49 *Rhizobium*

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49-1 INTRODUCTION

Bacteria in the genus *Rhizobium* are a diverse group that are similar not so much by biochemical characteristics, DNA base ratios, or morphology as by their ability to form nodules on some legumes. The classification of rhizobia on the basis of the legume nodulated presents many difficulties for the soil microbiologist but herein lies their importance to agronomists. Plant cross-inoculation groups persist as the basis for speciating *Rhizobium* in an age of more sophisticated classification methods (Vincent, 1977). Subgroups within the main cross-inoculation groups have been developed as an aid in inoculant production (Burton, 1979).

The approach in this chapter is to present principles and methods in a manner that enables a microbiologist with agronomic training to successfully conduct experiments involving the symbiotic N_2 fixation system of rhizobia and legumes. To accomplish this, material is presented on the microbiology of rhizobia, including enumeration, isolation, culturing, preservation, authentication, and recognition of strains. Since the main interest in rhizobia is due to their ability to symbiotically provide fixed N_2 to legumes, methods are also provided in this chapter for inoculum production, inoculation, measurement of nitrogenase activity using the C_2H_2–C_2H_4 assay, and measurement of N_2 fixation. Methods are included that are practical for most soil microbiology laboratories and of interest to many soil microbiologists. Notably missing are methods on measuring N_2 fixation by free-living rhizobia, measuring hydrogenase activity, and conducting genetic investigations. Interest in these areas was thought to be too limited to justify inclusion.

By use of the methods presented in this chapter, many scientists with little background in microbiology and agronomy may feel confident in

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working with rhizobia. However, experience has indicated this group of microorganisms is not easy to work with because of the classification method, their slow growth rate, and lack of a selective medium. Guidance from an experienced individual should be sought when possible.

49-2 CULTIVATION

49-2.1 Principles

Rhizobia are aerobic to microaerophilic bacteria requiring organic compounds as their source of C and energy and have the unique ability to form nodules on legume roots and produce nitrogenase. Optimum growth is in the 20 to 28°C range, with little growth or even death occurring at temperatures above 40°C (Vincent, 1977). Rhizobia can be divided into two groups according to their generation times on yeast extract-mannitol medium. The "fast growers" have a generation time of approximately 2 to 3 hours, whereas that of the "slow growers" is approximately 5 to 6 hours. The media used for culturing rhizobia may be defined or complex. Defined media have been used mainly in genetic and biochemical investigations, and one such medium is described by Bergersen (1961). Complex media are widely used in most other types of research and in inoculum production. A complex medium is described in section 49-2.2.

The growth medium must provide a sugar, needed inorganic nutrients, and growth factors and must be buffered against pH changes. Mannitol is a commonly used sugar, but others may be equally good or better for some strains (Graham 1976; Vincent, 1977; Kuykendall & Elkan, 1976). Yeast extract is used in complex media to provide growth factors, N, and micronutrients. Calcium carbonate is generally used to buffer against pH change, but it makes the medium cloudy because of its insolubility and results in a relatively high pH (7.8-8.3). Organic buffers have also been used (Cole & Elkan, 1973), but they are expensive for routine use.

49-2.2 Materials

The complex medium generally used for growth of rhizobia is a modification of medium 79 described by Fred and Waksman (1928). The medium is referred to as *yeast-mannitol agar* (YMA) and contains 10 g of mannitol, 0.5 g of potassium monohydrogen phosphate (K_2HPO_4), 0.2 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.1 g of sodium chloride (NaCl), 0.01 g of calcium carbonate ($CaCO_3$), 0.5 g of yeast extract powder, 15 g of agar, and 1,000 ml of distilled water. Leave the agar out to prepare *yeast extract-mannitol broth* (YMB). Adjust the pH to 7.0 with 1*N* hydrochloric acid (HCl) before autoclaving.

