PHENOTYPIC AND BIOCHEMICAL CHARACTERIZATION OF *BRADYRHIZOBIUM* AND *ENSIFER* SPP. ISOLATED FROM SOYBEAN RHIZOSPHERE

[1]Harpreet Kaur, Poonam Sharma, [1]Navprabhjot Kaur and B S Gill

[1]Department of Microbiology
Department of Plant breeding and Genetics
Punjab Agricultural University, Ludhiana 141004
poonam1963in@yahoo.co.in

ABSTRACT

A total of 15 rhizobia were isolated from soybean rhizosphere on yeast extract mannitol agar (YEMA) medium. The isolates were further subjected to morphological, cultural and biochemical characterization along with two reference culture (DS-1, National check) and SB271(Local check. Out of 15 isolates, 10 were selected as rhizobia on ketolactose medium (circular, light pink colonies), further 6 (LSER4,LSER5,LSER6,LSER7,LSER8,LSER9) were selected as fast and 4 (LSBR1, LSBR2, LSBR3, LSBR10) as slow growing isolates based on bromothymol blue (BTB) test. Fast and slow growing rhizobia were found to be positive for oxidase, urease, citrate utilization and catalase whereas negative reactions for methyl red and voges-proskauer test. The optimum physical condition for growth of fast and slow growing rhizobia was 28˚C at neutral pH (7.0). Four bradyrhizobial spp. and one *Ensifer* spp. showed hydrogen uptake (Hup^+) positive system with 0.01% TTC dye.

Keywords: *Bradyrhizobium,* Biochemical Characterization*,* Cultural diversity, *Ensifer,* Soybean

INTRODUCTION

Soybean (*Glycine max* L.) has been introduced in central part of India as pulse and oil seed crop. In Punjab, it is gaining importance as a diversifying crop in cereal dominating cropping system. It occupies an area of 930 million hectares with an annual production of 10.47 million tons and with an average yield of 1.2 tons/ha in India (Anonymous, 2010). In Punjab, it is particularly important, where grain legumes like soybean are being introduced to new land; limitations are arising for effective symbiotic nitrogen fixation (SNF) due to non-availability of suitable and compatible inoculants. SNF is a result of intimate mutualistic relationship between the root nodule bacteria and the host plant. Soybean can be nodulated by different rhizobia distributed in six species belonging to three different genera (Albareda *et al.,* 2009). Most of these bacterial species are in the Rhizobiacae family in the alpha-proteobacteria and are in either the *Rhizobium, Mesorhizobium, Ensifer*, or *Bradyrhizobium* genera (Weir, 2011). Despite considerable ability to fix atmospheric nitrogen, *Rhizobium* inoculation not always results in yield enhancement. Lack of response to inoculation can be attributed to intrinsic characteristics of both the host plant and bacteria as well as the sensitivity of symbiotic system due to environmental stresses such as temperature, dryness and low fertility of soil (Keyser and Li, 1992).Recently several workers have attempted (Annapurna *et al.,* 2007; Appunu *et al.,* 2008; Dhami and Prasad, 2009) the role of rhizobial inoculation on improvement in growth and yield of soybean.

Considering the above fact, an effort has been made to exploit the native rhizobial diversity for morphological, cultural and biochemical characterics for *Ensifer* and *Bradyrhizobium* species to be used as effective microbial inoculant in soybean.

MATERIALS AND METHODS

1) Procurement of Reference Culture

Two recommended cultures of *Bradyrhizobium* spp. DS-1 and SB-271 were procured from Indian Agricultural Research Institute (IARI), New Delhi and Department of Microbiology, Punjab Agricultural University, Ludhiana, respectively.

2) Collection of samples

Rhizospheric soil (1 kg) and five plant samples were collected from different parts of soybean growing areas of India (Table 1). Fifteen bold, pink, active nodules were collected randomly and placed in screw cap vials (10 ml) containing calcium carbonate as dessicant below cotton at the bottom and stored in refrigerator until isolation.

