Nodulation of *Sesbania* species by *Rhizobium* (*Agrobacterium*) strain IRBG74 and other rhizobia

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Summary

Concatenated sequence analysis with 16S rRNA, *rpoB* and *fusA* genes identified a bacterial strain (IRBG74) isolated from root nodules of the aquatic legume *Sesbania cannabina* as a close relative of the plant pathogen *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*). However, DNA:DNA hybridization with *R. radiobacter*, *R. rubi*, *R. vitis* and *R. huautlense* gave only 44%, 5%, 8% and 8% similarity respectively, suggesting that IRBG74 is potentially a new species. Additionally, it contained no *vir* genes and lacked tumour-forming ability, but harboured a *sym*-plasmid containing *nifH* and *nodA* genes similar to those in other *Sesbania* symbionts. Indeed, IRBG74 effectively nodulated *S. cannabina* and seven other *Sesbania* spp. that nodulate with *Ensifer* (*Sinorhizobium*)/ *Rhizobium* strains with similar *nodA* genes to IRBG74, but not species that nodulate with *Azorhizobium* or *Mesorhizobium*. Light and electron microscopy revealed that IRBG74 infected *Sesbania* spp. via lateral root junctions under flooded conditions, but via root hairs under non-flooded conditions. Thus, IRBG74 is the first confirmed legume-nodulating symbiont from the *Rhizobium* (*Agrobacterium*) clade. Cross-inoculation studies with various *Sesbania* symbionts showed that *S. cannabina* could form fully effective symbioses with strains in the genera *Rhizobium* and *Ensifer*, only ineffective ones with *Azorhizobium* strains, and either partially effective (*Mesorhizobium huakii*) or ineffective (*Mesorhizobium plurifarium*) symbioses with *Mesorhizobium*. These data are discussed in terms of the molecular phylogeny of *Sesbania* and its symbionts.

Introduction

Sesbania is a genus of approximately 60 species of tropical legume of which 40 have so far been reported to nodulate (Sprent, 2001). Many species occur naturally in wet or flooded soils and these have considerable potential as green manure in wetland rice production due to their ability to fix large quantities of N_2 (James *et al*., 2001 and references therein). *Sesbania* nodules may be induced by a variety of rhizobia, including *Azorhizobium* spp. (Dreyfus *et al*., 1988; Gonçalves and Moreira, 2004;

Moreira *et al.*, 2006; Lee *et al.*, 2008), *Ensifer* spp. (syn. *Sinorhizobium* spp.) (De Lajudie *et al.*, 1994; 1998; Boivin *et al.*, 1997; Chen and Lee, 2001; Sharma *et al.*, 2005), *Mesorhizobium* spp. (McInroy *et al.*, 1999; Bala *et al.*, 2002; Odee *et al.*, 2002; Vinuesa *et al.*, 2005) and *Rhizobium* spp. (Rana and Krishnan, 1995; Wang *et al.*, 1998; Wang and Martínez-Romero, 2000; Chen and Lee, 2001; Bala *et al.*, 2002; Sharma *et al.*, 2005; Vinuesa *et al.*, 2005). In addition to the 'standard' rhizobial types, strains of the genus *Agrobacterium* (which are now, rather controversially, included in the genus *Rhizobium*; Young *et al.*, 2001; Farrand *et al.*, 2003) have also been isolated frequently from *Sesbania* nodules (Tan *et al.*, 2001; Bala *et al.*, 2002; Odee *et al.*, 2002). The present study concerns one of these, *Rhizobium* strain IRBG74, isolated from root nodules of *Sesbania cannabina* (Retz.) Pers. (Biswas *et al.*, 2000). This strain has been used to promote the growth of rice, as one of a group of plant growth-promoting rhizobacteria (Biswas *et al.*, 2000). On the basis of its 16S rRNA gene sequence, it is most closely related to the plant pathogen *Rhizobium radiobacter* (syn *Agrobacterium tumefaciens*) (Tan *et al.*, 2001). Biswas and colleagues (2000) tagged IRBG74, isolated from rice roots, with a *gusA* reporter gene and found that it not only expressed *gus* activity on plates containing X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid), but could also nodulate *S. cannabina*. Although plant growth was strongly promoted, both acetylene reduction and ^{15}N isotope dilution assays found no activity associated with rice roots (Biswas *et al.*, 2000). On the other hand, using both PCR and Southern hybridization, Tan and colleagues (2001) detected a *nifH* gene in IRBG74, one of the components required for nitrogenase production. Although some rhizobia occasionally express nitrogenase activity in culture, only the genus *Azorhizobium* (Dreyfus *et al.*, 1988; Moreira *et al.*, 2006) and some nodulating species of *Burkholderia* (Elliott *et al.*, 2007) can grow on the products of nitrogen fixed *ex planta*. There are many reports of isolation of agrobacteria from nodules, but few have established whether or not these strains are capable of inducing nodules, and even if they can, whether or not the nodules are effective in fixing nitrogen.

The species *R. radiobacter* (Young *et al.*, 2001) now includes the two former agrobacteria, *Agrobacterium radiobacter* (avirulent) and *A. tumefaciens* (tumour forming) (Willems, 2006). Although in the laboratory *sym* genes have been mobilized into *R. radiobacter* and the recipient strain was able to form N_2-fixing nodules on common bean (*Phaseolus vulgaris* L) (Martínez *et al.*, 1987), there is little published evidence that any *Agrobacterium* or *Agrobacterium*-like strains can fix nitrogen in the wild, even if they induce nodulation (De Lajudie *et al.*, 1999; Mhamdi *et al.*, 2005; Wang *et al.*, 2006). Arguably

the best evidence prior to the present study comes from recent work on two strains of the causal agent of hairy root disease, *Rhizobium rhizogenes* (syn. *Agrobacterium rhizogenes*), ATCC11325^T and 163C, isolated from tumours on apple (*Malus domestica* Borkh.) and *Prunus persica* (L) Batsch respectively. These strains have been shown to harbour both *sym* and *vir* genes (Velázquez *et al.*, 2005). Moreover, the *sym* plasmid in both cases had *nodD* and *nifH* genes that were phylogenetically close to known bean symbionts, and the strains were able to produce tumours/hairy roots on tomato or nodules on common bean. However, although the bean plants accumulated significantly more N than uninoculated control plants without added N, the total N accumulated was half that of plants inoculated with *Rhizobium etli* CFN42, and there was no confirmation via either acetylene reduction assays (ARA) of nitrogenase activity or ^{15}N-based techniques that symbiotic N_2 fixation was responsible for this N accumulation (Velázquez *et al.*, 2005).

In this context, the aims of the present study were to clarify the phylogenetic position of strain IRBG74 within the genus *Rhizobium* and to obtain further evidence on its symbiotic properties with *S. cannabina* and other hydrophytic *Sesbania* spp. Because IRBG74 is only one of many bacteria that can nodulate species of *Sesbania*, we also compared its *nifH* and *nodA* genes with those of the four rhizobial genera known to nodulate *Sesbania* spp. from Africa, Asia and South America [i.e. *Azorhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium* and *Rhizobium*]. Finally, we compared the ability of all these bacteria to nodulate *S. cannabina* with that of IRBG74. The symbiotic properties (i.e. host range and *nodA* sequences) of IRBG74 and the other *Sesbania* symbionts are discussed in the context of a molecular phylogeny of *Sesbania*.

Results

Phylogenetic analysis of strain IRBG74 and the other Sesbania-*nodulating strains*