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49-2.3 Procedure

Combine all the ingredients of the medium recipe except the agar and $CaCO_3$ in a container large enough for the quantity of medium desired. After dissolving the ingredients, subdivide the medium to individual flasks of convenient size (500 ml), and fill each approximately half full (300 ml) with medium. Add agar (5 g) directly to the individual flasks. Rhizobia grow faster in pour plates containing 1.2% agar rather than 1.5% agar, but for the spread plate method, the firmer medium is desirable.

Add $CaCO_3$ (0.003 g) directly to individual flasks. Addition of $CaCO_3$ to the medium makes it slightly turbid but neutralizes the acids produced during growth of the rhizobia. It may be desirable to add more (up to 1 g/liter) to the medium when acid-producing strains of rhizobia (fast growers) are being grown on agar slants. If the rhizobia do not produce acid (most slow-growing rhizobia), the $CaCO_3$ may be left out of the medium because Ca in the yeast extract meets the nutritional needs. Incubate plates for rhizobial growth at temperatures between 25 and 30°C. Good growth is generally achieved at room temperature.

Inoculate YMB with a relatively young culture of rhizobia. The culture may be from a previously grown broth culture or an agar slant. Inoculate with adequate rhizobia to provide 1 to 10% of the final concentration of the quantity of cells if fast production is desired. Otherwise, it may take several days to weeks for the population to become 1×10^8 or more rhizobia per milliliter. This is due to an extended lag phase of growth and the slow growth rate of rhizobia. If a 1 to 10% inoculum is used, satisfactory growth should be achieved in 1 or 2 days or at the most 1 week even for slow-growing strains. If an agar culture older than 4 to 6 weeks is used as the inoculum directly to broth, an extended lag time of several days may occur. Therefore, it is desirable to first develop a young culture by inoculating another slant with a large inoculum. Inoculate the fresh slant with a visible quantity of inoculum on a loop if good growth is to be achieved in 1 or 2 weeks.

Rhizobial colonies on YMA may appear in 2 to 4 days for the fast-growing rhizobia and from 3 to 9 days for the slow-growing rhizobia depending on the physiological state of the rhizobia at the time of plating. Colonies appearing white to somewhat translucent, circular, and raised on the agar surface may be rhizobia. Sizeable colonies appearing 1 or 2 days after plating are not likely rhizobia. Broth cultures and plates of rhizobia should be relatively odorless except for the normal odors associated with the medium. Microscopic examination of cultures should reveal small rod-shaped organisms approximately 1 to 3 μm in length by 0.5 μm in diam. Rhizobia frequently are not uniform in size but bacteria that are distinctly cocci, spore forming, or in chains should be suspected of not being rhizobia. Cells are authenticated as rhizobia by demonstrating nodule-forming ability on a legume except in the specialized case when the strain loses its infectiveness (ability to form nodules).

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49-3 ISOLATION FROM NODULES

49-3.1 Principles

The most convenient method of obtaining rhizobia from nature is by isolation from root nodules. Contrary to popular belief, many of the bacteroids in nodules are viable (Gresshoff et al., 1977; Staphorst & Strijdom, 1972; Tsien & Schmidt, 1977). It is impractical to isolate rhizobia directly from the soil because of their fastidious growth requirements and the presence of numerous less fastidious fast-growing soil microorganisms. Nodules, on the other hand, generally contain only rhizobia. Some nodules, however, may contain more than one strain of *Rhizobium* (Lindemann et al., 1974) or may even contain other bacteria (Jansen van Rensburg & Strijdom, 1972). Nodules used for isolation should be in good physical condition to reduce the chance of bacteria other than rhizobia being present.

The portion of the nodule-containing rhizobia can be located by noting the area with the reddish pigment leghemoglobin. If the nodule is not potentially capable of fixing N_2, the bacteroid area may not be red but will likely differ in color from the remainder of the nodule. A nodule contains rhizobia within it but has many other microorganisms on its surface. These surface microorganisms must be prevented from contaminating the rhizobial portion of the nodule by surface sterilizing the nodule.

After bacteria have been isolated and are presumed to be rhizobia, they must be tested for authenticity. The only sure test is to demonstrate their ability to incite nodulation on a legume. Most often a bacterial isolate from a nodule of a particular legume would have to be tested only on the same legume. Authentication of an isolate as a rhizobial culture is absolutely essential.

