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2

3 *Paper for Forum issue*

4 CHAPTER 5

5 The biodiversity of beneficial microbe-host mutualism: The case of rhizobia**

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20 **This contribution is dedicated to the memory of Dr. Peter H. Graham, who

21 through his activities worldwide raised the interest in microbial genresources as the

22 foundation for improved agriculture and food. For a long

23 time he also served as the chair person for the Subcommittee for

24 *Agrobacterium* and *Rhizobium* of the International Committee for the Systematics

25 of Prokaryotes.

26 Abstract

27 Symbiotic nitrogen fixation is the main route for sustainable input of nitrogen
28 into ecosystems. Nitrogen fixation in agriculture can be improved by inoculation of legume
29 crops with suitable rhizobia. Knowledge of the biodiversity of rhizobia and of local
30 populations is important for the design of successful inoculation strategies. Soybeans are
31 major nitrogen-fixing crops in many parts of the world. Bradyrhizobial inoculants for soybean
32 are very diverse, yet classification and characterization of strains have long been difficult.
33 Recent genetic characterization methods permit more reliable identification and will
34 improve our knowledge of local populations. Forage legumes form another group of
35 agronomically important legumes. Research and extension policies valorising rhizobial
36 germplasm diversity and preservation, farmers training for proper inoculant use and legal
37 enforcement of commercial inoculant quality have proved a successful approach to promote
38 the use of forage legumes while enhancing biological N₂ fixation. It is worth noting that
39 taxonomically important strains may not necessarily be important reference strains for other
40 uses such as legume inoculation and genomics due to specialization of the different fields.
41 This article points at current knowledge on one hand and and gaps to be filled on the other,
42 for further interaction and improvement of a rhizobial commons.

43

44 Key Words: indigenous, diverse, adaptation, promiscuous, rhizobium, bradyrhizobium,
45 inoculant, competition, evolution, Chronos, Kairos

46

47

48 1. The importance of legumes

49 Symbiotic nitrogen fixation with rhizobia is the most important route for
50 sustainable nitrogen input into agro-ecosystems. The legume-rhizobium symbiosis
51 represents a significant basic model for symbiosis, evolution and differentiation in
52 agriculture for sustainable production and other fields. The signal exchange between rhizobia
53 and their host plants has been elucidated in great detail, including interacting proteins and
54 encoding genes, and is schematically depicted in Fig. 1. The role of root nodule bacteria for
55 growth promotion through biological nitrogen fixation (BNF) and the carryover of
56 environmental benefits in agro-ecosystems have been reported already in ancient times. The
57 most recent estimates of annual nitrogen fixation inputs by crop legumes, as given in a
58 recent report (Herridge et al., 2008), were 21.45 Tg, and the inputs of pasture and fodder
59 legumes 12-25 Tg. In comparison, the inputs for rice, sugar cane, non-legume cropland and
60 extensive savannas were estimated to be < 23.5 Tg, giving a total input from biological
61 nitrogen fixation (BNF) of 50-70 Tg in agricultural systems. Since symbiotic BNF in legumes is
62 mediated by nodule bacteria, here collectively called rhizobia, these bacteria account for at
63 least half of all biologically fixed nitrogen in agriculture. If we consider the efficiency of the
64 process, crop legumes fix on an average 66% of the nitrogen recovered in the crop, 23-176
65 kg N ha⁻¹, depending on the plant species, but also on the rhizobia present in the nodules.

66

67 Fig. 1.

68

69 The rhizobia have during the last decades received much attention, and the
70 number of bacterial species able to form nitrogen-fixing symbioses with legumes has
71 increased almost exponentially. The symbionts occur in several, even distantly related

72 bacterial genera (Supplementary table 1). The description of new species of rhizobia has
73 partly masked the fact that our knowledge of what makes a good symbiont is still very
74 vague.

75

76 2. Inoculation as a current and essential practice

77 Inoculation of legume seeds with rhizobia is perhaps the oldest agro-
78 biotechnological application. Inoculants are generally produced regionally or locally, making
79 use of selected bacterial strains and carrier materials available in that area. For example, in
80 Finland the inoculant producer Elomestari Ltd. provides rhizobium inoculants for clovers,
81 lucerne (alfalfa), fodder galega, lotus, pea, faba bean, vetches and lupins for the Nordic
82 market. They use locally available, neutralised and ground peat, suitable prepared to
83 accommodate rhizobia and give the preparation a long shelflife. Peat-based inoculants are
84 applied to legume seed as a slurry just before sowing. Other technological approaches are
85 liquid inoculants, pre-coated seed and granules. During the last decades, the technological
86 development has been along these lines, with emphasis laid on the utilisation of local waste
87 material as carriers on one hand and ease of use on the other (Ben Rehab et al., 2007;
88 Brockwell and Bottomley, 1995; Denton et al., 2009). Inoculation is recommended if the field
89 has no history of legume cultivation, especially if the plant is exotic to a new environment, or
90 if the soil is acidic, saline or otherwise hostile to rhizobia. The Finnish inoculant strains come
91 from Elomestari's own collection or from the HAMBI collection at the University of Helsinki.
92 They have been deposited by scientists who isolated the strains from the field and tested
93 them for efficiency on selected plants. In the case of the inoculant for lucerne, the strain still
94 in use today was isolated by myself (KL) in 1981 from a nodule in a lucerne field inoculated
95 with a commercial inoculant provided by the Nitragin Company in the U.S.

96 This is an example of how the microbial commons for rhizobium inoculants
97 evolve. The main purpose of the commons is the exchange of inoculant strains with highly
98 desirable properties, such as good field performance and stability of symbiotic properties in
99 culture. These strains are not necessarily otherwise well documented or used in taxonomy
100 or molecular biology, exceptions being for example the type strains of *Rhizobium galegae*,
101 HAMBI 540^T (Lindström, 1989) and *Sinorhizobium* (syn. *Ensifer*) *arboris* HAMBI 1552^T (Nick
102 et al. 1999), which were both originally identified as good symbiotic partners for their
103 respective host legumes in the field.

104

105 3. Knowledge from genomes and ecology

106 3.1. Core and accessory genome compartments

107 The approach in inoculant development was traditionally the isolation, testing
108 and selection of single strains with desired properties, such as high nitrogen fixation
109 efficiency in symbiosis with selected host plants. In cases where indigenous rhizobia capable
110 of nodulating the host were already present in soil but not very efficient, the capacity of the
111 inoculant to outcompete those strains became important. The selection of strains is still
112 hampered by the fact that even though the genes encoding nodulation and nitrogen fixation
113 in rhizobia are well-known (e.g. Franche et al., 2009), the genetic basis for symbiotic
114 effectiveness, competitiveness and tolerance to environmental stress factors are largely not
115 known. Milestones in unraveling the genetic basis of symbiosis in rhizobia were the cloning
116 of nodulation genes of *S. meliloti* strain 1021 (Long et al., 1982), another the completion of
117 the whole genome sequence of the same strain (Galibert et al., 2001). This important work
118 has not yet had practical field implications.

119 In an EU funded research project BACDIVERS (2003-2005; N° QLRT-2001-02097)
120 scientists and inoculant manufacturers got together in order to utilize modern phylogenetic
121 and genomic methods for dissection of the genomes of important inoculant strains as well as
122 taxonomic type strains. Focus was on genes involved in stress tolerance, which is an
123 important property for an inoculant strain while desiccating on the seed or later in a hostile
124 soil environment. Comparative genomic hybridizations revealed that *Sinorhizobium* strains
125 from the wild had significantly different genomes from that of the molecular reference strain
126 *Sinorhizobium meliloti* strain 1021, especially among genes involved in recombination and
127 DNA repair. Multilocus sequence analysis was applied and evaluated for taxonomic
128 purposes, especially for the genus *Sinorhizobium*, and found to be a valuable tool (Martens
129 et al., 2007, 2008) for rhizobium classification and speciation studies, giving similar but more
130 detailed information than classical methods, but no inoculant strains were yet included
131 among the strains studied.

132 Conceptually, the rhizobial genomes can be divided into compartments. The
133 core genome carries housekeeping genes necessary for proper functioning of the cell. The
134 phylogeny of housekeeping genes indicate speciation and forms a good biological basis for
135 rhizobium taxonomy (Martens et al., 2007, 2008; Vinuesa et al., 2008). The accessory
136 genome on the other hand is responsible for properties such as symbiosis and other kinds of
137 ecological niche adaptation (Young et al. 2006).