The 16S rRNA gene sequence was used in initial phylogenetic analyses to compare the four available sequences of IRBG74 with the most similar homologues derived from the NCBI database attributable to validly described species. The inferred tree showed that all four IRBG74 sequences grouped with a low bootstrap value (61%) in a clade with *R. radiobacter* NCIMB 9042 and NCIMB 13307 (Fig. 1), the type strains of *Agrobacterium radiobacter* and *A. tumefaciens* respectively. To explore these phylogenetic relationships further, two housekeeping genes, *fusA* and *rpoB*, were included in a concatenated analysis of nine rhizobial strains for which the three sequences (i.e. 16S rRNA, *fusA*, *rpoB*) were available. These nine strains included three of *R. radiobacter*
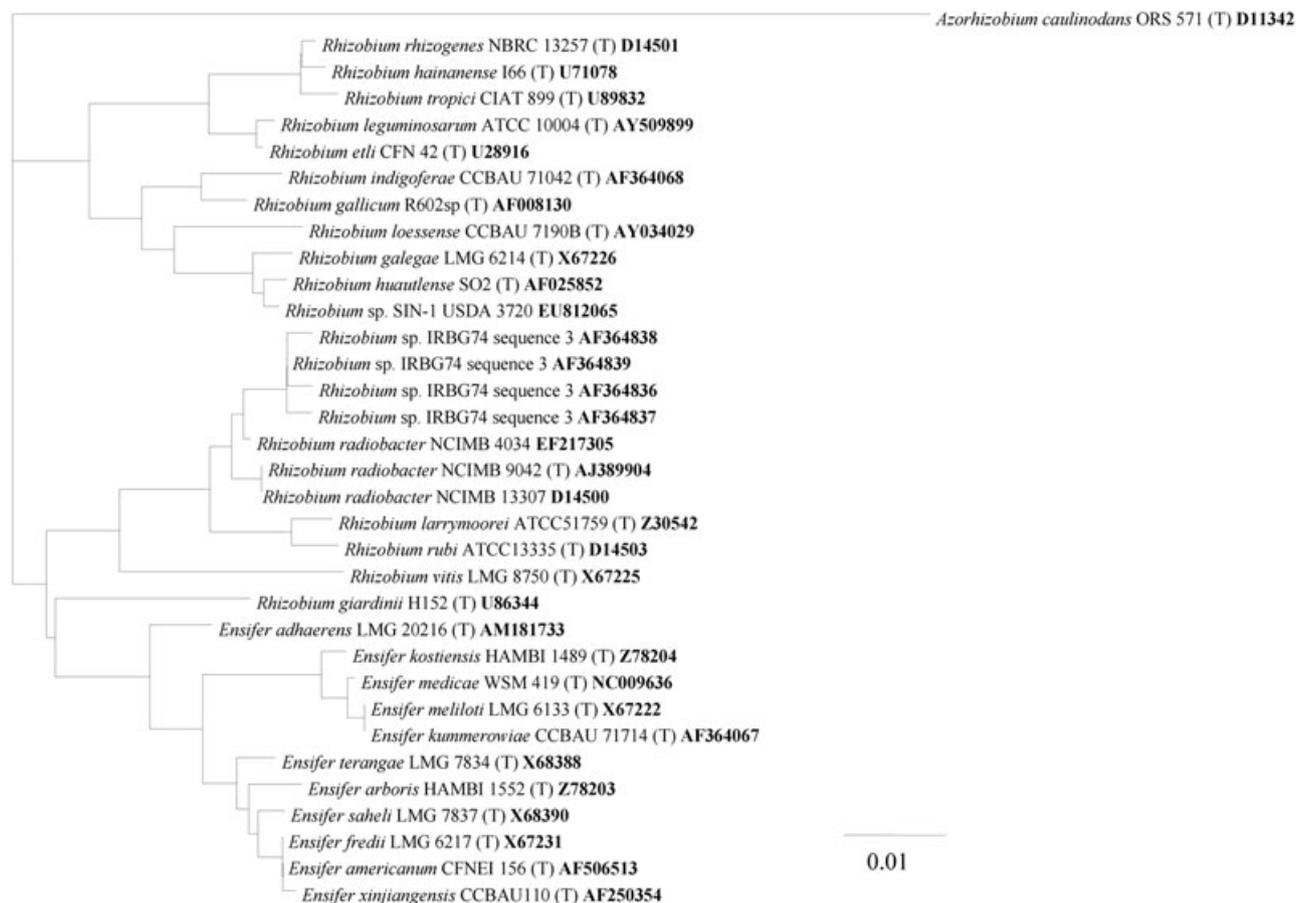
Fig. 1. Unrooted phylogenetic dendrograms based upon 16S rRNA sequences, 1356 bp. The comparisons were made using the maximum likelihood algorithm and the Tamura-Nei substitution model, bootstrap confidence percentages were calculated from 100 pseudoreplicates of the data set. Bootstrap support percentages are shown for branches recovered in more than 50% of the trees. The scale bar represents the number of nucleotide substitutions per site. Numbers in bold are GenBank accession numbers and T denotes the type strain of the species.

(NCIMB 9042, NCIMB 13307 and NCIMB 4034). From the inferred tree, IRBG74 clustered with a high bootstrap value (89%) with these three strains (Fig. S1), congruent with the 16S rRNA sequence analysis.

The similarity between the 16S rRNA gene sequence of IRBG74 and that of the *R. radiobacter* strains was 99%, suggesting that they may be conspecific. To resolve this, DNA:DNA hybridization studies were carried out against strains of *R. radiobacter* (the type strain, NCIMB 9042, NCIMB 13307 and NCIMB 4034) plus the type strains of closely related organisms identified by the analysis using the three housekeeping genes (16S rRNA, *fusA*, *rpoB*). The degree of hybridization of IRBG74 to the *R. radiobacter* strains NCIMB 13307 and NCIMB 4034 was 57% and 44% respectively. Against type strains of *R. rubi*, *R. vitis* and *R. huautlense*, hybridizations were much lower (8%, 5% and 8% respectively). None of these values approached the upper threshold value (70%) for the definition of a bacterial species (Wayne *et al.*, 1987). Moreover, strain IRBG74 did not form tumours on tobacco, and no *virD2* or *ipt* gene homologues could be

amplified by PCR, thus indicating the absence of the Ti plasmid. Therefore, these data support IRBG74 being placed within a species distinct from the other former *Agrobacterium* species now housed in the genus *Rhizobium* (Young *et al.*, 2001).

During the course of this work we isolated an additional strain, DUS1110, from *Sesbania exasperata* Kunth nodules collected during the study of James and colleagues (2001) (Table 1), and its 16S rRNA gene sequence (not shown) suggested that it was potentially related to *R. huautlense*, a 'water *Rhizobium*' species (Wang and Martínez-Romero, 2000) that has frequently been isolated from *Sesbania* nodules. It is represented in the present study by strains Ss121 and Se127 isolated, respectively, from nodules of *Sesbania sericea* (Willd.) Link and *S. exasperata* growing in seasonally flooded regions of Venezuela (Vinuesa *et al.*, 2005). The 16S rRNA sequence from another *Rhizobium* strain, SIN-1, originally isolated from *S. bispinosa* (Jacq.) W.F. Wight nodules by Rana and Krishnan (1995), suggested that it was also closely related to *R. huautlense* (Fig. 1). All the

Table 1. Rhizobial strains used in this study.

Strain	Original host	Country of origin	Reference
Rhizobium (*Agrobacterium*) sp. IRBG74	*S. cannabina*	Philippines	Tan *et al.* (2001)
			This study
Rhizobium (*Agrobacterium*) sp. IRBG74GUS	*S. cannabina*	Philippines	This study
Rhizobium sp. SIN-1	*S. bispinosa*	India	Rana and Krishnan (1995)
Rhizobium sp. DUS1110	*S. exasperata*	Brazil	This study
Rhizobium huautlense Se127	*S. exasperata*	Venezuela	Vinuesa *et al.* (2005)
Rhizobium huautlense Ss121	*S. sericea*	Venezuela	Vinuesa *et al.* (2005)
Azorhizobium caulinodans ORS571^T	*S. rostrata*	Senegal	Dreyfus *et al.* (1988)
Azorhizobium doebereinereae Br5401^T	*S. virgata*	Brazil	Moreira *et al.* (2006)
Mesorhizobium huakuii KFR647	*S. sesban*	Kenya	McInroy *et al.* (1999)
Mesorhizobium plurifarium Sp45	*S. punicea*	Venezuela	Vinuesa *et al.* (2005)
Ensifer saheli ORS609^T	*S. cannabina*	Senegal	Boivin *et al.* (1997)

other strains used in the following sections have been previously characterized using their 16S rRNA sequences and, in some cases, via sequences of other genes (see references in Table 1).

### Characterization of symbiosis-related genes of IRBG74 and other Sesbania microsymbionts

The presence of the *nifH* and *nodA* genes within the genome of IRBG74 was confirmed using specific PCR primers. Attempts were made to amplify the *nifH* and *nodA* genes of all the strains listed in Table 1, except for *A. caulinodans* ORS571 and *Rhizobium* sp. SIN-1, which were already available in the GenBank database. With the exception of the *nifH* gene of *R. huautlense* Se127 and the *nodA* gene from *Mesorhizobium huakuii* KFR647, amplified products of both genes were obtained for all strains. Amplicons were cloned, sequenced and utilized in GenBank database searches via BLASTN.

The phylogenetic analysis of the *nifH* sequence showed that IRBG74 nested in a cluster, with high bootstrap support (97%), containing a number of *Ensifer* isolates, but was most similar to *Ensifer saheli* ORS609^T (Fig. S2A), which was also originally isolated from *S. cannabina* (Boivin *et al.*, 1997). Also in this cluster were a number of other recognized type species including *Ensifer kostiense* and *Ensifer terangae*, as well as a sequence derived from the *Sesbania* strains DUS1110, Ss121 and SIN-1, all putatively identified as *Rhizobium* spp. (Vinuesa *et al.*, 2005; this study). In contrast, strains KFR647 and Sp45, previously identified as *Mesorhizobium* spp. by Odee and colleagues (2002) and Vinuesa and colleagues (2005) respectively, produced sequences that clustered with a number of *Mesorhizobium* sequences (Fig. S2A) and were clearly distinct from the *Ensifer* cluster. The *nifH* sequences of the two *Azorhizobium* strains, *A. caulinodans* ORS571 (Lee *et al.*, 2008) and *A. doebereinerae* Br5401 (accession number FJ223129), were 95% similar to each other, but they did not cluster with the other *Sesbania*-nodulating rhizobial strains (not shown).

The *nodA* sequences of IRBG74 and the *Sesbania*-nodulating *Rhizobium* strains Ss121, SIN-1, DUS1110 and Se127 clustered together, consistent with the *nifH* phylogeny, and also formed a group with a high bootstrap support (100%) with *E. saheli* ORS609 and several other *nodA* sequences derived from *Ensifer* strains isolated from *Sesbania* (Fig. S2B). The *nodA* sequence of Sp45 clustered with other mesorhizobial sequences (data not shown), reinforcing the findings from the *nifH* analysis that this organism and its *sym* genes are mesorhizobial in origin. *Mesorhizobium huaukii* strain KFR647, however, did not produce a PCR product for *nodA* despite nodulating *S. cannabina* and its original host, *S. sesban* (L) Merr. (Table 3, Fig. S4D). It did, however, produce a PCR product for *nodD* (GenBank accession number FJ514244), which was closest to other mesorhizobial strains, particularly *Mesorhizobium loti*. In the case of the azorhizobia, the *nodA* sequence of *A. doebereinerae* Br5401 (GenBank accession number FJ223128) was 95% similar to that of *A. caulinodans* ORS571 (Lee *et al.*, 2008), and neither of the *Azorhizobium* sequences clustered with any *nodA* sequences from the other known rhizobial genera.

