Chapter 31
Root Nodule Bacteria and Symbiotic Nitrogen Fixation

D. Prévost
Agriculture and Agri-Food Canada
Quebec, Quebec, Canada

H. Antoun
Laval University
Quebec, Quebec, Canada

31.1 INTRODUCTION

Symbiotic nitrogen fixation in plants occurs in root nodules of legumes and nonlegumes. The bacterium rhizobium is one of the most studied symbiotic nitrogen-fixing bacteria because it nodulates legumes, which are environmentally significant in soil N fertility management of cultivated lands. The majority of nonleguminous nodules belong to the *Alnus*-type symbiosis, in which the actinomycete *Frankia* is the microsymbiont. The cyanobacteria *Nostoc* or *Anabaena* nodulate the Cycadales, while the bacterium rhizobium forms *Parasponia*-type symbioses.

This chapter focuses on the methodology developed to study the rhizobium–legume symbiosis. The global success of legume production is due to the development of inoculation technologies and cropping systems by multidisciplinary teams. Microbiologists, soil scientists, plant physiologists, plant breeders, and agronomists contributed to this breakthrough. Increased knowledge in rhizobial ecology is mainly due to the development of molecular techniques. Moreover, the taxonomy of the microsymbiont rhizobium has considerably changed since the last edition of this chapter (Rice and Olsen 1993). The use of genotypic and phenotypic approaches, applied to isolates obtained from a large number of legume species and from different regions, resulted in reclassification of known rhizobial species and in an increased number of new species.

Symbiotic rhizobia belong to the α-subclass of Proteobacteria (α-rhizobia). However, some tropical legumes are nodulated by strains of *Burkholderia* and *Ralstonia* species belonging to the β-subclass of Proteobacteria. These strains evolved from diazotrophs through multiple lateral *nod* gene transfers, and this phenomenon seems to be widespread in nature (Chen et al. 2003). The current taxonomy of rhizobia (Rhizobia_Taxonomy 2006) includes the genera *Rhizobium*

(14 species), *Mesorhizobium* (10 species), *Azorhizobium* (1 specie), *Sinorhizobium*, which could be renamed as *Ensifer* (11 species), *Bradyrhizobium* (5 species), and six other genera (*Methylbacterium*, *Burkholderia*, *Ralstonia*, *Devosia*, *Blastobacter*, and *Ochrobacterium*).

In this chapter, the general term ''rhizobia'' will be used for the designation of bacteria that form nodules on legumes root and stem. Table 31.1 shows the rhizobial species associated to some indigenous and cultivated legumes. Recent classification of rhizobia isolated from legumes in tropic regions is not included.

TABLE 31.1 Some Indigenous and Cultivated Legumes in Canada and Their Nodulating Rhizobial Species

Legume species		
Latin name	**Common name**	**Rhizobial species**
Arachis hypogae	Peanut	*Bradyrhizobium* sp.[a]
Astragalus cicer	Cicer milkvetch	*Mesrohizobium* sp.[a]
Astragalus sinicus		*Mesorhizobium huakuii*
Astragalus adsurgens		*M. septentrionale, M. temperatum*
Cicer arietinum	Chickpea	*Mesorhizobium ciceri* *Mesorhizobium mediterraneum*
Galega	Goat's rue	*Rhizobium galegae*
Glycine max	Soybean	*Bradyrhizobium japonicum* *B. elkanii, B. liaoningense,* *Sinorhizobium fredii,* *S. xinjiangense*
Lathyrus spp.	Flat pea, tangier pea beach pea	*Rhizobium leguminosarum* bv. viceae
Lathyrus sativus	Chickling vetch, grass pea	*Rhizobium leguminosarum* bv. viceae
Lathyrus pratensis	Yellow vetchling	*Rhizobium leguminosarum* bv. viceae
Lens culinaris	Lentil	*Rhizobium leguminosarum* bv. viceae
Lotus corniculatus	Birsfoot trefoil	*Mesorhizobium loti*
Lupinus spp.	Lupine (white, blue, yellow)	*Bradyrhizobium* sp.
Medicago spp.	Alfalfa	*Sinorhizobium meliloti, S. medicae*
Melilotus spp.	Sweetclover (white, yellow)	*Sinorhizobium meliloti, S. medicae*
Onobrychis vivifolia	Sainfoin	*Rhizobium* sp.[a]
Oxytropis sp.		*Mesorhizobium* sp.[a]
Phaseolus vulgaris	Common beans	*Rhizobium leguminosarum* bv. phaseoli *R. gallicum, R. giardinii, R. etli*
Pisum sativum	Field, garden pea	*Rhizobium leguminosarum* bv. viceae
Securigera varia	Crownvetch	*Rhizobium* sp.[a]
Trifolium spp.	Clover	*Rhizobium leguminosarum* bv. trifolii
Vicia sativa	Common vetch	*Rhizobium leguminosarum* bv. viceae
Vicia villosa	Hairy vetch	*Rhizobium leguminosarum* bv. viceae
Vicia faba	Faba bean, broadbean	*Rhizobium leguminosarum* bv. viceae

Source: Adapted from Sahgal, M. and Johri, N., *Curr. Sci.*, 84, 43, 2003. Updated from http://www.rhizobia.co.nz/Rhizobia_Taxonomy.html.

[a] Species designation is still unknown for these legumes.

Methods commonly used for the isolation and estimation of rhizobial populations in soils and for the evaluation of symbiotic nitrogen fixation will be described to enable a scientist with little experience obtain reliable results. Examples of methodologies using genetic tools to directly isolate and estimate the size of rhizobial populations in soils will be briefly presented; references will be given for more complete information.

31.2 ISOLATION OF RHIZOBIA

Symbiotic rhizobia are common colonizers of the rhizosphere of both legume and nonlegume plants and in addition to legumes they are also endophytes of several nonlegumes like rice and maize (Sessitsch et al. 2002). However, nonsymbiotic rhizobia can also be present in soil (Sullivan et al. 1996) and therefore, the methodology described here is aimed at the isolation of nodule-forming rhizobia in legumes. Rhizobia can be isolated either by collecting nodules from field grown legumes or by inducing nodule formation by inoculating surface disinfected legume seeds with soil suspensions under aseptic laboratory conditions (see Section 35.3). Guidelines for collecting nodules and preserving them during a collecting trip have been described and discussed by Date and Halliday (1987) and by Somasegaran and Hoben (1994).

The procedure given here is a general description of the basic steps to be followed in obtaining a culture of rhizobia inhabiting the nodules of selected plant (Rice and Olsen 1993).

31.2.1 Material

1. Tools for excavating plants and removing roots—spade, garden trowel, knife, etc.
2. Plastic sampling bags.
3. Cooler and dry ice.
4. Collection vessel: (a) glass vial with screw cap (10 to 20 mL capacity) containing a desiccant (anhydrous calcium chloride or silica gel) occupying one-fourth the volume of the container, held in place by a cotton wool plug or (b) glass or plastic vial with screw cap containing 50% glycerol.
5. 95% (w/v) ethanol.
6. Disinfectant solution: 8% (w/v) sodium or calcium hypochlorite solution or 3% (v/v) hydrogen peroxide solution.
7. Sterile water, test tubes, and glass rods.
8. Petri dishes containing 20 mL yeast-extract mannitol agar (YMA):
   Composition (g L^{-1}): mannitol, 10.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; yeast extract, 1.0; agar, 15.0. Adjust pH to 7.0 with 1 *M* NaOH or HCl before autoclaving at 121°C for 15 min.
9. Screw cap tubes with slants of YMA; sterile mineral oil.
10. Cryovials-containing YMB (YMA without agar) with 25% glycerol.