49-3.2 Materials

1. Yeast-mannitol agar (YMA plates).
2. Ethyl alcohol 95%.
3. Sterile water.
4. Mercuric chloride ($HgCl_2$), 0.1% solution.
5. Nodulated root or nodules.
6. Scalpel or razor blade.
7. A straight pin or needle.

49-3.3 Procedure

Isolation is easiest from fresh nodules severed from the root about 0.5 cm on either side of the nodule with small scissors or a sharp knife. Preservation of nodules for a few weeks before isolation of rhizobia may be accomplished by keeping the nodules in capped vials in a freezer. An alternative method and a better one for many field collections is to store a few

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nodules in a vial containing a desiccant (J. C. Burton. 1980. Personal communication.). Place the nodules in a vial that contains a layer of silica gel or anhydrous $CaCl_2$ topped with a layer of cotton. The cotton separates the nodules from the desiccant. Nodules stored in this manner will dehydrate and may be kept for weeks at room temperature before isolations are made. Rehydrate the nodules for isolation by soaking them for a few minutes in sterile water.

Select firm nodules that appear in good physical condition. The nodule should be washed with water to remove all traces of soil. The surface of the nodule should then be sterilized by first rinsing in 95% ethanol and then soaking in 0.1% $HgCl_2$ or alternant sterilants for 3 to 4 min. Longer soaking may be necessary, but exercise caution because enough sterilant may be absorbed into the nodules to kill the rhizobia. Exposure time is especially critical with rehydrated nodules. Rinse the nodule repeatedly in sterile water to remove residual sterilant. Aseptically cut the nodule in half, remove a small portion of the bacterial tissue with a sterile needle, and streak for isolation on YMA plates. Plates must contain enough agar to last approximately 2 weeks without drying out. Incubating plates in plastic bags reduces drying during incubation. Incubate the plates at approximately 26°C for 1 to 2 weeks or until colonies are evident. Examine for the presence of colonies during the first 24 to 48 hours. Colonies forming in this period of time are likely not rhizobia, and their location on the plate should be marked to avoid confusing them with rhizobial colonies appearing later. To avoid contamination by actinomycetes and fungi that may develop after prolonged incubation, immediately transfer isolated colonies characteristic of rhizobia to agar slants or restreak them for isolation to determine purity.

49-4 MAINTENANCE OF CULTURES

49-4.1 Principles

Cultures of rhizobia must be maintained in a manner to ensure viability, minimize the possibility of mutations, and maintain their purity. Rhizobia may be stored in a viable state for several months on agar slants held at room temperature and protected from drying out. Most of the bacteria die during storage, but an adequate population survives for reisolation and culturing. Lowering the temperature of storage to 4°C after adequate growth has occurred greatly increases the percentage of rhizobia remaining viable and reduces the chance of mutants reproducing during storage. Some cells will remain viable for 1 year or longer if desiccation of the slant is prevented. Generally, new stock cultures should be made at least every 5 months for cultures maintained at room temperature and every year for refrigerated cultures. Screw-capped test tubes must be used for these relatively long storage periods or the slants will become too dry.

This method of storage is very simple but not highly desirable for long-term maintenance of cultures, because each time a culture must be trans-

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ferred to maintain viability, there is a chance for mutation and contamination to occur. If a culture is to be kept for more than a few years, an alternative method of storage is advisable.

Lyophilization is highly recommended for the storage of cultures for maintenance of viability and prevention of mutation (Vincent, 1970). However, many laboratories do not have the necessary equipment readily available. Where lyophilization apparatus is unavailable, a second method of drying a culture is employed. This method involves the use of porcelain beads on which rhizobia are stored in the presence of a desiccant. Storage in the dry state prevents growth during storage and extends survival (Norris, 1963). Even though the rhizobia are not dried by lyophilization, an adequate population for reisolation survives for many years even when stored at room temperature.