Plasmid profiles

The plasmid profile of IRBG74 (Fig. S3) was compared with that of the type strains of *E. saheli* ORS609, *R. radiobacter* NCIMB 13307 (formerly the type strain of *A. tumefaciens*), *R. radiobacter* NCIMB 9042 (current type strain of this species) and *Rhizobium* sp. SIN-1. Strain IRBG74, like *R. radiobacter* NCIMB 13307, had a single plasmid of approximately 700 kb. The *nodA* gene of IRBG74 hybridized with this plasmid and also with the plasmids derived from *E. saheli* ORS609 and *Rhizobium* sp. SIN-1, thus indicating that in IRBG74 it was, indeed, a symbiotic plasmid, and also that the *nodA* gene from this strain was very similar to that found in the other *Sesbania* symbionts, *E. saheli* ORS609 and *Rhizobium* sp. SIN-1.
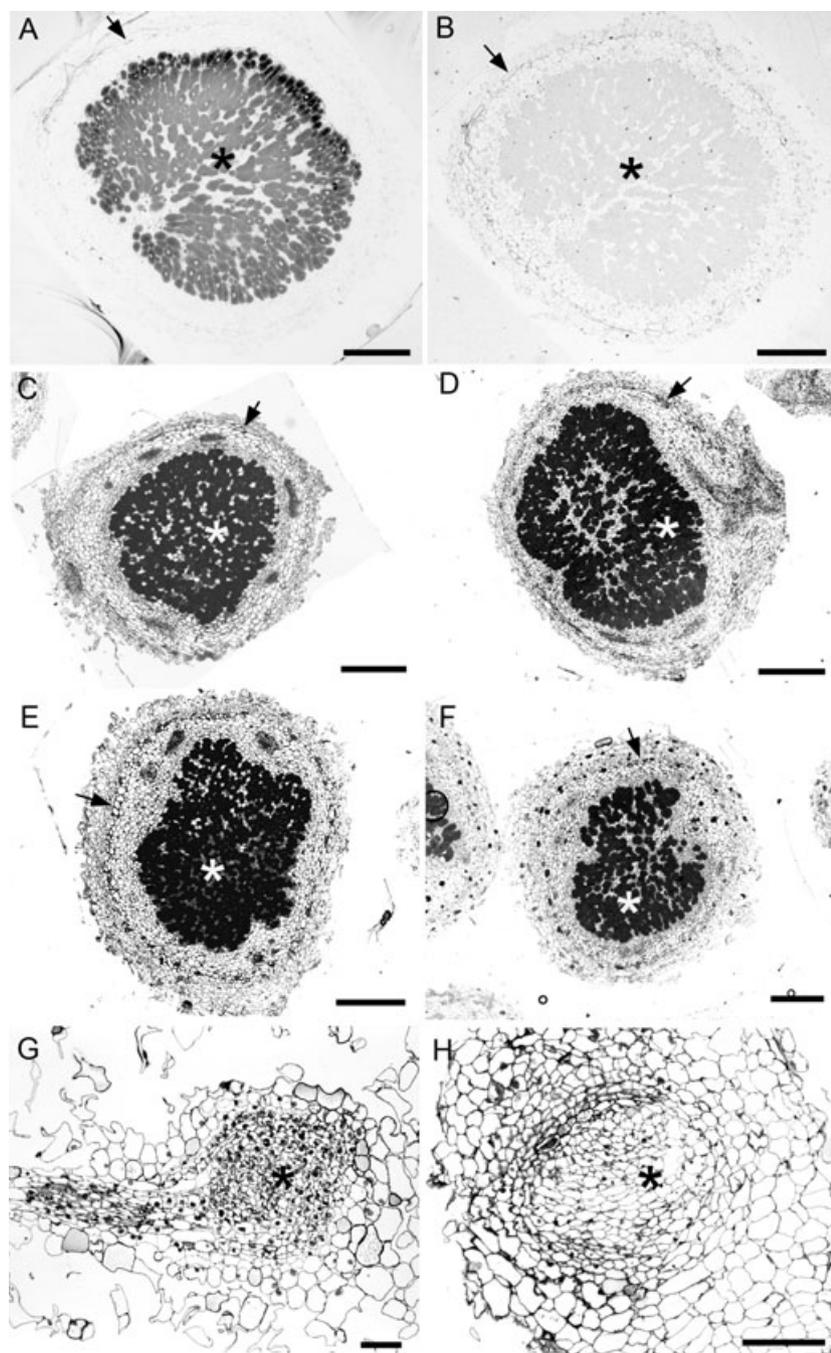
Fig. 2. Nodulation of *Sesbania* spp. by *Rhizobium* (*Agrobacterium*) strain IRBG74 at 30 days after inoculation (dai).
A. Nodule of *S. cannabina* immunogold labelled using an antibody raised against strain IRBG74 (followed by silver enhancement).
B. Serial section to A incubated in non-immune serum substituted for the IRBG74 antibody.
C–F. Effective nodules on, respectively, *S. bispinosa*, *S. exasperata*, *S. formosa* and *S. macrantha*.
G and H. Ineffective nodules (empty root outgrowths) on *S. sesban* (G) and *S. virgata* (H). The infected tissue is marked with a white or black asterisk and the sclereid layer with an arrow in the effective, N₂-fixing nodules in A–F. The smaller, ineffective nodules shown in G and H did not have any internal colonization by bacteria (black asterisks). Bars, 500 µm (A–F), 100 µm (G, H)

Nodulation of Sesbania *spp. and other legumes by IRBG74*

Effective nodules on the roots were indicated by plants being green and healthy at 60 days after inoculation (dai), by significant acetylene reduction activity, and by microscopic examination of nodules. On this basis, strain IRBG74 effectively nodulated eight of the 13 *Sesbania* species tested (*S. bispinosa*, *S. cannabina*, *S. exasperata*, *S. formosa* (F. Muell.) N.T. Burb., *S. grandiflora* (L)

Pers., *S. macrantha* Welw. ex Phillips and Hutch., *S. madagascariensis* Du Puy and Labat and *S. pachycarpa* DC.), formed ineffective nodules on *S. herbacea* (Mill.) McVaugh, small, ineffective 'bumps' on the roots of *S. rostrata* Beremek. and Oberm., *S. sesban* and *S. virgata* (Cav.) Pers., and no outgrowths on *S. punicea* (Cav.) Benth. (Table S2, Fig. 2). No nodules were formed on either of the 'promiscuous' legumes, *Macroptilium atropurpureum* (Moc. and Sessé ex DC.) Urb. or *Phaseolus vulgaris* (Table S2). Effective nodules were large (up to

Table 2. Effect of flooding on growth, nodulation and nitrogenase (acetylene reduction activity, ARA) of *Sesbania cannabina* and *S. bispinosa* (syn. *S. aculeata*) at 30 days after inoculation with *Rhizobium* (*Agrobacterium*) sp. strain IRBG74.

	Plant dry weight (mg)	Nodule No.	Nodule dry weight (mg)	ARA (nmol C_2H_4 per plant h^{-1})
S. cannabina				
Non-flooded	81.4 ± 7.5	9 ± 2	4.6 ± 0.4	247.8 ± 51.2
Flooded	187.6 ± 9.2	24 ± 3	26.3 ± 1.7	755.6 ± 89.9
S. bispinosa				
Non-flooded	16.0 ± 2.3	4 ± 1	0.7 ± 0.1	41.2 ± 11.0
Flooded	18.8 ± 3.3	12 ± 1	2.9 ± 0.3	84.4 ± 11.2

$n = 6$. Values are means ± SE.

4 mm in diameter), and red when cut open, indicating the presence of leghaemoglobin (Lb). Ineffective nodules were much smaller (< 2 mm in diameter), and white inside. Figure 2A–F show that effective nodules had structures similar to those reported for other *Sesbania* spp. (Harris *et al.*, 1949; Ndoye *et al.*, 1994; James *et al.*, 1996; 2001), i.e. the nodules had a central infected zone containing both infected and uninfected cells, an uninfected cortex with a ring of sclereid cells separating the inner and outer cortex, and a relatively transient meristem (Fig. 4C, and see the effective *S. virgata* nodule in Fig. S4A formed by *A. doebereinerae* Br5401), indicating an intermediate type of nodule between the classical determinate and indeterminate types (Ndoye *et al.*, 1994; Den Herder *et al.*, 2006). In contrast, the 'nodules' on *S. rostrata*, *S. sesban* and *S. virgata* (Fig. 2G and H) were root outgrowths formed at lateral root bases, and these contained no sign of infection within their central tissues. Immunogold labelling with an antibody raised against strain IRBG74 confirmed for each species that the bacterium occupying the central infected N_2-fixing tissue was, indeed, *Rhizobium* (*Agrobacterium*) sp. IRBG74 (e.g. *S. cannabina*; Fig. 2A and B).

Nodulation by IRBG74 on its original host, *S. cannabina*, grown under flooded conditions to simulate its natural wetland environment, was studied in more detail. Flooding greatly enhanced growth, nodulation and nitrogenase activity, giving two to three-fold increases in all parameters compared with non-flooded conditions (Table 2). Flooding also increased the nodulation and nitrogenase activity of *S. bispinosa*, but not its overall growth (as determined by plant dry weight). Indeed, *S. cannabina* was generally a much more robust plant than *S. bispinosa* under both flooded and non-flooded growth conditions (Table 2).