31.2.2 Procedure

1 With a spade, cut around the selected plant a block of soil with approximately 15 cm in diameter to a depth of at least 20 cm.

2 Pull the block, and carefully remove soil from roots. Avoid detaching secondary roots from the plant. This step can be greatly facilitated by immersion of the soil block in water and allowing soil to fall away. Using a sieve of an appropriate mesh, depending on nodule size, is useful to catch nodules that may become detached from the root.

3 If isolation can be performed within 24 h, put the roots with nodules in the sampling bag and transport them to the laboratory in the cooler. Fresh root nodules can be kept in the refrigerator overnight, and processed as indicated in step 6.

4 For a longer storage period, place the nodules in the collection vessel. Nodules preserved this way can last 6–12 months. However, as rhizobial recovery may vary depending on legume species and storage temperature, nodules should be processed within 3 weeks. Place dry nodules in water for 60 min to allow them to fully imbibe. Nodules stored for more than 3 weeks should be kept in the refrigerator overnight to imbibe.

5 Alternatively, in our laboratory, we observed that nodules can be preserved for more than 1 year at −20°C in vials containing about 50% glycerol. Moreover, if there is no time to take out nodules from roots at the sampling time, nodulated roots from pots or field can be preserved, wrapped in a paper towel imbibed in 50% glycerol, placed in a plastic bag, and stored at −20°C.

6 Immerse nodules for 5–10 s in 95% ethanol, and then in disinfectant for 3–4 min.

7 Remove disinfectant and rinse at least five times in sterile water.

8 Check the surface sterility of nodules by passing them on the surface of YMA plates. Discard isolates originating from a surface-contaminated nodule.

9 Crush each nodule with a sterile glass rod in a test tube. Add sterile water to make a turbid suspension and transfer a drop to YMA plates.

10 Streak the drop of suspension onto the agar surface so that suspension is progressively diluted.

11 Incubate the plates in an inverted position at the optimum temperature for the targeted rhizobial species (25°C–30°C). Make daily observation for the appearance of colonies typical of *Rhizobium* or *Bradyrhizobium* (Somasegaran and Hoben 1994).

12 Pick off and restreak well-isolated single colonies on fresh plates to obtain pure cultures. If more than one typical colony appears on a plate, each of these types should be taken to pure culture.

13 For short-term storage, transfer pure culture isolates to screw cap YMA agar-slant tubes. Incubate and completely cover rhizobial growth with cold sterile mineral oil. Keep in the refrigerator for up to 1 year. For longer storage period, suspend rhizobial cells in a cryovial-containing YMB with 25% glycerol and keep at −80°C.

14 Authenticate each pure culture isolate by confirming nodule-forming ability on test host plants grown under axenic conditions in growth pouches (see Section 31.3) or in Leonard jar assemblies (Vincent 1970; Gibson 1980).

31.2.3 Comments

1 Senescent nodules may contain fungi that can cause heavy overgrowth on agar plate. This problem can be reduced by adding 20 μg mL^{-1} cycloheximide to YMA. Prepare a fungicide stock solution by dissolving 0.5 g of cycloheximide in 25 mL of 95% (v/v) ethanol. Add 1.2 mL of fungicide stock solution to 1 L of YMA cooled to 50°C–55°C. Cycloheximide is very toxic if swallowed, inhaled, or absorbed through the skin.

2 Bacterial contaminants and β-rhizobia can be distinguished from α-rhizobia by incorporating Congo Red in YMA at a concentration of 25 μg mL^{-1} (to each L of YMA add 10 mL of a stock solution of 250 mg Congo Red in 100 mL of water). When incubated in the dark, α-rhizobia show little or no Congo Red absorption, they form colonies that are white, opaque, or occasionally pink, while other bacteria absorb the red dye, and their colonies are dark red. There are, however, exceptions, some strains of *Sinorhizobium meliloti* can absorb the dye strongly and α-rhizobia will absorb the dye if plates are exposed to light for an hour or more (Somasegaran and Hoben 1994).

3 Frequently, more than one colony type, with all morphological characteristics of rhizobia, will appear on one plate from one nodule. In some cases, these colony types will form nodules on the host, and in other cases, one or more types may be incapable of forming nodules. These types of contaminants are often ''latent'' in that they appear to be carried along in subsequent transfers without detection and will suddenly appear, particularly if the culture is grown under nutritional and physical stress. It is therefore extremely important that new isolates and cultures undergoing frequent transfer be thoroughly checked onto test plants and reisolation from fresh nodules.

31.3 ENUMERATION OF RHIZOBIA BY THE MOST PROBABLE NUMBER PLANT-INFECTION TECHNIQUE

The most probable number (MPN) plant-infection technique has been used for many years for the enumeration of rhizobia in soils. It has also been adapted for the enumeration of rhizobia in inoculants by the Canadian Food Inspection Agency (CFIA) for the legume inoculant testing program.

The MPN procedure relies upon the pattern of positive or negative nodulation responses of host plants inoculated with consecutive series of dilutions of sample (soil, inoculants,

preinoculated seeds) containing rhizobia. The method is based on the following major assumptions: (1) a single viable rhizobium cell inoculated onto its specific host in a nitrogen-free medium will cause nodule formation, (2) nodulation is the proof of infective rhizobia, (3) the validity of the test is demonstrated by the absence of nodules on uninoculated plants, and (4) absence of nodules on inoculated plants is proof of the absence of infective rhizobia. The procedure described below is adapted from the official ''Methods for testing legume inoculants and preinoculated seed products'' by CFIA (Anonymous 2005).

31.3.1 Materials

1 Disposable seed germination pouches (Mega International, St. Paul. Minneapolis): they are designed to observe root development and so, they can be advantageously used to replace pots and glassware. The pouch (16 × 20 cm) is made of a strong and transparent polyester film capable of withstanding steam sterilization at 100 kPa (15 lb $in.^{-2}$) for up to 20 min. Inserted in the pouch is a sleeve-like paper germination towel that is folded along the top edge into a trough and perforated to permit roots to escape from the seeding area. In practice, sterilization is not required, since the pouches are free of rhizobia. This type of pouch can be divided into two parts that permit two tests in the same pouch: the paper germination towel is cut into two and the polyester pouch is split by hermetically sealing the pouch itself. It is recommended to sterilize modified pouches.

2 Undamaged seeds of the appropriate legume species: they are surface disinfected by immersion in 95% ethanol for 30 s followed by either: (1) 10 min in 3%–5% hydrogen peroxide (H_2O_2) solution or (2) 10 min in 5% sodium hypochlorite (NaClO) solution, or (3) by immersion in conc. H_2SO_4 for 10 min. The seeds are then washed or rinsed thoroughly with at least five changes of sterile, distilled water.

3 Nitrogen-free plant nutrient solution: $CoCl_2 \cdot 6H_20$, 0.004 mg; H_3BO_3, 2.86 mg; $MnCl_2 \cdot 4H_2O$, 1.81 mg; $ZnSO_4 \cdot 7H_2O$, 0.22 mg; $CuSO_4 \cdot 5H_2O$, 0.08 mg; $H_2MoO_4 \cdot H_2O$, 0.09 mg; $MgSO_4 \cdot 7H_20$, 492.96 mg; K_2HPO_4, 174.18 mg; KH_2PO_4, 136.09 mg; $CaCl_2$, 110.99 mg; $FeC_6H_5O_7 \cdot H_2O$, 5.00 mg; distilled water, 1 L. Use HCl or NaOH 1.0 *M* solution to bring to pH 6.8 ± 0.1. Sterilize by autoclaving at 100 kPa for 20 min.