3) Isolation of and *Bradyrhizobium* and *Ensifer* spp. of soybean

a) Isolation from Rhizospheric soil samples of soybean

10 gm of soil sample from soybean rhizosphere were shaken in 90 ml sterilized distilled water for 10 minutes and serial dilutions were made in sterile water blanks. Pour plating was done on CRYEMA medium and plates were incubated at 28±2°C for 2-10 days.

b) Isolation of *Bradyrhizobium* and *Ensifer* spp. from soybean nodules

Isolation of rhizobia from root nodules was done by the method of Somasegaran and Hoben (1985). From each sample, two-three nodules were picked up and washed thoroughly with sterile distilled water. After washing, nodules were surface sterilized in 95 % alcohol for 30–40 seconds to remove wax coating if any and subsequently immerged in 4 % sodium hypochlorite for 3–4 minutes. Then nodules were immediately washed 5–6 times with sterile distilled water to remove traces of sodium hypochlorite. The surface-sterilized nodules were transferred to sterile tubes containing 100 µl sterile distilled water. Nodules were crushed with the help of sterile glass rod and were streaked with one loopful of milky suspension on CRYEMA medium containing 25 µg/ml Congo red and were incubated at 28±2°C in dark until growth appeared. Single unique colonies were picked up and were re-streaked on CRYEMA medium until pure culture was obtained.

4) Purification of *Bradyrhizobium* and *Ensifer spp*. of soybean

a) Growth on Congo red Medium

Rhizobium colonies appeared white, translucent, gummy, glistening, elevated and comparatively small with entire margins were selected in contrast to stained colonies of *Agrobacterium* on congo red medium which were red in color.

b) Gram staining

Gram staining was done to ensure purity and freedom from gram +ve bacteria. Gram-staining reaction was carried out by using a loopful of pure culture grown on YEM broth (yeast extract mannitol broth) and stained as per the standard Gram's procedure (Somasegaran and Hoben 1994).

c) Ketolactose test (Bernaerts and Deley 1963)

The principle of this test is based on the ability of *Agrobacterium*, a common contaminant of *Rhizobium* to produce ketolactase enzyme which converts lactose to ketolactose. Ketolactose would be detected by Benedict's reagent. *Rhizobium* cultures were streaked on lactose medium in the centre. After incubation for 4 days at 28±2°C, 5 ml of Benedict's reagent was poured in each Petriplate and kept at room temperature for 1-1½ hours. *Agrobacterium* growth was surrounded by yellowish zone of Cu_2O, whereas no such yellow zone was observed around the growth of rhizobia.

5) Morphological studies

The thick bacterial smear of all the isolates was gram stained and morphological characterization was done on the basis of colony morphology including shape,color and surface margin.

6) Differentiation between *Bradyrhizobium* and *Ensifer* spp. of soybean

Bromothymol blue (BTB) agar medium was used for differentiating *Bradyrhizobium* from *Ensifer* spp. of soybean.The cultures were streaked on BTB agar plates. BTB agar was made by adding 5 ml of (0.5% BTB in ethanol) to 1 litre of YEMA medium. The plates were incubated at 28±2ºC for 2-10 days. The change in color of medium was observed. The isolates were classified as slow growers (medium turns blue) or fast growers (medium turns yellow) on their reaction on YEMA supplemented with BTB (Somasegaran and Hoben 1994).

Purified colonies were then transferred to YEMA medium slants. These slants were incubated at 28±2°C for 2-10 days and stored in refrigerator at 4°C for further studies.

7) Biochemical studies

Biochemical characterization was done on the basis of oxidase, catalase, citrate utilization, methyl red (MR), voges-proskauer (VP), urease and nitrate reduction test as per standard procedure (Cappuccino and Sherman 1992).

8) Growth Kinetics

Growth kinetics was done by examining the effect of temperature and pH on isolates.

a) Effect of pH

Tolerance to pH extremes was determined by inoculating 10^8 log cells per ml from exponentially growing YEM liquid cultures into flasks containing 50 ml portions of YEMB. pH of the medium was adjusted to 4.0, 7.0 and 9.0 by using 1 N HCl or NaOH.

The tubes were incubated at 30°C for 14 days and scored for growth. Test was performed in triplicate.

b) Effect of temperature

Cultures were grown on slants containing YEMA medium at three different temperatures: 10°C, 28°C and 42°C and kept for 3-5 days. They were examined for presence or absence of growth.

9) Screening of *Bradyrhizobium/Ensifer* sp. for Hydrogenase uptake system (Hup).