49-4.2 Materials

1. Rubber-lined screw-capped vials (4 by 2 cm).
2. Sterile culture tubes plugged with absorbent cotton.
3. Glass wool.
4. Unglazed porcelain beads (25,000/kg), 3 mm (McDaniel Refractory Porcelain Co., Beaver Falls, Pa.).
5. Cultures.
6. Silica gel desiccant with indicator.
7. Solution of sterile 10% maltose.
8. Sterile yeast extract-mannitol broth (YMB) (recipe in section 49-2.2).

49-4.3 Procedure

Add approximately 5 g of silica gel to each vial, place a 1-cm layer of glass wool over the desiccant, and tamp down. Place approximately 20 porcelain beads on the glass wool. Limit the number to 20 beads so that excess liquid culture can be easily absorbed by the desiccant. Cover the entire assembly with Al foil, and heat them for 1 to 2 hours at 160 to 170°C in a forced draft oven for sterilization and evaporation of moisture. Autoclave the caps separately to prevent burning of the rubber liners. After the vials and caps are sterilized, handle them aseptically to prevent contamination.

Make a heavy suspension of the culture to be preserved by adding 1 ml of sterile YMB and 1 ml of a sterile 10% maltose solution to a slant, and using a sterile loop, mix the culture with the liquid. Remove the cotton plug from an empty sterile tube, and aseptically transfer the porcelain beads from a vial to the tube. Pipette 1 ml of the culture suspension into the tube, and replace the cotton plug. Shake the tube so that the beads become saturated with the culture. Invert the tube so that the excess liquid drains into the absorbent cotton plug. Remove the plug, and pour the beads into the vial containing the desiccant. Screw the cap on tightly. As long as some of the desiccant remains absorbent (as indicated by color), the culture should remain viable for years when stored at room temperature. Prepare two vials

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of each culture. Keep one set of cultures for long-term storage and the other set as working stocks.

To grow a new culture, remove a bead aseptically, and add it to quantities of broth up to 50 ml. After incubation at 25 to 30°C, a population exceeding 1×10^8 rhizobia/ml of broth should be reached.

49-5 ENUMERATION OF RHIZOBIA IN SOIL AND INOCULANTS

49-5.1 Principles

Enumeration of rhizobia in soil by routine plating procedures generally is not possible because rhizobia grow relatively slowly and many more numerous soil microorganisms grow rapidly on YMA. However, with inoculants, if the rhizobial population is large relative to that of the other microorganisms present, dilution may make enumeration by plating possible.

The most probable number (MPN) method described in Chapter 39 is generally used to enumerate rhizobia in the presence of large numbers of other microorganisms. Soil or other materials containing rhizobia is diluted in a liquid, and dilutions are used to inoculate legume roots. This method was used to measure rhizobial populations by Wilson (1926). The presence of rhizobia in a given experimental unit, inoculated by a given dilution, is indicated by the formation of at least one nodule on a root. Obviously, only rhizobia capable of nodulating the host legume will be enumerated. The MPN method is widely used, and techniques have been described by Date and Vincent (1962), Brockwell (1963), Weaver and Frederick (1972), and Brockwell et al. (1975).

The method requires the growth of legumes under conditions such that a single rhizobial cell can multiply and form at least one nodule on the root. This assumption is not always valid and must be checked for each legume and system utilized (Boonkerd & Weaver, 1982). Brockwell (1963) and Brockwell et al. (1975) suggest that vermiculite as the support medium may lead to underestimation of the population but that mathematical procedures may be used to correct for this (Grassia & Brockwell, 1978). Thompson and Vincent (1967) reported that low rhizobial populations may be underestimated due to the large amount of soil present in low dilutions. The bacteria must become dispersed in the diluent for accurate results. Rhizobia in peat inoculants easily desorb and become dispersed in the diluent (Weaver, 1979).

49-5.2 Materials

1. Disposable plastic growth pouches (American Scientific Products, Div. of American Hospital Supply Corp., McGaw Park, Ill.).
2. Pouch holders (record racks are convenient).