4 Sterile buffer diluent solution: peptone, 1.0 g; KH_2PO_4, 0.34 g; K_2HPO_4, 1.21 g; distilled water, 1 L. pH = 7.0 ± 0.1.

5 Stomacher (a paddle blender) and Stomacher bags, or professional Waring Blender. Basic models are adequate for regular cell dispersion. These can be purchased from various sources.

6 Growth chamber or room providing 16,000 lux, 22°C during 16 h light period and 18°C during the dark period, and relative humidity at 65%–70%.

31.3.2 Procedure

1 Add 30 mL of sterile plant nutrient solution to each pouch and place pouches in rack.

2 Place aseptically 20 small (e.g., alfalfa, clover) or 15 intermediate (e.g., sainfoin) surface disinfected seeds directly in the trough of each pouch and incubate in darkness at 20°C (for about 2 days) until the radicles have elongated to 0.5–1.0 cm. In the case of large seeds (e.g., pea, soybean), pregerminate surface disinfected seeds either on three layers of Kimpak germination paper or in sterile humid vermiculate and incubate at 20°C (for about 2 days) until the radicles have elongated to 0.5–1.0 cm; then, place five seedlings carefully in the trough of the pouch, by introducing each radicle into a perforation. Growth units are ready for inoculation. For convenience, they can be kept in a fridge (4°C) for up to 1 week, before their inoculation.

3 A 10-fold dilution (w/v) of the soil sample is prepared as follows: Place 10 g of soil into 90 mL of sterile buffer diluent in a Waring Blender and disperse for 2 min at 12,600 rpm or in a Stomacher bag and disperse for 1 min. Transfer a 10 mL aliquot of the soil suspension to a bottle containing 90 mL of diluent and shake for 5 min. Tenfold serial dilutions (v/v) using a minimum 1 mL buffer-inoculant suspension in buffer diluent, are made as required, depending on the expected number of rhizobia in the soil sample. Soils in which legumes have been grown may contain about 10^4 rhizobia per gram of dry soil, and therefore, the 10-fold dilution series should be carried out to the 10^{-1} or 10^{-2} level before starting the fivefold dilution series.

4 Fivefold dilution series are prepared by mixing 1.0 mL of the final 10-fold dilution and 4.0 mL of sterile phosphate-peptone buffer. Six or seven consecutive fivefold dilutions are made and 4 mL of each dilution level is used to inoculate four growth pouches (1 mL per pouch), except for the last dilution where five pouches are inoculated. Practically, fivefold dilutions and inoculation can be done simultaneously in the following convenient way: take 4 mL of the first fivefold dilution (which has 5 mL) and inoculate four growth pouches with 1 mL in each applied to the root zone of the plantlets. Add 4 mL of diluent to the remaining 1 mL in the dilution tube, mix and take 4 mL of this new dilution for inoculation of other pouches as just described. Repeat these steps until the last fivefold dilution, and use all the last 5 mL to inoculate five pouches. Leave an uninoculated control pouch between each set of four or five inoculated pouches.

5 Place rack of pouches in the growth chamber, and water aseptically with sterile distilled water as required. Examine after 3 weeks for the presence of nodules; legume species that show slow development (e.g., soybean) are examined after 4 weeks. Controls must be free of nodules for the test to be meaningful. Record results as ''+'' for a growth unit (pouch) showing nodulated plants or ''−'' for a growth unit showing no nodules.

31.3.3 Calculation of the MPN of Rhizobia

1 Record the number of positive growth units (pouches) at each dilution level, from the least to the most dilute. This will yield to a six-digit code: a typical result could be: 4,4,4,1,1,0. Locate this series of number on the MPN table (Table 31.2) and read the corresponding number of rhizobia. A computer software program, Most Probable Number Enumeration System (MPNES), is also available and useful in assigning population estimates to codes which are

TABLE 31.2 Most Probable Number (MPN) of Nodule Bacteria Calculated from the Distribution of Positive (Nodulated) Growth Units in a Plant-Infection Test Based on a Fivefold Dilution Series

Number of positive (nodulated) growth units							
Fivefold dilution series						MPN of nodule bacteria	
1:5	1:25	1:125	1:625	1:3,125	1:15,625[a]	Estimate	Confidence limits (95%)
0	1	0	0	0	0	1.0	0.1–7.7
0	2	0	0	0	0	2.1	0.5–9.2
0	3	0	0	0	0	3.0	0.9–10.6
1	0	0	0	0	0	1.1	0.2–7.9
1	1	0	0	0	0	2.3	0.6–9.6
1	2	0	0	0	0	3.5	1.1–11.9
1	3	0	0	0	0	4.9	1.6–14.6
2	0	0	0	0	0	2.6	0.6–10.1
2	1	0	0	0	0	4.0	1.2–12.8
2	2	0	0	0	0	5.5	1.9–16.0
2	3	0	0	0	0	7.2	2.7–19.6
3	0	0	0	0	0	4.6	1.5–14.1
3	1	0	0	0	0	6.5	2.3–18.0
3	2	0	0	0	0	8.7	3.3–23.0
3	3	0	0	0	0	11.3	4.4–29.2
4	0	0	0	0	0	8.0	3.0–21.5
4	1	0	0	0	0	11.4	4.4–29.5
4	2	0	0	0	0	16.2	6.2–42.4
4	3	0	0	0	0	24.2	9.0–64.9
4	4	0	0	0	0	40.4	15.3–106.6
4	0	1	0	0	0	10.8	4.2–28.1
4	1	1	0	0	0	15.1	5.8–39.2
4	2	1	0	0	0	21.5	8.1–57.4
4	3	1	0	0	0	32.8	12.2–87.9
4	0	2	0	0	0	14.1	5.4–36.6
4	1	2	0	0	0	19.6	7.4–51.9
4	2	2	0	0	0	28.3	10.5–76.1
4	3	2	0	0	0	43.6	16.6–114.2
4	0	3	0	0	0	18.1	6.9–47.7
4	1	3	0	0	0	25.2	9.4–67.6
4	2	3	0	0	0	36.4	13.7–96.8
4	3	3	0	0	0	56.5	21.9–146.0
4	4	1	0	0	0	5.7×10	$2.2–14.7 \times 10$
4	4	2	0	0	0	8.1×10	$3.1–21.2 \times 10$
4	4	3	0	0	0	12.1×10	$4.5–32.4 \times 10$
4	4	4	0	0	0	20.2×10	$7.6–53.3 \times 10$
4	4	0	1	0	0	5.4×10	$2.1–14.0 \times 10$
4	4	1	1	0	0	7.5×10	$2.9–19.6 \times 10$
4	4	2	1	0	0	10.8×10	$4.0–28.7 \times 10$
4	4	3	1	0	0	16.4×10	$6.1–43.9 \times 10$
4	4	0	2	0	0	7.1×10	$2.7–18.3 \times 10$
4	4	1	2	0	0	9.8×10	$3.7–26.0 \times 10$
4	4	2	2	0	0	14.1×10	$5.3–38.1 \times 10$
4	4	3	2	0	0	21.8×10	$8.3–57.1 \times 10$
4	4	0	3	0	0	9.1×10	$3.4–23.8 \times 10$

(continued)

TABLE 31.2 (continued) Most Probable Number (MPN) of Nodule Bacteria Calculated from the Distribution of Positive (Nodulated) Growth Units in a Plant-Infection Test Based on a Fivefold Dilution Series