Hydrogenase uptake expression of *Bradyrhizobium/Ensifer* spp. was done on defined medium of Maier *et al.,* (1978) and YEMA medium for the expression of hydrogenase in free living rhizobia with Triphenyl Tetrazolium Chloride (TTC) dye (0.01% W/V). The plates were incubated at 28±2°C for 2 days. Production of TTC dye was examined up to 10 days. *Rhizobium* cultures possessing Hup^+ system showed red coloration; whereas Hup^- strains were unable to reduce TTC, showed no red coloration and remained as colorless.

RESULTS AND DISCUSSION

Fifteen rhizobia were isolated from different soil and nodule samples of soybean rhizosphere. Phenotypically isolated (Table 2) rhizobial colonies were cream colored with slime/mucoid transparent appearance on CRYEMA plates with marked distinction from red colored colonies of *Agrobacterium.*Further conformity of rhizobia was performed on ketolactose agar showed, 10 isolates were negative for the production of 3 ketolactose from lactose and remaining 5 isolates belonged to *Agrobacterium* due to yellow zone of Cu_2O around the colonies. Production of 3-ketolactose from lactose is limited to species of *Agrobacterium* (Fig 1). Similarly, Gachande and Khansole (2011) isolated *Rhizobium japonicum* and *Bradyrhizobium japonicum* colonies, which were circular in shape with whitish pink color on CRYEMA medium. Absence of 3 ketolactose in rhizobial colonies on CRYEMA was also in agreement with earlier work carried out by Sadowasky *et al.,* (1983) and Sharma *et al.,* (2010) in soybean rhizobia.Further purified isolates were classified as fast (turn medium yellow) and slow growing (turn medium blue) rhizobia on YEMA supplemented with BTB (Fig 2).

The rhizobial isolates in the current study were further tested on YEMA plates containing BTB indicated that fast growing isolates (six) were found to produce yellow colonies due to acid production on the medium with high amount of mucus after 2 days of incubation (Table 3). Whereas, remaining four isolates along with reference strains DS 1 and SB 271 produced blue color colonies, which indicated the presence of alkali producers, considered as slow growing rhizobia. The use of YMA-BTB medium for categorizing indigenous soybean root nodulating fast and slow growing rhizobia based on acid/alkali production was also carried out by Saeki *et al.,* (2005) in Vietnam and Sharma *et al.,* (2010) in India. On BTB agar plates both fast and slow growing rhizobia formed circular, convex, colonies. The isolates were classified tentatively as fast (medium turn yellow) and slow growers (medium turn blue) based on their reaction on the yeast extract mannitol agar supplemented with bromothymol blue (Somasegran and Hoben 1994). The conformity on YEM agar plates containing bromthymol blue showed that six cultures produced yellow color due to acid production and were classified as fast growers (*Ensifer*) and six cultures (four isolates and two reference cultures) produced blue color due to alkali production and were found to be slow growers (*Bradyrhizobium*). These isolates were similar in terms of reaction on the YEMA (BTB) when compared with reference strains which produced yellow and blue color in fast and slow growing strains, respectively according to Hungria *et al.,* (2001). Out of six fast growers, four *Ensifer* species (LSER 6, LSER 7, LSER 8, LSER 9) and one *Bradyrhizobium* spp. (LSBR 3) produced gum. Our results indicated that 66% *Ensifer* spp. produced gum which is in close agreement with Baoling *et al.,* (2007).Both the fast and slow growing rhizobia were found to be positive for oxidase, urease, citrate utilization and catalase activity (Table 4).

Our findings congruence with Gachande and Khansole (2011) as reported for soybean rhizobia. Sadowasky *et al.,* (1983) reported that production of 3 ketolactose from lactose is limited to species of *Agrobacterium*, a genus that is closely related to *Rhizobium.* Nitrate is reduced to nitrite producing ammonia. Gachande and Khansole (2011) suggested rapid nitrate utilization by slow growing root nodule bacteria. Mahana *et al.,* (2000) also reported catalase activity in some isolates of *Rhizobium* from *Vigna mung*. It showed negative chemical reaction for methyl red, voges-proskauer and 3 ketolactose productions.