138 Symbiotic nodulation functions are encoded partly by accessory genes found in
139 almost all rhizobia ("common nodulation genes") and partly by genes encoding host
140 specificity. These genes evolved under host constraint and clearly belong to the accessory
141 gene pool (Suominen et al., 2001). By using microarrays, Ruberg et al. (2003) demonstrated
142 how osmotic stress influenced the gene expression in strain 1021. Over one hundred genes

143 were identified showing significant changes in gene expression resulting from the osmotic
144 upshift. Among these genes repression was observed for genes related to motility and
145 chemotaxis, genes encoding amino acid biosynthesis enzymes and genes involved in iron
146 uptake whereas some genes involved in transport of small molecules and genes related to
147 polysaccharide biosynthesis were induced. Jensen et al. (2005) demonstrated the role of
148 trehalose transport in competitiveness of *Sinorhizobium meliloti* strains on *Medicago sativa*,
149 emphasizing the important role of different saccharides in the ecology of rhizobia. These
150 genes, which are involved in adaptation to certain environmental conditions, might belong to
151 both the core and the accessory gene pools. Further studies should clarify their roles and
152 hopefully assist the development of inoculation strategies.

153 Biogeographical studies of *S. meliloti* populations revealed that their genetic
154 composition varied in diverse gene centers, such as Siberia, China and Central Asia
155 (Roumiantseva et al., 2002). Several recent studies also addressed biogeography, but did not
156 yet answer questions related to agronomy (e.g. Vinuesa et al. 2008). Thus, genes important
157 for effective, high-yielding nitrogen fixation or responsible for e.g. competitiveness have not
158 yet been properly pinpointed, they obviously vary depending on bacteria, hosts and the
159 environment, and are possibly part of the accessory genome. Further studies into genome
160 composition and evolution in combination with biogeographic approaches are likely to shed
161 more light on the genetics of symbiotic nitrogen fixation in nature.

162

163 3.2. Chronos and Kairos evolution of bacterial genomes

164 Also the evolution of micro-organisms can be viewed conceptually from two
165 different perspectives, which are here termed '*Chronos*' and '*Kairos*' evolution. Both
166 words stem from the old Greek, who recognized two different words for 'time'. Chronos

167 evolution is characterized through its long-term perspective; slowly evolving genes and
168 genomes leading to speciation, when suitable genes are used as molecular clocks or
169 chronometers (Turner and Young, 2000). Evolutionary events can be fixed on a time scale in
170 chronological order. In contrast, Kairos evolution is fast, takes place under special conditions,
171 leads to niche adaptation and cannot be used to measure evolutionary time. Thus, Kairos is
172 the time when big events take place, often in a short measurable time. The hypothesis is
173 that chronos evolution occurs among all DNA sequences (core and accessory genomes)
174 within an organism, while Kairos evolution impacts most effectively the accessory genome.
175 Speciation in rhizobia is mainly Chronos evolution of the core genome, whereas symbiotic
176 interactions with plants and other kinds of niche adaptation are largely determined by
177 accessory genes, subjected to both Chronos and Kairos evolution (for a review, see Dresler-
178 Nurmi et al., 2009). A classical example of Kairos evolution is the study by Sullivan et al.
179 (1995), in which the symbiotic island from an inoculant *Mesorhizobium* strain spread into a
180 native population of non-nodulating rhizobia, which thereby became symbiotic.

181

182

183

184 4. Microbial commons in the context of rhizobia

185 4.1. *Where to search for inoculant strains*

186 With this brief background information in mind, what is the best place to look
187 for good inoculant strains to be used in agro-biotechnology? Vavilov (1926) proposed that
188 the greatest diversity of agricultural plants was to be found in their gene centers (centers of
189 origin). Thus, the gene centers of the plants would be the places to look for a wide range of
190 diversity among strains. Indeed, we have shown that the diversity of both plants and

191 rhizobia of the species *Galega orientalis* was greater in the gene center of the plant than in
192 other places (Andronov et al. 2003). In this very host specific symbiosis the plant seems to be
193 an important determinant for rhizobial diversity. In other systems the situation might be
194 different. With less specific, suitable symbiotic genes available, local populations
195 representing even diverse species might quickly adapt to newly introduced plant species and
196 varieties via Kairos evolution. The potential for great plasticity has been detected in all
197 rhizobial genomes sequenced (reviewed by MacLean et al., 2007). Work with inoculant
198 strain development on diverse continents have demonstrated the importance of diversity of
199 indigenous rhizobial populations for symbiotic nitrogen fixation on one hand and the success
200 of inoculation on the other.

201

202 *4.2. Purposes of strain exchange*

203 A main function of the rhizobial microbial commons is type strain deposition in recognised
204 collections with public databases (e.g. ATCC, DSMZ, LMG, HAMBI) and exchange for
205 comparative taxonomic purposes. The taxonomic type strains and representatives of
206 described species mainly stem from collection efforts aiming at sampling the biodiversity of
207 rhizobia from diverse host plants and geographic regions. Such strains are also material for
208 population genetic studies aiming at revealing patterns of Chronos and Kairos evolution
209 though for this purpose many more strains than what is required for species descriptions
210 should be used. Such collections are often kept by individual researchers and not available
211 to the whole scientific community. Obstacles for free exchange of rhizobial collections are
212 nowadays protection of intellectual property rights by restriction of strain exchange based
213 on the Cartagena protocol. Even though molecular markers form the core in species
214 delineations, molecular biological studies and whole genome sequencing do not normally

215 require strain exchange, since scientists work with "in house" model strains. Inoculant
216 strains for agriculture are not regularly deposited in culture collections, but mainly
217 maintained by companies or research institutes. These strains are not often listed in public
218 databases and thus only available locally or via personal contacts. In conclusion, strain
219 exchange for taxonomic purposes is well organised and readily accessible to the scientific
220 community. However, collections of strains with agro-bioechnological interest are mainly
221 regional or local and less accessible for exchange.

222

223 5. Biodiversity of soil rhizobia

224 5.1. *Indigenous rhizobia*

225 Indigenous rhizobia are those found naturally in the soil of a given locality. A
226 great diversity occurs in most soils and an enhanced population size occurs where
227 compatible legumes are grown and the soil is fertile (Zengeni et al., 2006). Rhizobia are very
228 diverse at species and strain levels. One soil may contain various species and various strains
229 within a species (Bala et al., 2001), while also similar isolates may be found in distant places
230 (Abaidoo et al., 2007). When the bacteria are not dormant, the accessory genomes of
231 indigenous soil rhizobia probably undergo frequent Kairos evolution. A challenge for
232 agriculture is to match rhizobia and legume crops for optimal performance either by having
233 plant genotypes adapted to local rhizobial populations or by inoculation with effective
234 strains adapted to prevailing environmental conditions and with good competitive ability
235 against local, less effective strains.

236

237 5.2. *Diversity and specificity*

238 The plant family Leguminosae, the members of which rhizobia form symbioses

239 with is very diverse ranging from field grain annual legumes such as soybean to perennial
240 trees such as *Sesbania*. Whilst there may be cross-inoculation of strains compatible with any
241 given legume (Abaidoo et al., 2007), the wide diversity of rhizobia requires more precise
242 matching in the symbioses. This specificity of an association enables maximisation of
243 nitrogen fixation. Subsequently, grain legumes can yield up to about 300 kg ha⁻¹ year⁻¹ whilst
244 some tree legumes fix as much as 600kg N ha⁻¹ per year (Giller, 2001) with well matched
245 symbionts. For example, a wide diversity of soybean nodulating rhizobia has been found in
246 Zimbabwean soils (Musiyiwa et al., 2005; Vance et al., 1988). However, only three out of the
247 129 isolates obtained from one study had higher nitrogen fixation efficiency than the
248 standard commercial strain MAR 1491 used in the country. This emphasises the possibility of
249 closer matching of strain and crop for improved symbiotic efficiency by careful strain
250 selection.

251 The occurrence of a wide diversity of strains increases the opportunity of a
252 legume host finding a compatible rhizobium in any particular soil. Some smallholder farmers
253 prefer using promiscuous varieties of soybean as opposed to the higher yielding specific
254 varieties because of the challenges they face in getting access to inoculants (Mpeperekwi et
255 al., 2000; Musiyiwa et al., 2005). Many developing countries do not have inoculant factories
256 and therefore indigenous rhizobia become an important resource in their natural state.

