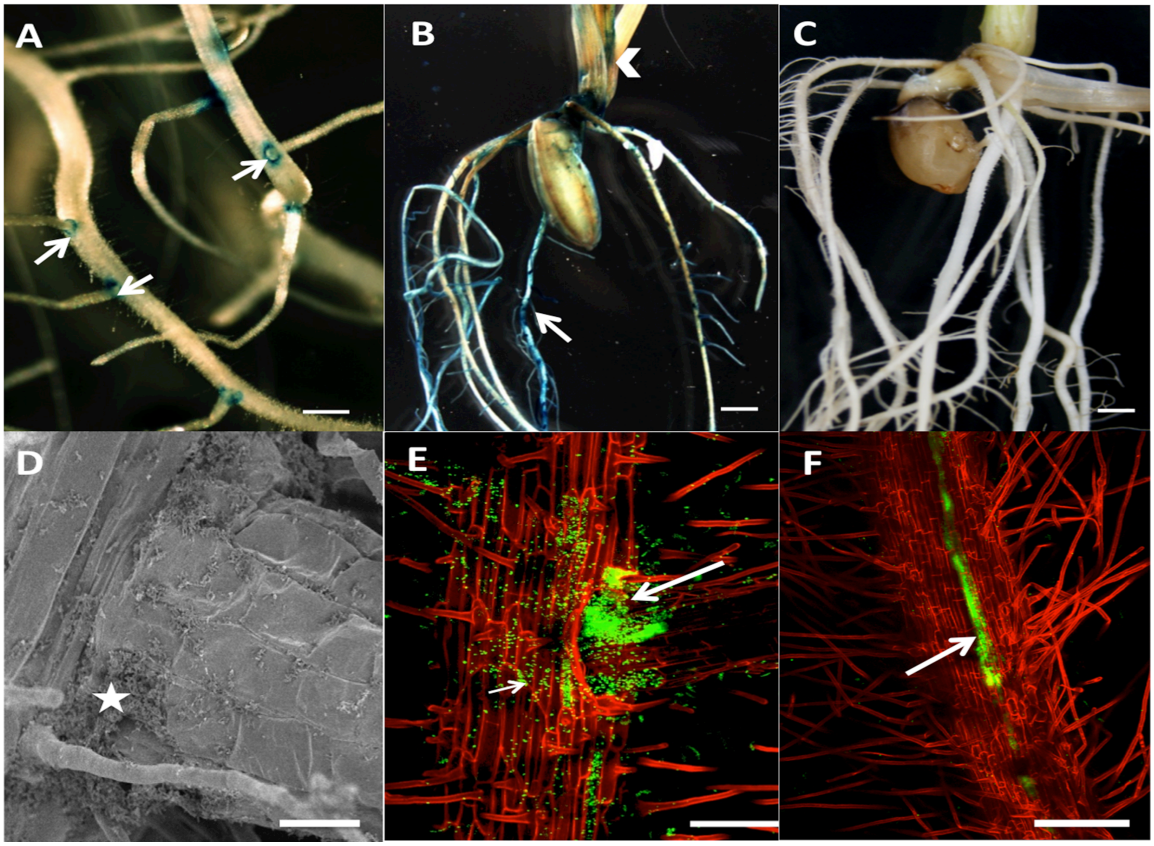


Fig. 6 Colonization of rice cultivar Nipponbare under submerged conditions by *Rhizobium* sp. IRBG74 marked with GUS and GFP. (A) At 3 days after inoculation (DAI), GUS staining was localized at the point of lateral root emergence (arrows) indicating crack entry. Scale bar = 0.5 mm. (B) At 10 DAI, the GUS staining can be seen throughout the primary roots as well as lateral roots. Scale bar = 1 mm. (C) GUS staining was not detected on the roots of control non-inoculated plants. Scale bar = 1 mm. (D) Scanning electron micrograph showing dense colonization of rice at the lateral root emergence (star). Scale bar = 25 μm. (E) Confocal laser scanning micrograph (CLSM) showing localized colonization of GFP-expressing *Rhizobium* sp. IRBG74 at the point of lateral root emergence (large arrow) as well as its presence on rice root surface (small arrow). Scale bar = 25 μm. (F) Confocal laser scanning micrograph (CLSM) showing the

presence of GFP-expressing *Rhizobium* sp. IRBG74 in the internal rice tissues. The rice root was stained with propidium iodide, which fluoresces red.

When the inoculated rice seedlings were grown under aerobic conditions, bacteria expressing GFP were also observed within root hairs (Fig. 7 A-C arrow) including the contiguous basal portion of the trichoblast from which the root hair has emerged and the cells adjacent to the infected root hairs (Fig. 7C and 7D arrow and star). In contrast to *S. cannabina*, where *Rhizobium* sp. IRBG74 induces root hair curling, we did not observe circumstances of this occurring in rice under these conditions. Further examination of rice root transversal sections showed that *Rhizobium* sp. IRBG74 colonizes the epidermal cells (small arrows), the exodermis (large arrow), as well as intercellular spaces and the aerenchyma in the cortical region (Fig. 7E; thin arrows). Additionally, examination of the GUS-stained stem sections demonstrated that *Rhizobium* sp. IRBG74 had colonized the internal tissues of the stem and was localized in the aerenchyma (large arrow) and stele (small arrow) (Fig. 7 F, G & H).

In order to further confirm the microscopic observations showing enodophytic colonization of rice by *Rhizobium* sp. IRBG74, we re-isolated *Rhizobium* sp. IRBG74 from the interior of inoculated plants by surface-sterilizing roots and shoots, and then quantified the bacteria using plate counts at different time points (3, 7, and 10 and 20 DAI). Bacteria were found inside roots and shoots in significant amounts, although at lower levels than in the non-surface-sterilized ones (Table 2). Total bacterial counts in both roots and shoots indicate that colonization continues to increase up to 20 DAI. However, the internal colonization pattern differed between plant organs. Inside the roots,

the *Rhizobium* sp. IRBG74 population increased between 3 and 7 DAI, while the internal colonization of the shoot was delayed and occurred between 7 and 10 DAI (Table 2).

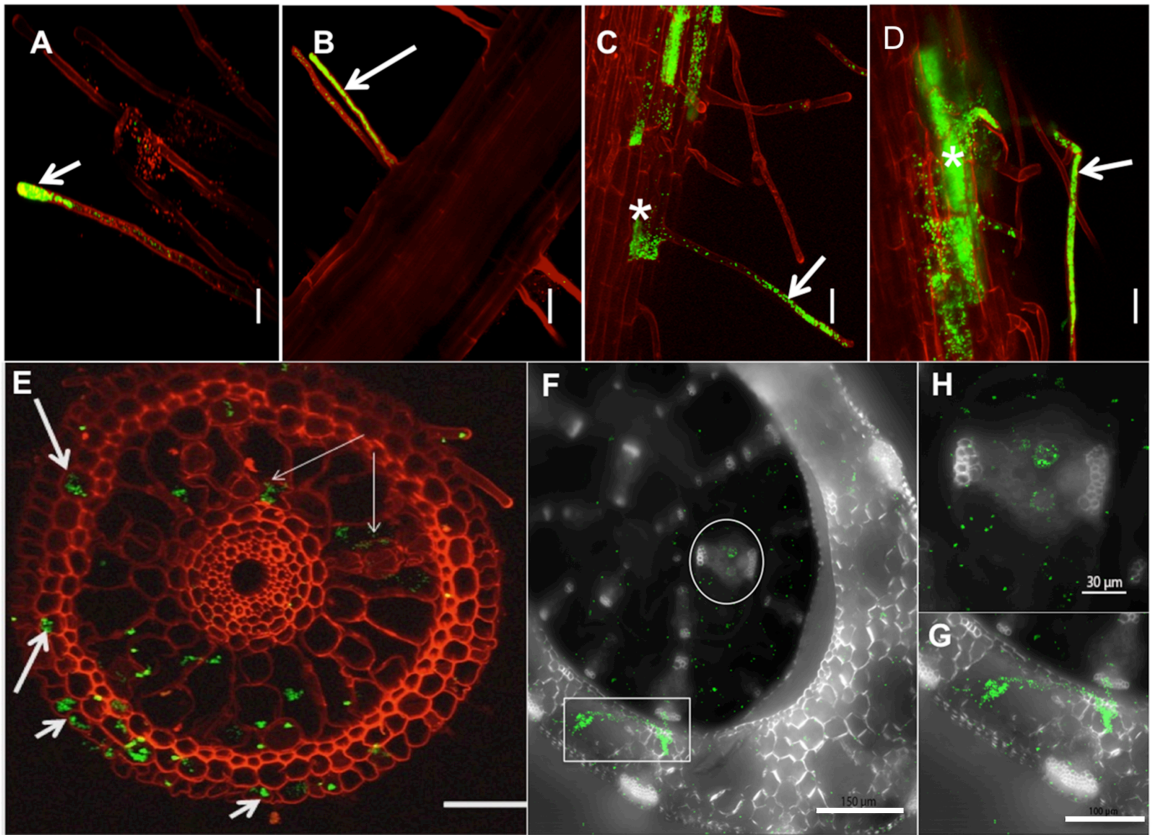


Fig. 7 *Rhizobium* sp. IRBG74 colonizes Nipponbare via root hairs and is endophytic under aerobic conditions. (A) Green fluorescence can be seen at the tip of a root hair (arrow). Scale bar = 50 µm. (B) Green fluorescing bacteria are present throughout the root hairs (arrow) Scale bar = 50 µm. (C &D) Bacteria expressing GFP were also observed within root hairs including the contiguous basal portion of the trichoblast (star) from which the root hair has emerged and the cells adjacent (arrow head) to the infected root hair. Scale bar = 50 µm. (E) CLSM of a transverse section of a rice root showing bacteria at the root surface (small arrow), inside the epidermal cells (large arrow) as well

as inside the arenchyma (thin arrows). Scale bar = 100 μm. (F) Transverse section showing green fluorescing bacteria in rice stem (circle and rectangle). Scale bar = 150 μm. (G) Inset of image F showing the localization of bacteria in aerenchyma. Scale bar = 100 μm. (H) Inset of figure (F) showing the localization of bacteria in Xylem (arrowhead) Scale bar = 30 μm.

Table 2. Surface and endophytic colonization of rice cultivar Nipponbare by *Rhizobium* sp. IRBG74

Days after inoculation (DAI)	Total bacterial counts (cfu X 10^3 mg^{-1} fresh weight)			
	Roots		**Shoots**	
	Surface	Internal	Surface	Internal
3 DAI	520 ± 25	0.15 ± 0.03	0.22 ± 0.08	0.12 ± 0.05
7 DAI	1000 ± 85	35 ± 4.5	6.2 ± 0.85	0.17 ± 0.03
10 DAI	2500 ± 75	58 ± 6.8	38 ± 5.2	5.9 ± 0.95
20 DAI	3400 ± 90	65 ± 7.2	150 ± 7.0	7.5 ± 1.5

Results are mean ± standard deviation of numbers obtained from 3 plants inoculated and incubated separately.

***Rhizobium* sp. IRBG74 is an endophyte of an indica rice cultivar**

The above results were obtained using the japonica cultivar Nipponbare. To determine if *Rhizobium* sp. IRBG74 can also colonize and become endophytically established within an indica rice cultivar, we inoculated bacteria onto surface-sterilized seeds of cultivar Moroberekan, an aluminum-tolerant indica rice variety, that was previously shown to support N_2-fixation by the rice endophyte *Herbasprillum seropedicae* Z67 (Gyaneshwar *et al.*, 2002). Dr. Euan K. James at the James Hutton Institute, Dundee, UK, performed these experiments. As our results with *O. sativa* ssp. Japonica suggested that the lower stem was heavily colonized by *Rhizobium* sp. IRBG74 (Fig. 2), we focused our attention on this tissue. The stems showing GUS activity were fixed with glutaraldehyde, sectioned and analyzed by light and transmission electron microscopy (TEM) (James *et al.*, 2002). The bacteria were observed inside epidermal cells (Fig. 8A) and occasionally in cortical cells (Fig. 8B), but the majority of bacteria were located in the intercellular spaces between cortical cells, especially those close to the endodermis (Fig. 8B). A few bacteria were also observed in the xylem (Fig. 8C). The identity of the bacteria inside the rice tissues was confirmed by TEM and immunogold labeling using a polyclonal antibody against *Rhizobium* sp. IRBG74 (Cummings *et al.*, 2009). Intense labeling of the bacteria present both outside and inside the rice stems was observed (Fig. 8D), further confirming that *Rhizobium* sp. IRBG74 forms an endophytic association with rice. Taken together, these results demonstrate that *Rhizobium* sp. IRBG74 forms endophytic associations with *O. sativa* ssp. indica.

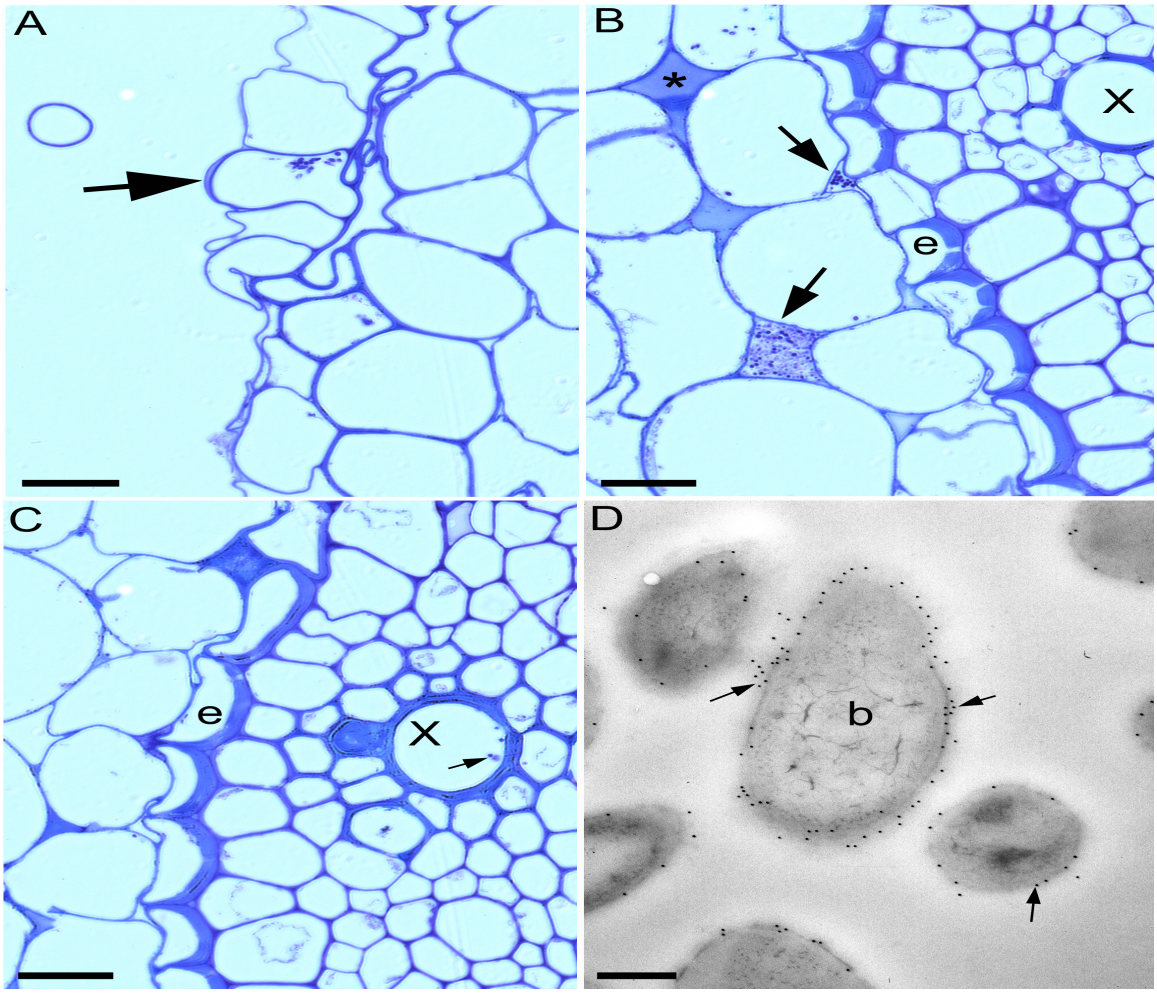


Fig. 8 Endophytic colonization of the lower stem of a seedling of rice cultivar Moroberekan by *Rhizobium* sp. IRBG74. (A) Light microscopy (LM) showing colonization of an epidermal cell (large arrow). (B) LM showing colonization of cortical intercellular spaces (arrows) close to the endodermis (e). Also note that some other spaces are apparently filled with material that stains with toluidine blue (*). (C) LM showing a few bacteria (arrows) in a xylem vessel (X). (D). Transmission electron microscopy (TEM) of bacteria (b) in the intercellular spaces. The bacteria were

immunogold labeled (small arrows) with an antibody specific to *Rhizobium* sp. IRBG74 (see Cummings et al. 2009 for more details). Serial sections treated with non-immune serum in place of the antibody were unlabeled (not shown). Bars, 20 μm (A – C). Scale bar = 200 nm (D). The light and transmission electron microscopy was performed by Dr. Euan K. James at The James Hutton Institute, Dundee, Scotland.