A more detailed study of the interaction between four *Sesbania* spp. and *Rhizobium* (*Agrobacterium*) sp. IRBG74 wild-type (WT) and glucuronidase (GUS)-tagged strains was carried out under both flooded (Fig. 3) and non-flooded conditions (Fig. 4). Plants were harvested at 7

and 15 dai and stained with X-Gluc to detect the location of the bacteria on the roots. All four species examined, i.e. *S. cannabina* (Fig. 3A), *S. bispinosa* (Fig. 3B), *S. rostrata* (Fig. 3C) and *S. sesban* (Fig. 3D), showed clear evidence of root colonization by IRBG74GUS by 7 dai with particularly intense staining at lateral root junctions (e.g. Fig. 3A, C and D). Staining was also intense in the root hair zone close to root tips, even on *S. bispinosa* (Fig. 3B), which is known to be infected via cracks at lateral root junctions (Rana and Krishnan, 1995). Both WT and GUS-tagged strains induced visible nodules on *S. cannabina* by 15 dai (Fig. 3E and F); the WT (control) strain showed no blue staining after treatment with X-Gluc (Fig. 3F). Functional nodules were also formed on *S. bispinosa* by IRBG74GUS (not shown), but only small 'bumps' were formed on *S. rostrata* and *S. sesban* by 15 dai (as described previously for the WT strain; Fig. 2G and H). Under non-flooded conditions, IRBG74 infected *S. cannabina* via root hairs (Fig. 4A and B), and nodule development thereafter was as described previously for other *Sesbania* spp., i.e. with the development of an apical meristem containing newly divided cells being penetrated by infection threads which 'released' bacteria into symbiosomes (Fig. 4C). These bacteria subsequently developed into bacteroids as the host cells expanded (Fig. 4C and D). Using an antibody raised against pea Lb (Fig. 4D), which had previously been shown to recognize Lb in N_2-fixing nodules on *S. rostrata* infected by *A. caulinodans* (James *et al.*, 1996), nodules on *S. cannabina* infected by IRBG74 were shown to contain this O_2-carrying protein, which is essential for the functioning of the symbiosis. Functional nodules were not formed on either *S. rostrata* or *S. sesban* after inoculation by IRBG74 under non-flooded conditions. However, there was clear evidence of infection of these species by IRBG74 in both root hairs and epidermal cells (Fig. 4E and F), with massive colonization of the 'nodule' and root surfaces by the bacteria, and even the formation of infection thread-like structures within *S. rostrata* root hairs (Fig. 4E).

Nodulation of S. cannabina *by other* Sesbania microsymbionts

A range of *Sesbania* symbionts, many originally isolated from species that had been tested for their nodulation by IRBG74 (Table 3), were inoculated on to *S. cannabina* (Fig. S4, Table 3). Neither *Azorhizobium* strain (ORS571 or Br5401) nodulated *S. cannabina* effectively, although *A. doebereinerae* Br5401 formed effective nodules on its original host, *S. virgata* (Fig. S4). No nodules were formed on *S. cannabina* by *A. caulinodans* ORS571 and those formed by *A. doebereinerae* Br5401; Fig. S4B) were small bumps without any internal colonization by the bacteria, and were similar to those formed on *S. rostrata*, *S. sesban* and *S. virgata* by IRBG74 (Figs 2G and H and
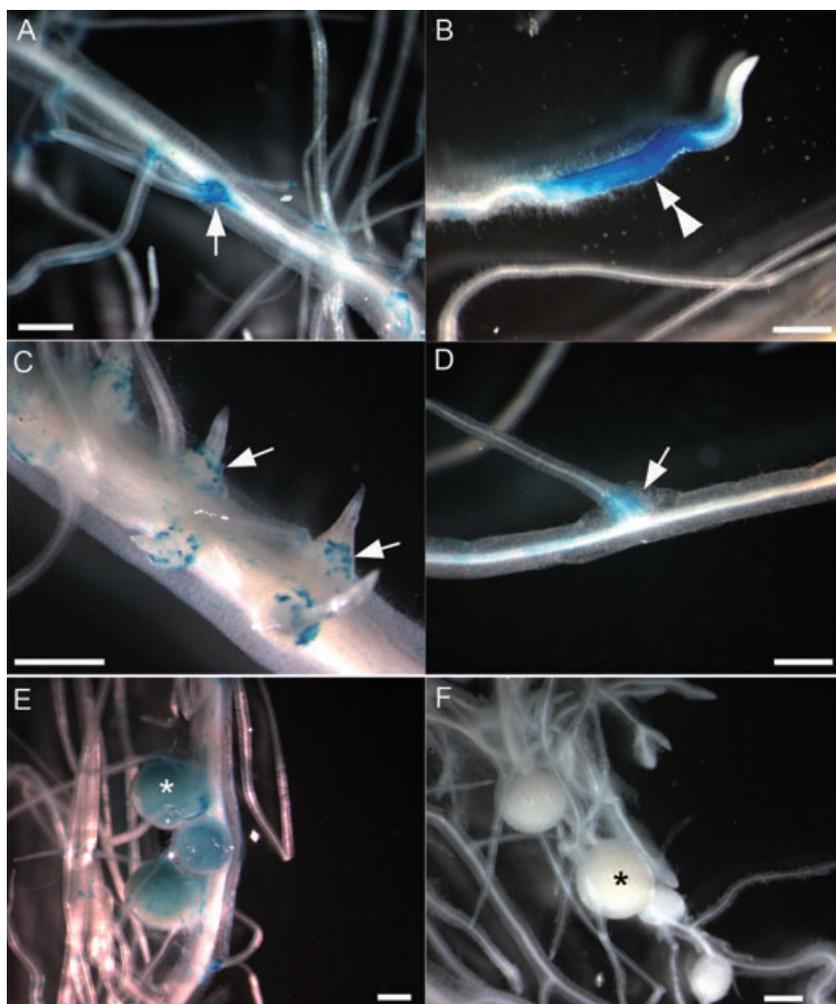
Fig. 3. 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) staining revealing *Rhizobium* (*Agrobacterium*) sp. strain IRBG74GUS (A–D) colonizing roots of *S. cannabina* (A), *S. bispinosa* (B), *S. rostrata* (C) and *S. sesban* (D) at 7 days after inoculation (dai), and GUS-tagged (E) and wild-type (F) IRBG74 colonizing roots of *S. cannabina* at 15 dai. All plants were grown under flooded conditions in sterile tubes. Note that the blue colouration on the young roots is located at the lateral root junctions (arrows in A, C, D) and also on the root hair zone close to the tip (double arrowhead in B). The mature nodules infected with the GUS-tagged strain are stained blue after treatment with X-Gluc (* in E), whereas those infected with the WT strain are white after the same treatment (* in F). Bars, 100 μm.

4E and F). The two *Mesorhizobium* strains differed in their ability to nodulate *S. cannabina*; *M. huakuii* KFR647, which formed fully effective nodules on its original host, *S. sesban* (Fig. S4C), formed partially effective nodules on *S. cannabina* (Fig. S4D), while *Mesorhizobium plurifarium* Sp45, which can effectively nodulate *S. punicea* (Vinuesa *et al.*, 2005), only formed ineffective nodules (small bumps similar to Fig. S4B). In contrast to the *Azorhizobium* and *Mesorhizobium* strains, all of the *Rhizobium-Agrobacterium-Sinorhizobium* strains produced effective nodules on *S. cannabina*, regardless of their geographical origins. They included *Rhizobium* sp. SIN-1 (Fig. S4E), which was isolated from *S. bispinosa* in India (and confirmed to nodulate it by Rana and Krishnan, 1995), and the two strains isolated from the South American wetland species, *S. exasperata*, *Rhizobium* sp. DUS1110 from Brazil (this study) and *R. huautlense* Se127 from Venezuela (Vinuesa *et al.*, 2005), both of which could also effectively nodulate their original host (e.g. DUS1110; Fig. S4F). The same was also true of the other *R. huautlense* strain from Venezuela, Ss121 (not shown), which was originally

isolated from *S. sericea* by Vinuesa and colleagues (2005). *Ensifer* (*Sinorhizobium*) *saheli* ORS609 from Senegal also effectively nodulated *S. cannabina*, as expected, because it was originally isolated from it (Boivin *et al.*, 1997).