Table 1: Location of rhizospheric soil and root nodule samples

Sample number	Location of Soybean Rhizospheric soil
1	Bangalore
2	Pune
3	Indore
4	Pantnagar
5	Dharwad
Soybean plant sample with nodules (Punjab)	
6	P. A. U farm I
7	P. A. U farm II
8	P. A. U farm III
9	Ladhowal I
10	Ladhowal II
11	Ladhowal III
12	Ladhowal IV
13	Ladhowal V
14	Kharar
15	Roopnagar

Table 2: Morphological characters of soybean rhizobia

Character	Result
Shape	Circular
Color	Creamy white translucent, slime
Surface margin	Regular/Entire
Gram's nature	Gram –ve

Table 3: Differentiation of fast (*Ensifer* spp.) and slow grower (*Bradyrhizobium* spp.) soybean rhizobia on YEMA (BTB) medium

Isolates	Color produced on BTB agar	Fast/Slow grower
LSBR 1	Blue	Slow
LSBR 2	Blue	Slow
LSBR 3	Blue	Slow
LSER 4	Yellow	Fast
LSER 5	Yellow	Fast
LSER 6	Yellow	Fast
LSER 7	Yellow	Fast
LSER 8	Yellow	Fast
LSER 9	Yellow	Fast
LSBR 10	Blue	Slow
DS 1	Blue	Slow
SB 271	Blue	Slow