Nod factor dependent colonization of rice by *Rhizobium* sp. IRBG74

The formation of nodules by a rhizobium in a host legume requires recognition of a nodulation (Nod) factor produce by that rhizobium. As *Rhizobium* sp. IRBG74 forms classic nitrogen fixing nodules in *S. cannabina*, it confirms that *Rhizobium* sp. IRBG74 has genetic machinery to synthesize and secrete the nod factor. In legume, when a nod factor binds to its cognate receptor, the signal transcends through a pathway involving activation of a set of plant genes (common symbiotic pathway genes or CSP genes) required for symbiotic association with arbuscular mycorrhizal fungi (AM fungi).

Cereals form symbiosis with mycorrhizae and since rice requires the homologs of legume CSP pathway genes for mycorrhizal infection (Chen *et al*., 2007, 2008, 2009; Gutjhar *et al*., 2008), we hypothesized that the nod factors in IRBG74 may have a role in rice IRBG74 colonization and may involve the legume CSP homolog genes.

To test this hypothesis, we first checked expression of the *nodA* (involved in nod factor backbone synthesis) in *Rhizobium* sp. IRBG74 in the presence and absence of rice using semi-quantitative RT-PCR (Fig 9E). Transcripts for *nodA* were detected in the bacteria in the presence of rice. Expression of *rpoB* was used as an internal control.

To test if *Rhizobium* sp. IRBG74 requires nod factor for rice colonization, a *nodABC* deletion mutant was generated and a rice colonization study was performed. Interestingly, significantly less GUS activity was detected on the rice root colonized with the *nodABC* deletion mutant (Fig. 9B &D), when compared to the wild type *Rhizobium* sp. IRBG74 (Fig. 9A & C) . The decrease in colonization of rice by the *nodABC* deletion mutant was further verified via quantification of colony-forming units (cfu g^{-1} fresh rice root), which showed more than a 5-fold reduction in rice colonization by the mutant as compared to the parental strain (Fig. 9F).

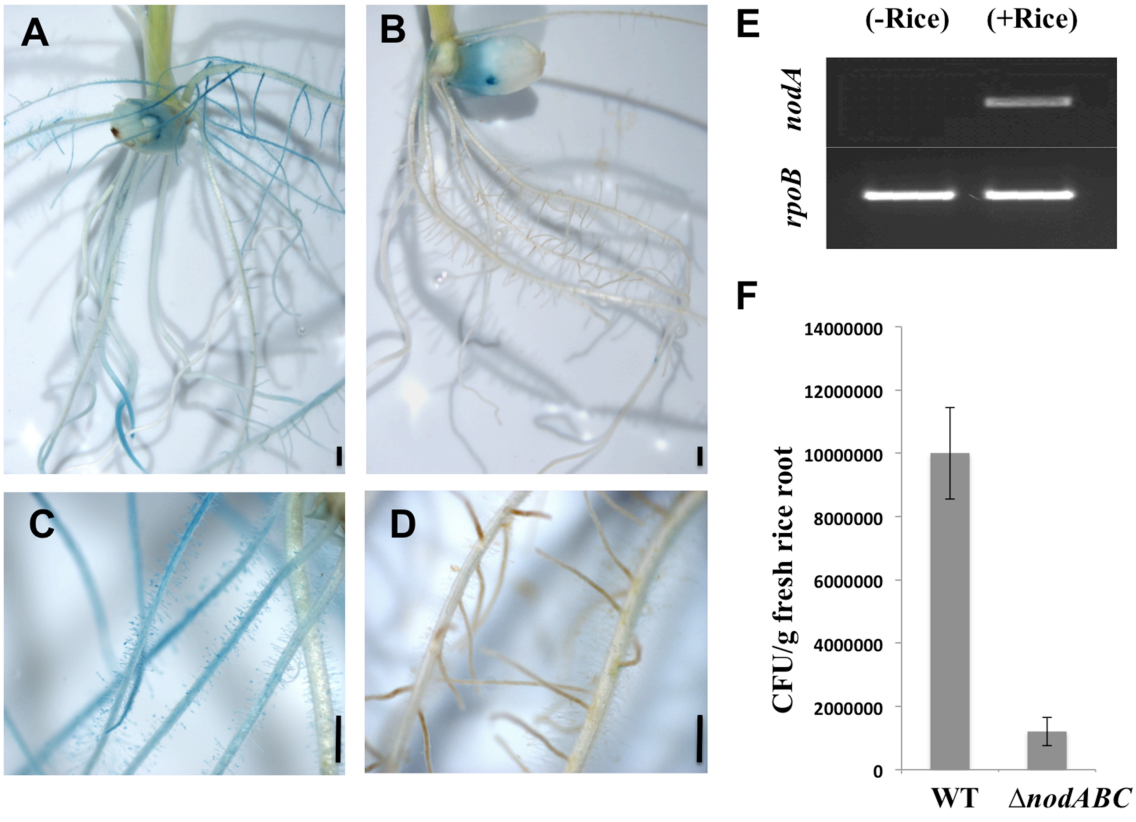


Fig. 9 Nod factor dependent colonization of rice by *Rhizobium* sp. IRBG74 and RT-PCR analysis of *nodA* expression. (A & C) GUS staining of rice roots colonized by the wild type strain. Scale bar = 1mm. (B & D) Substantially less GUS staining was observed

on the roots colonized by the *nodABC* deletion mutant. Scale bar = 1 mm. (E) Transcript for *nodA* was detected in *Rhizobium* sp. IRBG74 grown in the presence of rice, but not in the absence of rice. *rpoB* was used as an internal control. (F) Counts of bacteria re-isolated from inoculated roots showed significantly less colonization by the *nodABC* deletion mutant as compared to the WT strain. Results are mean ± standard deviation of 3 plants inoculated with independent bacterial cultures.

Rhizobium* sp. IRBG74 *genome

Though rice doesn't have the specific nod factor perception receptor, experiments in this study suggest that the colonization of rice by *Rhizobium* sp. IRBG74 is Nod factor dependent. So, we hypothesized that the *Rhizobium* sp. IRBG74 Nod factor is structurally similar to that of mycorrhizal LCO like signal and like *Parasponia*, rice also possesses a plant receptor capable of identifying LCOs from both *Rhizobia* as well as from *Arbuscular mycorrhiza.* (Op den Camp *et al.*, 2011).

To deduce the structure of *Rhizobium* sp. IRBG74 nod factor, and to know whether other symbiotic specific genes are present in *Rhizobium* sp. IRBG74 we performed the complete genome sequencing of *Rhizobium* sp. IRBG74. The genome of *Rhizobium* sp. IRBG74 (5,464,982 bp) consists of a 5,540 predicted coding DNA sequences (CDS), which are distributed on a circular chromosome (2,844,565 bp, 59.30% G+C content), a linear chromosome (2,035,452 bp, 59.29% G+C content), and a symbiotic plasmid, pIRBG74a (584,965 bp, 57.48% G+C content). Plasmid pIRBG74a, a *repABC*-family plasmid, contains many symbiotic specific genes including, *nod*, *nif* and *fix*.

These results are consistent with those of earlier studies (Cummings *et al.*, 2009) and show that *Rhizobium* sp. IRBG74 is a naturally occurring unique species in the *Rhizobium*/*Agrobacterium* clade that likely obtained nodulation capabilities by acquisition of the pIRBG74a symbiosis plasmid by horizontal gene transfer. The availability of this genome sequence will help determine the mechanisms by which *Rhizobium* sp. IRBG74 forms endophytic and growth-promoting associations with rice, an important cereal crop.

The *Rhizobium* sp. IRBG74 *rffB* mutant is defective in rice colonization

To identify genetic determinants important for rice colonization by *Rhizobium* sp. IRBG74, we generated random transposon-insertion mutants, tagged them with GUS, and then screened them for their inability to colonize rice using GUS staining. Out of ca. 500 mutants screened, we isolated one mutant that showed a marked decrease in colonization (Fig. 10B) as compared to its parental strain (Fig. 10A). The decrease in colonization was verified via quantification of colony-forming units (CFU), which showed more than a 1000-fold reduction in rice colonization by the mutant as compared to the parental strain (Fig. 10C). To identify the transposon-insertion site in the mutant, the genomic DNA flanking the transposon was cloned and sequenced using transposon-specific primers. The DNA sequence was then used for a homology search against the genome sequence of *Rhizobium* sp. IRBG74 (Crook *et al.*, 2013). The sequence analysis found that the transposon had disrupted a gene showing high similarity (>99% identical) to *A. tumefaciens rffB,* encoding dTDP-D-glucose-4, 6-dehydratase, that is part of a cluster of four genes involved in the synthesis of O-antigen of bacterial LPS (Fig. 11A). RffB synthesizes dTDP-rhamnose, which is important for the synthesis of O-specific

lipopolysaccharide (LPS) in many Gram-negative bacteria, including *Rhizobium* and *Agrobacterium*. Earlier studies have shown that LPS is a major determinant of symbiotic competence in rhizobia (Gage 2004; Becker *et al*., 2005; Jones *et al*., 2007) as well as for effective colonization of plants by beneficial plant-associated bacteria, such as *Pseudomonas putida* (de Weger *et al*., 1989) and *Herbaspirillum seropedicae* (Balsanelli *et al*., 2010, 2013). It is thus not surprising that our mutant screen would identify a LPS mutant.

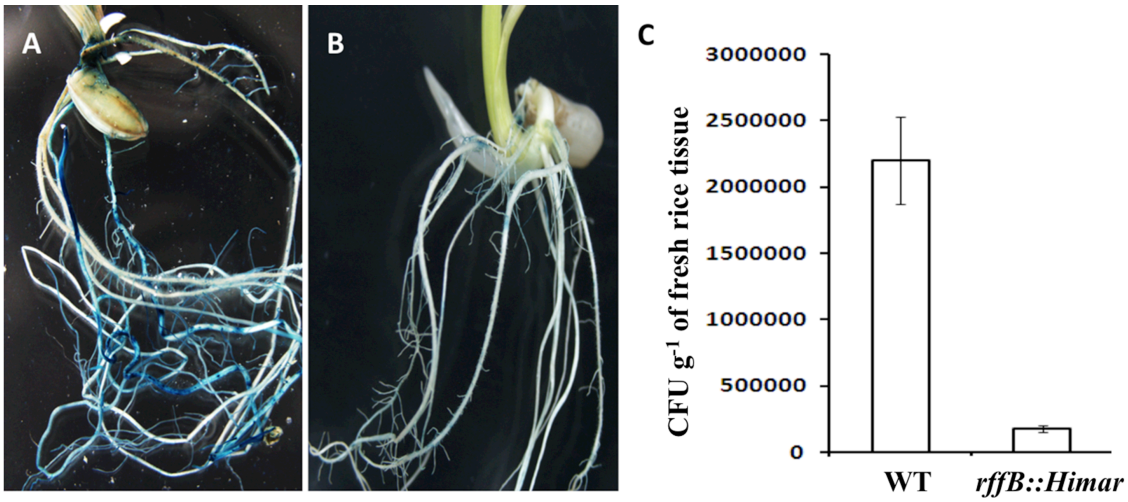


Fig. 10 Rice root colonization by *Rhizobium* sp. IRBG74 and the *rffB* mutant.

(A) GUS staining on rice roots colonized by the wild type strain. Scale bar = 1 mm. (B) Substantially less GUS staining was observed on the roots colonized by the *rffB* mutant. Scale bar = 1 mm. (C) Counts of bacteria re-isolated from inoculated roots showed significantly less colonization by the *rffB* mutant as compared to the parental strain. Results are mean ± standard deviation of 3 plants inoculated with independent bacterial cultures.

The *Rhizobium* sp. IRBG74 *rffb* mutant synthesizes an altered LPS that lacks rhamnose

To determine if the *rffb* mutant produces altered LPS due to the transposon insertion, total LPS was extracted using the hot phenol water method and resolved by SDS-polyacrylamide gel electrophoresis, silver staining and comparison to LPS from the wild-type strain. The LPS from the wild type strain showed an intense low molecular band consisting of lipid A and the LPS core (Fig. 11B, black bar), and a high molecular weight band corresponding to the O-antigen (Fig. 11B, white bar inside the boxed). In accordance with the insertional inactivation of *rffB*, the LPS of the mutant lacked the higher molecular weight band corresponding to the O-antigen of LPS (Fig. 11B, Box) suggesting a defect in the synthesis of the O-antigen by the *rffB* mutant similar to that reported for the *rffB* mutant of *H. seropedicae* (Balsanelli *et al.*, 2010).

Analysis of monosaccharides of the LPS from the wild type and *rffB* mutant strains using gas chromatography-mass spectrometry showed that the wild type LPS contained glucose (45.7%), rhamnose (42.6%), ribose (7.9%), mannose (1.7%), galactose (0.9%), N-acetyl glucosamine (0.4%) and KDO (0.7%) (Table 3). In contrast, LPS from the *rffb* mutant completely lacked rhamnose and contained higher concentrations of glucose (85.7%), but was not affected in other sugars, indicating that insertional inactivation of *rffB* specifically altered the rhamnose content of the LPS (Table 3).

Rhizobium sp. _rffB_ mutant is not affected in growth, motility, or survival in the rice rhizosphere

Earlier studies have shown that LPS modifications are correlated with reduced bacterial motility and reduced growth rates (Ormeño-Orrillo *et al.*, 2008; Balsanelli *et al.*, 2010). As these phenotypes are important for host colonization, we examined the swimming motility and growth rate of the *rffB* mutant and its parental strain in rice growth media supplemented with mannitol and ammonium chloride as carbon and nitrogen sources, respectively. No significant differences were observed in motility of the *rffB* mutant and the parental strain (Fig. 11C) or in their growth rate in YM liquid medium, with both strains showing a doubling time of ~75 minutes.