Table 49-1. Nutrient solutions for growing plants with and without N.†

Stock solution	Chemical	Concentration	Quantity of stock used per liter
Without N			
1	KH_2PO_4	34 g/liter	1 ml
2	$MgSO_4 \cdot 7H_2O$	123 g/liter	1 ml
3	K_2SO_4	65 g/liter	1 ml
4	$CaSO_4 \cdot 2H_2O$		0.1 g
5‡	$FeCl_3 \cdot 6H_2O$	1.4 g/liter	1 ml
	Na_2H_2EDTA	1.7 g/liter	
6	KCl	0.75 g/liter	1 ml
	H_3BO_3	124 mg/liter	
	$MnSO_4 \cdot H_2O$	67 mg/liter	
	$ZnSO_4 \cdot 7H_2O$	46 mg/liter	
	$CuSO_4 \cdot 5H_2O$	10 mg/liter	
	H_2MoO_4 (88% MoO_3)	[illegible] mg/liter	
With N			
3A§	KNO_3	[illegible] g/liter	1 ml
	$(NH_4)_2SO_4$	133 g/liter	

† The nutrient solution is a modification of that of Munns (1968). Adjust to pH 6.5 by adding 1*N* KOH.
‡ Dissolve the two chemicals separately in 500 ml of water before combining.
§ For complete nutrient solution, substitute solution 3A for 3.

3. Legume seed.
4. Nitrogen-free nutrient solution (Table 49-1).
5. Drinking straws.
6. Ethanol solution, 70%; saturated solution of calcium hypochlorite [$Ca(OCl)_2$]; or a 10% mixture in water of a commercial bleach composed of 5% sodium hypochlorite (NaOCl).
7. Dilution blanks (100 ml) made from the plant nutrient solution.
8. Culture of rhizobia.
9. Samples with rhizobial populations to be enumerated

49-5.3 Procedure

All materials must be rhizobia free, and extreme care must be taken to prevent contamination of plant growth units and to ensure that airborne rhizobia do not fall into the containers. Commercial plastic pouches for growth of plants are 16 cm wide by 17 cm high and are provided with a paper wick (Porter et al., 1966). Economy of space and equipment may be affected by dividing the pouches into two compartments for large-seeded legumes and four compartments for small-seeded legumes. Remove the paper wicks, and use a plastic heat sealer to divide the pouch. Vertically cut the wicks to the appropriate size, and insert them into the new compartments. The wicks should not extend to the top of the pouches because it increases the chance for contamination and evaporative loss of water. Pouches have been rhizobia free when they arrive from the manufacturer. Place the pouches in a record rack to hold them while seeding, inoculating, and growing seedlings (Fig. 49-1).