Number of positive (nodulated) growth units							
Fivefold dilution series						MPN of nodule bacteria	
1:5	1:25	1:125	1:625	1:3,125	1:15,625[a]	Estimate	Confidence limits (95%)
4	4	1	3	0	0	12.6×10	$4.7–33.8 \times 10$
4	4	2	3	0	0	18.2×10	$6.9–48.4 \times 10$
4	4	3	3	0	0	28.2×10	$10.9–73.0 \times 10$
4	4	4	1	0	0	2.9×10^2	$1.1–7.3 \times 10^2$
4	4	4	2	0	0	4.1×10^2	$1.6–10.6 \times 10^2$
4	4	4	3	0	0	6.0×10^2	$2.3–16.2 \times 10^2$
4	4	4	4	0	0	10.1×10^2	$3.8–26.6 \times 10^2$
4	4	4	0	1	0	2.7×10^2	$1.0–7.0 \times 10^2$
4	4	4	1	1	0	3.8×10^2	$1.5–9.8 \times 10^2$
4	4	4	2	1	0	5.4×10^2	$2.0–14.4 \times 10^2$
4	4	4	3	1	0	8.2×10^2	$3.1–22.0 \times 10^2$
4	4	4	0	2	0	3.5×10^2	$1.4–9.2 \times 10^2$
4	4	4	1	2	0	4.9×10^2	$1.8–13.0 \times 10^2$
4	4	4	2	2	0	7.1×10^2	$2.6–19.0 \times 10^2$
4	4	4	3	2	0	10.9×10^2	$4.2–28.6 \times 10^2$
4	4	4	0	3	0	4.5×10^2	$7.7–11.9 \times 10^2$
4	4	4	1	3	0	6.3×10^2	$2.3–16.9 \times 10^2$
4	4	4	2	3	0	9.1×10^2	$3.4–24.2 \times 10^2$
4	4	4	3	3	0	14.1×10^2	$5.4–36.7 \times 10^2$
4	4	4	4	1	0	14.3×10^2	$5.5–36.9 \times 10^2$
4	4	4	4	2	0	20.3×10^2	$7.8–53.0 \times 10^2$
4	4	4	4	3	0	30.2×10^2	$11.2–81.3 \times 10^2$
4	4	4	4	4	0	50.5×10^2	$19.0–133.8 \times 10^2$
4	4	4	4	0	1	13.5×10^2	$5.2–35.3 \times 10^2$
4	4	4	4	1	1	18.8×10^2	$7.2–49.0 \times 10^2$
4	4	4	4	2	1	26.9×10^2	$10.1–71.8 \times 10^2$
4	4	4	4	3	1	41.0×10^2	$15.3–110.2 \times 10^2$
4	4	4	4	0	2	17.7×10^2	$6.8–45.9 \times 10^2$
4	4	4	4	1	2	24.5×10^2	$9.2–65.0 \times 10^2$
4	4	4	4	2	2	35.3×10^2	$13.1–95.4 \times 10^2$
4	4	4	4	3	2	54.4×10^2	$20.6–143.8 \times 10^2$
4	4	4	4	0	3	22.6×10^2	$8.6–59.7 \times 10^2$
4	4	4	4	1	3	31.4×10^2	$11.7–84.7 \times 10^2$
4	4	4	4	2	3	45.5×10^2	$17.0–121.4 \times 10^2$
4	4	4	4	3	3	70.6×10^2	$27.1–184.2 \times 10^2$
4	4	4	4	4	1	7.1×10^3	$2.7–18.6 \times 10^3$
4	4	4	4	4	2	10.1×10^3	$3.8–27.0 \times 10^3$
4	4	4	4	4	3	15.1×10^3	$5.4–42.6 \times 10^3$
4	4	4	4	4	4	25.2×10^3	$8.6–74.0 \times 10^3$
4	4	4	4	4	5	$>35.5 \times 10^3$	

[a] Five growth units inoculated with 1 mL aliquots from this dilution level.

not included in published MPN tables (Woomer et al. 1990). It is available from University of Hawaii, NifTAL Project, 1000 Holomua Avenue, Paia, HI 96779, USA.

2 MPN estimate refers to the number of rhizobia present in 1 mL of the 10-fold dilution used to make the fivefold dilution for plant inoculation and it is assumed that the inoculation volume is 1.0 mL. To determine the number of viable rhizobia per gram of soil/inoculant, multiply the MPN estimate by the reciprocal of the level of the 10-fold dilution (before inoculation) that was used to start the fivefold dilutions. This number can be adjusted with soil humidity when results are expressed per gram of dry soil.

31.3.4 Comments

1 It is important to manipulate aseptically seeds and growth units with sterile devices during all steps to avoid contamination by rhizobia. Moreover, special care should be taken when watering pouches during test. Plastic drinking straws placed at a corner inside the pouch facilitate watering and provide rigidity to the pouch.

2 When this method is used to estimate number of rhizobia in liquid or solid legume inoculant products, the level of the 10-fold dilution required to start the fivefold dilution should be about 10^5–10^6. For preinoculated seeds, the starting dilution is made by placing 100 seeds in 100 mL diluent (1:1), 10-fold dilutions can be done depending on the expected number of rhizobia; the MPN number found is per seed.

31.4 DIRECT ISOLATION AND ENUMERATION OF RHIZOBIA FROM SOILS

The use of host plant for the isolation of rhizobia is an indirect procedure that has the disadvantage to recover only strains that have the ability to compete for nodulating the host plant. For ecological study of natural populations, the direct recovery of rhizobia on a semiselective medium has been shown to be satisfactory in studies with *S. meliloti*, *Rhizobium leguminosarum*, and *Bradyrhizobium japonicum*. The semiselective media have been developed to limit the growth of soil microorganisms and favor the growth of rhizobial species. In the case of *S. meliloti* and *R. leguminosarum,* colonies are usually identified by hybridization with a rhizobium species-specific DNA probe.

For scientists not familiar with molecular biology techniques, it is recommended to obtain training from colleagues using these techniques. Thus, detailed molecular biology techniques will not be described here; they can be found in manufacturer's instructions, in bench-top guides (Caetano-Anollés and Gresshoff 1997), and more advanced molecular biology books (Sambrook et al. 1989; Sambrook and Russell 2001).

The basis of colony hybridization is to transfer or replicate bacterial colonies to membranes (nitrocellulose filters, nylon or Whatman No. 541 filter papers). The membranes are lyzed to immobilize DNA, and they are hybridized with a specific DNA-labeled probe (radioactive or nonradioactive). Colonies that are bound to the probe are detected by autoradiography (for radioactive probe) or with IgG conjugated to a reporter molecule (for nonradioactive probe).

31.4.1 Isolation and Enumeration of *S. meliloti*

Medium AS (Nutrient-Poor Agar Medium) (Bromfield et al. 1994)

Composition (g L^{-1}): yeast extract, 0.1; tryptone, 0.4; $CaCl_2$, 0.1; NaCl, 5.0; cycloheximide, 0.15; Congo Red, 0.025; agar, 10.0. Congo Red is prepared as a stock solution and added to the medium before autoclaving at 121°C, 15 min. Cycloheximide is prepared as a stock solution sterilized by membrane filtration and added to the autoclaved molten media.