LPS is a structural component of Gram-negative bacteria, and defects in LPS can lead to sensitivity to antimicrobial compounds and detergents (Nikaido 2003). Additionally, LPS mutants of *R. tropici* (Ormeño-Orrillo *et al.*, 2008) and *H. seropedicae* (Balsanelli *et al.*, 2010) were found to be susceptible to antimicrobial compounds produced during plant defense, leading to a decrease in plant colonization ability (Ormeño-Orrillo *et al.*, 2008). To determine if the *rffB* mutant of *Rhizobium* sp. IRBG74 was similarly sensitized to plant-produced antimicrobials in the rice rhizosphere, we determined its survival in the rice root zone by viable counts at different DAI. This showed that the survival level of the *rffB* mutant was not significantly different from the wild type at 5 and 10 DAI (Fig. 11D). Taken together, these results suggest that the rice colonization defect of the *rffB* mutant is not due to pleiotropic defects associated with altered LPS, but it is likely that the rhamnose-containing O-antigen is involved in the interactions with rice that lead to surface and endophytic colonization.

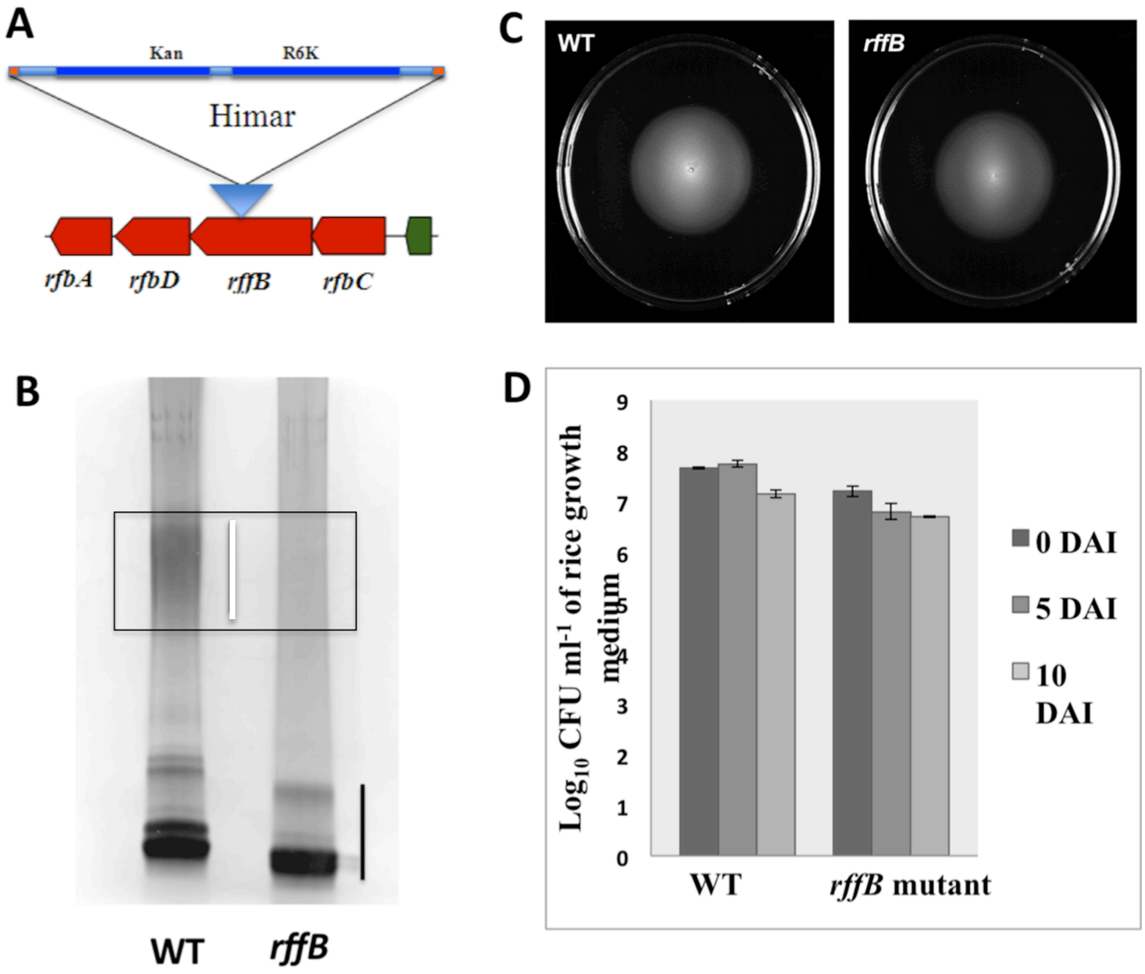


Fig. 11 Characterization of the *rffB* mutant of *Rhizobium* sp. IRBG74.

(A) The transposon insertion was found in *rffB*, which is part of a cluster containing genes for rhamnan O-antigen synthesis. (B) SDS-PAGE analysis of purified LPS showing the lack of higher molecular weight O-antigen in the *rffB* mutant as compared to the wild type (WT). (C) The *rffB* mutant did not show any significant differences in motility as compared to the WT. (D) No significant differences were observed in the survival of the *rffB* mutant in the rice rhizosphere as compared to the WT.

Table 3. Monosaccharide composition of LPS isolated from *Rhizobium* sp. IRBG74 and its *rffB* mutant.

Monosaccharide	**Wild type**	***rffB:Himar***
Glucose	45.7	85.7
Rhamnose	42.6	ND
Ribose	7.6	9.9
Mannose	1.7	1.9
Galactose	0.9	1.2
N-acetyl glucosamine	1.1	0.6
KDO	0.4	0.7

Sugars are expressed as Mol% of total carbohydrate.

The *rffB* mutant is also defective in nodulating *S. cannabina*

LPS synthesis is involved in rhizobial-legume interactions and subsequent nodule formation (Beker *et al.*, 2005). Rhizobial LPS has been hypothesized to act as an inhibitor of the plant defense response, or as being important for rhizobial adaptation to the nodule environment. Specifically, a knockout of dTDP-ramnose synthase in *Azorhizobium caulinodans* was shown to be arrested in intracellular multiplication and nitrogen fixation (Gao *et al.*, 2001; Mathis *et al.,* 2005). It was postulated that rhamnose-containing LPS of *A. caulinodans* ORS571 acts as a communication signal for promoting an effective symbiosis with *S. rostrata* (*Mathis et al.*, 2005). As *Rhizobium* sp. IRBG74 is also a *Sesbania* symbiont, and since both *Sesbania*-nodulating symbionts synthesize rhamnose-containing LPS, we tested the nodulation and symbiotic properties of the IRBG74 *rffB* mutant on *S. cannabina*. It formed only a few white nodules (6-10 nodules plant^{-1}) as compared to the parental strain that formed large pink nodules (15-20 nodules plant^{-1}) (Fig. 12, A and E). Staining of the nodules revealed that nodules elicited by a GUS-tagged variant of the *rffB* mutant showed very little GUS activity, as compared to nodules from the GUS-tagged parental strain, that turned intense blue (Fig. 12, B and F). In accordance with the GUS staining pattern, no fluorescent bacteria were detected in the nodules induced by a GFP-tagged variant of the *rffB* mutant as compared to extensive fluorescence in nodules containing the wild type bacteria tagged with GFP (Fig. 12, C and G). The white nodules formed by the *rffB* mutant were most likely defective in symbiotic nitrogen fixation, as indicated by the pale green chlorotic leaves of the inoculated plants compared to the dark green leaves of plants nodulated with the wild type strain (Fig. 12, D and H). To further determine the nature of defects associated with

the *rffB* mutant, the fixed nodules were examined by light and electron microscopy and compared to the nodules of the wild type strain. Light microscopic analysis showed that the *rffB* mutant infected the nodules but the nodule infection was much lower than the wild type, and the nodules showed signs of degradation (Fig 13). Further analysis using transmission electron microscopy revealed that the *rffB* mutant formed symbiosomes that were enlarged as compared to the wild type and the bacteroids showed signs of senescence indicating lack of symbiotic effectiveness (Fig 13). The light and transmission electron microscopy was performed by Dr. Euan K. James at The James Hutton Institute, Dundee, Scotland. These results show that the rhamnose-containing LPS of *Rhizobium* sp. IRBG74 is critical for successful nodule occupancy in a manner similar to that reported for LPS in the *A. caulinodans* symbiosis with *S. rostrata* (Mathis *et al.*, 2005).

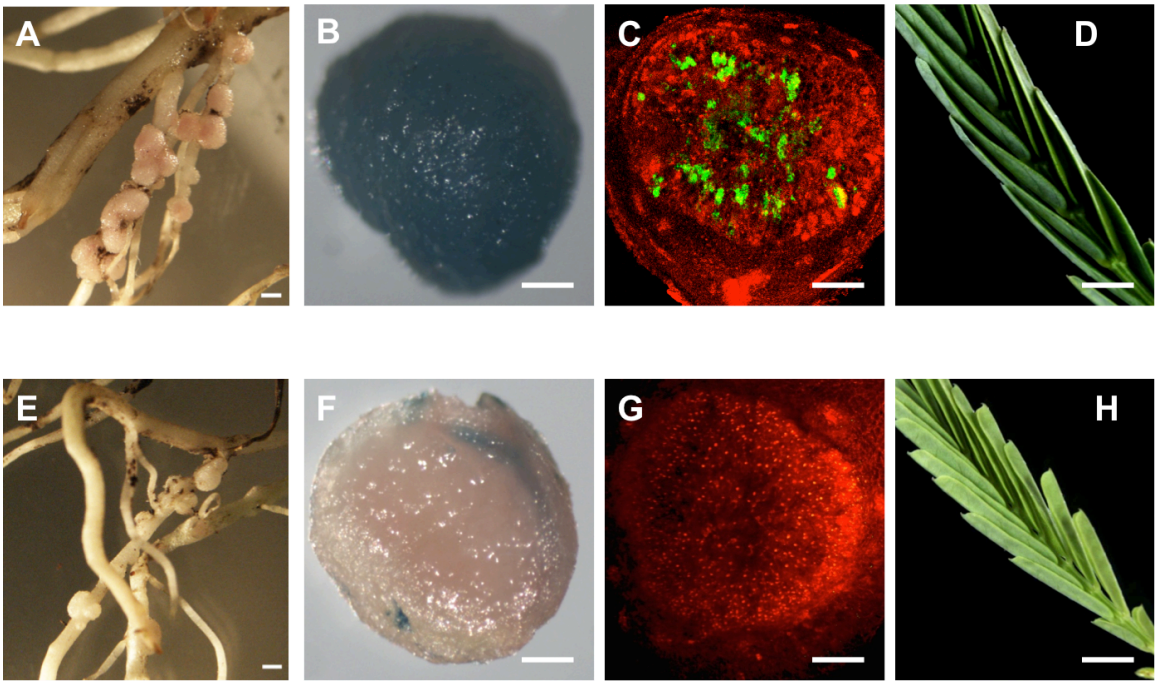


Fig. 12 The *Rhizobium* sp. IRBG74 *rffB* mutant is defective in symbiosis with *S. cannabina*. (A-D) The symbiosis of *S. cannabina* with WT *Rhizobium* sp. IRBG74. (A) WT forms numerous pink nodules. Scale bar = 1mm. (B) Nodule showing intense GUS staining. Scale bar =500 µm. (C) GFP fluorescing WT bacteria inside a nodule. Scale bar = 300 µm. (D) Evidence of effective N-fixation (green leaves). Scale bar =500 µm. (E-H) The interaction between *S. cannabina* with *rffB* mutant. (E) *rffB* mutant induces small white nodules in *Sesbania*. (F) *Sesbania* nodule induced by *rffB* mutant showed very little GUS staining. Scale bar =500 µm. (G) Almost undetectable amount of green fluorescing mutant bacteria inside the nodules. Scale bar = 300 µm. (H) Pale green leaves indicating an N-deficiency in plant. Scale bar =500 µm.

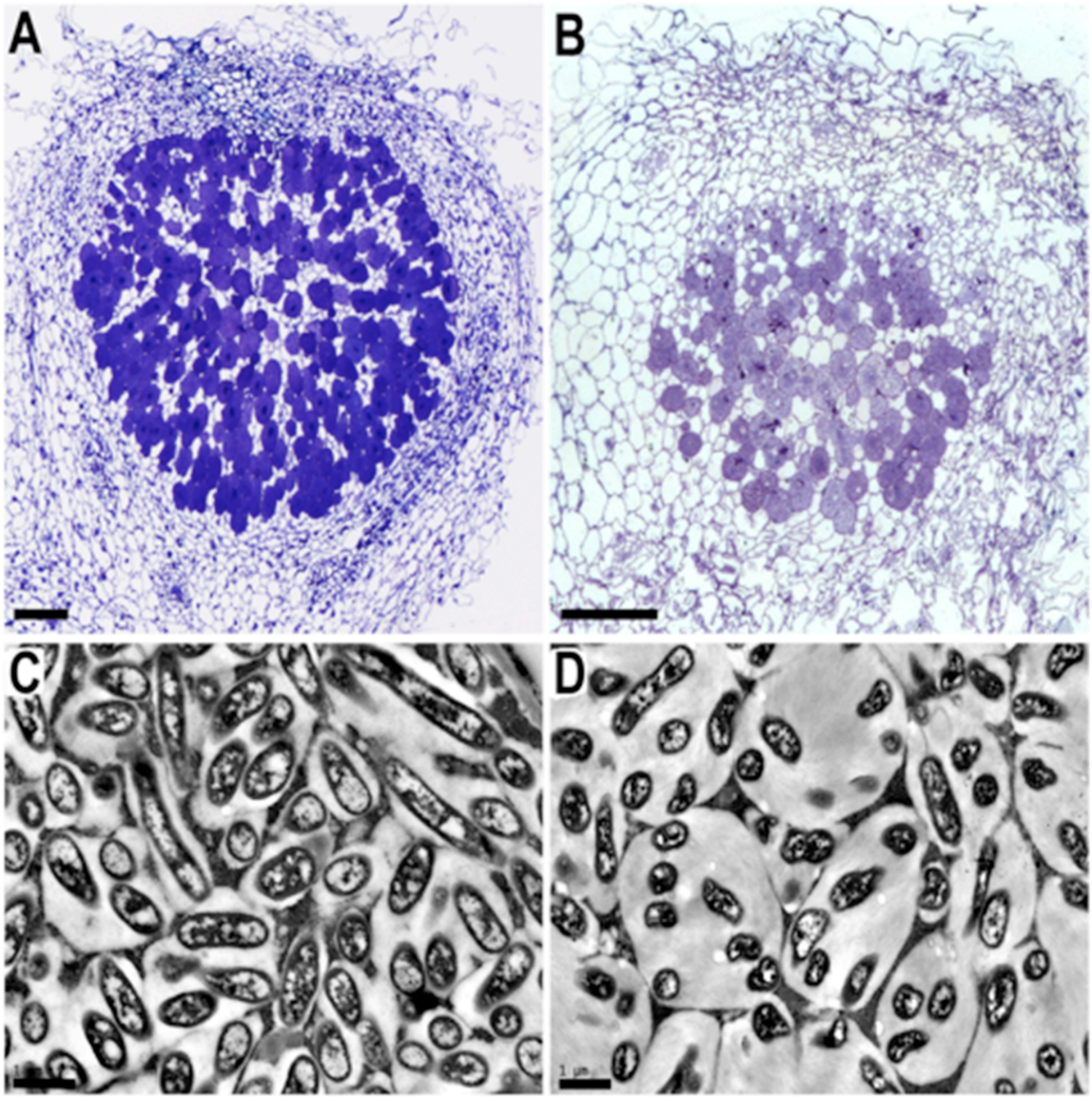


Fig. 13 Light and electron micrographs of *S. cannabina* nodules induced by wild type and the *rffB* mutant of *Rhizobium* sp. IRBG74. (A, B) Light micrograph showing a semi-thin section of a nodule infected with wild type bacteria (A) and the *rffB* mutant (B). (C, D) Transmission electron micrograph showing ultra-thin section of nodules infected with wild type bacteria (C), and the *rffB* mutant (D). Scale Bar = 20 µm (A, B) and 1 µm (C, D).