Using *S. cannabina* as a test host, a more detailed comparison of strain IRBG74 with other *Sesbania*-nodulating strains was performed. The bacteria selected were *A. doebereinerae* Br5401, *M. huakuii* KFR647, *Rhizobium* sp. DUS1110 and *E. saheli* ORS609[T]. IRBG74 was found to be as effective in nodulation, N_2 fixation and plant growth promotion (dry weight accumulation) as the other *S. cannabina* strain, ORS609, but was slightly less effective in promoting the growth of *S. cannabina* than *Rhizobium* sp. DUS1110 (which, interestingly, had much lower nitrogenase activity than either IRBG74 or ORS609) (Table 3). Surprisingly, however, was the fact that *A. doebereinerae* strain Br5401, although it could not form effective N_2-fixing nodules on *S. cannabina*, was capable of promoting dry matter accumulation to a level equal to that of the symbiotically effective strains (Table 3), thus suggesting that it has plant growth-promoting rhizobacterium
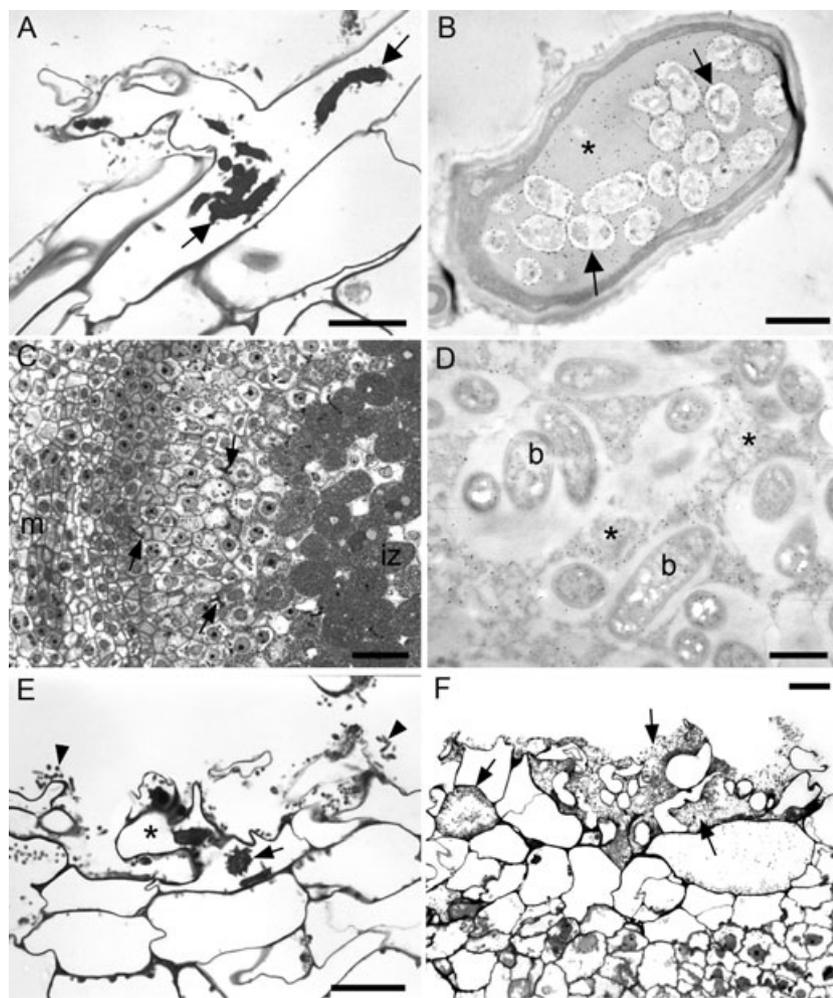
Fig. 4. Infection and development of nodules on *S. cannabina* (A–D), *S. rostrata* (E) and *S. sesban* (F) after inoculation with *Rhizobium* (*Agrobacterium*) sp. strain IRBG74. All plants were grown in non-flooded vermiculite/perlite under non-sterile conditions and were harvested at either 7 (A and B) or 15 (C–F) days after inoculation (dai).
A and B. Light microscopy (A) and transmission electron microscopy (TEM) (B) of infection threads within root hairs (arrows in A). The bacteria (arrows) in B were immunogold labelled with an antibody against strain IRBG74, and the labelling can be observed on the bacterial surface, but also within the infection thread matrix, which is composed of a host-derived glycoprotein impregnated with bacterial exopolysaccharide.
C. Transient nodule meristem (m) showing newly divided cells being invaded by infection threads (arrows).
D. TEM of a N$_2$-fixing cell with bacteroids (b) surrounded by host cell cytoplasm that has been immunogold labelled with an antibody against leghaemoglobin (*).
E. Infection threads (arrows) within a root hair (*) on an empty ineffective nodule formed on the root of *S. rostrata*. Note the bacteria (arrowheads) associated with the epidermal cells.
F. Surface of an ineffective nodule on *S. sesban* that is heavily colonized by bacteria (arrows). Bars, 5 µm (A), 2 µm (B, D), 20 µm (C), 10 µm (E, F).

(PGPR) properties, although, it should be noted that at the time of harvest (30 days) the *S. cannabina* plants were showing symptoms of N-deficiency (i.e. yellowing of the leaves). The plants inoculated with *M. huakuii* KFR647 showed highly variable nodulation ranging from no nodules through root bumps to partially effective nodules (Fig. S4D), and the mean dry weight accumulation was not significantly different to uninoculated *S. cannabina* (Table 3).

Discussion

Phylogeny of the core genome of Rhizobium *(Agrobacterium) sp. IRBG74*

The primary objectives of this study were (1) to understand the phylogenetic relationship of the rice growth-promoting strain IRBG74 to validly published species of *Rhizobium* and *Agrobacterium* and (2) to confirm whether or not it is able to nodulate and fix N$_2$ in association with

Table 3. Nodulation, nitrogenase activity and dry weight accumulation of *Sesbania cannabina* at 30 days after inoculation with rhizobial strains isolated from various *Sesbania* spp.

Strain	Original host	Number of nodules	ARA (µmol C$_2$H$_4$ per plant h^{-1})	Total dry weight (mg)
Rhizobium (*Agrobacterium*) IRBG74	*S. cannabina*	43 ± 6	18.57 ± 6.77	293 ± 59*
Azorhizobium doebereinereae Br5401^T	*S. virgata*	Several small bumps	0	354 ± 32*
Mesorhizobium huakuii KFR647	*S. sesban*	7 ± 2	2.87 ± 1.23	153 ± 19
Rhizobium sp. DUS1110	*S. exasperata*	55 ± 5	3.55 ± 0.32	441 ± 27*
Ensifer saheli ORS609T	*S. cannabina*	52 ± 5	16.14 ± 1.27	360 ± 32*
Uninoculated		0	0	130 ± 11

Values are means ± SE (*n* = 6) and those marked with * are significantly greater than the uninoculated plants at *P* < 0.01 using analysis of variance.

Sesbania spp. Strain IRBG74 was originally isolated from nodules of the aquatic legume *S. cannabina* and classified on the basis of 16S rRNA sequence analysis as a member of the revised *Rhizobium* genus (Young *et al.*, 2001), and the closest organisms on the basis of the 16S rRNA gene similarities were *R. radiobacter* strains NCIMB9402 and 13307 (Tan *et al.*, 2001). Here, we repeated and extended the analyses of Tan and colleagues (2001) to include additional data (Fig. S1). A concatenated approach was deployed using two universally conserved protein-coding genes, *rpoB* and *fusA*, which are useful alternatives to 16S rRNA sequences in determining the relationship between both divergent and highly related lineages (Santos and Ochman, 2004). The phylogenetic tree derived from the concatenated sequence matrix under a maximum likelihood approach offered the opportunity to infer a phylogeny based on the total evidence, from which the underlying species tree could be estimated. This tree supports the phylogeny estimated from the 16S rRNA sequence, namely that strain IRBG74 clusters, with high bootstrap support (89%), with three validly described strains of *R. radiobacter*, including the type strain NCIMB9042. However, the inferred tree does not identify whether IRBG74 is a strain of *R. radiobacter* or a unique species. To accomplish this, DNA–DNA hybridization, which remains the 'gold standard' to differentiate between these alternatives, was used. Our data demonstrated that against closely related strains of the *Rhizobium* genus, the DNA–DNA similarity was greatest against two strains of *R. radiobacter,* NCIMB9042, and NCIMB4034 (a recently described strain that has been used as a PGPR in agricultural systems; Humphry *et al.*, 2007), although neither of these strains is diazotrophic. Nevertheless, the similarity was significantly lower than the 70% threshold (Wayne *et al.*, 1987) required for designating IRBG74 as a strain of *R. radiobacter* or, indeed, any of the other species examined. These observations therefore support the inclusion of IRBG74 as a novel species within the *R. radiobacter*–*R. rubi* clade, and, to our knowledge, is the first N_2-fixing bacterium to be identified from among these species. However, these data should be interpreted with caution, because there are potential issues (which have yet to be resolved) in using DNA hybridization to circumscribe bacterial species in isolates with large accessory genomes (Young *et al.*, 2006). Moreover, this study was on a single strain (i.e. IRBG74), and several strains should be studied before a robust species description can be developed.

Phylogeny of symbiosis-related genes and host range

Analyses of two genes essential for symbiotic N_2 fixation, *nifH* and *nodA*, indicated that they had a distinct evolutionary history to the conserved 16S rRNA, *fusA* and *rpoB*

'housekeeping' loci. Alignment of the *nifH* and *nodA* sequences against homologous genes in GenBank revealed that both were most closely related to homologues present in members of the genus *Ensifer*, particularly those that had been isolated from *Sesbania* spp. The *nifH* gene of IRBG74 showed highest similarity to homologues from *E. saheli* strain ORS609 (also isolated from *S. cannabina*; De Lajudie *et al.*, 1994; Boivin *et al.*, 1997), whereas the *nodA* gene sequence was most similar to those of *E. saheli* bv. *sesbaniae* strain ORS611 and *E. terangae* bv. *sesbaniae* (although it did also hybridize with the *nodA* gene from *E. saheli* ORS609). The other *Rhizobium* strains isolated from *Sesbania* nodules (Ss121, Se127, SIN-1 and DUS1110) also contained *nifH* and *nodA* genes that were very similar to those of the *Ensifer* symbionts, and a phylogenetic relationship between the *nifH* genes of *Rhizobium* symbionts of *Sesbania* and those of *E. saheli* was previously noted by Vinuesa and colleagues (2005). It therefore appears that although the core genome of IRBG74 is essentially 'agrobacterial', its accessory symbiosis-related genome is similar to that of a wide range of *Rhizobium*/*Ensifer Sesbania* symbionts.