257

258 *5.3. Access to indigenous rhizobia*

259 Success stories of the exploitation of indigenous rhizobia by farmers with no
260 access to inoculants have been reported. The International Institute of Tropical Agriculture
261 (IITA) has bred promiscuous varieties through crossing specific American varieties with
262 promiscuous Asian varieties of soybean (Vanlauwe and Giller, 2006). Conversely erratic

263 nodulation and nitrogen fixation in Zimbabwe and Zambia by these crosses have also been
264 reported (Mpepereki et al., 2000; Sanginga, 2003). It was concluded that it may be
265 impossible to develop a promiscuous soybean variety that nodulates in all regions of the
266 Guinean savannah. Additionally, striving for nodulation is not sufficient because some
267 nodulating, non-nitrogen fixing associations occur. Due to the heterogeneous nature of
268 native rhizobia populations it is difficult to access this resource in its natural condition and
269 lack of knowledge regarding the genetics of symbiotic effectiveness hampers development.

270 Population sizes of indigenous rhizobia compatible with the legume crop of
271 choice are often very low in economically significant soils. Population sizes ranging from 2 to
272 4200 cells per gram of soil were reported in a study across nine African countries (Abaidoo et
273 al., 2007). The numbers were enhanced by previous cropping of the compatible legumes.
274 About 38% of isolates from this experiment were as effective as USDA 110 when inoculated
275 on soybean, forming a rich source for inoculant development yet emphasizing the need to
276 supplement soil rhizobia for economic production of specific legumes by inoculation.

277

278 *5.4. Population dynamics*

279 Unfortunately, adaptation of indigenous rhizobial populations to local
280 environments, which is a big advantage for selection of inoculant strains, may pose a
281 challenge to productivity. The adaptability of indigenous rhizobia to their environment
282 results in high levels of saprophytic competence (Zengeni et al., (2006). Sometimes
283 indigenous rhizobia may be found in greater numbers than those of the inoculated strains
284 which are also limited in mobility. This is a challenge that must be overcome by the inoculant
285 strains and it raises the standard of the inoculant required with regards to competitiveness
286 and nitrogen fixation (Tas et al., 1995). Rhizobia may also be an indicator of soil properties.

287 Indigenous rhizobia are affected by soil acidity, temperature, moisture and other factors and
288 therefore the diversity may be an indicator of the soil condition. Fields which receive
289 consistent fertility management and legume cropping host higher rhizobial numbers and
290 diversity. The application of manure has been noted to increase rhizobial numbers in soil
291 populations (Zengeni et al., 2006).

292

293 *5.5. indigenous rhizobia as sources for inoculants*

294 All inoculant strains originate in indigenous rhizobial pools. Thus, although
295 indigenous rhizobia may pose challenges of competition to inoculated strains, they are an
296 important resource that must be preserved by integrated soil fertility approaches. Continual
297 isolation and characterisation to identify new isolates offers the opportunity to improve BNF
298 with minor limits geographically to the areas of use. It may be difficult to find an isolate
299 bearing all the required attributes to the desired level. Indigenous rhizobia are a source for
300 genes that may be required for the enhancement of rhizobial performance using molecular
301 biological techniques (Appunu and Dhar, 2008). A prerequisite for this approach is more
302 insight into the genetic makeup of rhizobia and the dynamics of their genomes.

303 It is important to continually isolate higher nitrogen fixing isolates to be used as
304 inoculant strains from the wide diversity of indigenous rhizobia. There have also been
305 reports of inoculant strains losing their symbiotic properties (Weaver and Wright, 1987). A
306 wide diversity of isolates ensures a sustainable source of replacement strains and may be
307 developed into strains for commercial use (Musiyiwa et al., 2005).

308

309 *6. Bradyrhizobium strains as inoculants for soybean*

310 *6.1. Importance of soybean*

311 Soybean is a major agricultural crop worldwide (Table 1). It is used for human
312 food and livestock feed and increasingly additional uses for soybean oil are being developed,
313 including biodiesel and industrial products such as inks, plastics, building materials and
314 lubricants (<http://www.ilsoy.org/soybean-uses/new-uses/>). These increasing demands
315 together with an increased competition for land with the corn ethanol industry, fuel a
316 constant drive for improved yields (Graham and Vance, 2000; Salvagiotti et al. (2008).

317

318 Table 1

319

320 6.2. *Bradyrhizobia* as inoculants

321 High yielding soybean plants require a lot of nitrogen and it is estimated that
322 BNF can cover 60 to 70% of the nitrogen requirement of the plant (Herridge et al., 2008).
323 Salvageotti et al. (2008) compared studies with a wide range of yield levels and
324 environments and concluded that on average, about 50 to 60% of nitrogen required by
325 soybean plants is provided by BNF. They point to a lack of data to assess the real
326 contribution of varying levels of belowground nitrogen and call for more research to
327 elucidate whether optimised BNF systems can sustain optimal yields with minimal input of
328 additional nitrogen.

329 The main rhizobial partners of soybean are the slow-growing bradyrhizobia and
330 particularly the species *B. japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1992) and
331 *B. liaoningense* (Xu et al., 1995). The latter species is very close to *B. japonicum* (Van
332 Berkum and Fuhrmann, 2000). In addition, the moderately fast-growing *Mesorhizobium*
333 *tianshanense* (Chen et al., 1995) and the fast-growing *Sinorhizobium (Ensifer) fredii* (Scholla
334 and Elkan, 1984) and *S. xinjiangense* (Chen et al., 1988) also nodulate soybean and can be as

335 effective as bradyrhizobia in suitable ecological conditions (Albareda et al., 2009). Possible
336 synergistic effects promoting soybean plant growth by bradyrhizobia with other bacteria
337 such as some strains of *Pseudomonas* (Chebotar et al., 2001), *Bacillus thuringiensis* (Mishra
338 et al., 2009) and *Azospirillum brasilense* (Cassan et al., 2009) have also been documented.

339 Because soils do not always contain a sufficiently large population of suitable
340 rhizobia, inoculation of soybean crops is a widespread practice. When a legume is
341 introduced into an area there is the opportunity to co-introduce rhizobia that are adapted to
342 that environment. Some important environmental constraints include acidity and salt
343 tolerance, drought and high temperature tolerance (Musiyiwa et al., 2005).

344 Commercial inoculant production is an important agricultural industry (Catroux
345 et al., 2001) and selected bradyrhizobial inocula with superior symbiotic capacities are
346 produced to improve production and grain yields. The success of such introduced strains to
347 establish a symbiotic relationship and persist in a field is dependant on local soil conditions
348 and the presence of competing indigenous rhizobial strains (Botha et al., 2004; Brockwell et
349 al., 1995; Sadowsky, 2000). This competition may comprise effective colonizers that are less
350 effective nitrogen fixers. Commercial inoculant strains may evolve quickly via kairos
351 evolution in the soil (Farooq and Vessey, 2009). Over time, genetic exchange may dilute the
352 beneficial capacities of introduced strains (Bottomley et al., 1994; López-García et al., 2002)
353 or introduced strains may disappear completely without repeated inoculation (Obaton et al.,
354 2002), though it has also been reported that strains are highly adaptable to new
355 environments (Alves et al., 2003; Andrade et al., 2002). There are even reports from Brazil of
356 re-isolated rhizobia that had become more competitive in their new environment while they
357 maintained their nitrogen fixation (Alves et al., 2003). Knowledge of the local soil community

358 is therefore indispensable to assess the potential benefits of an inoculation strategy (Catroux
359 et al., 2001; McInnes et al., 2004; Obaton et al., 2002).

360

361 6.3. Bradyrhizobium diversity

362 In the absence of local soybean nodulating bradyrhizobia, e.g. when newly
363 introducing the crop in a particular region, inoculation can be very effective (Botha et al.,
364 2004). However, a resident population of nodulating bradyrhizobia may significantly reduce
365 the chances of a successful competition by inoculant strains (Jansen van Rensburg and
366 Strijdom, 1985; Okogun and Saniya, 2003), with local conditions such as soil composition,
367 pH, temperature and land use also influencing the outcome (Botha et al., 2004; Obaton et
368 al., 2002; Rahmani et al., 2009; Sadowsky, 2000).

369 Studies characterizing the local rhizobial flora have resulted in the presence in
370 national as well as specialist culture collections of many rhizobial strains that are of local and
371 regional importance, but that are often not identified to species level. These strains
372 represent a major biodiversity resource and are very diverse. However, identifying them has
373 long been a serious challenge because of the slow growth of bradyrhizobia and their poor
374 performance in many standardized biochemical tests. As a consequence, the classification of
375 bradyrhizobia is less well developed than that of most other rhizobia; however, molecular
376 methods are now leading to more comprehensive characterizations (Rivas et al., 2009; Van
377 Berkum and Fuhrmann, 2009). Particularly among soybean symbionts, 17 different
378 serogroups were described by 1965 (Date and Decker, 1965), 12 of them belonging to *B.*
379 *japonicum* and five to *B. elkanii*. This diversity was confirmed by rRNA internally transcribed
380 spacer (ITS) sequence divergence (Van Berkum and Fuhrmann, 2000) and further analysis
381 with ITS data from *B. liaoningense* revealed a very close relationship of this species with *B.*

382 *japonicum* (Willems et al., 2001) with reticulate evolutionary patterns obscuring species
383 boundaries (Van Berkum and Fuhrmann, 2009). Molecular methods now allow a more
384 precise identification and characterization of strains and this will contribute to an improved
385 classification for *Bradyrhizobium* as well as to more successful design of inoculation
strategies and quality control of inoculant strains (McInnes et al., 2004; Rickli Binde et al., 2009).