Soil dilution for inoculation: 10-fold dilutions are made in sterile water, the initial 10-fold dilution being mixed for 15 min on a wrist action shaker. Appropriate dilutions levels (1:1000 and 1:100) are then made, depending on the expected soil bacterial and rhizobial populations and 0.1 mL is used to inoculate AS agar plates. Good results are obtained when about 100–300 cfu (colony-forming units) are growing per plate. About 7 to 100 rhizobial colonies per plate can be easily detected by hybridization. When using 1:100 and lower soil dilutions, the medium AS can be supplemented with 0.05% sodium deoxycholate and 12.5 m*M* Tris–HCl, pH 8.0 (Barran et al. 1997) to reduce the total number of soil bacterial colonies.

Colony Blot Hybridization for *S. meliloti*

A replica of the arrangement of colonies on the agar plate is made by pressing a membrane (Whatman filter paper or nitrocellulose filter) on the surface of the plate to lift the colonies. This replica is treated with alkali to lyze the cells, and the paper is then hybridized to a labeled probe (^{32}P). Those colonies that have bound to the probe are identified by autoradiography. Two probes have been used successfully for the detection of *S. meliloti*. The probe pRWRm61 derived from the insertion element IS*Rm*5 hybridizes strongly to total genomic DNA of the majority of *S. meliloti* strains, except few strains from Australia or from eastern Mediterranean (Wheatcroft et al. 1993; Barran et al. 1997). The probe pSW95 derived from the *nodH* gene of *S. meliloti* strain RCR2011 (SU47) (Debelle and Sharma 1986) is highly species-specific and has been used for the detection of Canadian *S. meliloti* isolates (Bromfield et al. 1994).

31.4.2 Isolation of *R. leguminosarum*

Medium MNBP (Louvier et al. 1995) and LB (Miller 1972)

The medium MNBP reduces colony counts of total soil bacteria by 88%–95%. LB medium (on which *R. leguminosarum* is unable to grow) is used as counter selection step. Colonies growing only on MNBP are identified by colony blot hybridization. This technique is very long, suitable for the isolation of *R. leguminosarum*, but not practical for their enumeration. This procedure offers the advantage of reducing the number of ''putative'' rhizobium colonies to be tested as compared to a nonselective medium.

MNBP composition (g L^{-1}): $Na_2HPO_4 \cdot 12H_2O$, 0.045; $MgSO_4 \cdot 7H_2O$, 0.10; $FeCl_3$, 0.02; $CaCl_2$, 0.04; mannitol, 1.00; NH_4NO_3, 0.005; biotin, 0.0005; thiamine, 0.0005; Ca pantothenate, 0.0005; bacitracin, 0.025; penicillin G, 0.003; cycloheximide, 0.10; benomyl, 0.005; pentachloronitrobenzene, 0.0035; agar, 15.0. Adjust pH to 6.8 and autoclave at 121°C, 15 min. Vitamins and antimicrobial substances are prepared in stock solutions in deionized water and sterilized by membrane filtration. They are added to sterile and molten agar.

LB composition (g L^{-1}): Tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; agar, 15.0. Adjust pH to 7.2 and autoclave at 121°C, 15 min.

TY composition (g L^{-1}): Tryptone, 5.0; yeast extract, 3.0; $CaCl_2 \cdot H_2O$, 0.87; agar, 12.0. Adjust pH to 6.8–7.2 with 1 *M* NaOH. A precipitate forms after autoclaving at 121°C, 15 min.

Soil dilutions for inoculation: 0.1 mL of soil dilutions (10^{-3}) is plated on medium MNBP (25 plates). After 2–3 days growth, mucous-spreading colonies are excised (about 30% of the agar plate surface) to prevent overgrowth. The remaining colonies in each plate are transferred by velveting replication to LB and MNBP plates. The colonies growing only on MNBP plates (about 300 per plate) are transferred to TY agar slopes for further identification by hybridization. With the soils tested during method development, about 20 colonies per plate showed strong homology. The use of this approach reduces the number of colonies to be tested as compared to a nonselective media.

Colony Blot Hybridization for *R. leguminosarum*

Colonies obtained from MNBP plates and kept on TY agar are grown 48 h in 200 μL of TY liquid medium in microplates. From each microplate well, 25 μL of these cultures (OD 650: 0.2–0.3) is spotted on nylon filters. After lysis, the nylon filters are hybridized with one of the *nod* gene probes: pIJ1246, specific for *R. leguminosarum* bv. viciae; pIJ1098, specific for *R. leguminosarum* bv. phaseoli; pRt587, specific for *R. leguminosarum* bv. trifolii (Laguerre et al. 1993). The probes are labeled with digoxigenin-dUT, hybridized, and detected using a kit according to the manufacturer's instructions.

31.4.3 Isolation and Quantification of *Bradyrhizobium* Species

Medium BJMS (Tong and Sadowsky 1994)

This medium allows the isolation of *B. japonicum* and *B. elkanii* from soils, on the basis of their resistance to more than 40 ug of the metal ions Zn^{2+} and Co^{2+} per mL. There is no need to identify colonies by hybridization, the medium is selective for *Bradyrhizobium* and does not allow the growth of *Rhizobium* sp.

Composition (g L^{-1}): HM salts medium (Na_2HPO_4, 0.125; Na_2SO_4, 0.25; NH_4Cl, 0.32; $MgSO_4 \cdot 7H_2O$, 0.18; $FeCl_3$, 0.004; $CaCl_2 \cdot 2H_2O$, 0.013; *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid, 1.3; 2-(*N*-morpholino)ethane sulfonic acid, 1.10); the pH is adjusted to 6.6 with 5 *M* NaOH (Cole and Elkan 1973). This solution is supplemented with: yeast extract, 10.0; L-arabinose, 10.0; Na-gluconate, 10.0; BG (brilliant green), 0.001; pentachloronitrobenzene (PCNB), 0.5; $ZnCl_2$, 0.83; $CoCl_2$, 0.88. The BG and heavy metals are prepared in stock solutions sterilized by filtration. PCNB is prepared as a 10% solution in acetone, then, it can be added to autoclaved 0.05% Triton X-100 to aid in the suspension. These substances are added to the sterile and molten medium.

Soil dilutions for inoculation: Before making serial dilutions, rhizobia are extracted from soils in the following way: 10 g of soil are placed in 95 mL of gelatin–ammonium phosphate solution (1% gelatin in water, adjusted to pH 10.3 and hydrolyzed by autoclaving, 10 min; Kingsley and Bohlool 1981), containing 0.5 mL of Tween 80 and 0.1 mL of silicone antifoam AF72 (General Electric Co., Waterford, New Jersey). Suspensions are shaken on a wrist action shaker for 30 min, and settled for an additional 30 min. The upper aqueous

phase is transferred to a sterile tube from which serial 10-fold dilutions are made for agar plate inoculation.

Comments: The number of contaminants is low (2–5 colonies per plate at the 10^{-1} soil dilution). *Bradyrhizobium* forms small and whitish colonies. However, some *Bradyrhizobium* of serocluster 123 (e.g., strain USDA123) are inhibited at these concentrations; if this serotype is expected, the media can be modified by lowering concentrations of BG (0.0005 g L^{-1}) and PCNB (0.25 g L^{-1}) or by substituting PCNB by cycloheximide (0.1 to 0.2 g L^{-1}).

Isolation of *B. japonicum* and Other Slow-Growing Rhizobia (Gault and Schwinghamer 1993)

Selective media containing various combinations of antibiotics and heavy metal compounds that are toxic to most soil bacteria including *Rhizobium* species are proposed; the effectiveness of combinations may vary according to the soil type. The basic medium SG used for growth of *B. japonicum* is supplemented with antibiotics and fungicides.