Purified LPS from wild type IRBG74 rescues the rice colonization defect of the *Rhizobium* sp. IRBG74 *rffB* mutant

Our results indicated that LPS is important for establishment of endophytic colonization of both legume and rice hosts by *Rhizobium* sp. IRBG74. To confirm that LPS is the main determinant of this phenotype, we determined the colonization of rice and *S. cannabina* by the wild type and *rffB* mutant in the presence of purified LPS extracted from the wild type or the *rffB* mutant. The *rffB* mutant containing the GFP plasmid (Cheng and Walker, 2008) was inoculated onto surface-sterilized seeds of rice and *S. cannabina* with or without LPS and the colonization was determined by GFP fluorescence, SEM and cell counts. Addition of LPS extracted from the wild type strain led to a significant enhancement of bacterial attachment on inoculated rice roots (Fig. 14B) as compared to roots that were supplemented with the mutant LPS (Fig. 14C) or to the untreated roots (Fig. 14A). Analysis using SEM further confirmed these observations, as addition of wild type LPS led to increased bacterial attachment on rice roots as compared to the untreated roots (Fig. 14D), and the bacteria were found to be present in a biofilm-like matrix (Fig. 14E). In contrast, addition of the mutant LPS did not promote bacterial attachment or matrix production (Fig. 14F). These microscopic observations were confirmed by determining the bacterial counts that showed significantly higher numbers of bacteria on the roots treated with wild type LPS as compared to the mutant LPS or no LPS (Table 4). These results confirm that rhamnose-containing LPS is an important determinant for successful *Rhizobium* sp. IRBG74-rice interactions.

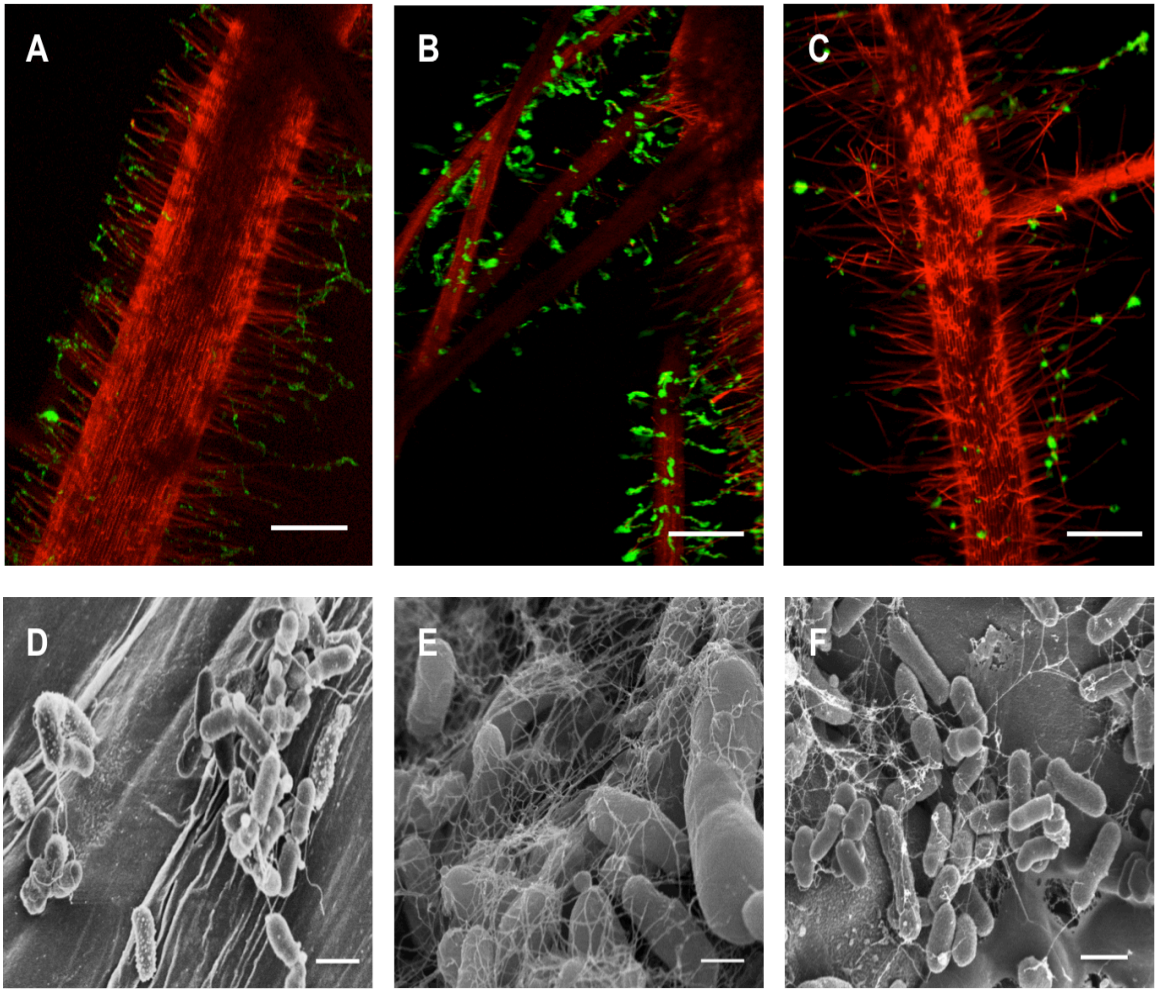


Fig. 14 Colonization of rice by the *rffB* mutant in the presence of purified LPS.

(A) Roots inoculated with the GFP marked *rffB* mutant show sparse amount of green fluorescing bacteria on their surfaces. Scale bar = 600 µm. (B) Supplementation of LPS from the WT strain leads to an increase in GFP fluorescence, when compared to roots inoculated with the *rffB* mutant alone. Scale bar = 600 µm. (C) Supplementation of LPS from the *rffB* mutant strain does not lead to increased colonization by the mutant. Scale bar = 600 µm. (D) SEM showing the *rffB* mutant on roots not treated with LPS. Scale bar = 1 µm (E) SEM of the *rffB* mutant on rice roots treated with WT LPS. Scale bar = 0.5 µm. (F) *rffB* mutant on rice root treated with LPS extracted from the *rffB* mutant. Scale

bar = 1 μm. Note the increased amount of a filamentous-like matrix in the presence of LPS extracted from the WT strain but not from the *rffB* mutant.

Table 4. Effect of LPS addition on the endophytic colonization of rice by wild type and *rffB* mutant strains of *Rhizobium* sp. IRBG74.

Strain	**Treatment**	**CFU × 10^3 mg^{-1} of fresh rice tissue**
WT	Water	1214 ± 25
WT	WT LPS	1152 ± 52
rffB	Water	14 ± 2
rffB	WT LPS	1022 ± 34
rffB	*rffB* LPS	29 ± 12

Results are mean ± standard deviation of at least 3 plants inoculated with independent bacterial cultures.

Supplementation of LPS restores the defects in early steps but not nodulation and symbiotic nitrogen fixation of the *Rhizobium* sp. IRBG74 *rffB* mutant

Earlier studies have suggested that LPS is important for proper progression of endosymbiosis between *A. caulinodans* and *S. rostrata* (Mathis *et al.*, 2005). To determine if purified LPS can rescue the nodulation and symbiotic defect of the *Rhizobium* sp. IRBG74 rffB mutant, we studied colonization of *S. cannabina* by the *rffB* mutant in the presence or absence of LPS, and compared it to the colonization of the wild type strain. Analysis using confocal microscopy showed that at 3DAI, the *rffB* mutant colonized *S. cannabina* roots at a much lower level than the wild type strain (Fig 15A and B). Supplementation with LPS led to significant enhancement of bacterial numbers on the roots as observed by increase in fluorescence (Fig 15C). At 10 DAI, the *rffB* mutant was still much lower in number but still showed root hair deformation similar to the wild type (Fig 15 D and E). Supplementation with LPS resulted in much higher colonization and root hair deformation (Fig 15F). However, addition of purified LPS did not affect the total number of nodules formed or the dry weight of the plants inoculated with the *rffB* mutant (Table 5).

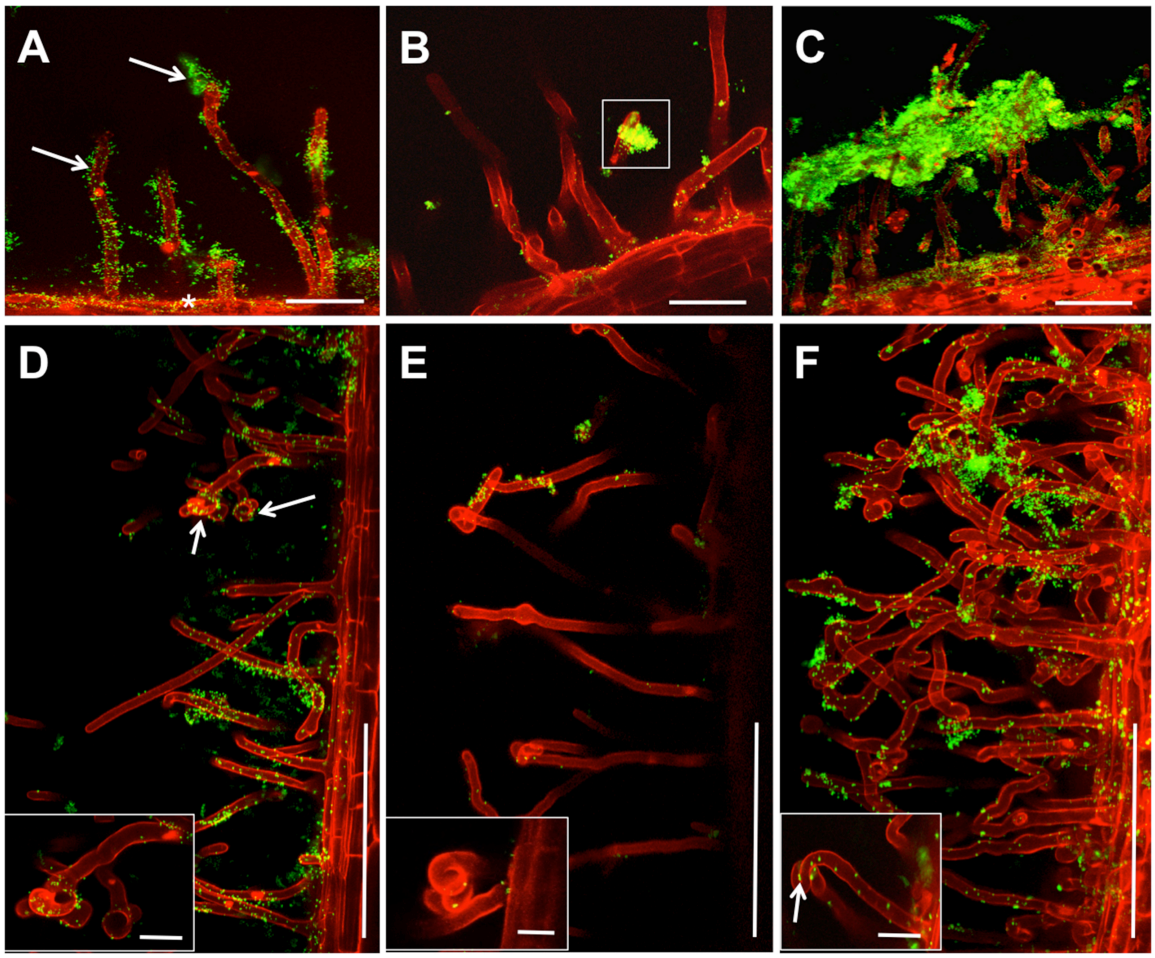


Fig. 15 Effect of LPS supplementation on the colonization of *Sesbania cannabina* by the *rffB* mutant. (A) At 3DAI, wild type *Rhizobium* sp. IRBG74 (wildtype) was found to be closely associated with *Sesbania cannabina* root hair and on the root surface. (B) gfp-expressing *rffB* mutant formed small aggregations (box) on *Sesbania cannabina* root hairs, but were found to be significantly lower in number when compared to the wildtype. (C) Supplementation of the *rffB* mutant with wild type LPS during *sesbania cannabina* colonization study results in enhanced aggregation of gfp-expressing *rffB* mutant on *Sesbania cannabina* root surface. (D) At 10 DAI, gfp-expressing wild type *Rhizobium* sp. IRBG74 colonized the deformed root hair (arrow); Inset showing the formation of shepherd crook structure. (E) Very few gfp-expressing *rffB* mutant bacteria around a

deformed *Sesbania cannabina* root hair; Inset showing the formation of root hair curling in the presence of the *rffB* mutant. (F) Increased aggregation of the gfp-expressing *rffB* mutant and root hair deformation in the presence of purified WT LPS on *Sesbania cannabina* root surface; inset showing the entrapped bacteria (arrow) in curled root hair. Scale bars = 75 µm (A-C), 150 µm (D-F) and 15 µm for insets.

Table 5. Nodulation of *S. cannabina* by *Rhizobium* sp. IRBG74 and its *rffB* mutant

Strain	No. Nodules (Size)	Plant Dry Weight (mg)
None	0	126 ± 16^{a}
Wild type	30 ± 5 (large)	255 ± 24^{b}
rffB	12 ± 6 (small)	165 ± 13^{c}
rffB + WT LPS	10 ± 5 (small)	150 ± 10^{c}

Results are mean ± standard deviation of 3 plants inoculated with independent bacterial cultures. Values in a column with different letters are statistically different with $p<0.05$.

Discussion

Although many rhizobial strains are able to enhance the growth of cereals, very little is known about the bacterial determinants for beneficial rhizobial-cereal interactions. In the present study, interactions between *Rhizobium* sp. IRBG74 and rice were analyzed and we found that *Rhizobium* sp. IRBG74 showed extensive colonization of rice roots at 7-10 DAI, especially at the point of lateral root emergence. This pattern of colonization is similar to that observed with other rice endophytes, such as *H. seropedicae* (James *et al*., 2002; Roncanto-Maccari *et al*., 2003) and *Azoarcus* (Egener *et al*., 1998). We used microscopy and immunogold labeling to demonstrate that *Rhizobium* sp. IRBG74 is a *bona fide* rice endophyte, as it is able to colonize the interior of both roots and stems. Akin to legumes, *Rhizobium* sp. IRBG74 accumulated especially around root hairs on rice and extensively colonized them internally, but unlike in legumes no root hair deformation was observed. Based on the pattern of surface colonization during the early stages of colonization as shown by SEM, and by intense GUS staining at the points of lateral root emergence, it is likely that *Rhizobium* sp. IRBG74 penetrated through the cracks at the sites of lateral root emergence and then migrated up to the shoots. Indeed, the points of lateral root emergence are prominent sites for entry of another rice endophyte, *H. seropedicae* (James *et al*., 2002).