387

388 7. Root nodule bacteria for forage legume growth promotion

389 7.1. Importance of forage legumes

390 Cow milk and cattle meat rank the first and third, respectively, of food and
391 agricultural commodities in the world (Table 2). Forage legumes are essential for an efficient
392 animal-based agriculture worldwide. They have been the foundation for dairy and meat
393 production for centuries as rich sources of protein, fibre, and energy (Graham and Vance,
394 2003). Besides providing high quality feed for livestock, they are a key component for
395 sustainability of crop-pasture rotations. Legumes offer the potential to enhance the
396 productivity as well as the sustainability of mixed, inter-cropped and rotational cropping
397 systems (Hardarson and Atkins, 2003). Their value lies greatly in their ability to fix nitrogen
398 (N_2) in symbiosis with soil bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*,
399 *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium* (Table1) (Graham, 2008; Graham and
400 Vance, 2003; Gualtieri and Bisseling, 2000; Vance, 1998).

401

402 Table 2

403

404 Biological N_2 fixation is second only to photosynthesis as the most important
405 biochemical process on earth. It can provide substantial amounts of N_2 to plants and soil,

406 which reduces the need for industrial fertilizers (Carlsson and Huss-Danell, 2003). Use of
407 legumes in pastures and for soil improvement dates back to the Romans, with Varro (37 BC;
408 cited by Fred et al., 1932) noting "Legumes should be planted in light soils, not so much for
409 their own crops as for the good they do to subsequent crops" (Graham and Vance, 2003).

410 Perennial forage legumes are usually more effective and derive higher
411 percentages of their N₂ from the atmosphere than most grain legume species (Hardarson
412 and Atlins, 2003). When grown in mixtures with grasses, these species take a large fraction
413 of their N₂ from the atmosphere, with average field measurements over 80% (Carlsson and
414 Huss-Danell, 2003). Reported rates of N₂ fixation in above-ground plant tissues are in the
415 range of up to 373 kg N ha⁻¹ year⁻¹ in red clover (*Trifolium pratense* L.), 545 kg N ha⁻¹ year⁻¹
416 in white clover (*T. repens* L.), 350 kg N ha⁻¹ year⁻¹ in alfalfa (*Medicago sativa* L.) and 138 kg N
417 ha⁻¹ year⁻¹ in birdsfoot trefoil (*Lotus corniculatus* L.) (Carlsson and Huss-Danell, 2003;
418 Gregerson et al., 1999; Vance et al., 1988).

419 In Uruguay, improved pastures integrated by forage legumes, are the primary
420 base of agriculture, dairy and livestock production (Rebuffo et al., 2006). Perennial cultivated
421 pastures cover an area of 1.400.000 has, while natural grasslands over sown with legumes
422 occupy approximately 650.000 ha (DIEA/MGAP, 2009). Total area sown to forage legumes
423 accomplishes over 2 million has, which represents 15% of agricultural land. Dairy farms
424 occupy an area of 750.000 ha, 50% of which is sown with legume-based pastures in mixture
425 with grasses (mainly alfalfa, birdsfoot trefoil and clovers) (DIEA/MGAP, 2009; Rebuffo et al.,
426 2006). Extensive cattle production is sustained on natural grasslands improved with over
427 sown exotic legumes (mainly *Lotus* spp., with *L. subbiflorus* representing 87.6%) DIEA/MGAP,
428 2002; Rebuffo et al., 2006).

429 The most remarkable feature in the utilization of forage legumes in Uruguay
430 has been the impact on the effective management of N in the environment through N supply
431 to natural and agro-ecosystems. Their use has largely reduced N fertilization requirements
432 while improving farmer profitability. Consequently, forage legume–rhizobia symbiosis does
433 have a significant effect on Uruguayan economy (Rebuffo et al., 2006). One ton of urea - the
434 most utilized N fertilizer - costs approximately \$420 and supplies 460 kg N. With average
435 estimates of N₂ fixation in forage legumes of about 230 kg N ha⁻¹ year⁻¹, \$210 are saved per
436 hectare (Montáñez et al. 2003). Thus, considering 2 million hectares sown with perennial
437 legumes, Uruguay accomplishes savings in the order of 420 million dollars per year through
438 reducing imports of N fertilizer (a figure close to 50 % of total annual meat exports).

439

440 *7.2. Rhizobia as inoculants for forage legumes: the Uruguayan system*

441 The perennial strategy of most temperate forage legumes like alfalfa
442 (*Medicago sativa* L.), trefoils (*Lotus* spp.) and clovers (*Trifolium* spp.), relies on the success of
443 stand establishment and early development of healthy root systems to achieve high dry
444 matter yields and optimal productivity. Microbial-based strategies that improve forage
445 legume productivity, optimize N₂ fixation, conserve soil N and augment the pool of soil N for
446 the benefit of rotational non-leguminous crop, have been exploited worldwide through
447 rhizobial inoculant technology (Brockwell and Bottomley, 1995; Carlsson and Huss-Danell,
448 2003; Catroux et al., 2001).

449 Although commercial rhizobial inoculant production and use is worldwide
450 extended, Catroux et al. (2001) concluded that their quality remains poor despite the
451 available technologies. These authors stated that legal requirements and controls can
452 improve the quality of inoculants and thus their efficacy. As observed in countries with

453 standards and government control, the trend is to increase the quality using sterile carriers
454 or liquid inoculants in order to avoid contaminants and to keep high numbers of rhizobia in
455 the packages for at least 1 year storage. Uruguay, together with Brazil, Canada and France,
456 has been recognized as one of the countries with regulatory authorities responsible for the
457 quality control services, supported by appropriate legislation (Brockwell and Bottomley,
458 1995; Lupawayi et al., 2000).

459 The Uruguayan system for biological N₂ fixation technology has been recently
460 described by Montáñez et al. (2003). Created in 1960, the key for its success has been the
461 implementation of a national government-supported strategy, based on a strong functional
462 relationship among public research, private industry and farmers. Regulatory authorities
463 (Ministry of Livestock, Agriculture & Fisheries) are supported by appropriate legislation on
464 inoculant registration, quality control and use. Main activities of the official Laboratory of
465 Soil Microbiology and Inoculant Control consist on: (i) characterization and selection of
466 rhizobial strains, (ii) preservation of the culture collection as the source of high quality
467 germplasm for research, (iii) strain supply to the industry and (iv) quality surveillance of
468 commercial inoculants. High quality standards are achieved using sterile peat carrier as well
469 as liquid formulations, with numbers of viable rhizobia in the packages mandatory high (2 x
470 10⁹ rhizobia /g peat), as pointed by Lupwayi et al. (2000). Today, four local manufacturers
471 share the inner market with four imported brands, while Uruguayan high-quality rhizobial
472 inoculants are exported to other South American countries. As a result of research and
473 extension policies, farmers have adopted the inoculation technology to a 100% extent.

474 As mentioned before, Uruguay bases its improvement of forage supply on the
475 temperate legumes alfalfa, clovers and trefoils. The main rhizobial partner of alfalfa is the
476 fast-growing species *Sinorhizobium (Ensifer) meliloti* (Vance et al., 1988); the fast-growing

477 species *Rhizobium leguminosarum* biovar *trifolii* nodulates *Trifolium* spp. (Gualtieri and
478 Bisseling, 2000), while the moderately fast-growing *Mesorhizobium loti* and slow growing
479 *Bradyrhizobium* sp. nodulate *Lotus* species (Díaz et al., 2005; Gregerson et al., 1999).
480 Selected strains with superior symbiotic capacities need to be isolated and developed as
481 inoculants (Hardarson and Atkins, 2003; Lupawyi et al., 2000). Periodical assessment of
482 commercial strains under field conditions is essential (Hardarson and Atkins, 2003) and the
483 need to monitor culture variability to maintain the quality of legume inoculants has been
484 also emphasized (Bloem et al., 2002). In Uruguay, *S. meliloti* strain U45 (isolated from alfalfa,
485 Uruguay) was formerly used for alfalfa commercial inoculant. However, variant cultures of
486 this strain exhibited a range of N₂-fixing effectiveness and competitiveness when inoculated
487 onto two alfalfa cultivars (Bloem et al., 2002). Therefore, it has been currently substituted by
488 U143 (MCH3, isolated from alfalfa, Uruguay). *M. loti* strain U226 is used for *Lotus*
489 *corniculatus*, while strain U531 (NC3, isolated from *L. subbiflorus*, Uruguay) is used for *L.*
490 *subbiflorus*.