SG agar medium composition (g L^{-1}): KH_2PO_4, 0.35; K_2HPO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.15; NaCl, 0.10; $CaCl_2 \cdot 2H_2O$, 0.08; biotin, 0.05; thiamin, 0.0003; Na-gluconate, 3.0; Difco yeast extract, 1.70; $(NH_4)_2SO_4$, 1.0. An effective combination of inhibitors to add is (mg L^{-1}): tetracycline, 10–15; rifampicin, 4–10; chloramphenicol, 15–25; cycloheximide, 50–60; pimafucin, 40. The addition of $ZnSO_4 \cdot 7H_2O$, 80–130, or $NiSO_4 \cdot 6H_2O$, 40–80, may add selectivity in some soils. Stock solutions of antibiotics and chemicals are prepared in water and sterilized by membrane filtration.

Soil dilutions: 2 g of soil sample is added to 10 mL of sterile water, thoroughly agitated and allowed to sediment for 3 h before drawing off the supernatant suspension. This suspension is serially diluted and 0.5 mL is surface-spread on the agar medium. Soil dilution can be made according to each laboratory procedure.

Comments: Although it is not proposed as a counting method, there is generally good agreement between this method and MPN plant-infection technique.

31.5 DIRECT DETECTION OF RHIZOBIA

Culture-independent methods involving polymerase chain reaction (PCR)-based approaches have potential for specific detection of rhizobia in the environment (soils, nodules, roots, inoculants) without the step of cultivation. Depending on the discrimination level (species or strain detection), it is possible to identify specific DNA (oligonucleotoides from total DNA, chromosomal, or symbiotic genes) which can be used in PCR-based protocols or in hybridization methods, as seen in Section 31.4.

The variations in rhizobial genome were initially studied to determine the diversity and to type and identify rhizobia from culture collections. Genes coding for 16S rRNA are used to identify rhizobia at the species and higher levels, while intergenic spacer (IS;16S–23S rDNA IGS) genes allow the differentiation of strains within a same species. DNA fingerprints obtained by using repetitive extragenic palindromic (REP) and enterobacterial repetitive intergeneric consensus (ERIC) primers have been used to identify and classify members of several rhizobial species (Laguerre et al. 1996). Symbiotic genes are useful to type and

classify rhizobia, as shown by the use of *nod* probes (from nodulation genes) in hybridization protocols in Section 31.4.1 and Section 31.4.2. Insertion sequences (IS) or repeated DNA sequences (RS) are used for strain identification and for evaluating the genetic structure of populations (Hartmann et al. 1992).

PCR-based protocols require firstly the extraction of microbial DNA from environment samples. In soils, this step may be more difficult than anticipated, as soils have complex composition. Compounds in the DNA extracts may inhibit subsequent PCR amplification and different DNA extraction methods affect the abundance and composition of bacterial community (Martin-Laurent et al. 2001). In nodules, DNA extraction is not necessary, as PCR can been preformed directly from crushed nodules (Tas et al. 1996). However, DNA extracted from nodules was used in a microarray assay (Bontemps et al. 2005). Recent advances on isolation of DNA and detection of DNA sequences in environmental samples have been recently published (Kowalchuk et al. 2004).

Although PCR is recognized as the most sensitive qualitative method for the detection of specific DNA in environmental samples, its quantification has become restricted to the clinical area (Jansson and Leser 2004). Until now, there is no standardized and robust screening tool for the direct detection and counting of rhizobia in soils. Only few protocols have been developed to trace specific strains, such as in competition studies for nodulation (Tas et al. 1996) or in root colonization (Tan et al. 2001).

31.6 SYMBIOTIC NITROGEN-FIXING EFFICIENCY OF RHIZOBIA

An efficient *Rhizobium* is a strain that is able to compete in the field with other indigenous rhizobia for the colonization of the rhizosphere of its homologous legume partner, under various soil physical and chemical conditions. This efficient strain will form many large nitrogen-fixing nodules on the roots of the plant host that will supply, for most legumes, from 70% to 90% of the plant need in nitrogen. Thus, the best way to estimate the symbiotic efficiency of rhizobial isolates is to do plant inoculation trials in field plots. However, as only about 10% of field-isolated strains are very efficient, if we aim at developing inoculant strains, the symbiotic efficiency of a large number of isolates has to be tested. Because of that, a first screening is performed under artificial axenic conditions in tubes, growth pouches, Leonard jars, or pots filled with sand or vermiculite or a mixture of both. These laboratory methods allow the identification of strains with high N_2-fixing ability, but they do not reflect the competitive ability of the strains. This can be alleviated by performing assays in potted field soils (Somasegaran and Hoben 1994). However, the real symbiotic efficiency of a strain cannot be determined without field plot inoculation trials. All legume inoculation experiments require prior elaboration of a proper experimental design. For information concerning the methodology and experimental designs for screening for effective strains, field site selection and preparation, we refer the readers to the book of Somasegaran and Hoben (1994). Usually treatments in addition to selected rhizobial strains include uninoculated and nitrogen fertilized controls (Vincent 1970). As commercially available legume inoculants include very efficient strains of rhizobia intensively tested under field conditions, if available for your area such an inoculant will be a proper control.

Although rhizobia have several plant growth promoting mechanisms of action (Antoun and Prévost 2005), symbiotic N_2 fixation is the most important mechanism in legumes. The different methods used for measuring symbiotic N_2 fixation in legumes were appraised by Azam and Farooq (2003).

31.6.1 Dry Matter Yield

As biological N_2 fixation is a major source of nitrogen for legumes, this biological activity is directly linked to dry matter yields in several legumes. This simple and inexpensive method is ideal in particular for field-based studies where other methods like the acetylene reduction technique are very variable. Harvested fresh matter (shoots, roots, or pods) are dried in an oven at 70°C until it reaches a constant weight (approximately 48 h). With forage legumes like alfalfa (*Medicago sativa*) it is necessary to take the shoots dry matter yield of a second cut, since it gives most information required to evaluate correctly the symbiotic efficiency of a *Sinorhizobium meliloti* isolate (Bordeleau et al. 1977).

31.6.2 Nodule Index

Since nodules are the site of N_2 fixation, a rapid visual nodule index based on nodule number ranging from 0 (absence of nodules) to 5 (the highest observed number of nodules) was frequently used to evaluate the efficiency of the symbiotic N_2 fixation of rhizobial isolates. However, as inefficient strains also can form numerous nodules, the following nodule index taking into account nodule number, size, and color is a more accurate evaluation (Ben Rebah et al. 2002):

$$\text{Nodulation index} = A \times B \times C \leq 18$$

Nodule size	Value *A*
Small	1
Medium	2
Large	3

Nodule color	Value *B*
White	1
Pink	2

Nodule number	Value *C*
Few	1
Several	2
Many	3

Nodules harboring efficient rhizobia are usually large and they contain leghemoglobin and are colored pink to red. Nodules formed by inefficient rhizobia are small and white. A legume inoculated with a very efficient strain of rhizobia will have a nodule index ranging from 12 to 18, while an inefficient strain will produce a nodule index of 6 or less. This nodule index will be very useful in screening isolates in Leonard jars, growth pouches, or pot experiments. This index will not be practical to use with field trials, because recuperation of roots with all nodules requires time consuming archaeological methodology.