Distinct and divergent evolutionary histories between symbiotic and housekeeping genes have been reported in other rhizobia (Vinuesa *et al.*, 2005). This phenomenon is most likely explained by the high mobility of the plasmid-borne genes in *Rhizobium* and close relatives such as *Ensifer* (Turner *et al.*, 2002; Bailly *et al.*, 2007). In the case of *Agrobacterium*, this mobility has been exemplified in laboratory experiments in which strains have had rhizobial *sym* plasmids mobilized into them, and the genetically modified *Agrobacterium* strains thus formed have consequently shown a limited ability to nodulate legumes (Hirsch *et al.*, 1985; Martínez *et al.*, 1987; Van de Wiel *et al.*, 1988). Interestingly, and in spite of the apparent ease with which *Agrobacterium* can acquire *sym* genes in the laboratory, fully effective N_2-fixing agrobacterial symbioses with legumes had not been demonstrated and/or discovered (Mhamdi *et al.*, 2005; Velázquez *et al.*, 2005; Wang *et al.*, 2006) until the present study of strain IRBG74, which is therefore the first confirmed naturally occurring agrobacterial symbiont of legumes to be fully described in both genetical and symbiotic terms.

Rhizobium strain IRBG74 showed a range of symbiotic phenotypes on the different *Sesbania* species tested, from no nodulation on *S. punicea*, through ineffective nodulation on *S. herbacea*, *S. rostrata*, *S. sesban* and *S. virgata* to effective nodulation on *S. bispinosa*, *S. cannabina*, *S. exasperata*, *S. formosa*, *S. grandiflora*, *S. madagascariensis*, *S. macrantha* and *S. pachycarpa*. There appears to be no obvious geographical link between those species that do nodulate and those that do not, other than that all of the Asian *Sesbania* spp. nodulated effectively. Indeed, our data suggest that symbioti-

cally effective N$_2$ fixation is observed only with those species that can also nodulate with *Rhizobium* or *Ensifer*. Host range in rhizobia is determined by 'decorations' on the structure of the lipoprotein oligosaccharide 'nod factors' transcribed by the nodulation genes, such as *nodA* (Sprent, 2001; Kobayashi and Broughton, 2008), and so it is likely that the host range of IRBG74 (and the other symbionts in this study) is reflected more in its *nodA* than its core genome phylogeny. The *nodA* phylogenies suggest that the *Rhizobium* isolates studied here have acquired their symbiosis-related genes by lateral gene transfer from an *Ensifer* sp. (Fig. 3B), with a potential candidate being *E. saheli*. In the case of IRBG74, this is further supported by the fact that the type strain of *E. saheli*, ORS609, which was originally isolated from *S. cannabina* (De Lajudie *et al.*, 1994), has a similar host range (e.g. it is capable of effectively nodulating many of the *Sesbania* species tested positive in the present study for symbiotic nodulation with IRBG74, such as *S. bispinosa*, *S. formosa*, *S. grandiflora* and *S. pachycarpa*; Boivin *et al.*, 1997). On the other hand, unlike ORS609 (Boivin *et al.*, 1997), strain IRBG74 was unable to form effective nodules with those *Sesbania* spp. that establish symbiotic partnerships with azorhizobia (*S. rostrata*, *S. virgata*; Table 3) and mesorhizobia (*S. punicea*, *S. sesban*), thus suggesting that although they share similarities in their *nodA* sequences, the *nod* genes of IRBG74 are not identical to those of ORS609.

On the plant side, the host of IRBG74, *S. cannabina*, has shown a clear preference for symbionts with *nodA* sequences in the *Ensifer-Rhizobium* clade, but it also has the ability to nodulate (albeit, only partially effectively) with mesorhizobial strains from *Sesbania*, such as *M. huakuii* KFR647. Unfortunately, the sequence of *nodA* from KFR647 could not be obtained, but its *nodD* gene sequence was different from the aforementioned *Ensifer-Rhizobium* clade, and the *nodA* sequence of *M. plurifarium* Sp45, which only forms ineffective nodules on *S. cannabina*, was also distant from this clade, thus lending support to the suggestion that *S. cannabina* has a preference for *Ensifer/Rhizobium* symbionts with plasmid-borne symbiosis-related genes. On the other hand, *S. cannabina* may not be typical in this respect, as other studies of *Sesbania* have demonstrated that both mesorhizobial and (sino)rhizobial symbionts are found within the same species, e.g. in *S. sericea* (Vinuesa *et al.*, 2005) and *S. sesban* (Bala *et al.*, 2002; Sharma *et al.*, 2005). Of the two species that are known to nodulate with *Azorhizobium*, *S. rostrata* appears to be more capable of forming effective symbioses with other rhizobia (such as *E. saheli* and *E. terangae*; De Lajudie *et al.*, 1994; Boivin *et al.*, 1997), than *S. virgata*, which seems to be capable of forming effective symbioses only with *A. doebereinerae* (Gonçalves and Moreira, 2004; this study). *Sesbania*

punicea also appears to be highly conservative in its choice of symbiont, as evidenced by the lack of nodulation with IRBG74 (this study) or even with the other mesorhizobial strain from *S. sesban*, *M. huakuii* KFR647 (E.K. James, unpubl. data), and the data so far obtained about this species suggest that it can nodulate only with *M. plurifarium* (Vinuesa *et al.*, 2005). Interestingly, the highly selective nature of *S. virgata* and *S. punicea* with regard to symbionts in comparison with other *Sesbania* spp. was illustrated by a rhizobial soil 'trapping' study in Brazil in which they were the only *Sesbania* spp. that failed to nodulate in uninoculated soils (Veasey *et al.*, 1997). In contrast, good nodulation was obtained with the relatively more promiscuous *S. exasperata*, *S. sesban* and *S. tetraptera* Hochst. ex Baker.

Infection and nodulation of S. cannabina *and other* Sesbania *spp.*

Stem nodulation, or stem-borne lateral root base nodulation [as it should more correctly be called (Den Herder *et al.*, 2006)], in the genus *Sesbania* has so far only been confirmed in *S. rostrata* (Dreyfus and Dommergues, 1981). However, the crack entry infection process via adventitious roots that leads to the initial formation of aerial stem nodules on *S. rostrata* has also been observed in flooded roots of this species (Ndoye *et al.*, 1994; Goormachtig *et al.*, 2004) and *S. bispinosa* (Rana and Krishnan, 1995), and it also probably occurs on the other hydrophytic *Sesbania* spp. regardless of the type of rhizobial symbiont. The present study of *Rhizobium* (*Agrobacterium*) sp. IRBG74 suggests that a crack entry infection probably occurs with *S. cannabina* under flooded conditions, whereas under non-flooded conditions, as in *S. rostrata* (Goormachtig *et al.*, 2004), it nodulates via a 'standard' root hair infection pathway. It remains to be seen if the other *Sesbania* spp. in this study also switch from crack entry under flooded conditions to root hair infection under the non-flooded conditions described by Goormachtig and colleagues (2004) for *S. rostrata*. However, given that the subsequent nodule development and structure of N$_2$-fixing *Sesbania* nodules is so distinctive and uniform across the genus (this study, Harris *et al.*, 1949; Dreyfus and Dommergues, 1981; Ndoye *et al.*, 1994; Boivin *et al.*, 1997; James *et al.*, 2001), it is possible that all the hydrophytic species share common strategies in terms of rhizobial infection.

Concluding remarks: is there a plant phylogenetical component to nodulation of Sesbania *by different symbionts?*

This study confirms the earlier observations of Tan and colleagues (2001), using a number of techniques, that a

bacterium (IRBG74) isolated from nodules on the wetland legume *S. cannabina* is phylogenetically a strain of '*Agrobacterium*', that it is not a phytopathogen, but a plant growth-promoting diazotroph. Although this is by no means the first report of a non-pathogenic *Agrobacterium* strain being isolated from nodules (e.g. Mhamdi *et al.*, 2005; Wang *et al.*, 2006), the fact that IRBG74 possesses a *sym*-plasmid with symbiosis-specific genes (e.g. *nodA*) and can effectively nodulate its original host and fix N_2 to the benefit of the growth of the plant to the same degree as 'conventional' rhizobia, is a novel observation. Indeed, it could be argued that despite its core genome being that of *Agrobacterium* (Tan *et al.*, 2001; this study), IRBG74 behaves in all respects (including its infection processes) like typical legume-nodulating rhizobia, and thus supports the recent decision to incorporate all *Agrobacterium* strains into the genus *Rhizobium* (Young *et al.*, 2001). It is likely that as further rhizobia are isolated from more legumes, particularly those in the tropics, the incidence of potentially genuine symbiotic '*Agrobacterium*' isolates will increase (e.g. see Bala *et al.*, 2002).