491

492 7.3. Diversity of indigenous rhizobia nodulating alfalfa and *Lotus* spp.

493 The need for rhizobial strains with enhanced N₂ fixation and tolerance to
494 edaphic soil constraints (i.e. pH soil) has been repeatedly emphasized (Catroux et al., 2001 ;
495 Graham and Vance, 2003; Langer et al., 2008). Several authors studied the occurrence,
496 diversity and symbiotic properties of alfalfa-nodulating strains isolated from acid soils of
497 Uruguay and Argentina (Castro-Sowinski et al., 2002a; Del Papa et al., 1999; Segundo et al.,
498 1999). Mid-acid tolerant strains (MAT) able to grow at pH 5.5 but not at pH 5.0 and acid-
499 tolerant (AT) strains able to grow at pH 5.0 were characterized. Ten percent of the
500 indigenous *S. meliloti* population in Uruguayan soils was tolerant to acidic conditions and

501 PCR analysis of the strains suggested that considerable diversity is present. Symbiotic
502 analysis of the strains confirmed that they have the potential to improve the growth of
503 alfalfa in acidic soils (Castro-Sowinski et al., 2002a) and may be considered for inoculant
504 production (Segundo et al., 1999). Mid-acid tolerant strains have been also characterized for
505 laccase activity and melanin production. Interestingly, plant growth-promoting effect in rice
506 by a laccase-producing *S. meliloti* strain when co-inoculated with *Azospirillum brasilense* was
507 observed [Castro-Sowinski et al., 2002b).

508 Symbiotic effectiveness and ecological characterization of indigenous rhizobia
509 nodulating *Lotus* spp. has been extensively studied in Uruguay. Immunological, biochemical
510 and genetic properties were described for a large collection of strains (Baraibar et al., 1999;
511 Díaz et al., 1995; Irisarri et al., 1996; Monza et al., 1992; Monza et al., 1997; Monza et al.,
512 2006). Based on colony type and growth rates, isolates from nodules of *Lotus* spp. were
513 separated into two groups corresponding to slow- and fast-growing strains, the first group
514 nodulating *L. subbiflorus* (Irisarri et al., 1996) and the second *L. corniculatus* (Monza et al.,
515 1992; Monza et al., 1997), respectively. Partial 16S rDNA gene sequencing revealed that fast-
516 growing strains could be identified as *Mesorhizobium loti* species and the slow-growers as
517 *Bradyrhizobium* sp. (Monza et al., 2006).

518 Although specificity is not yet completely defined in the genus *Lotus*, local
519 studies have demonstrated its occurrence within indigenous population, with different level
520 of efficiency (Díaz et al., 2005). Irisarri et al. (1996) found that all of the isolates effectively
521 nodulating *L. subbiflorus*, were unable to form effective nodules on *L. corniculatus*. Similarly,
522 Baraibar et al. (1999) and Monza et al. (1992) found that all of the isolates effectively
523 nodulating *L. corniculatus* induced small and ineffective nodules in *L. subbiflorus*. Inability of
524 the inoculant strains to successfully compete with established rhizobia populations in soil

525 has been frequently reported and agronomical implications need to be considered (Díaz et
526 al., 2005). Results reported by Baraibar et al. (1999) also proved that 83% of the indigenous
527 rhizobia nodulating *Lotus* spp. were acid tolerant in culture medium (pH 4.5) and supported
528 the importance of selecting among them the most efficient and resistant strains to be
529 included in the inoculants. As an example, the overwhelming increase in the area of natural
530 grasslands over sown with *L. subbiflorus*, especially adapted to acid soils, has been largely
531 sustained in the selection of the indigenous strain U531 (NC3) for commercial inoculant.

532

533 7.4. Final remarks

534 Research and extension policies valorising rhizobial germplasm study and
535 preservation, farmers training for proper inoculant use and legal enforcement of commercial
536 inoculant quality have proved a successful approach to promote the use of forage legumes
537 while enhancing biological N₂ fixation at a national scale. Interdisciplinary and inter-
538 institutional approaches should be strengthened on a global scale to recognize the ecology
539 of forage legume microbes as a key tool for developing sustainable agricultural systems.

540

541 8. Perspectives

542 The rhizobium-legume symbiosis is important in many fields, not only in
543 agriculture for sustainable production, but also in basic biology as a model for symbiosis,
544 evolution and differentiation. With the global interest in biodiversity rhizobia have also
545 become both friends and foes of taxonomists, and a debate about the naming of species is
546 going on since a few years (Lindström and Young, 2009). The diverging interests (choice of
547 diverse model/type strains) of taxonomists, molecular biologists and agronomists has
548 hampered research into inoculant development, since molecular biologists study certain

549 model strains and species, taxonomist describe new species using other type and
550 representative strains whereas many good inoculant strains are poorly described from
551 molecular and taxonomic points of view. Intellectual property rights will in the future protect
552 also rhizobial biodiversity resources from unfair economical exploitation, but the
553 development might as well lead to increased protectionism which will further hamper free
554 distribution and movement of strains for scientific and agronomic purposes.

555 However, the examples presented in this article show that much effort is put
556 into increasing the use of BNF and thus sustainability in agriculture. It is our hope that the
557 information about the well-functioning Uruguayan system in combination with the extensive
558 local knowledge in Africa, and the knowledge of current taxonomy as well as filling existing
559 gaps in the knowledge of population genetics, rhizobial genomes and Chronos and Kairos
560 evolution, could help bring the diverse fields of rhizobium research together and prompt
561 further development of the rhizobial commons.

562

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566

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568

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818 FIGURE LEGENDS

819 Fig. 1. Signal exchange in rhizobium-plant symbiosis. Flavonoids produced by the host plant
820 induce rhizobial nod genes. This leads to production of Nod factors. The insert shows an
821 infection thread passing the root cortex toward a cluster of dividing cells that will become a
822 root promordium. (Reprinted from Schultze and Kondorosi 1998, with permission).

823

824 Fig.2. Evolution in bacteria: Chronos evolution is the evolution of the bacterial core genome
825 and mediates speciation. Kairos evolution is rapid evolution of the accessory genome and
826 mediates ecological niche adaptation, for example symbiotic adaptation. The filled circles
827 represent bacteria and the small symbols transfer of genetic elements between bacteria.

828 TABLES

829 Table 1. Soybean cultivation: area cultivated and quantities produced in

830 2007. Source: FAOSTAT database accessed at 24 August 2009

831 (<http://faostat.fao.org/site/567/default.aspx#ancor>)

832

	Area (k Ha)	Production (k Tonnes)
World	90.000	220.533
Africa	1.210	1.254
Europe	1.893	2.630
Asia	19.479	27.183
North America	27.131	75.556
South America	40.393	113.747
Oceania	13	32

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834

835 Table 2. Dairy and meat production: quantities produced in 2007, ranked by value among

836 the 20 most important food and agricultural commodities (value rank number in brackets).