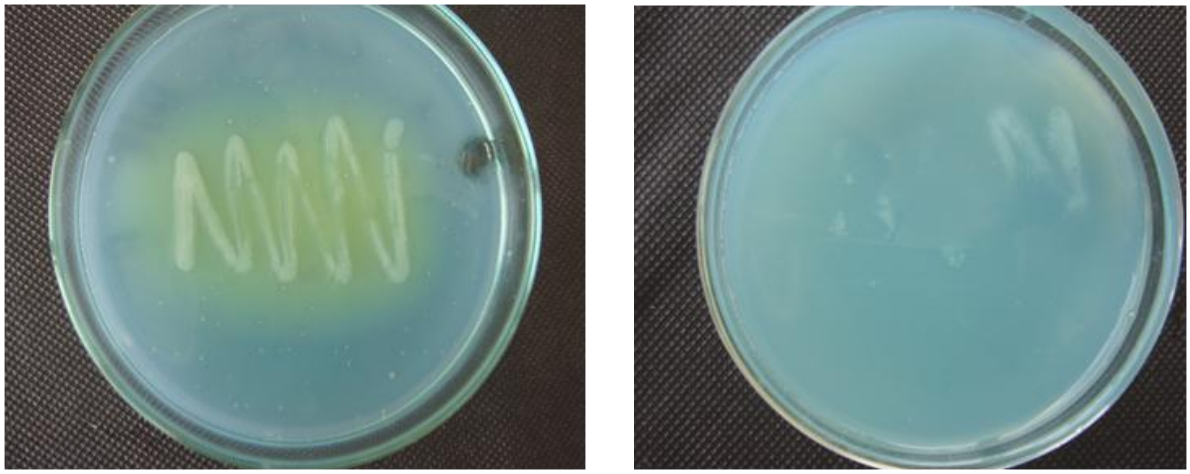

Fig 1: Differentiation between (a) *Agrobacterium* and (b) *Rhizobium*

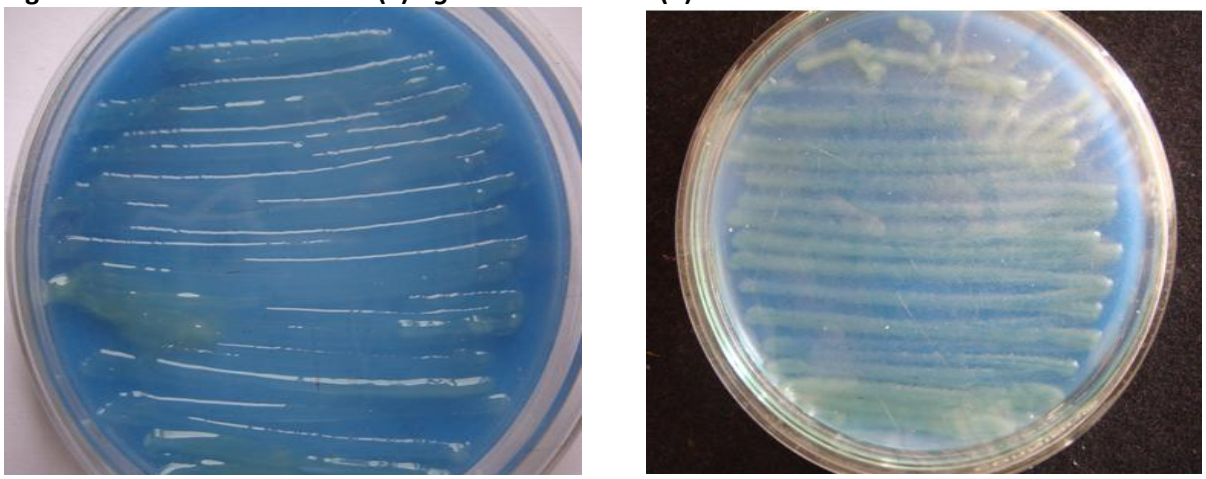

Fig 2: Differentiation between (a) *Bradyrhizobium* and (b) *Ensifer* species

Table 4: Biochemical characteristics of *Bradyrhizobium* and *Ensifer* sp. of soybean

Characteristics		% of isolates showing reactions	
		Bradyrhizobium spp.	*Ensifer* spp.
Oxidase		100	100
Catalase		100	100
Citrate utilization		100	100
Methy red (MR)		0	0
Voges Proskauer (VP)		0	0
Nitrate reduction (NR)		100	100
Urease		100	100
3 Ketolactose production		0	0
Temperature	**10°C**	0	0
	28°C	100	100
	42°C	0	0
pH	**4.0**	0	0
	7.0	100	100
	9.0	0	0

Table 5: Expression of hydrogenase uptake system in different *Bradyrhizobium* and *Ensifer spp.* of soybean

Bradyrhizobium/Ensifer isolates	Presence of Hup^+ /Hup^- system
LSBR 1	+
LSBR 2	+
LSBR 3	-
LSER 4	-
LSER 5	-
LSER 6	-
LSER 7	+
LSER 8	-
LSER 9	-
LSBR 10	+
DS 1	-
SB 271	+

Cultures were evaluated for tolerance to three temperature regimes- 10°C, 28°C and 42°C (Table 4). At 28°C, all cultures were able to grow, at 10°C very little growth was observed and at 42°C growth was absent.The results are supported with the findings of Gaur (1993) and Harwani (2006) who reported that the optimum temperature for growth of root nodulating bacteria ranged from 25°C - 30°C. However, inhibitory effect of elevated temperature (42°C) was clearly visible on the growth response of *Bradyrhizobium/Ensifer* spp. since no growth was recorded.

This result corroborates the previous findings of Werner (1992) where survival of the majority of *Brady*h*izobium* strains reduced drastically above 40°C. Our results indicated that cells were able to grow only at pH 7.0(Table 4).No growth was observed in YEMA medium with pH 4.0 and 9.0. Also cells were unable to grow when incubated at 37°C even at pH 7.0. Thus medium with pH 7.0 and temperature 28°C are the optimum parameters for growth of the organism. Our results were in agreement with previous studies (Gao *et al.,* 1994; Kucuk *et al.,* 2006; Baoling *et al.,* 2007). The presence of Hup system in rhizobia is helpful in providing an energy efficient, nitrogen fixing symbiosis in legumes. Three bradyrhizobial species (LSBR 1, LSBR 2, LSBR10), reference culture SB 271 (*Bradyrhizobium* spp.) and only one *Ensifer* spp. (LSER 7) showed red coloration on Maier's *et al* and YEMA media amended with 0.01% TTC dye (Table 5).This study demonstrated the occurrence of native diversity of *Bradyrhizobium* and *Ensifer* spp. for exploiting their potential as rhizobial inoculant in new areas of soybean.

LITERATURE CITED

Albareda M, Rodrigues DN and Temprano FJ, 2009. Use of *Sinorhizobium* (*Ensifer*) *fredii* for soybean [*Glycine max* (L.)] inoculants in South Spain. *Eur. J. of Agri.,* **30**: 205-211.

Annapurna K, Balakrishnan N and Vital L, 2007. Verification and rapid identification of soybean rhizobia in Indian soils. *Curr. Microbiol.,* **54**: 287-291.