Most legumes so far studied for their rhizobial infection processes appear to be infected via root hairs. However, it is now clear that crack entry and/or direct epidermal infection are also quite common mechanisms by which rhizobia can enter their legume hosts, and they may represent an evolutionary "default" infection process, even in

legumes that are normally infected via root hairs (Madsen *et al*., 2010). The natural occurrence of crack entry is particularly common (and often predominant) in aquatic and flooding-tolerant legumes, such as those in the hydrophytic genera *Aeschynomene*, *Neptunia* and *Sesbania* (Goormachtig *et al*., 2004; Bonaldi *et al*., 2011; Bomfeti *et al*., 2012). Interestingly, some species in these genera, with *Sesbania* being of particular relevance with regard to the present study, are capable of "dual" rhizobial infection processes which, depending on the degree of root/stem submergence, tend to be either via root hair infections under aerobic (non-flooded) conditions or via crack entry under flooded conditions (Goormachtig *et al*., 2004; Cummings *et al*., 2009; Bomfeti *et al*., 2012; this study). What is also clear from these studies is that the rhizobia that infect semi-aquatic *Sesbania* spp. are also highly plastic in terms of their ability to infect their legume hosts, and taken together with the new observations for rice and *Rhizobium* sp. IRBG74 presented in the present study, suggest that under flooded conditions aquatic rhizobia, such as *Rhizobium* sp. IRBG74 and *Azorhizobia,* utilize similar mechanisms to infect/colonize both their legume hosts and rice (this study; Gopalaswamy *et al*., 2000). Although *Rhizobium* sp. IRBG74 was inoculated into the rooting medium, it was subsequently found to colonize the internal tissues of stems indicating ascending migration of *Rhizobium* sp. IRBG74 within rice tissues. Colonization of the xylem might explain the ability of rhizobia to migrate efficiently from roots to shoots. This pattern of colonization has been observed with other rhizobial rice endophytes, such as *R. leguminosarum* bv. *trifolii* (Prayitno *et al*., 1999) and *Sinorhizobium* (*Ensifer*) *meliloti* 1021 (Chi *et al*., 2005). A similar migration in rice tissues (and localization in the xylem in many, but not all cases) have been reported for other rhizobial and non-rhizobial

endophytes of rice, such as *Az. caulinodans* (Gopalaswamy *et al*., 2000), *Bradyrhizobium* sp. (Chaintreuil *et al*., 2000), *R. leguminosarum* bv. *trifolii* (Prayitno *et al*., 1999), *S. meliloti* (Chi *et al*., 2005) and *H. seropedicae* (James *et al*., 2002; Gyaneshwar *et al*., 2002; Roncanto-Maccari *et al*., 2003). Our results indicate that rhizobial endophytic colonization in the stems of rice is a common phenomenon that needs to be further studied.

A mutant of *Rhizobium* sp. IRBG74 deficient in rice colonization was identified via random transposon mutagenesis. This mutant was found to contain a transposon insertion in *rffB*, a gene that was shown to be involved in the synthesis of UDP-rhamnose, an essential component of LPS in *A. tumefaciens* (De Castro *et al*., 2006). Considering the close phylogenetic relationship of *Rhizobium* sp. IRBG74 to *A. tumefaciens,* it is likely that *Rhizobium* sp. IRBG74 produces a rhamnan-O-antigen similar to the one produced by *A. tumefaciens,* and the *Rhizobium* sp. IRBG74 *rffB* mutant is indeed defective in O-antigen synthesis. Supplementation of rice seeds with LPS from the wild type strain restored the rice colonization ability of the *Rhizobium* sp. IRBG74 *rffB* mutant, thus indicating that the rhamnose moiety of LPS made by *Rhizobium* sp. IRBG74 is likely to be of importance for its recognition by rice as a potentially beneficial endophyte, and for the subsequent internalization of the bacteria. These results are consistent with the observations that rhamnose-containing LPS is important for the endophytic colonization of maize by the beneficial bacterium *H. seropedicae* (Balsanelli *et al*., 2010) and that maize lectins, which bind LPS, mediate *H. seropedicae*-maize interactions (Balsanelli *et al*., 2013).

Earlier studies have shown that the LPS-O-antigen is important for nodulation of legume-hosts by various rhizobia (Becker *et al*., 2005; Noel *et al*., 2004; Ojdea *et al*., 2010). In particular, a rhamnan-O-antigen of *Sinohizobium* sp. NGR234 is specifically induced by plant-produced flavonoids and is important for symbiotic interactions with many legumes (Reuhs *et al*., 2005). In accordance with these studies, the *rffB* mutant formed small and ineffective nodules on *S. cannabina* that contained prematurely senescing bacteroids, but it remains to be determined if the rhamnan-LPS of *Rhizobium* sp. IRBG74 is induced during its interactions with rice and *S. cannabina*.

In legume, when a nod factor is perceived via its cognate receptor, the signal transcends via activation of a set of plant genes. In legumes these genes are also involved in AMS, and thus considered as the common symbiotic pathway genes or CSP genes. As rice requires the homologs of legume CSP pathway genes for mycorrhizal infection (Chen *et al*., 2007, 2008, 2009; Gutjhar *et al*., 2008) and IRBG74 is capable of infecting legume (*Sesbania*) and is also an aggressive colonizer of rice, we tested the possibility of nod factor dependent rice colonization by *Rhizobium* sp. IRBG74. First, we checked the expression of *Rhizobium* sp. IRBG74 *nodA* (involved in nod factor backbone synthesis) gene in the rice rhizosphere, and then tested the importance of *Rhizobium* sp. IRBG74 nod factor in rice colonization by making a Δ*nodABC* mutant and performing a rice colonization study with the nod factor defective mutant. Surprisingly, we were able to detect the expression of *nodA* in *Rhizobium* sp. IRBG74 grown in the presence of rice and also found that the nod factor defective mutant of *Rhizobium* sp. IRBG74 is also defective in rice colonization. These results strongly suggest that, the *Rhizobium* sp. IRBG74 nod factor is important in the rice- *Rhizobium* sp. IRBG74 interaction and plays

a pivotal role in successful colonization. However, whole genome transcriptome analysis of rice in the presence of *Rhizobium* sp. IRBG74 purified nod factor is required to further deduce the role of nod factor and to understand the mode of interaction.

The genome sequence of *Rhizobium* sp. IRBG74 reveals that it contains a linear and a circular plasmid similar to those *of A.tumifecian* C 58 and *Agrobacterium sp*. H13-3. In addition, it also contains a symbiosis specific plasmid (pIRBG74a) with a large number of *nod*, *nif*, and *fix* genes, required for nitrogen fixation within legume hosts. In accordance with the previous study (Cummings *et al.*, 2009), we agree on the fact that *Rhizobium* sp. IRBG74 is a naturally occurring unique species in the *Rhizobium*/*Agrobacterium* clade and most likely it obtained its nodulation capabilities by acquisition of the pIRBG74a symbiosis plasmid by horizontal gene transfer. The availability of this genome sequence will open a plethora of opportunities to determine the mechanisms by which *Rhizobium* sp. IRBG74 forms endophytic and growth-promoting associations with rice.

The fact that the *Rhizobium* sp. IRBG74 mutant defective in rhamnan-LPS synthesis was not only significantly affected in its ability to colonize rice (both surface and internally), but was also defective in its ability to colonize *S. cannabina* suggests a similar role of LPS in initial colonization of both legume and cereal hosts. However, even though the initial mechanisms by which they are colonized have similarities, the mode of action of *Rhizobium* sp. IRBG74 on each of the two hosts is very different i.e. it acts as a non-diazotrophic epiphytic/endophytic plant growth-promoting rhizobacteria on rice, and as an endosymbiotic N_2-fixing nodulator in *S. cannabina*.

Chapter III

Role of a novel ABC transporter in endophytic colonization of rice and nodulation of *Sesbania cannabina* by *Rhizobium* sp. IRBG74

Introduction

Bacterial adherence to environmental surfaces and formation of a well-organized multicellular structure is commonly known as a biofilm. Rhizobia form biofilms on plant surfaces during symbiosis, which help them to mitigate common environmental stresses, like nutrient limitation and desiccation, and give a competitive advantage in that rhizosphere. (Pérez-Montaño *et al.*, 2014; Ramey *et al.*, 2004; Rinaudi and Giordano, 2010). The structure of a rhizobial biofilm may vary from small aggregations to an extensive biofilm, depending on the properties of the plant root surface, availability of water and nutrients in the rhizosphere and the proclivity of that *Rhizobium* to colonize a plant. (Pérez-Montaño *et al.*, 2014; Ramey *et al.*, 2004). Though the role of biofilms in rhizosphere survivability is well-studied in bacteria from the genera *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Rhizobium*, there are only a few studies describing the role of rhizobial biofilms in symbiosis.

As *Rhizobium* sp. IRBG74 forms extensive biofilm on rice root surfaces, we hypothesized that the biofilm formation is an intrinsic component of the *Rhizobium* sp. IRBG74- rice interaction. To study this interaction, a transposon insertion mutant library of *Rhizobium* sp. IRBG74 was screened to identify biofilm defective mutants. We identified a mutant (BD1, biofilm defective mutant 1), which showed impaired substrate attachment and defective biofilm formation on biotic and abiotic surfaces. When tested

for colonization and nodulation efficiency in rice and *Sesbania* respectively, the biofilm defective mutant showed a severe defect in colonization of both legume and cereal hosts.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in the study are summarized in Table 6. The media and growth conditions used for the maintenance of the strains are the same as described in Materials and Methods section in Chapter II.

Table 6: Strains, plasmids and primers used in this study

Strains/Plasmids/ Primers	Description	Reference
Strains		
IRBG74-GUS	Wild type *Rhizobium* sp. IRBG74 marked with pCAM121 (*papH-gusA*) Spec^r	Cummings et al. (2009)
IRBG74-GUS-GFP	*Rhizobium* sp. IRBG74-GUS containing pHC60	This study
thiQ::Himar	*Rhizobium* sp. IRBG74-GUS *thiQ::Himar* mutant Kan^r	This study
thiQ::Himar-GFP		This study
Δ*thiQ*	*Rhizobium* sp. IRBG74-GUS *thiQ* deletion mutant Gen^r	This study
thiQ::Himar complemented	*Rhizobium* sp. IRBG74-GUS *thiQ::Himar* mutant with pMBC223-*thiBPQ* Tet^r	This study
E. coli β2155	*thrB*1004 *pro thi strA hsdsS lacZ* ΔM15 (F9 *lacZ* ΔM15 *lacI^q traD*36 *proA*1 *proB*1) Δ*dapA*::*erm* (Erm^r) *pir*::RP4 [::*kan* (Km^r) from SM10]	Dehio and Meyer (1997)

Plasmids		
pHC60	IncP Tet^r, *gfp* under the control of a constitutive *lac* promoter	Cheng and Walker (1998)
pMiniHimar	*mini-HimarRB1* transposon, R6K *ori*	Bouhenni *et al*. (2005)
pCAM121	Sm/Sp, Ap; mTn5SS*gusA2l* (P*aph-gusA-trpA* ter) in pUT/mini-Tn5 Sm/Sp	Wilson *et al*. (1995)
pENTR™/D-TOPO® Vector	Entry Vector for TOPO cloning Kan^r	Life Technologies
pMBC219	The gateway destination vector derivative of the suicide plasmid pJQSK200 Gen^r	Quandt and Hynes (1993)
pMBC223	The gateway destination vector derivative of the expression plasmid pRF771Tet^r	Wells and Long (2002)
Primers		
Himar 1	CATTTAATACTAGCGACGCCATCT	Bouhenni *et al*. (2005)
Himar 615	TCGGGTATCGCTCTTGAAGGG	Bouhenni *et al*. (2005)
k2	CGGTGCCCTGAATGAACTGC	Datsenko and Wanner (2000)
kt	CGGCCACAGTCGATGAATCC	Datsenko and Wanner (2000)
thiQ upstream_F	CACCCGGCCTCATCTTCATGCTTTG	This study
thiQ upstream_R	GCAAAGAAATCCGCTGTCGCGAGCTGCAC TGCGAAATCGTC	This study

thiQ downstream_F	GACGATTTCGCAGTGCAGCTCGCGACAGC GGATTTCTTTGC	This study
thiQ downstream_R	GAGACTGGTTTCTGGACGGTATAG	This study
P_*thiBPQ* F	CACCTCGAGACATCGAGAGCCTTCTTC	This study
thiBPQ R	TTTGTTGACGTTCGTGAGTG	This study
*thiQ*_RT_FP	TGTCCACGGCCTTCACATTT	This study
*thiQ*_RT_FP	GCAGGATTTCGACCTCGACT	This study
*rpoB*_RT_FP	CGAGTTCGACGCCAAGGATA	This study
*rpoB*_RT_RP	ACGCGGTAGATGTCGAACAG	This study

Colonization of rice and *S. cannabina* by *Rhizobium* sp. IRBG74

The colonization studies of rice and Sesbania by *Rhizobium* sp. IRBG74 were performed using the same methods as described in Chapter II.

Enumeration of *Rhizobium* sp. IRBG74 colonizing rice roots and stems

The colonization of rice roots and stems was quantified as described earlier in the Materials and Methods section in Chapter II.

Microscopic studies of plant infection and colonization by *Rhizobium* sp. IRBG74

The sample preparations for microscopic studies were performed as described earlier in the Materials and Methods section in Chapter II.

Construction of a *Rhizobium* sp. IRBG74 mutant library

The random insertion mutant library of *Rhizobium* sp. IRBG74 was constructed using a modified mariner transposon miniHimar (pMiniHimarRB1) as described earlier in the Materials and Methods section in Chapter II.

Screening of a biofilm defective mutant

Substrate attachment and biofilm formation ability of the mutants were tested on 96 well polystyrene plates. Overnight grown mutants on YM plates were separately inoculated in to 200 µl of fresh YM media in each well of a 96 well plates. After incubation at 30˚ C for 48 h the optical density (OD) of the cells were measured at 600 nm using a spectrophotometer. Planktonic cells from the 96 well plates were gently pipetted out of the well and discarded. The wells were then washed with sterile distilled water and stained with 1% crystal violet (CV) for 10 min. After 10 min the wells were washed twice with sterile distilled water and dried briefly, before adding 95% ethanol. The optical densities of the CV in 96 well plates were measured at 570 nm using a spectrophotometer.

Biofilm preparation

The substrate attachment and biofilm formation ability of the bacteria were also tested on other abiotic and biotic surfaces using different microscopic techniques as described in Chapter II. For the preparation of biofilms on glass and plant surfaces, the bacteria were revived on a freshly prepared YM plate containing suitable antibiotics and then inoculated into 10 ml of YM media and grown overnight for the preparation of seed

culture. The bacteria were re-inoculated in fresh YM from the seed culture at a 1:1000 ratio and grown until the OD_{600} reached 0.6. The grown bacterial cultures were pelleted by centrifugation and washed twice with sterile distilled water.

For preparing biofilm on glass surfaces, sterile glass coverslips were used. The coverslips were placed in glass petriplates and inoculated with bacteria. The entire setup was incubated at 30˚C for 48 - 72 hours for proper formation of biofilms on glass surfaces.

For preparing biofilms on plant (sesbania and rice) surfaces, plants were germinated aseptically as described in Chapter II. After germination, plants were transferred to sterile Magenta boxes containing 30 ml of plant growth medium and inoculated with 3×10^7 cells. Plant samples were harvested at different time points to observe the formation and maturation of the biofilm.

Identifying the transposon insertion sites

Genes disrupted by the transposon were identified by PCR amplification and by sequencing of genomic regions flanking the transposon as described earlier in the Materials and Methods section in Chapter II.