This is also the first study to compare a nodulation gene (in this case, *nodA*) within a range of *Sesbania* symbionts. The *nodA* gene phylogeny, which groups the bacteria into three distinct clades (ie. *Azorhizobium*, *Mesorhizobium* and *Rhizobium* [*Agrobacterium*]/*E. saheli*), also appears linked to these microsymbionts' host range. Indeed, the present study, together with data from previous published work, have indicated three definable 'groups' of *Sesbania* spp. in terms of their propensity to nodulate with symbionts harbouring particular *nodA* gene types. Groups 1 and 2 consist of only one species each, i.e. the two South American species, *S. punicea* and *S. virgata*, which both have a high specificity for a very narrow range of symbionts (*M. plurifarium* and *A. doebereinerae* respectively) harbouring *nodA* sequences that are very different from each other and from the other *Sesbania* symbionts examined in the present study. Group 3, on the other hand, is a large group that contains several non-selective ('promiscuous') species that can nodulate with a wide range of symbionts in the *Rhizobium* [*Agrobacterium*]/*E. saheli* clade harbouring similar *nodA* genes (as well as, in some cases, with *M. huakuii* and *A. caulinodans*). The species in Group 3 include *S. cannabina* and *S. sesban*, and given their ability to nodulate effectively with strain IRBG74 (this study) and/or *S. saheli* ORS609 (Boivin *et al.*, 1997), probably also include *S. exasperata*, *S. formosa*, *S. grandiflora*, *S. macrantha* and *S. pachycarpa*. This group also includes *S. herbacea*, the original source of *R. huautlense* (Wang *et al.*, 1998), but which can also nodulate with *Mesorhizobium* strains and IRBG74 (Wang and Martínez-Romero, 2000; this study), and *S. sericea*, which, along with *S. cannabina* and *S. exasperata*, nodulates with *R. huautlense* strains (Vinuesa *et al.*, 2005). Although both

the Group 1 and 2 species are South American in origin, the three nodulation groups appear to be independent of geography, as other South America spp. are present in Group 3 (e.g. *S. exasperata*) and, indeed, the Group 3 species come from all parts of the tropical world. This therefore leaves open the possibility that the nodulation preferences (and *nodA* types) are actually linked to plant phylogeny, and this is demonstrated by a tree inferred from nrDNA Internal Transcribed Spacer 1 and 2 sequences of several *Sesbania* spp., including all those used in the present study (Fig. 5). This preliminary study has shown that although the genus is monophyletic (F.T. Farruggia, unpublished), it contains two distinct clades that appear to match the symbiont preference/*nodA* groups described above. For example, both the selective species, *S. punicea* and *S. virgata*, are present in one of the clades, whereas all the promiscuous species and/or those species that can nodulate with IRBG74 and other members of the *Rhizobium* [*Agrobacterium*]/*E. saheli nodA* group of *Sesbania* symbionts are in the other clade. Further studies of *nod* genes of symbionts from other members of the 60 plus species in the genus should confirm if the heterogeneity in symbiont preference is, indeed, linked to the molecular phylogeny of *Sesbania*.

Experimental procedures

Culture conditions and DNA–DNA hybridizations

All strains used in this study (Table 1) were routinely grown in yeast mannitol broth (YMB; Vincent, 1970). DNA–DNA hybridizations of strain IRBG74 against *R. radiobacter* NCIMB 9042, *R. vitis* LMG8750, *R. rubi* LMG 17935 and *R. huautlense* LMG 18254 were carried out by the identification service of DSMZ (Braunschweig, Germany) as described by De Ley and colleagues (1970), with the modification described by Huss and colleagues (1983) and Escara and Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

The amplification, cloning and sequencing of the 16S rRNA, housekeeping and symbiotic genes

The 16S rRNA genes of IRBG74, SIN-1 and DUS1110 were amplified by PCR using recombinant *Taq* polymerase (Life Technologies) and a pair of primers designed from *Escherichia coli* rRNA positions, 8-27F and 1509-1491R (Weisburg *et al.*, 1991). Reaction and PCR conditions were as described by Humphry and colleagues (2001). Amplification of the housekeeping genes from strain IRBG74 and the *Agrobacterium* type strains shown in Fig. 1 was performed using the protocol described by Santos and Ochman (2004). The primers employed were rpoBBDUP1, rpoBBDUP4, rpoB-BJDN2 and rpoBBJDN4 for the *rpoB* genes and fusAF and fusAR for the *fusA* sequence (Santos and Ochman, 2004).
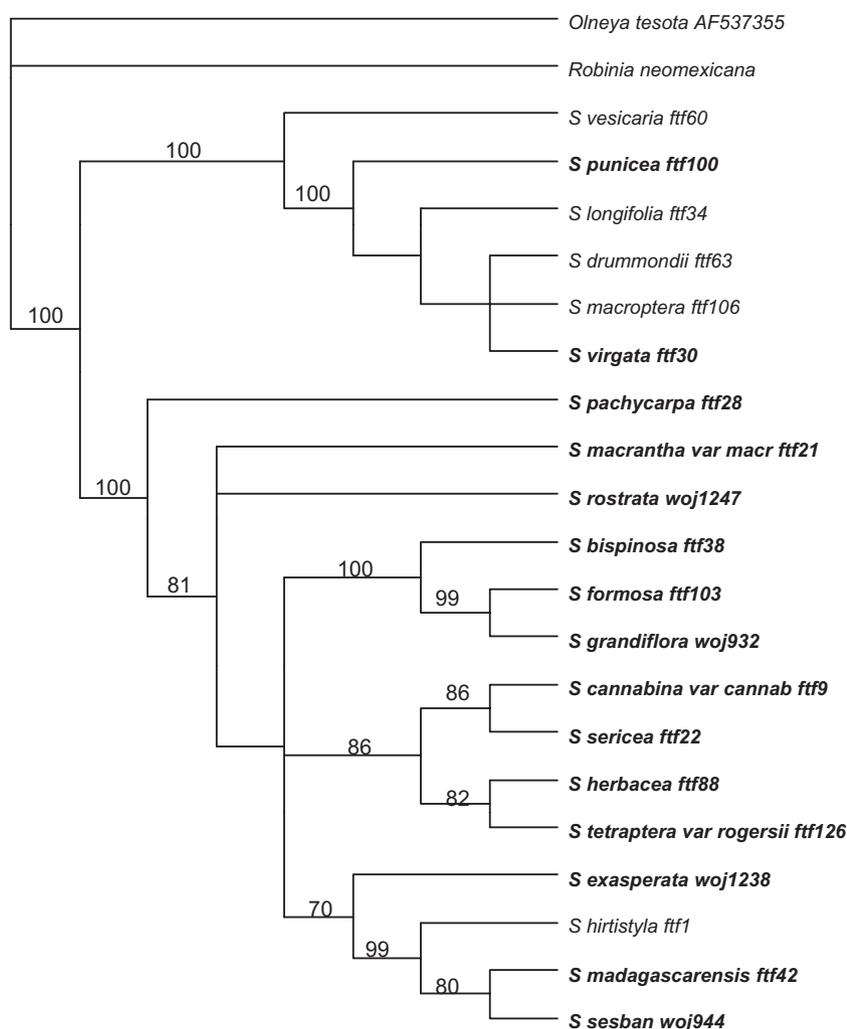
Fig. 5. *Sesbania* phylogeny inferred from sequence data of nrDNA Internal Transcribed Spacer 1 and 2 (including nrDNA 5.8S). Maximum parsimony (MP), as implemented in PAUP* 4.0b10 (Swofford, 2002), resulted in four most parsimonious trees at 588 steps. The strict consensus of these four trees is included here. Searches were conducted using random additions and TBR branch swapping with a maximum of 10 000 trees. Clade support was estimated by non-parametric bootstrap resampling (Felsenstein, 1985). Bootstrap values were derived from 1000 replicates using one random addition per replicate and branch swapping options as in standard analyses. Resulting bootstrap support is indicated above each well-supported node. Species examined for nodulation by *Rhizobium* (*Agrobacterium*) sp. IRBG74 are indicated in bold.

The PCR protocols to amplify *nifH* products from all the strains except *A. doebereinerae* Br5401 were performed according to the method of Poly and colleagues (2001) with primer pair PolF and PolR. The amplification of *nodA* sequences (except that of *A. doebereinerae* Br5401) was performed using the methods described by Haukka and colleagues (1998), with primers nodA-1 and nodA-2. The primers used in this study are detailed in Table S1. Amplification products were visualized using electrophoresis in a 1% agarose gel and stained with SYBR safe (Invitrogen). For each 16S rRNA, *fusA*, *rpoB*, *nifH* and *nodA* reaction, the amplified products from multiple independent inserts were cloned into pGEM-T 'easy' plasmid (Promega) and sequenced (Lark Technologies).

To obtain *A. caulinodans* Br5401 *nifH* and *nodA* sequences, cells were grown in YM broth and genomic DNA was isolated as described by Wheatcroft and Watson (1998). Two microlitres of genomic DNA was used as a template in a 25 μl reaction volume containing 25 mM TAPS-HCl, 50 mM KCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 0.2 mM of each of dATP, dCTP, dGTP and dTTP, 0.5 μM of each primer (Table S1) and 1 U of Phusion Taq DNA polymerase (New England Biolabs). The PCR conditions employed were initial denaturation at 95°C for 4 min followed by 35 cycles of 95°C for 45 s, 51°C for 45 s, 68°C for 1 min and a final extension at 72°C for 7 min. The amplified products were purified and sequenced directly using either the *nifH* or *nodA* primers.

The DNA sequence from 16S rRNA, *rpoB*, *fusA*, *nifH* and *nodA* genes from strain IRBG74 were aligned with sequences obtained from GenBank using CLUSTAL W (Benson *et al.*, 1998). The 16S rRNA sequences were checked for intragenic recombination using Chimerae as implemented in RDP2. The 16S rRNA, *fusA* and *rpoB* sequences were concatenated with BIOEDIT (Hall, 1999) and a phylogenetic tree inferred using the maximum likelihood approach with PhyML 3.0 (Guindon *et al.*, 2005). The appropriate nucleotide substitution model, GTR plus gamma, was selected using FindModel (Posada and Crandall, 2001). The robustness of the ML topology was inferred by non-parametric bootstrap tests with 100 pseudoreplicates using PhyML. The Neighbour-joining phylogenetic trees for *nifH* and *nodA* were performed with the Phylip package. The analysis included SEQBOOT, DNADIST, NEIGHBOR, CONSENSE (PHYLIP 3.5c package; Felsenstein, 1993) and TREEVIEW (Page, 1996) as described by Humphry and colleagues (2001).