31.6.3 Total-N Difference (Rice and Olsen 1993)

This is a relatively simple procedure, commonly used when only total-N analysis is available. The amount of N fixed by legumes is estimated from the difference in N yield between legume and a nonfixing (reference) plant grown in the same soil under the same conditions as the legume. The most suitable reference plant is an unnodulated plant of the legume being tested (Bremer et al. 1990). This can be achieved only when the soil in which the experiment

is being conducted contains no rhizobia which form effective nodules on the legume. The use of a nonnodulating isoline of the legume provides a suitable reference plant that gives reliable results (Smith and Hume 1987), but nonlegume plants have also been used successfully (Bell and Nutman 1971; Rennie 1984). The major assumption with this method is that the legume and the reference plant assimilate the same amount of soil N. However, differences in soil N uptake because of differences in root morphologies may result in erroneous estimates of N_2 fixation.

The procedure is simple in that all that is required is the inclusion of the reference crop treatment in the design of the field experiment. Care must be used in sampling to ensure that as much of the above-ground portion of the plants as possible is harvested without contaminating the plant material with soil. The plant samples are then processed and analyzed for total-N by the Kjeldahl method. Calculate the total yield from the percent total-N in the plant material and the dry matter yield, and obtain the difference in total-N yield between the legume and the reference crop, which will give the estimate of N_2 fixation.

31.6.4 Acetylene Reduction Assay

In nodules, bacteroids are the *Rhizobium* cells producing nitrogenase, the enzyme responsible for the reduction of N_2 to NH_3. Several other compounds are also reduced by nitrogenase, including acetylene (C_2H_2) reduced to ethylene (C_2H_4). The acetylene reduction assay (ARA) involves the enclosure of excised nodules, detached root systems, or whole plants in a closed container containing 10% C_2H_2. The size of the container will vary according to the plant material under study (glass tubes to mason jars). After specific incubation periods the C_2H_4 produced is quantified in the container by using a gas chromatograph equipped with hydrogen flame ionization detector, which can detect very low concentrations of C_2H_4. Nitrogenase activity is usually expressed as μmol C_2H_4 plant^{-1} h^{-1} or μmol C_2H_4 g^{-1} nodule fresh or dry weight h^{-1}. Standard procedure and calculation for the ARA test were previously described (Turner and Gibson 1980; Somasegaran and Hoben 1994; Weaver and Danso 1994). This method is very sensitive, and is greatly affected by plant disturbance (Singh and Wright 2003). The ARA does not measure total nitrogenase activity because the assay conditions themselves cause decline in nitrogenase activity, and thus ARA cannot be used to calculate the exact amount of N_2 fixed. However, ARA is a valuable tool to assess relative differences in nitrogenase activity in the *Rhizobium*/legume symbioses (Vessey 1994), in pot-grown legumes but not in field-based studies (Minchin et al. 1994). ARA is a nondestructive method that can be very useful when selecting plants for N_2 fixation traits, since it allows later production of seeds from the same plant (Hardarson 2001). The ARA assay is about 1000 times more sensitive than the $^{15}N_2$, and furthermore, it is cheap and simple in its application (Knowles and Barraquio 1994).

31.6.5 Methods Involving ^{15}N

The ^{15}N isotope dilution method involves the growth of N_2 fixing and nonfixing reference plants in soil labeled with ^{15}N labeled inorganic or organic fertilizers. A nitrogen-fixing plant will have lower ^{15}N enrichment as compared to a nonfixing plant due to assimilation of unlabeled N_2 from the air (Hardarson 1994). The amount of N_2 accumulated during a growing season can be calculated using this methodology.

Calculation of the %N derived from the atmosphere (%Ndfa) can be made using the following equation:

$$\%\text{Ndfa} = (1 - \%\text{Ndff}_F/\%\text{Ndff}_{NF}) \tag{31.1}$$

Where $\%Ndff_F$ and $\%Ndff_{NF}$ are %N derived from fertilizer or tracer by fixing (F) and nonfixing (NF) plants receiving the same amount and enrichment of ^{15}N (Hardarson 1994).

The ^{15}N natural abundance method is based on the same principle as the isotope dilution method, but no ^{15}N-enriched material is added to soil (Weaver and Danso 1994). During N turnover reactions in soil, ^{14}N is preferentially lost into the atmosphere which results in a slightly higher $^{15}N/^{14}N$ ratio in soil than atmospheric N_2. Thus nitrogen-fixing plants have lower ^{15}N enrichment than nonfixing plants and this has been used to measure biological nitrogen fixation.

Detailed methodology on the use of ^{15}N isotope dilution method and ^{15}N natural abundance is described by Hardarson (2001) and Weaver and Danso (1994).

REFERENCES

Anonymous 2005. Methods for testing legume inoculant and preinoculated seed products. Fertilizers Act, Section 23, Regulations, Canadian Food Inspection Agency, Canada.

Antoun, H. and Prévost, D. 2005. Ecology of plant growth promoting rhizobacteria. In Siddiqui, Z.A., Ed. *PGPR: Biocontrol and Biofertilization*. Springer, New York, NY, pp. 1–38.

Azam, F. and Farooq, S. 2003. An appraisal of methods for measuring symbiotic nitrogen fixation in legumes. *Pak. J. Biol. Sci*. 18:1631–1640.

Barran, L.R., Bromfield, E.S.P., and Whitwill, S.T. 1997. Improved medium for isolation of *Rhizobium meliloti* from soil. *Soil Biol. Biochem*. 29: 1591–1593.

Bell, F. and Nutman, P.S. 1971. Experiments on nitrogen fixation by nodulated Lucerne. *Plant Soil Special Vol*.: 231–234.

Ben Rebah, F., Prévost, D., and Tyagi, R.D. 2002. Growth of alfalfa in sludge-amended soils and inoculated with rhizobia produced in sludge. *J. Environ. Qual*. 31: 1339–1348.

Bontemps, C., Golfier, G., Gris-Liebe, C., Carrere, S., Talini, L., and Boivin-Masson, C. 2005. Microarray-based detection and typing of the rhizobium nodulation gene *nodC*: Potential of DNA arrays to diagnose biological functions of interest. *Appl. Environ. Microbiol*. 71: 8042–8048.

Bordeleau, L.M., Antoun, H., and Lachance, R.A. 1977. Effets des souches de *Rhizobium meliloti* et des coupes successives de la luzerne (*Medicago sativa*) sur la fixation symbiotique d'azote. *Can. J. Plant Sci*. 57: 433–439.

Bremer, E., Van Kessel, C., Nelson, L., Rennie, R.J., and Rennie, D.A. 1990. Selection of *Rhizobium leguminosarum* strains for lentil (*Lens culinaris*) under growth room and field conditions. *Plant Soil* 121: 47–56.

Bromfield, E.S.P., Wheatcroft, R., and Barran, L.R. 1994. Medium for direct isolation of *Rhizobium meliloti* from soils. *Soil Biol. Biochem*. 26: 423–428.

Caetano-Anollés, G. and Gresshoff, P.M. 1997. *DNA Markers: Protocols, Applications, and Overviews*. Wiley-Liss Inc., New York, NY, 364 pp.

Chen, W-M., Moulin, L., Bontemps, C., Vandamme, P., Béna, G., and Boivin-Masson, C. 2003. Legume symbiotic nitrogen fixation by β-proteobacteria is widespread in nature. *J. Bacteriol*. 185: 7266–7272.

Cole, M.A. and Elkan, G.H. 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrob. Agents Chemother*. 4: 248–253.

Date, R.A. and Halliday, J. 1987. Collection, isolation, cultivation and maintenance of rhizobia.

In Elkan, G.H., Ed. *Symbiotic Nitrogen Fixation Technology*. Marcel Dekker, New York, NY, pp. 1–27.