Southern Hybridization

Approximately 10 µg of bacterial chromosomal DNA was digested with *Bam*HI and separated on a 0.8% agarose gel. The gel was depurinated with 5-10 volumes of 0.25 M HCl and the DNA was denatured using a solution containing 1.5 M NaCl and 0.5 N NaOH. The DNA transfer from gel to a nylon membrane was done overnight at room

temperature using 20X SSC buffer. The probe used for hybridization was synthesized by PCR using primers k2 and kt listed in Table 6, which annealed to the neomycin transferase gene of pMiniHiMar (Bouhenni *et al.*, 2005). The probe was labeled with DIG-dUTP using a Biotin 3′ End DNA Labeling Kit according to the manufacturer's instructions (Thermo Scientific). Hybridization was performed overnight with shaking at 55°C in the North2South® Hybridization buffer (Thermo Scientific). After hybridization, the membrane was washed three times with North2South® Stringency wash buffer (2 × SSC, 0.1% SDS) for 15 to 20 min at 37˚ C. Probe hybridization was detected using North2South® Chemiluminescent detection kit (Thermo Scientific).

Construction of the *thiQ* deletion mutant

To construct a deletion mutant of *thiQ*, Gateway® Cloning Technology (Life Technologies) was employed to clone a 2 kb IRBG74 Δ*thiQ* PCR amplicon into the destination vector pMBC219. The deletion vector pMBC219 [pJQ200SK (Quandt and Hynes, 1993) modified with the Gateway® cloning cassette from pDEST24™ (Invitrogen)] was kindly provided by Matthew Crook (University of Wisconsin–Madison). Briefly, PCR primers were designed to amplify approximately 1kb upstream and 1 kb downstream sequence of IRBG74 *thiQ* gene. Overlap extension PCR (OE-PCR) was used to generate the IRBG74 *thiQ* deleted (Δ*thiQ*) amplicon. The Δ*thiQ* amplicon was then cloned into pENTR™/D-TOPO® Vector (entry vector) using manufacturer's (Life Technologies) protocol. The vector containing the IRBG74 Δ*thiQ* fragment was then chemically transformed into One Shot® TOP10 chemically competent *E. coli* cells using manufacturer's protocol and selected on LB plates containing 50 µg

ml^{-1} of kanamycin. Kanamycin resistant TOP10 *E. coli* colonies were inoculated in LB broth and the entry vector containing 2 kb IRBG74 Δ*thiQ* fragments were isolated and confirmed by PCR. Using LR cloning technique, the 2 kb IRBG74 Δ*thiQ* PCR fragment was then transferred to the destination vector pMBC219. The destination vector containing IRBG74 Δ*thiQ* was then chemically transformed into β2155 cells and selected on LB gentamycin 50 μg ml^{-1} plates. (Dehio and Meyer, 1997). For efficient transfer of destination vector (containing Δ*thiQ* fragment) to the IRBG74 *gusA* strain the process of conjugation was employed. The single recombinants of *Rhizobium* sp. IRBG74 containing IRBG74 Δ*thiQ* fragment were then selected on LB gentamycin 150 μg ml^{-1}plates and confirmed with PCR. Sucrose selection was performed on YM minimal media plate, containing 10% sucrose, to obtain the double recombinants. The double recombinants obtained were then tested for their gentamycin sensitivity to confirm loss of the destination vector. Then sucrose resistant and gentamycin sensitive IRBG74 colonies were screened for Δ*thiQ* using PCR.

Complementation of the *thiQ::Himar* mutant

The entire *thi* operon including the 500 bp upstream DNA region was amplified using the primers (P_thiBPQ F and thiBPQ R) listed in Table 6 and high fidelity Phusion polymerase (Thermo Scientific). The 5.8 kb amplicon was then cloned into destination vector pMBC223 (a derivative of pRF771) using Gateway technology, transferred into chemically competent β2155 (Dehio and Meyer, 1997), and selected on LB tetracycline 10 μg ml^{-1} plates. The expression vector pMBC223 [pRF771 (Wells and Long, 2002) modified with the Gateway® cloning cassette from pDEST24™ (Invitrogen)] was kindly

provided by Matthew Crook (University of Wisconsin–Madison). The destination vector containing the *thi* operon was then transferred from β2155 *E. coli* cells to IRBG74 thiQ Himar mutant by the process of conjugation. The transconjugants were then selected on YM minimal media plate containing tetracycline 10 μg ml^{-1}.

RNA isolation

To isolate bacterial RNA in the presence of naringenin, the strains were grown to mid-logarithmic phase in YM minimal medium containing 50 μm naringenin. The cells were then harvested and RNA was isolated using TRIzol reagent (Sigma). The RNA concentrations were determined by UV/Vis spectrophotometry. RNA samples were further treated with RNase free DNase (Promega) to eliminate any DNA contamination.

Reverse Transcription PCR

cDNA synthesis and RT-PCR were performed using the AccessQuick RT-PCR system (Promega) as follows. The DNase treated RNA sample (0.3 μg) was used as template for cDNA synthesis at 45°C for 45 min. PCR amplification was performed using primers listed in Table 1 as follows: 2 min of incubation at 94°C, followed by 25 cycles of amplification with denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. RNA samples that had not been subjected to reverse transcription were used as negative controls and *rpoB* was used as the internal control.

Sensitivity to Flavonoids

The bacterial strains were grown in YM in the presence of a synthetic flavonoid, naringenin. Varying concentrations of naringenin ranging from 50-200 μM were used for the flavonoid sensitivity experiments. The strains were allowed to grow overnight and the absorbance was measured at 600 nm.

Results

Rhizobium sp. IRBG74 forms an extensive biofilm on rice root surface

When studying the colonization pattern of *Rhizobium* sp. IRBG74 with rice, we observed that the initial response of *Rhizobium* sp. IRBG74 is to form a biofilm-like structure on the rice root surface. In order to obtain a high resolution image of these cellular aggregation on the rice root surface, SEM was performed on plant samples inoculated with *Rhizobium* sp. IRBG74. *Rhizobium* sp. IRBG74 was found to form an extensive biofilm on rice root surfaces (Fig 16 A-C). The bacteria was found to be embedded in an extracellular fibrillar matrix which is not evident in other rice colonizing bacteria, such as *Azoarcus* sp. DQS4 (Fig 16 D). This suggests that the formation of biofilm is an intrinsic part of the *Rhizobium* sp. IRBG74- rice interaction.

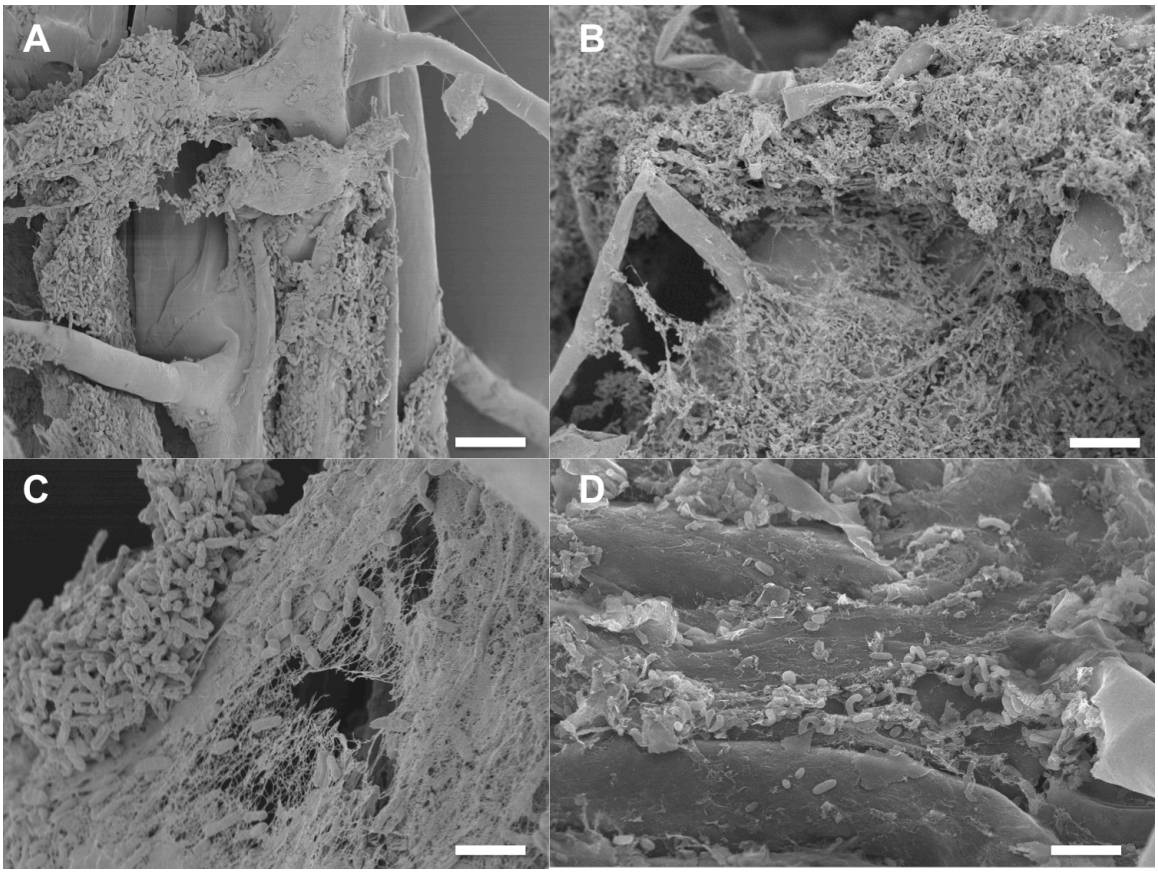
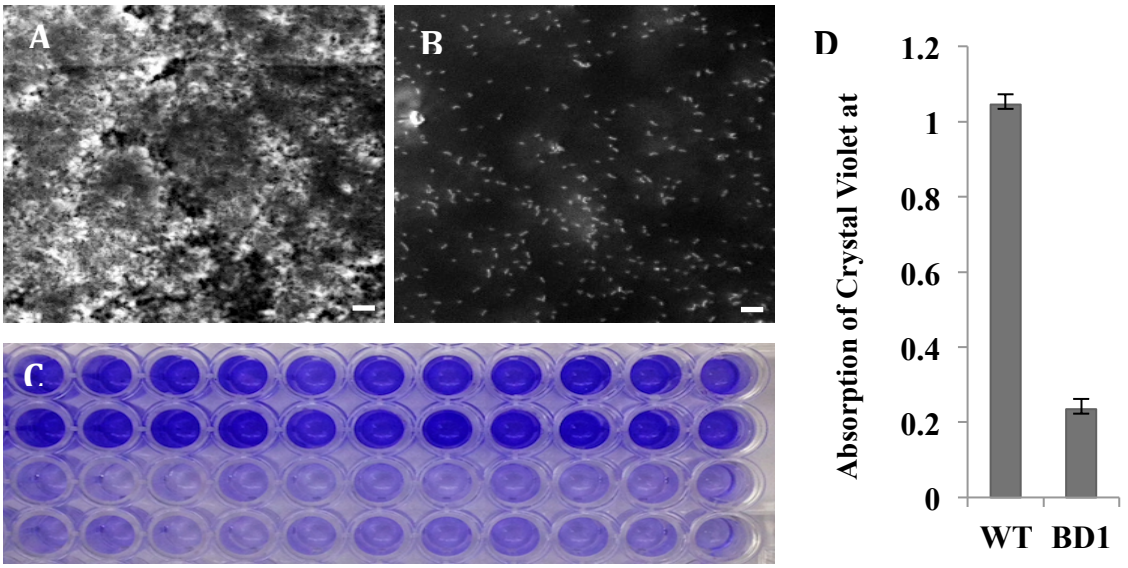


Fig. 16 *Rhizobium* sp. IRBG74 forms an extensive biofilm on the surfaces of the rice roots. (A-C) Scanning electron micrographs showing biofilm formed by *Rhizobium* sp. IRBG74 on the surface of rice roots, where rhizobia were embeded in a fibrilar extra cellular matrix. (A &B) Scale bar =20 μm; (C) Scale bar =5 μm. (D) Rice inoculated with *Azoarcus* sp. DQS4, where aggrigation of bacteria on the rice surface is markedly different than IRBG74. Formation of extra cellular fibrillar matrix was not observed during the surface colonization of rice by DQS4. Scale bar =5 μm.

Identification of mutants defective in biofilm formation on biotic and abiotic surfaces

The mutant library of *Rhizobium* sp. IRBG74 was screened for biofilm defect in 96 well polystyrene plates. Approximately 790 independent mutants were screened for biofilm defects by growing in YM media for 48 hours in separate wells, followed by staining with CV and taking the absorbance at 570 nm.

Screening for biofilms resulted in identification of one mutant [biofilm deficient 1 (BD1)], which showed a severe biofilm defect on 96 well polystyrene plates (Fig 17C) as well as on glass surfaces (Fig 17B), compared to WT (Fig 17A,C). Quantification of biofilms showed at least 5-fold reduction in biofilm formation in the mutant compared to WT *Rhizobium* sp. IRBG74 (Fig 17D).



Results are mean ± standard deviation of 10 wells inoculated with bacterial cultures.

Fig. 17 The BD1 mutant was defective in biofilm formation.

(A) Biofilm formed by *Rhizobium* sp. IRBG74 on a glass surface. (B) BD1 failed to form

a biofilm on a glass surface. Scale bar =50 μm. (C) Crystal violet staining of biofilm formed by *Rhizobium* sp. IRBG74 (Top two rows) and BD1 (Bottom two rows). (D) Absorption of crystal violet at 570 nm by *Rhizobium* sp. IRBG74 and BD1.

During plant colonization studies, BD1 showed defective surface colonization on *Sesbania* (Fig 18B) as well as on the surface of rice root surface (Fig 18D) compared to WT, which was able to form extensive multilayer cellular aggregation on both *Sesbania* (Fig 18A) and rice root surfaces (Fig 18C).

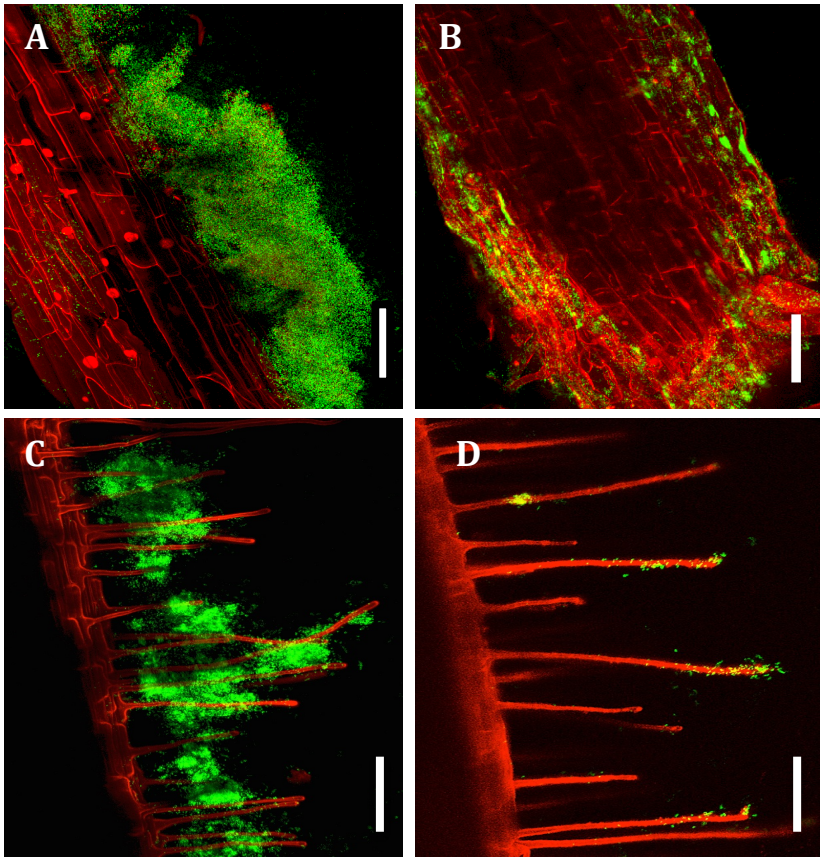


Fig. 18 Confocal microscopy showing that BD1 defective in biofilm formation on tested biotic surfaces. (A, C) GFP-expressing *Rhizobium* sp. IRBG74 on *Sesbania cannabina* (A) and rice (C) root surfaces. (B, D) GFP-expressing BD1 mutant on *Sesbania cannabina* (B) and rice (D) root surface. BD1 failed to form multilayered

cellular aggregations and biofilms on both, *Sesbania* as well as rice root surfaces, compared to WT. Scale bar =100 μm.