837 Source: FAOSTAT database accessed at 30 September 2009

838 (<http://faostat.fao.org/site/339/default.aspx>)

839

	Cow milk (Tonnes)	Cattle Meat (Tonnes)
World	566850186 (1)	55491236 (3)
Africa	26578917 (4)	4879269 (1)
Europe	208615099 (1)	10721290 (3)
Asia	144423727 (5)	11957639 (10)
North America	92335414 (2)	12422756 (1)
South America	54413949 (4)	12431029 (1)
Oceania	25131511 (1)	680764 (4)
Uruguay	1576000 (2)	572748 (1)

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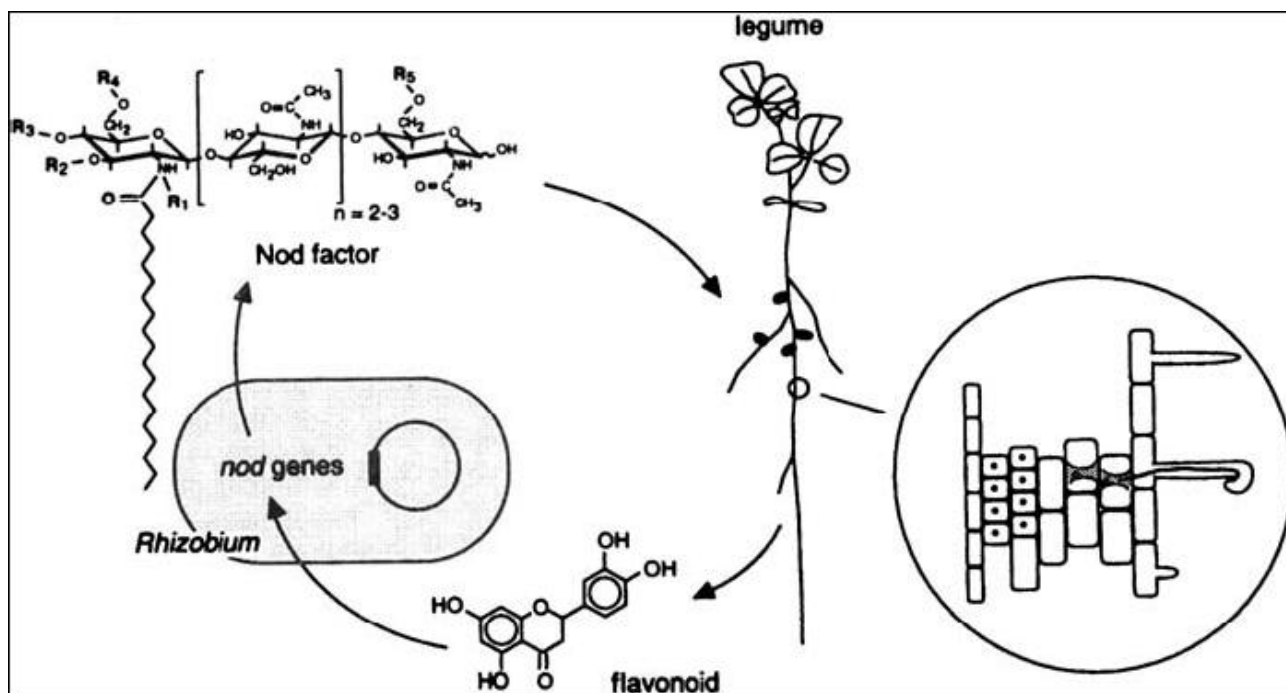
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844 FIGURES

845 Figure 1.

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850 Fig. 1. Signal exchange in rhizobium-plant symbiosis. Flavonoids produced by the host plant
 851 induce rhizobial nod genes. This leads to production of Nod factors. The insert shows an
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 853 root primordium. (Reprinted from Schultze and Kondorosi 1998, with permission).

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Supplementary Table 1: Species of rhizobia and the plants they nodulate

Name ¹	Host plants nodulated	References
Alphaproteobacteria		
<i>Azorhizobium caulinodans</i>	<i>Sesbania rostrata</i>	Dreyfus et al., 1988
<i>Azorhizobium doebereineriae</i>	<i>Sesbania virgata</i>	Moreira et al., 2006
<i>Bradyrhizobium betae</i>	Isolated from deformed roots of <i>Beta vulgaris</i> ; unable to reproduce root deformations on <i>Beta vulgaris</i> ; unable to nodulate <i>Glycine max</i> and <i>Pachyrrhizus ahipa</i>	Rivas et al., 2004
<i>Bradyrhizobium canariense</i> bv. <i>genistearum</i>	<i>Adenocarpus</i> spp., <i>Chamaecytisus proliferus</i> , <i>Spartocytis supranubius</i> , <i>Lupinus</i> spp., <i>Teline</i> spp., <i>Ornithopus</i> spp.	Vinuesa et al., 2005
<i>Bradyrhizobium elkanii</i>	<i>Glycine</i> spp., <i>Macroptilium atropurpureum</i> , <i>Vigna</i> spp.	Kuykendall, 2005
<i>Bradyrhizobium iriomotense</i>	Isolated from nodules of <i>Entada koshunensis</i> . Nodulates <i>Macroptilium atropurpureum</i>	Islam et al., 2010
<i>Bradyrhizobium japonicum</i> bv. <i>glycinearum</i>	<i>Glycine</i> spp., <i>Macroptilium atropurpureum</i>	Jordan, 1982; Vinuesa et al., 2005
<i>Bradyrhizobium japonicum</i> bv. <i>genistearum</i>	<i>Adenocarpus</i> spp., <i>Chamaecytisus proliferus</i> , <i>Spartocytis supranubius</i> , <i>Lupinus</i> spp., <i>Teline</i> spp., <i>Ornithopus</i> spp.	Vinuesa et al., 2005
<i>Bradyrhizobium japonicum</i> group I	<i>Crotalaria pallida</i> , <i>Trifolium repens</i> , <i>Trifolium fragiferum</i>	Liu et al., 2007
<i>Bradyrhizobium japonicum</i> group Ia	<i>Crotalaria pallida</i> , <i>Mimosa pudica</i> , <i>Trifolium fragiferum</i> , <i>Trifolium repens</i>	Liu et al., 2007
<i>Bradyrhizobium jicamae</i>	<i>Pachyrrhizus erosus</i> , <i>Lespedeza</i> spp.	Ramirez-Bahena et al., 2009
<i>Bradyrhizobium liaoningense</i>	<i>Glycine soja</i> , <i>Glycine max</i>	Xu et al., 1995
<i>Bradyrhizobium pachyrrhizi</i>	<i>Pachyrrhizus erosus</i>	Ramirez-Bahena et al., 2009
<i>Bradyrhizobium yuanmingense</i>	<i>Lespedeza cuneata</i> , <i>Vigna unguiculata</i> , <i>Glycyrrhiza uralensis</i> , <i>Phaseolus lunatus</i> , <i>Vigna mungo</i> , <i>Vigna radiata</i> , <i>Vigna unguiculata</i>	Appunu et al., 2009; Ormeño-Orrillo et al., 2006; Yao et al., 2002
<i>Devosia neptuniae</i>	<i>Neptunia natans</i>	Rivas et al., 2003
<i>Devosia yakushimensis</i>	Isolated from nodules of <i>Pueraria laobata</i> ; no nodulation reported	Bautista et al., 2010
<i>Ensifer adhaerens</i> (genomovar A)	<i>Sesbania grandiflora</i> , <i>Medicago sativa</i> , <i>Leucaena leucocephala</i> , <i>Pithecellobium dulce</i> , <i>Lotus arabicus</i>	Merabet et al., 2010; Willems et al., 2003

Name¹	Host plants nodulated	References
<i>Ensifer arboris</i>	<i>Acacia senegal</i> , <i>Prosopis chilensis</i>	Nick et al., 1999
<i>Ensifer fredii</i>	<i>Glycine max</i> , <i>Glycine soja</i> , <i>Vigna unguiculata</i> , <i>Cajanus cajan</i>	Chen et al., 1988
<i>Ensifer garamanticus</i>	<i>Agyrolobium uniflorum</i> , <i>Medicago sativa</i>	Merabet et al., 2010
<i>Ensifer kostiense</i>	<i>Acacia senegal</i> , <i>Prosopis chilensis</i>	Nick et al., 1999
<i>Ensifer kummerowiae</i>	<i>Kummerowia stipulacea</i>	Wei et al., 2002
<i>Ensifer medicae</i>	<i>Medicago trunculata</i> , <i>Medicago</i> spp.	Rome et al., 1996
<i>Ensifer meliloti</i>	<i>Medicago</i> spp., <i>Melilotus</i> spp., <i>Trigonella</i>	Dangeard 1926; Rome et al., 1996; Yan et al., 2000
“ <i>Ensifer mexicanus</i> ”	<i>Acacia angustissima</i> , <i>Acacia cochliacantha</i> , <i>Leucena leucocephala</i> , <i>Phaseolus vulgaris</i>	Lloret et al., 2007
<i>Ensifer morelense</i>	<i>Leucaena leucocephala</i>	Wang et al., 2002
<i>Ensifer numidicus</i>	<i>Agyrolobium uniflorum</i> , <i>Lotus creticus</i>	Merabet et al., 2010
<i>Ensifer saheli</i>	<i>Sesbania</i> spp.	de Lajudie et al., 1994
<i>Ensifer terangae</i>	<i>Sesbania</i> spp., <i>Acacia</i> spp.	de Lajudie et al., 1994
<i>Ensifer terangae</i> bv. <i>sesbaniae</i>	<i>Sesbania</i> spp.	Lortet et al., 1996
<i>Ensifer terangae</i> bv. <i>acaciae</i>	<i>Acacia</i> spp.	Lortet et al., 1996
<i>Ensifer xinjiangense</i>	<i>Glycine max</i>	Chen et al., 1988
<i>Mesorhizobium albiziae</i>	<i>Albizia kalkora</i> , <i>Albizia julibrissin</i> , <i>Glycine max</i> , <i>Leucaena leucocephala</i> , <i>Phaseolus vulgaris</i>	Wang et al., 2007
<i>Mesorhizobium alhagi</i>	<i>Alhagi sparsifolia</i> , <i>Sophora alopecuroides</i> , <i>Glycyrrhiza inflata</i> , <i>Medicago sativa</i> , <i>Indigofera endecaphylla</i> , <i>Vicia cracca</i> , <i>Sophora flavescens</i>	Chen et al., 2010
<i>Mesorhizobium amorphae</i>	<i>Amorpha fruticosa</i> , <i>Cicer arietinum</i>	Rivas et al., 2007; Wang et al., 1999b
<i>Mesorhizobium australicum</i>	<i>Biserrula pelecinus</i> , <i>Astragalus membranaceus</i> , <i>Macroptilium atropurpureum</i>	Nandasena et al., 2009
<i>Mesorhizobium camelthorni</i>	<i>Alhagi sparsifolia</i> , <i>Sophora alopecuroides</i> , <i>Glycyrrhiza inflata</i> , <i>Medicago sativa</i>	Chen et al., in press.
<i>Mesorhizobium caraganae</i>	<i>Caragana microphylla</i> , <i>Caragana intermedia</i>	Guan et al., 2008
<i>Mesorhizobium chacoense</i>	<i>Prosopis alba</i>	Velázquez et al., 2001