Plasmid profiles and Southern blotting of nodA *genes*

Plasmid profiles were studied on horizontal gels using the modified Ekhardt technique as described by Kuykendall and colleagues (1996). Gels were blotted onto nylon membranes and DNA fixed by UV cross-linking. Southern blotting was carried out using a *nodA* probe of IRBG74 prepared using the PCR amplified gene products described above and labelled by random priming using the Dig-High prime system (Roche). Hybridization conditions were as described by Turner and colleagues (2002). Hybridization was detected using the anti-dioxigenin kit with the chemiluminescent substrate CSPD (Roche) according to the manufacturer's instructions.

Phytopathogenic testing

The ability of IRBG74 to form crown galls on young tobacco (*Nicotiana tabacum*) plants was tested by wounding stems and inoculating the wounds as described by Moore and colleagues (2001). The PCR methods used to amplify the characteristic *VirD2* and *ipt* gene portions found in functional 'Agrobacterial' Ti and Ri plasmids were those described by Haas and colleagues (1995).

Nodulation of Sesbania *spp. by IRBG74*

Seeds of *Sesbania* spp. and *M. atropurpureum* cv. Siratro (Table 3) were surface sterilized and their dormancy broken by treating them with concentrated sulphuric acid for 20 min (Elliott *et al.*, 2007). *Phaseolus vulgaris* cv. Contender seeds were surface sterilized by immersion in 70% ethanol for 10 min and germinated by placing them in the dark on wet paper towels. The seedlings were grown in pots with a 1:1 mixture of vermiculite and perlite in a greenhouse according to Elliott and colleagues (2007). The plants were watered with either N-free nutrient solution or tap water so that the potting medium was moist but not flooded. At 5 days after sowing, they were inoculated with a culture of *Rhizobium* (*Agrobacterium*) strain IRBG74 grown to log phase. Plants were then inspected at two weekly intervals for signs of nodulation, and at 60 dai the plants were tested for nitrogenase activity using the acetylene reduction assay (ARA) according to Elliott and colleagues (2007). Any nodules were then removed, counted, and their dry weights determined, with some nodules taken for light and electron microscopy studies (see below).

A second experiment was set up to compare the ability of two *Sesbania* spp. (*S. bispinosa*, *S. cannabina*) inoculated with IRBG74 to nodulate and fix N$_2$ under flooded conditions. In this case, two sets of seedlings from each species were sown into pots as described above, and at 5 days after sowing, both sets were inoculated with IRBG74, but one set was also flooded so that the whole of the developing root system was submerged. The plants were harvested at 30 dai, and were examined for nodulation, nitrogenase activity and dry weight accumulation.

Infection and nodulation of Sesbania *spp. with WT and GUS-marked strains of IRBG74*

Further nodulation experiments using a GUS-tagged strain of IRBG74 were performed under sterile flooded conditions in tubes according to Elliott and colleagues (2007). Strain IRBG74 was marked according to Gyaneshwar and colleagues (2001) using *Escherichia coli S17.1* containing transposon-based GUS marker pCAM120 (Tn5ssgusA20), which has the *gusA* gene under the control of a constitutive kanamycin-resistance gene promoter (Wilson *et al.*, 1995). Seeds of *S. bispinosa*, *S. cannabina*, *S. rostrata* and *S. sesban* were prepared for germination as described above, washed thoroughly with sterile distilled water and allowed to germinate on YMB plates. Seedlings free of visual contamination were used for inoculation with the *gusA*-marked strain. Bacteria were grown on YMB medium supplemented with spectinomycin until an optical density of 0.6. The cells were then harvested, washed twice with normal saline and resuspended in saline. The seedlings were carefully placed into the N-free liquid medium in the tubes, and were inoculated 5 days later. The plants were harvested at 7 and 15 dai for staining to detect GUS activity according to Gyaneshwar and colleagues (2001). Another experiment was set up in parallel, but in this case the seedlings were grown under non-flooded conditions in pots filled with vermiculite/perlite (see above for details), and were inoculated with WT IRBG74 5 days after sowing. At harvesting (7 and 15 dai), the *Sesbania* roots (and nodules, if present) were examined by light and transmission electron microscopy according to James and colleagues (2001) and Elliott and colleagues (2007). Sections were immunogold labelled according to James and colleagues (1996), either with a polyclonal antibody raised against IRBG74 (diluted 1:500) or with a polyclonal antibody (diluted 1:100) raised against Lb purified from pea (*Pisum sativum*) nodules (Van de Wiel *et al.*, 1988). The IRBG74 antibody was tested for specificity via an enzyme-linked immunosorbent assay (ELISA) with a range of common soil and plant-associated bacteria according to Gyaneshwar and colleagues (2001), and it was also tested via immunogold labelling of sections of nodules formed on *Sesbania* spp. by all the bacteria listed in Table 1. No significant ELISA or immunogold reaction was obtained with any bacterium except for IRBG74 and its derivative strain, IRBG74GUS.

Nodulation of S. cannabina *by other* Sesbania-*nodulating rhizobia*

All the WT strains listed in Table 1 were inoculated on to seedlings of *S. cannabina*. Although all the strains, with the exception of DUS1110 (*S. exasperata*) and KFR647 (*S. sesban*), are known to be symbionts of their original hosts (see references cited in Table 1), they were also inoculated on to their original hosts (depending on availability of seeds) to confirm their symbiotic effectiveness. The plants were grown under sterile flooded conditions in glass tubes (as for the experiment using the GUS-tagged IRBG74 strain; see above). The plants were harvested at 60 dai, and were scored for presence of nodules, plant health (i.e. green shoots), ARA and nodule structure. From the results of this initial screening process, a more extensive experiment was set up to compare the symbiotic performance (growth, nodulation and nitrogenase activity) of *Rhizobium* (*Agrobacterium*) sp. IRBG74 on *S. cannabina* with representative strains from each of the four different genera of rhizobia known to

nodulate *Sesbania* spp. (i.e. *Rhizobium* sp. DUS1110, *A. doebereinerae* Br5401, *Mesorhizobium huakii* KFR647 and *Sinorhizobium* (*Ensifer*) *saheli* ORS609; Table 1). Plants 'inoculated' with sterile YMB alone served as controls. The plants were grown under controlled environmental growth conditions for 30 days in pots filled with flooded vermiculite/perlite under a 12-h day, at a day/night temperature of 28/21°C and an irradiance of 1500 μE m^{-2} s^{-1}. At harvest, nitrogenase activity (ARA) was measured, nodules were counted, and total plant dry weights were determined.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Maximum likelihood phylogram inferred from concatenated 16S rRNA + *rpoB* + *fusA* sequences estimated using the GTR +G substitution model for nine *Sesbania* isolates. Bootstrap support for 100 pseudoreplicates of the data set are provided at the corresponding nodes. The scale bar represents the number of nucleotide substitutions per site. Numbers in bold are GenBank accession numbers and T denotes the type strain of the species.

Fig. S2. Phylogenetic dendrograms based upon (A) *nifH* – 561 bp (B) *nodA* – 525 bp sequences of IRBG74. The Jukes and Cantor algorithm and the Neighbour-joining method were employed; bootstrap confidence percentages were calculated from 1000 replicate trees and shown on the branches if these occurred in more than 50% of the trees. The scale bar represents nucleotide substitutions per site. Numbers in bold are GenBank accession numbers and T denotes the type strain of the species.

Fig. S3. Plasmid profile of *Rhizobium* sp. IRBG74 compared with bacterial type strains with the most similar chromosomal and symbiotic gene sequences. Lane A – *Ensifer saheli* ORS 609, lane B – *Rhizobium* sp. SIN-1, lane C – *Rhizobium* sp. IRBG74, lane D – *R. radiobacter* NCIMB 13307, lane E – *R. radiobacter* NCIMB 9042(T). The size standard was produced using the plasmids of *Rhizobium leguminosarum* biovar *viciae* strain 3841, for which the sizes are already known. Arrows indicate the symbiotic plasmids.

Fig. S4. Nodulation of *S. cannabina* and other *Sesbania* species by various rhizobial strains. (A) *S. virgata* + *Azorhizobium doebereinerae* Br5401. (B) *S. cannabina* + *A. doebereinerae* Br5401. (C) *S. sesban* + *Mesorhizobium huakuii* KFR647. (D) *S. cannabina* + *M. huakuii* KFR647. (E) *S. cannabina* + *Rhizobium* sp. SIN-1. (F) *S. exasperata* + *Rhizobium* sp. DUS1110. The infected tissue is marked with a white asterisk in the effective, N$_2$-fixing nodules in A, C, D, E, F. The smaller, ineffective nodule shown in B did not have any internal colonization by bacteria (black asterisk), but there was dense colonization of the epidermal tissue (arrow). Note in D that compared with the other effective nodules those formed by *M. huakuii* KFR647 on *S. cannabina* are more sparsely populated by infected, N$_2$-fixing cells. The transient meristem in a nodule on *S. virgata* is indicated by an arrow in A. Bars, 500 μm (A, C–F), 100 μm (B).

Table S1. Primers used in this study.

Table S2. Nodulation of *Sesbania* spp. and other legumes at 60 days after inoculation with *Rhizobium* (*Agrobacterium*) sp. strain IRBG74.

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