Anonymous, 2010. All India Coordinated Research Project on Soybean.Directorate of soybean research.Indian council of agricultural research.Khandwa road, Indore (M.P) Pp.ix**.**

Appunu C, Sen D, Singh MK and Dhar B, 2008. Variation in symbiotic performance of *Bradyrhizobium japonicum* strains and soybean cultivars under field conditions. *J .Centra. Euro. Agri.,* **9**: 185-190.

Baoling H, ChengQun L, Bo W and LiQin F, 2007. A rhizobia strain isolated from root nodule of gymnosperm *Podocarpusm acrophyllus. Sci.Chin. Ser C-Life Sci.,* **50**: 1-6.

Bernaerts MJ and Deley J, 1963. A biochemical test for crown gall bacteria. *Nature,* **197**:406-407.

Cappuccino JC and Sherman N, 1992. *Microbiology: A Laboratory Manual*. Pp 125-79. New York.

Dhami N and Prasad BN, 2009. Increase in root nodulation and crop yield of soybean by native *Bradyrhizobium japonicum* strains*. J. Plant. Sci.,* **6**: 1–3

Gachande BD and Khansole GS, 2011. Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn and *Bradyrhizobium japonicum* of soybean. *Bioscience Discovery,* **2**: 1-4

Gao JL, Sun JG, Li Y, Wang ET and Chen WX, 1994. Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province.*Chin. Int. J. Syst. Bacteriol.,* **44**: 151-158.

Gaur YD, 1993. Microbiology, Physiology and Agronomy of nitrogen fixation.Legume –*Rhizobium* symbiosis. *Proc. Indian. Natl.Sci. Acad.,* **59**: 333-358.

Harwani D, 2006. Biodiversity and efficiency of bradyrhizobial strains and arbuscular mycorrhizal fungi of soybean cultivars grown in Harotiregion of Rajasthan. Ph. D. Thesis, Maharshi Dayanand Saraswati University, Ajmer, India.

Hungria M, Campo RJ, Chueire LMO, Grange L and Megias M, 2001. Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil. *Biol. Ferti. Soils.,* **33**: 387–394

Keyser HH and Li F, 1992. Potential for increasing biological nitrogen fixation in soybean. *Pl soil,* **141**: 119-35.

Kucuk C, Kivanc M and Kinaci E, 2006. Characterization of *Rhizobium* sp. isolated from Bean. *Turk. J. Biol.,* **30**: 127-132.

Mahana SK, Garg R and Parvateesam M, 2000. Cultural and Biochemical Characteristics of root nodule bacteria from induced mutants of *Vigna mung* L. Seed Pathology Pp. 417-421. Printwell publications, Jaipur.

Maier RJ, Campbell NER, Hanus JF, Simpson FB, Russel SA and Evans HJ, 1978. Expression of hydrogenase activity in free living *Rhizobium japonicum. Proc. Natt. Acad. Sci. USA.* **75**: 3258-3262.

Sadowsky MJ, Keyser HH and Bohlool BB, 1983. Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. *Int. J. Systematic. Bacteriol.,* **33**: 716–722.

Saeki Y, Kaneko A, Hara T, Suzuki K, Yamakawa T, Nguyen MT, Nagatomo Y and Akao S, 2005. Phylogenetic analysis of soybean-nodulating rhizobia isolated from alkaline soils in Vietnam. *Soil. Sci. Plant. Nutr.,* **51**: 1043–1052.

Sharma MP, Srivastava K and Sharma SK, 2010. Biochemical characterization and metabolic diversity of soybean rhizobia isolated from Malwa region of Central India.*Plant. Soil. Environ.,* **56**: 375–383

Somasegaran P and Hoben HJ, 1985. *Methods in Legume-Rhizobium Technology*. NifTAL project and MIRCEN. Pp. 1-52 Department of Agronomy, 2nd Soil Science Hawaii Institute Tropical Agriculture Human research, Univ Hawaii Manoa.

Somasegaran P and Hoben HJ, 1994. *Handbook for Rhizobia: Methods in legume-Rhizobium Technology*. Pp 1- 450. Springer-Verlag, New York.

Weir BS, 2011. The current taxonomy of rhizobia. New Zealand rhizobia website. http://www.rhizobia.co.nz/taxonomy/rhizobia.html.

Werner D, 1992. The *Bradyrhizobium/Rhizobium* Fabales symbiosis. In: Werner D (ed) *Symbiosis of plants and microbes*. Pp. 49-151 Chapman and Hall, London.