Name¹	Host plants nodulated	References
<i>Mesorhizobium ciceri</i>	<i>Cicer arietinum</i>	Nour et al., 1994
<i>Mesorhizobium gobiense</i>	<i>Astragalus filicaulis</i> , <i>Lotus frondosus</i> , <i>Lotus tenuis</i> , <i>Oxytropis glabra</i>	Han et al., 2008a
<i>Mesorhizobium huakuii</i>	<i>Astragalus sinicus</i> , <i>Robinia pseudoacacia</i>	Chen et al. 1991; Ulrich and Zaspel, 2000
<i>Mesorhizobium loti</i>	<i>Lotus</i> spp., <i>Anthyllis vulneraria</i> , <i>Lupinus densiflorus</i> , <i>Robinia pseudoacacia</i>	Jarvis et al., 1982, Ulrich and Zaspel, 2000
<i>Mesorhizobium mediterraneum</i>	<i>Cicer arietinum</i>	Nour et al., 1995
<i>Mesorhizobium metallidurans</i>	<i>Anthyllis vulneraria</i>	Vidal et al., 2009
<i>Mesorhizobium opportunistum</i>	<i>Biserrula pelecinus</i> , <i>Astragalus adsurgens</i> , <i>Astragalus membranaceus</i> , <i>Lotus peregrinus</i> , <i>Macroptilium atropurpureum</i>	Nandasena et al., 2009
<i>Mesorhizobium plurifarum</i>	<i>Acacia</i> spp., <i>Leucena</i> spp., <i>Prosopis juliflora</i> , <i>Chamaecrista ensiformis</i>	de Lajudie et al., 1998b
<i>Mesorhizobium robiniae</i>	<i>Robinia pseudoacacia</i>	Zhou et al., in press.
<i>Mesorhizobium septentrionale</i>	<i>Astragalus adsurgens</i> , <i>Phaseolus vulgaris</i> , <i>Glycine max</i> , <i>Leucaena leucocephala</i> , <i>Macroptilium atropurpureum</i> , <i>Lotus corniculatus</i> , <i>Robinia pseudoacacia</i>	Gao et al., 2004; Han et al., 2008a
<i>Mesorhizobium shangrilense</i>	<i>Caragana</i> spp., <i>Glycyrrhiza uralensis</i> , <i>Astragalus adsurgens</i> , <i>Vigna unguiculata</i> , <i>Vigna radiata</i> , <i>Phaseolus vulgaris</i>	Lu et al., 2009b
<i>Mesorhizobium tarimense</i>	<i>Lotus frondosus</i> , <i>Lotus tenuis</i>	Han et al., 2008a
<i>Mesorhizobium temperatum</i>	<i>Astragalus adsurgens</i> , <i>Phaseolus vulgaris</i> , <i>Vigna unguiculata</i> , <i>Glycine max</i> , <i>Leucaena leucocephala</i> , <i>Medicago sativa</i> , <i>Lotus corniculatus</i>	Gao et al., 2004
<i>Mesorhizobium thiogangeticum</i>	Isolated from the rhizosphere of <i>Clitoria ternatea</i> although it did not nodulate this host	Ghosh and Roy, 2006
<i>Mesorhizobium tianshanense</i>	<i>Glycyrrhiza</i> , <i>Sophora</i> , <i>Caragana</i> , <i>Halimodendron</i> , <i>Swainsonia</i> , <i>Glycine</i> , <i>Cicer arietinum</i>	Chen et al., 1995; Rivas et al., 2007
<i>Methylobacterium nodulans</i>	<i>Crotalaria</i> spp.	Jourand et al., 2004
<i>Ochrobactrum cytisi</i>	<i>Cytisus scoparius</i>	Zurdo-Pineiro et al., 2007

Name¹	Host plants nodulated	References
<i>Ochrobactrum lupini</i>	<i>Lupinus albus</i> , <i>Lupinus honoratus</i>	Trujillo et al., 2005
<i>Phyllobacterium trifolii</i>	<i>Trifolium pratense</i> , <i>Trifolium repens</i> , <i>Lupinus albus</i>	Valverde et al., 2005
<i>Phyllobacterium ifriqiyense</i>	Isolated from root nodules of <i>Lathyrus numidicus</i> and <i>Astragalus algerianus</i> ; no nodulation data	Mantelin et al., 2006
<i>Phyllobacterium leguminum</i>	Isolated from root nodules of <i>Astragalus algerianus</i> , <i>Agyrolobium uniflorum</i> ; no nodulation data	Mantelin et al., 2006
<i>Rhizobium alamii</i>	<i>Medicago ruthenica</i> (most strains isolated from <i>Arabidopsis</i> rhizosphere)	Berghe et al., 2009
<i>Rhizobium alkalisoli</i>	<i>Caragana intermedia</i> , <i>Caragana microphylla</i> , <i>Phaseolus vulgaris</i> , <i>Vigna radiata</i>	Lu et al., 2009a
<i>Rhizobium cellulosityticum</i>	Isolated from poplar saw dust. One strain forms ineffective nodules on <i>Medicago sativa</i>	Garcia-Fraile et al., 2007
<i>Rhizobium daejeonense</i>	Isolated from a cyanide treatment bioreactor; nodulates <i>Medicago sativa</i>	Quan et al., 2005
<i>R. etli</i> bv. <i>mimosae</i>	<i>Phaseolus vulgaris</i> , <i>Mimosa affinis</i> , <i>Leucaena leucocephala</i>	Wang et al., 1999a
<i>R. etli</i> bv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>Phaseolus coccineus</i>	Segovia et al., 1993; Souza et al., 1994
<i>Rhizobium fabae</i>	<i>Vicia faba</i>	Tian et al., 2008
<i>Rhizobium galegae</i>	<i>Galega officinalis</i> , <i>Galega orientalis</i>	Lindström, 1989
<i>R. galegae</i> bv. <i>officinalis</i>	<i>Galega officinalis</i>	Radeva et al., 2001
<i>R. galegae</i> bv. <i>orientalis</i>	<i>Galega orientalis</i>	Radeva et al., 2001
<i>R. gallicum</i> bv. <i>gallicum</i>	<i>Phaseolus vulgaris</i> , <i>Phaseolus coccineus</i> , <i>Macroptilium atropurpureum</i> , <i>Onobrychis viciifolia</i> , <i>Leucaena leucocephala</i>	Amarger et al., 1997
<i>R. gallicum</i> bv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>Macroptilium atropurpureum</i>	Amarger et al., 1997
<i>R. giardinii</i> bv. <i>giardinii</i>	<i>Phaseolus vulgaris</i> , <i>Macroptilium atropurpureum</i> , <i>Leucaena leucocephala</i>	Amarger et al., 1997
<i>R. giardinii</i> bv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>Macroptilium atropurpureum</i>	Amarger et al., 1997
<i>R. giardinii</i>	<i>Desmanthus illinoensis</i> , <i>Desmanthus leptolobus</i> , <i>Dalea purpurea</i> , <i>Psoralea esculenta</i> , <i>Prosopis juliflora</i>	Beyhaut et al., 2006