Identification of the disrupted gene in the biofilm defective mutant of *Rhizobium* sp. IRBG74

The gene disrupted by the transposon insertion in the biofilm defective mutant of *Rhizobium* sp. IRBG74 was identified by PCR amplification and by sequencing of genomic regions flanking the transposon. Comparison of the DNA sequence to the genome of *A. tumefaciens* using BLAST revealed that the transposon has inserted into a putative ABC transporter gene annotated as *thiQ* (involved in the transport of thiamine in *E. coli* and *Salmonella*). The transposon insertion site was reconfirmed by PCR amplification of *thiQ* from the mutant and WT *Rhizobium* sp. IRBG74 using *thiQ* gene specific primers. The *thiQ* amplicon from the mutant showed a shift in size (Fig 19A) due to the presence of the transposon within *thiQ* gene.

To determine if the observed biofilm defective phenotype of the BD1 mutant is due to transposon insertion in *thiQ* and not in any other gene, a Southern blot of DNA isolated from biofilm defective mutant and hybridization with a specific probe for neomycin resistance gene, present inside the transposon was performed. The results demonstrate that the mutant had a single integrated copy himar transposon (Fig 19B).

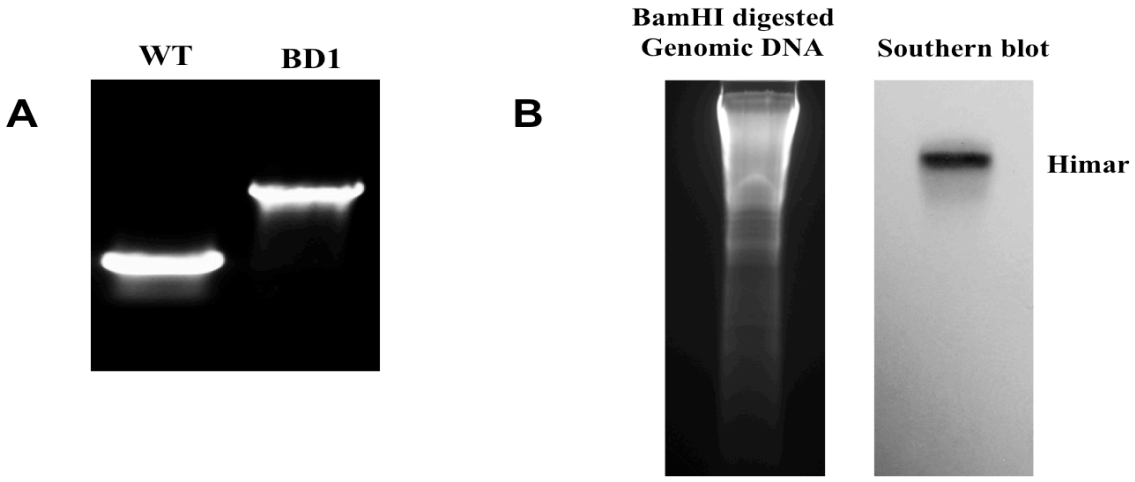


Fig. 19 Single integrated copy of Himar transposon in BD1. (A) Larger amplicon of *thiQ* in BD1 confirms insertion of himar in *thiQ* when compared to wild type (WT). (B) Agarose gel analysis of *BamHI* digested genomic DNA from BD1 mutant (left panel). Southern blot of *Rhizobium* sp. *thiQ::Himar* showing single inserted copy of the Himar transposon.

Characterization of the *thiQ* gene in *Rhizobium* sp. IRBG74

As insertional inactivation of the *thiQ gene* resulted in a severe biofilm defect in *Rhizobium* sp. IRBG74, we wanted to test if the biofilm defect of *Rhizobium* sp. *thiQ::Himar* can be restored by genetic complementation. The biofilm defect in *thiQ::Himar* mutant was restored back to wild type levels by plasmid-based complementation, observed on 96 well plate surfaces. To further confirm the role of the *thiQ* gene in biofilm formation we constructed a fresh in-frame deletion of *thiQ* gene in IRBG74-GUS (IRBG74-GUS- Δ*thiQ*). Δ*thiQ* showed a similar phenotype as the *thiQ::Himar* mutant, suggesting that mutation in *thiQ* resulted in the biofilm defect (Fig 20).

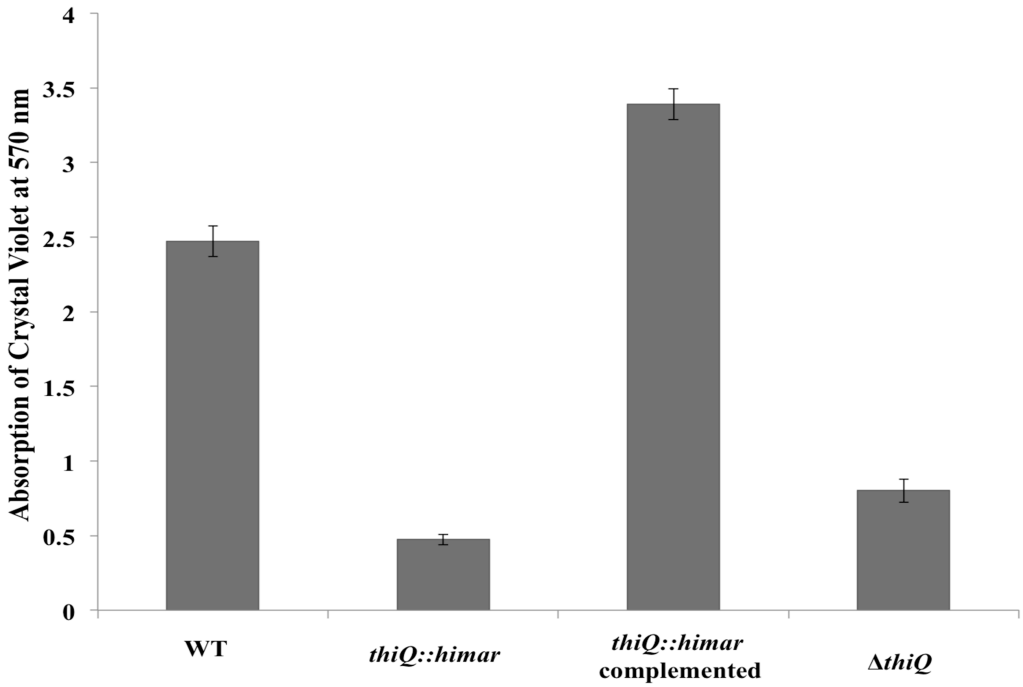


Fig. 20 Mutation in *thiQ* results in a biofilm defect. Biofilm defect of the *thiQ::Himar* mutant was restored back to wild type levels by genetic complementation with the entire *thi* operon. Inframe deletion of the *thiQ* gene in (Δ*thiQ*) reduced the biofilm formation by at least 5-7 fold.

Inactivation of the *thiQ* gene results in defective rice root colonization

To further understand the role of *thiQ* in rice root colonization, histochemical GUS staining followed by enumeration of bacteria from inoculated rice plants were performed. The *thiQ* himar and the Δ*thiQ* mutants showed a marked decrease in colonization (Fig. 21B, D) as compared to its wild type strain (Fig. 21A). The decrease in colonization was restored back by complementation with *thi* operon (Fig. 21C). The colonization phenotype was further quantified and expressed as colony-forming units g^{-1} of fresh rice

root (cfu g^{-1} of fresh rice root), which showed more than a 15-fold reduction in rice colonization by the mutant as compared to the wild type strain (Fig. 21E).

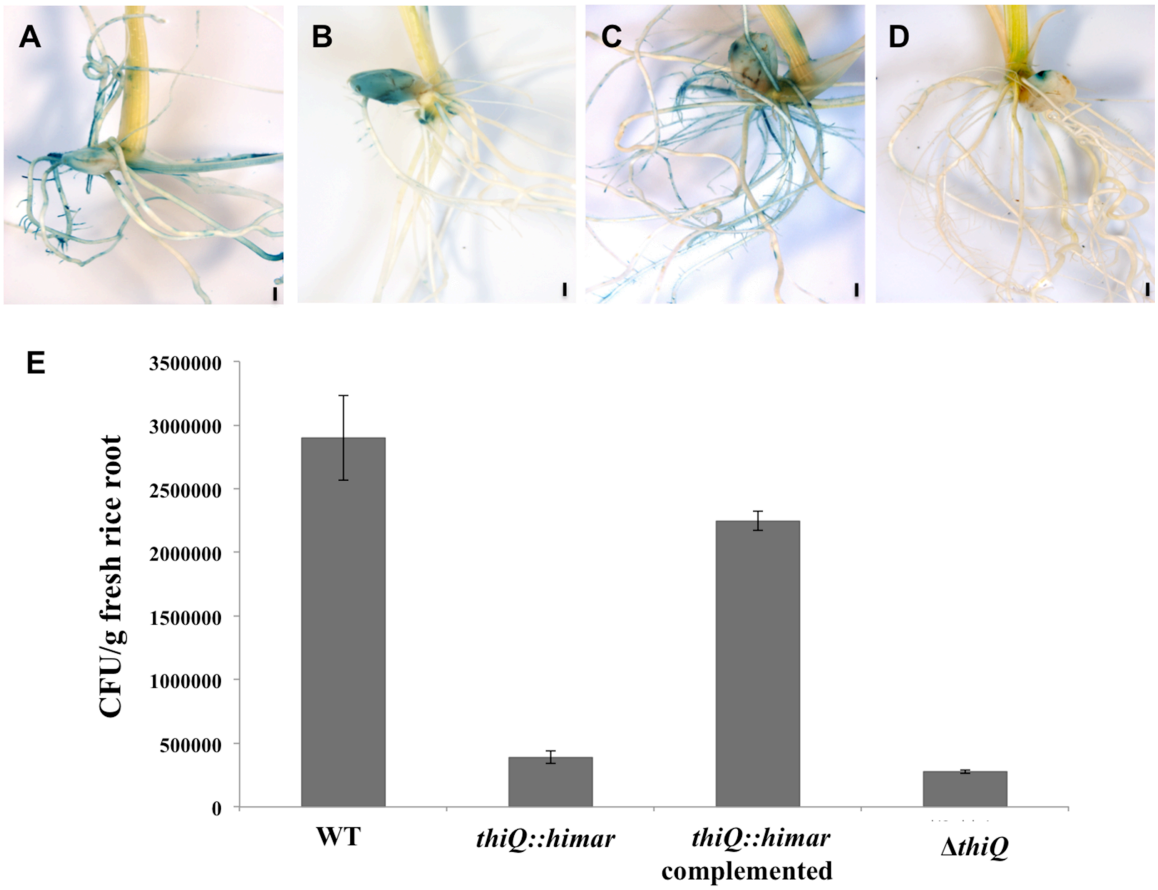


Fig. 21 Rice root colonization by *Rhizobium* sp. IRBG74 and the *thiQ* mutants. (A) GUS staining on rice roots colonized by the wild type strain. Scale bar = 1mm. (B & D) Substantially less GUS staining was observed on roots colonized by the *thiQ::Himar* and Δ*thiQ* mutants. Scale bar = 1mm. (C) The colonization defect in the *thiQ::Himar* mutant was restored in the complemented strain. Scale bar = 1mm. (E) Counts of bacteria re-isolated from inoculated roots showed significantly less colonization by the Δ*thiQ*

mutant as compared to the wild type strain. A significantly higher number of bacteria were recovered from the rice roots inoculated with the *thiQ::Himar* complemented.

Inactivation of the *thiQ* gene results in defective nodulation of *Sesbania*

As *Rhizobium* sp. IRBG74 is a *Sesbania* symbiont we tested the nodulation properties of the *thiQ* mutant on *S. cannabina*. Interestingly, *thiQ* mutant had lost the ability to form nodules in its host legume (*Sesbania*), suggesting that *thiQ* is involved in the symbiotic association of *Rhizobium* sp. IRBG74 with its host.

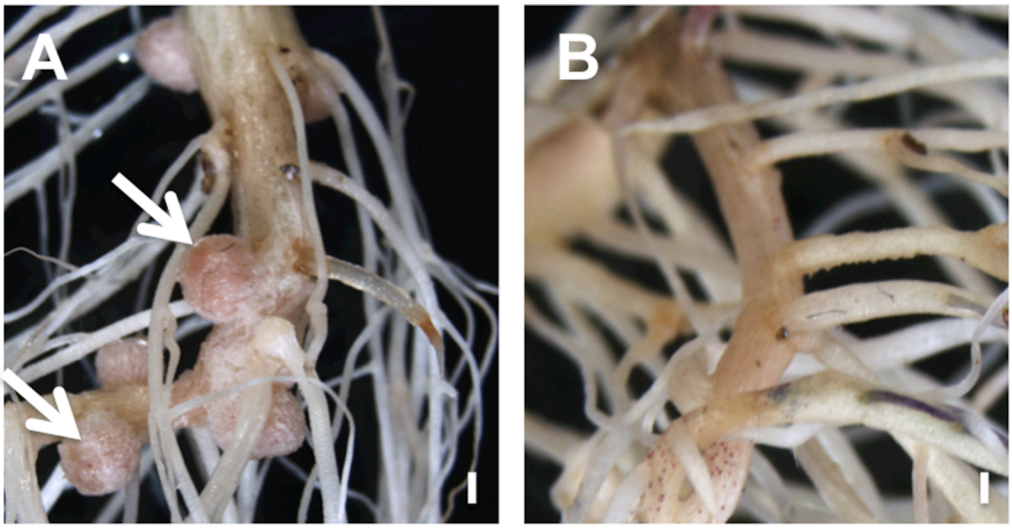


Fig. 22 The *Rhizobium* sp. IRBG74 *thiQ* mutant is defective in symbiosis with *S. cannabina*.

(A) WT formed large pink nodules (shown by arrows) on the roots of *Sesbania*. Scale bar = 1mm. (B) No functional nodules were detected on the roots of *Sesbania* that were inoculated with the *thiQ* himar mutant. Scale bar =1mm.

_thi_Q is likely involved in flavonoid transport in _Rhizobium_ sp. IRBG74

Often the no nodule phenotype is the result of a disruption in the very early stages of nodule organogenesis, which depends on synthesis and recognition of a Nod factor (Mergaert *et al.*, 1997; Perret *et al.*, 2000). Due to the absence of nodules in *Sesbania cannabina* inoculated with *thiQ::Himar*, we consider the possibility of a defect in Nod factor production. To evaluate Nod factor production in the *thiQ::Himar* mutant, *nodA* transcript levels were determined by semi-quantitative RT-PCR. The transcript for *nodA* was not detected in *thiQ* himar mutant (Fig 23A).