Debelle, F. and Sharma, S.B. 1986. Nucleotide sequence of *Rhizobium meliloti* RCR2011 genes involved in host specifity of nodulation. *Nucl. Acid Res*. 14: 7453–7472.

Gault, R.R. and Schwinghamer, E.A. 1993. Direct isolation of *Bradyrhizobium japonicum* from soil. *Soil Biol. Biochem*. 25: 1161–1166.

Gibson, A.H. 1980. Methods for legumes in glasshouse and controlled environment cabinets. In Bergersen, F.J., Ed. *Methods for Evaluating Biological Nitrogen Fixation*. John Wiley & Sons, New York, NY, pp. 139–184.

Hardarson, G. 1994. International FAO/IAEA programmes on biological nitrogen fixation. In Graham, P.H., Sadowsky, M.J., and Vance, C.P., Eds. *Symbiotic Nitrogen Fixation*. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 189–202.

Hardarson, G. 2001. Use of ^{15}N to quantify biological nitrogen fixation in legumes. In *IAEA-TCS-14. Use of Isotope and Radiation Methods in Soil and Water Management and Crop Nutrition*. International Atomic Energy Agency, Vienna, Austria, pp. 58–70.

Hartmann, A., Gatroux, G., and Amarger, N. 1992. *Bradyrhizobium japonicum* strain identification by RFLP analysis using the repeated sequence RSα. *Lett. Appl. Microbiol*. 15: 15–19.

Jansson, J.K. and Leser, T. 2004. Quantitative PCR of environmental samples. In Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D.L., and van Elsas, J.D., Eds. *Molecular Microbial Ecology Manual*, 2nd Ed., Vol. 1. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 445–463.

Kingsley, M.T. and Bohlool, B.B. 1981. Release of *Rhizobium* spp. from tropical soils and recovery for immunofluorescence enumeration. *Appl. Environ. Microbiol*. 42: 241–248.

Knowles, R. and Barraquio, W.L. 1994. Free-living dinitrogen-fixing bacteria. In Weaver, R.W., Angle, S., and Bottomley, P., Eds. *Method of Soil Analysis, Part 2—Microbiological and Biochemical Properties*. Soil Science Society of America, Madison, WI, pp. 179–197.

Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D.L., and van Elsas, J.D. 2004. *Molecular Microbial Ecology Manual*, 2nd Ed., Vol. 1. Kluwer Academic Publishers, Dordrecht, the Netherlands. 849 pp.

Laguerre, G., Bardin, M., and Amarger, N. 1993. Isolation from soil of symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. *Can. J. Microbiol*. 39: 1142–1149.

Laguerre, G., Mavingui, P., Allard, M.-H., Charnay, M.-P., Louvrier, P., Mazurier, S.-I., Rigottier-Gopis, L., and Amarger, N. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: Application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol*. 62: 2029–2036.

Louvier, P., Laguerre, G., and Amarger, N. 1995. Semiselective medium for isolation of *Rhizobium leguminosarum* from soils. *Soil Biol. Biochem*. 27: 919–924.

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G., and Catroux, G. 2001. DNA extraction from soils: Old bias for new microbial diversity analysis methods. *Appl. Environ. Microbiol*. 67: 2354–2359.

Miller, J.H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 466 pp.

Minchin, F.R., Witty, J.F., and Mytton, L.R. 1994. Reply to 'Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay' by J.K. Vessey. *Plant Soil* 158: 163–167.

Rennie, R.J. 1984. Comparison of N balance and ^{15}N isotope dilution to quantify N_2 fixation in field grown legumes. *Agron. J*. 76: 785–790.

Rhizobia_Taxonomy 2006. [Online] Available htpp://www.rhizobia.co.nz/Rhizobia Taxonomy.html [March 2006]

Rice, W.A. and Olsen, P.E. 1993. Root nodule bacteria and nitrogen fixation. In Carter, M.R., Ed. *Soil Sampling and Methods of Analysis*.

Canadian Society of Soil Science, Lewis Publishers, Boca Raton, FL, pp. 303–317.

Sahgal, M. and Johri, N. 2003. The changing face of rhizobial systemics. *Curr. Sci.* 84: 43–48.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Woodbury, NY.

Sambrook, J. and Russell, T.W. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press. Woodbury, NY. Paperback edition or online. Available http://www.MolecularCloning.com [March 2006]

Sessitsch, A., Howieson, J.G., Perret, X., Antoun, H., and Martínez-Romero, E. 2002. Advances in *Rhizobium* research. *Crit. Rev. Plant Sci.* 21: 323–378.

Singh, G. and Wright, D. 2003. Faults in determining nitrogenase activity. *J. Agron. Crop Sci.* 189: 162–168.

Smith, D.L. and Hume, D.J. 1987. Comparison of assay methods for nitrogen fixation utilizing white bean and soybean. *Can. J. Plant Sci.* 67: 11–20.

Somasegaran, P. and Hoben, H.J. 1994. *Handbook of Rhizobia. Methods in Legume-Rhizobium Technology*. Springer-Verlag, New York, NY, 450 pp.

Sullivan, J.T., Eardly, B.D., Van Berkum, P., and Ronson, C.W. 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. *Appl. Environ. Microbiol.* 62: 2818–2825.

Tan, Z., Hurek, T., Vinuesa, P., Müller, P., Ladha, J.K., and Reinhold-Hurek, B. 2001. Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S–23S ribosomal DNA intergenic spacer targeted-PCR. *Appl. Environ. Microbiol.* 67: 3655–3664.

Tas, E., Leinonen, P., Saano, A.S., Räsänen, L.A., Kaijalainen, S., Piippola, S., Hakola, S., and Lindström, K. 1996. Assessment and competitiveness of rhizobia infecting *Galega orientalis* on the basis of plant yield, nodulation, and strain identification by antibiotic resistance and PCR. *Appl. Environ. Microbiol.* 62: 529–535.

Tong, Z. and Sadowsky, M.J. 1994. A selective medium for the isolation and quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains from soils and inoculants. *Appl. Environ. Microbiol.* 60 (2): 581–586.

Turner, G.L. and Gibson, A.H. 1980. Measurement of nitrogen fixation by indirect means. In Bergersen, F.J., Ed. *Methods for Evaluating Biological Nitrogen Fixation*. John Wiley & Sons, New York, NY, pp. 111–138.

Vessey, J.K. 1994. Measurement of nitrogenase activity in legume root nodules: In the defense of the acetylene reduction assay. *Plant Soil* 158: 151–162.

Vincent, J.M. 1970. *A Manual for the Practical Study of Root-Nodule Bacteria*. Blackwell Scientific, Oxford, UK.

Weaver, R.W. and Danso, S.K.A. 1994. Dinitrogen Fixation. In Weaver, R.W., Angle, S., and Bottomley, P., Eds. *Method of Soil Analysis, Part 2—Microbiological and Biochemical Properties*. Soil Science Society of America, Inc., Madison, WI, USA, pp. 1019–1045.

Wheatcroft, R., Bromfield, E.S.P., Laberge, S., and Barran, L.R. 1993. Species specific DNA probe and colony hybridization of *Rhizobium meliloti*. In Palacios, R., Mora, J., and Newton, W.E., Eds. *New Horizons in Nitrogen Fixation*. Kluwer, Boston, MA, USA, p. 661.

Woomer, P., Bennett, J., and Yost, R. 1990. Overcoming the inflexibility of most-probable number procedures. *Agron. J.* 82: 349–353.