Name ¹	Host plants nodulated	References
<i>Rhizobium hainanense</i>	<i>Desmodium</i> spp., <i>Centrosema pubescens</i> , <i>Stylosanthes guyanensis</i> , <i>Tephrosia candida</i> , <i>Acacia sinuate</i> , <i>Zornia diphylla</i> , <i>Macroptilium lathyroides</i>	Chen et al., 1997
<i>Rhizobium huautlense</i>	<i>Sesbania herbacea</i> , <i>Leucaena leucocephala</i>	Wang et al., 1998
<i>Rhizobium indigoferae</i>	<i>Indigofera</i> spp.	Wei et al., 2002
<i>Rhizobium (Agrobacterium) larrymoorei</i>	Isolated from aerial tumours of <i>Ficus benjamina</i> . No nodulation data	Bouzar and Jones, 2001
<i>Rhizobium leguminosarum</i>	<i>Pisum</i> spp., <i>Lathyrus</i> spp., <i>Vicia</i> spp., <i>Lens</i> spp., <i>Phaseolus</i> spp., <i>Trifolium</i> spp., <i>Robinia pseudoacacia</i> , <i>Carmichaelia australis</i> , <i>Clanthus puniceus</i> , <i>Sophora chathamica</i>	Frank 1889; Ramirez-Bahena et al., 2008; Ulrich and Zaspel, 2000; Weir et al., 2004;
<i>Rhizobium loessense</i>	<i>Astragalus</i> spp.	Wei et al., 2003
<i>Rhizobium lusitanum</i>	<i>Phaseolus vulgaris</i> , <i>Macroptilium atropurpureum</i> , <i>Leucaena leucocephala</i>	Valverde et al., 2006
<i>Rhizobium mesosinicum</i>	<i>Albizia julibrissin</i> , <i>Kummerowia stipulacea</i> , <i>Kummerowia striata</i> , <i>Dalbergia</i> spp.	Lin et al., 2009
<i>Rhizobium miluonense</i>	<i>Lespedeza</i> spp.	Gu et al., 2008
<i>Rhizobium mongolense</i> ²	<i>Medicago ruthenica</i> , <i>Phaseolus vulgaris</i>	van Berkum et al., 1998
<i>Rhizobium multihospitium</i>	<i>Lotus</i> spp., <i>Alhagi toum</i> , <i>Astragalus</i> spp., <i>Halimodendron halodendron</i> , <i>Oxytropis</i> spp., <i>Robinia pseudoacacia</i> , <i>Sophora pecuriodes</i> , <i>Caragana jubata</i> , <i>Lathyrus odoratus</i> , <i>Vicia hirsuta</i>	Han et al., 2008b
<i>Rhizobium oryzae</i>	Isolated from surface-sterilized roots of <i>Oryza alta</i> . Strains nodulate <i>Phaseolus vulgaris</i> and <i>Glycine max</i>	Peng et al., 2008
<i>Rhizobium phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>Trifolium repens</i>	Ramirez-Bahena et al., 2008
<i>Rhizobium pisi</i>	<i>Pisum sativum</i> , <i>Trifolium repens</i> , <i>Phaseolus vulgaris</i>	Ramirez-Bahena et al., 2008
<i>Rhizobium (Agrobacterium) radiobacter</i>	Pathogenic strains on various plants as well as non-pathogenic strains. No nodulation reported ³	Young et al., 2005
<i>Rhizobium (Agrobacterium) rhizogenes</i>	Pathogenic strains on various plants as well as non-pathogenic strains. No nodulation reported ³	Young et al., 2005

Name ¹	Host plants nodulated	References
<i>Rhizobium (Agrobacterium) rubi</i>	Pathogenic on <i>Rubus</i> spp. No nodulation data	Young et al. 2005
<i>Rhizobium selenitireducens</i>	no data	Hunter et al., 2007
<i>Rhizobium soli</i>	Isolated from soil, no plant hosts reported	Yoon et al., in press.
<i>Rhizobium sullae</i>	<i>Hedysarum coronarium</i>	Squartini et al., 2002
<i>Rhizobium tibeticum</i>	<i>Trigonella archiducis-nicolai</i> , <i>Medicago lupulina</i> , <i>Medicago sativa</i> , <i>Melilotus officinalis</i> , <i>Phaseolus vulgaris</i> , <i>Trigonella foenum-graecum</i>	Hou et al., 2009
<i>Rhizobium tropici</i>	<i>Phaseolus vulgaris</i> , <i>Leucaena</i> spp., <i>Robinia pseudoacacia</i> , <i>Gliricidia sepium</i>	Acosta-Durán et al., 2002; Martínez-Romero et al., 1991; Ulrich and Zaspel, 2000
<i>Rhizobium tubonense</i>	Isolated from <i>Oxytropis glabra</i> but unable to renodulate this host. Effective nodules on <i>Vigna unguiculata</i> and <i>Medicago sativa</i>	Zhang et al., in press.
<i>Rhizobium (Allorhizobium) undicola</i>	<i>Neptunia natans</i>	de Lajudie et al., 1998a
<i>Rhizobium vignae</i>	<i>Astragalus dahuricus</i> , <i>A. oxyglottis</i> , <i>Vigna radiata</i> , <i>Desmodium microphyllum</i>	Ren et al., in press.
<i>Rhizobium (Agrobacterium) vitis</i>	Non-pathogenic or pathogenic on <i>Vitis</i> spp. or other dicotyledonous plants. No nodulation data	Young et al., 2005
<i>Rhizobium yanglingense</i> ²	<i>Gueldenstaedtia multiflora</i> , <i>Coronilla varia</i> , <i>Amphicarpaea trisperma</i>	Tan et al., 2001
<i>Shinella kummerowiae</i>	Isolated from the root nodules of <i>Kummerowia stipulacea</i> , but unable to renodulate this host	Lin et al., 2008
“ <i>Sinorhizobium abri</i> ” ⁴	<i>Abrus precatorius</i>	Ogasawara et al., 2003
<i>Sinorhizobium americanum</i> ⁴	<i>Acacia</i> spp.	Toledo et al., 2003
“ <i>Sinorhizobium indiaense</i> ” ⁴	<i>Sesbania rostrata</i>	Ogasawara et al., 2003
Betaproteobacteria		
<i>Burkholderia caribensis</i>	<i>Mimosa pudica</i> , <i>Mimosa diplotricha</i>	Chen et al., 2003; Vandamme et al., 2002
<i>Burkholderia dolosa</i>	One strain isolated from <i>Alysicarpus glumaceus</i>	Vandamme et al., 2002; Vermis et al., 2004
<i>Burkholderia mimosarum</i>	<i>Mimosa pigra</i> , <i>Mimosa scabrella</i>	Chen et al., 2006
<i>Burkholderia nodosa</i>	<i>Mimosa bimucronata</i> , <i>Mimosa scabrella</i>	Chen et al., 2007
<i>Burkholderia phymatum</i>	<i>Machaerium lunatum</i> , <i>Mimosa invisa</i>	Elliott et al., 2007b; Vandamme et al., 2002
<i>Burkholderia sabiae</i>	<i>Mimosa caesalpinifolia</i>	Chen et al., 2008

Name ¹	Host plants nodulated	References
<i>Burkholderia tuberum</i>	<i>Aspalathus carnosa</i> , <i>Cyclopia</i> spp., <i>Macroptilium atropurpureus</i>	Elliott et al., 2007a; Vandamme et al., 2002
<i>Cupriavidus taiwanensis</i>	<i>Mimosa pudica</i> , <i>Mimosa diplotricha</i> , <i>Mimosa pigra</i>	Chen et al., 2001; Chen et al., 2003; Chen et al., 2005
<i>Herbaspirillum lusitanum</i>	<i>Phaseolus vulgaris</i>	Valverde et al., 2003

¹ Names in quotation marks have been published outside Int. J. Syst. Evol. Microbiol. and have not yet been validated.

² Silva et al. (2005) proposed that *R. mongolense* and *R. yanglingense* be considered as a biovar (bv. orientale) of *R. gallicum*.

³ Strains formerly classified as agrobacteria are sometimes present in nodules, but have not been shown to induce nodulation on their own unless a symbiotic plasmid is present (Velazquez et al., 2005; Wang et al., 2006).

⁴ This species belongs to the *Ensifer* phylogenetic cluster but was described before the transfer of *Sinorhizobium* to *Ensifer* (Young et al., 2003).

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