Synthesis of nod factor is contingent on the activation of rhizobial NodD [a transcriptional activator of other *nod* genes involved in Nod factor production and side chain decoration (Mergaert *et al.*, 1997; Perret *et al.*, 2000)] by plant flavonoids. A defect in transportation of plant flavonoid may results in defective Nod factor production. To test the hypothesis that the *thi* operon is involved in the transportation of flavonoid in *Rhizobium* sp. IRBG74, we performed the flavonoid cytotoxicity test, as flavonoids are cytotoxic to bacteria at high concentration (Ulanowska *et al.*, 2006; Xu and Lee, 2001), on *Rhizobium* sp. IRBG74 and *thiQ::Himar* in the presence of varying concentration of naringenin.

The wild type strain was found to be more susceptible to flavonoids at high concentrations when compared to the mutant (Fig 23B). These results suggest that the *thiQ* mutant is impaired in flavonoid transport, and thus can withstand high concentrations of flavonoids.

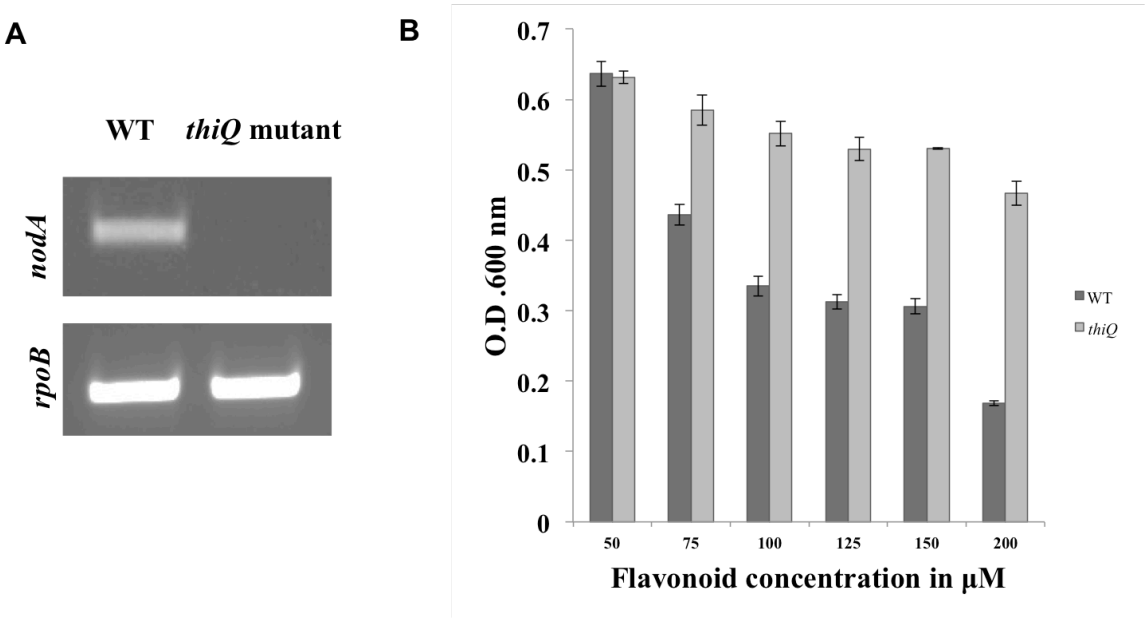


Fig. 23 Role of *thiQ* in plant flavonoid transport

(A) Semi quantitative RT-PCR analysis suggesting that the *thiQ::Himar* mutant is impaired in *nod* gene expression. Transcript for *nodA* could not be detected in the *thiQ* mutant. *rpoB* levels were used as internal control. (B) The wild type strain was found to be more susceptible to flavonoids at higher concentrations compared to the *thiQ::Himar* mutant.

Discussion

During the initial stages of root colonization, biofilm formation on plant root surfaces by rhizobia is very prominent and important. The ability to form a biofilm helps rhizobia to thrive in adverse rhizospheric condition like desiccation, nutrient limitation, etc. and gives it a competitive advantage over other microbes present in that niche (Pérez-Montaño *et al.*, 2014; Ramey *et al.*, 2004; Rinaudi and Giordano, 2010).

When grown in hydroponic culture, *Rhizobium* sp. IRBG74 forms an extensive biofilm on the surface of rice roots, similar to that of its symbiotic partner *Sesbania cannabina*. After initial attachment, *Rhizobium* sp. IRBG74 synthesizes an extracellular fibrillar matrix and embeds itself in it to form an extensive biofilm on the root surface. This type of cellular aggregation and extracellular matrix formation was observed in different rhizospheric bacteria in which the bacteria are embedded in a self-produced matrix composed of proteins, nucleic acids, and EPS (Danhorn and Fuqua, 2007; Rodríguez-Navarro *et al.*, 2007). However, the role of biofilms during a plant-microbe interaction has not yet been studied in a rhizobium capable of colonizing cereal as well as a legume.

In order to study the role of biofilms in colonization of cereals and legumes by *Rhizobium* sp. IRBG74, a rapid biofilm screening of the mutant library of *Rhizobium* sp. IRBG74 was performed in 96 well plates. A mutant defective in biofilm formation on this abiotic surface was identified.

When rice colonization and *Sesbania* nodulation studies were performed with WT and the biofilm-defective mutant of *Rhizobium* sp. IRBG74, the mutant showed

significantly less colonization and no nodulation phenotype in *Sesbania*. The rice colonization defect of the mutant is probably due to a defect in substrate attachment, as it is well established that attachment to the plant root surface is required for subsequent endophytic colonization (Meneses *et al.*, 2011).

Rhizobium legume symbiosis, which results in formation of root nodules, is a highly specific and multistep process. Disruption in any of these steps results in ineffective nodule formation or sometimes complete absence of nodules on the legume plants. Often the no nodule phenotype is the result of a disruption in the very early stages of nodulation. As both biofilm defects and the no nodule phenotype in legumes indicates disruption in the early stages of plant microbe interaction, we were interested to know what gene was disrupted in the BD1 mutant of *Rhizobium* sp. IRBG74.

PCR and sequencing of the adjacent chromosomal DNA of the transposon insertion in the biofilm defective mutant of *Rhizobium* sp. IRBG74 resulted in identification of *thiQ* as the disrupted gene. The *thiQ* gene is a part of a putative operon involved in transportation of thiamine in *A. tumefaciens*.

Based on RT-PCR analysis of the *nodA* transcript in *thiQ:: Himar* mutant and sensitivity to flavonoid of the wild type strain, *thiQ* is likely to be involved in active transport of flavonoids in *Rhizobium* sp. IRBG74. The transport of flavonoids into the WT *Rhizobium* sp. IRBG74 results in activation of NodD, which then transcriptionally activate the IRBG74 *nod* genes required for Nod factor synthesis. Disruption in the transport of flavonoid in *thiQ::Himar* mutant results in the absence of Nod factor synthesis and a communication blockage between *thiQ::Himar* and *Sesbania*. However,

further experiments need to be performed to confirm the role of *thiQ* in flavonoid transport.

Concluding Remarks

This study has established the *Sesbania*-nodulating *Rhizobium* sp. IRBG74 as a rice endophyte. It has been shown to infect the rice plant via cracks in lateral root junctions and through root hairs. Nodulation factors (Nod factors) that have been known to be the major player in rhizobium legume symbiosis were also shown to be required for rice rhizobial interaction. It will be interesting to see if the rhizobial interaction with rice also involves the signal transduction pathway (Common symbiotic pathway) that is known to be involved in rhizobium legume symbiosis and Arbuscular mycorrhizal symbiosis.

We have also demonstrated that biofilm formation and rhamnose rich lipopolysaccharide are indispensable for successful interaction of *Rhizobium* sp. IRBG74 with rice. However more experiments need to be performed to understand the mechanism of *thiQ* mediated biofilm formation, and also, to explore the possibility of the lipopolysaccharide serving as a signaling molecule in the rice-rhizobial interaction.

The ability of *Rhizobium* sp. IRBG74 to colonize both cereals and legumes makes the interaction unique. This duality in its beneficial interactions with two very different plant types also raises the possibility that *Rhizobium* sp. IRBG74 could be used as a model system to compare more closely the genetics underpinning legume and non-legume infection processes, with the ultimate aim of producing more effective (i.e. more productive in N-accumulation and biomass production) N_2-fixing associations between rhizobia and cereals.

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Curriculum Vitae

Shubhajit Mitra

Education

Ph.D. Biological Sciences, Area of Specialization: Molecular Microbiology, 2009-2014.
University of Wisconsin, Milwaukee, USA.

M. Sc. in Biotechnology, 2005-2007.
Bangalore University, India.

Advance Diploma in Genetic Engineering, 2005-2006.
Jain Institute of Vocational and Advanced Studies, India.

Research Experience

Doctoral Research, *University of Wisconsin-Milwaukee*

Synopsis

During my PhD I have studied the interaction of *Rhizobium* sp. IRBG74 with *Sesbania cannabina* (legume) and rice (cereal) to identify the key events and the common genetic determinants to realize the potential of symbiotic nitrogen fixation in major food crops.

Rhizobium sp. IRBG74 is the first known, naturally occurring, nitrogen fixing symbiont in the *Agrobacterium*/*Rhizobium* clade that nodulates the semi-aquatic legume *Sesbania cannabina* and is also a rice growth promoting rhizobacterium. Using GUS and GFP reporter system we have established *Rhizobium* sp. IRBG74 as a classic nitrogen-fixing symbiont of *Sesbania cannabina* and a bona fide rice endophyte, capable of colonizing root as well as the shoot. As per our understanding we know that this bacterium is involved in a molecular crosstalk with rice and requires the rice common symbiotic pathway (CSP) genes to associate endophytically. In order to determine the mechanism by which *Rhizobium* sp. IRBG74 forms endophytic and growth-promoting associations with rice, we sequenced the genome of *Rhizobium* sp. IRBG74, which revealed the presence of plasmid pIRBG74a (a *repABC*- family plasmid containing *nod*, *nif*, and *fix* genes involved in symbiosis) in addition to a circular and a linear chromosome.

Before IRBG74 genome was sequenced, using random transposon mutagenesis we created a mutant library of *Rhizobium* sp. IRBG74 and identified some of the genetic determinants involved in root nodule formation (in its host legume *Sesbania cannabina*) and rice colonization. The availability of the complete genome of *Rhizobium* sp. IRBG74 allowed us to further characterize the genes using site-directed mutagenesis and genetic complementation.

Publication

- **Mitra Shubhajit**, Mukherjee Arijit, Euan K. James, Seema Das, Heather Owen, Pallavolu M. Reddy, Jean-Michel Ané and Prasad Gyaneshwar. *Rhizobium* (Agrobacterium) sp. IRBG74 requires rhamnose rich LPS for endophytic colonization of rice as well as nodulation of *Sesbania cannabina*. ***Journal of Experimental Botany*** **(2014) [Communicated]**
- Hukam Singh Gehlot, Nisha Tak, Muskan Kaushik, **Shubhajit Mitra**, Wen-Ming Chen, Nicole Poweleit, Dheeren Panwar, Neetu Poonar, Rashmita Parihar, Alkesh Tak, Indu Singh Sankhla, Archana Ojha, Satyawada Rama Rao, Marcelo F. Simon, Fabio Bueno dos Reis Junior, Natalia Perigolo, Anil K. Tripathi, Janet I. Sprent, J. Peter W. Young, Euan K. James, and Prasad Gyaneshwar. An invasive *Mimosa* in India does not adopt the symbionts of its native relatives. ***Ann Bot*** **(2013)** 112 (1): 179-196 [**PMID: 24265489]**
- Matthew B. Crook*****, **Shubhajit Mitra***, Jean-Michel Ané, Michael J. Sadowsky, and Prasad Gyaneshwar. Complete Genome Sequence of the Sesbania Symbiont and Rice Growth-Promoting Endophyte Rhizobium sp. Strain IRBG74. ***Genome Announc***. **November/December (2013)** 1:e00934-13 **(*Joint First Author)[PMID: 23712450]**
- **Mitra Shubhajit**, Parida Adwaita and Prasad Gyaneshwar. A novel flavonoid transporter in *Rhizobium* sp. IRBG74 is involved in biofilm formation and nodulation in *Sesbania cannabina*. **[In Preparation]**

Technical Skills

Microscopy – Laser Confocal Scanning Microscopy, Scanning Electron Microscopy, Transmission Electron Microscopy

Microbiology - Culturing and aseptic techniques, enumeration and identification of bacteria, media and buffer preparation, antibiotic assay (MIC)

Molecular biology and bacterial genetics

Isolation and Purification **-** DNA, RNA, Protein and LPS
Amplification - PCR, OE PCR, RT-PCR
Gene Delivery **–** Conjugation, electroporation, transformation.
Mutagenesis **–** Insertional mutagenesis, Site-directed mutagenesis
Separation & Detection **–** Agarose gel electrophoresis, SDS-PAGE, Western blot, Southern blot, LPS profiling, Gas chromatography.

Bioinformatics – ApE-a plasmid editor, BioCyc, NCBI BLAST and other basic bioinformatics tools.

Plant handling – Germination and maintenance of plant under aseptic, gnotobiotic and green house condition.

Presentations

Invited Lecture

Interaction of *Rhizobium* sp. IRBG74 with Rice to Extend the Possibility of Biological Nitrogen Fixation in Cereals. ***Biological Sciences Colloquium Series,***

UWM, Wisconsin, Milwaukee, **2014.**

Posters

Rhizobium sp. IRBG74 utilizes common mechanisms for endophytic colonization of *Sesbania cannabina* and rice. Shubhajit Mitra, Arijit Mukherjee, Matthew Crook, Euan K. James, Michael Sadowsky, Jean-Michel Ané, and Prasad Gyaneshwar. ***The 22nd North American Symbiotic Nitrogen Fixation Conference,*** Minnesota, **2013.**

Identification of Genetic Determinants involved in Rice Rhizobial Interaction and Role of Lipopolysaccharide during Colonization of Rice by *Rhizobium* sp. IRBG74. ***113th ASM General Meeting***, Denver, Colorado, **2013.**
Role of Lipopolysaccharide and Biofilm Formation during Endophytic Colonization of Rice and Nodulation of *Sesbania cannabina* by *Rhizobium* sp. IRBG74, ***72nd Annual Meeting of the North Central Branch American Society for Microbiology,*** Fargo, North Dakota, **2012.**
Identification of Genetic Determinants Involved In Rice Rhizobial Interaction. ***Biological Sciences Research Symposium, UWM***, Wisconsin, Milwaukee, **2011.**

Awards and honors

2014: Chancellors Graduate Student Award for exceptional academic records
2013: Chancellors Graduate Student Award for exceptional academic records
2013: Ruth Walker Graduate Student Grant- In Aid Award for "Outstanding Achievement in Biological sciences"
2013: UWM Graduate School **Travel Award**
2012: Chancellors Graduate Student Award for exceptional academic records
2011: Chancellors Graduate Student Award for exceptional academic records
2011: Best Poster Award (Graduate Student), Biological Sciences Research Symposium, UWM
2010: Chancellors Graduate Student Award for exceptional academic records
2009: Chancellors Graduate Student Award for exceptional academic records

Experience

Teaching

2009: Anatomy and Physiology
2010 – 2014: Introduction to Biology

Mentoring Undergraduate researchers

2013 – 2014: Brigit Blemberg
2013 – 2014: Sydnee Rausch

Affiliations/Memberships

2013 – Present: American Society for Microbiology
2009 – Present: Graduate Organization of Biological Students, UWM
2014 – UWM Badminton Club (President)