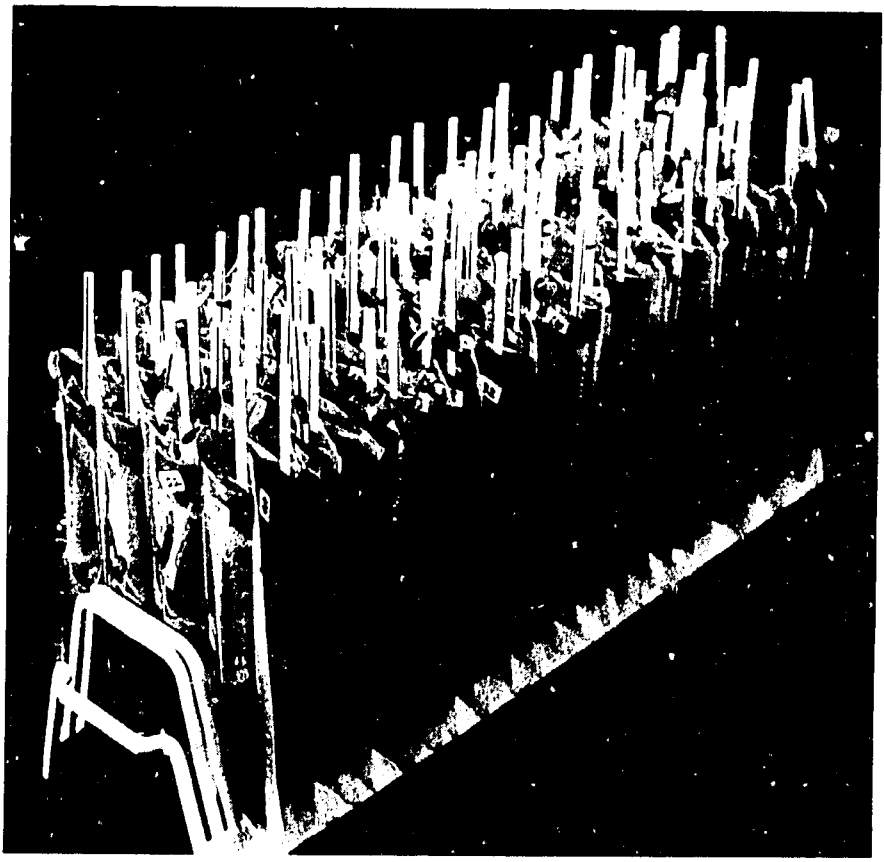

Fig. 49-1. Arrangement of divided plastic pouches used in making MPN counts of rhizobia.

Kill the rhizobia on the seed surface by soaking the seed in one of the bactericidal solutions for 2 min. Rinse the rhizobia-free seed with ample amounts of water to remove any residual bactericide. Use high-quality seeds, because the moist conditions in the trough of the pouches encourage the development of fungi. Viability of planted seeds is ensured by germinating large seeded legumes before placing them in the pouches. Place one or two seeds of large-seeded legumes in each trough; however, several seeds of small-seeded legumes may be used.

Peanuts (*Arachis hypogaea* L.) do not perform well in the pouches unless the roots are shielded from light. The pouches may be made opaque by spraying them with black paint or covering them with Al foil. This makes the use of pouches less practical for enumeration of peanut rhizobia. It may be more practical to use the plant growth units described in section 49-6.3 for use in effectiveness tests.

Plastic drinking straws greatly facilitate watering and help prevent contamination of pouches. Cut the straws so that they extend half way down the pouch and 1 or 2 cm above the pouch. Use great care during the addition of the nutrient solution so that the pouches are not contaminated by splash-

ing or transfer of rhizobia via the tip of the watering device. Add approximately 50 ml of N-free nutrient solution to each individual pouch. Add proportionately less to each compartment. Make certain the nutrient solution is rhizobia free.

Include both positive and negative controls. The positive controls consist of plants inoculated with a culture of rhizobia to be certain plant growth conditions are appropriate for nodulation. Negative controls are pouches that are not inoculated and serve to indicate if cross contamination occurs during plant growth.

When seedlings are established, inoculate by adding 1 ml of the appropriate serial dilution onto the root system within the pouch. This method ensures that healthy seedlings are present for the test. Do not use containers with poor germination.

Grow the plants for approximately 1 week after nodules are observed on the positive controls. Generally 2 weeks after inoculation is adequate for small-seeded legumes and 3 weeks for large-seeded legumes. Record each container as positive (one or more nodules present) or negative (no nodules present).

The MPN of rhizobia in a sample is computed by procedures outlined by Cochran (1950) and Finney (1947). Alternatively for a particular dilution series and number of replications per dilution, statistical tables have been prepared. Alexander (1965) in Chapter 39 provides tables for a 10-fold dilution series inoculating five replications per dilution. Brockwell (1963; Brockwell et al., 1975) provides tables for employing a fivefold dilution series and inoculating four replications per dilution. Brockwell (1963; Brockwell et al., 1975) also provides tables for a 10-fold dilution series and inoculating three replications per dilution. Narrowing the dilution series and increasing the number of replications per dilution decreases the confidence limit on the MPN (Table 39-2).

49-6 NITROGEN FIXATION

49-6.1 Principles

The agronomic interest in rhizobia is due to the N_2-fixing ability (effectiveness) of these bacteria. Their effectiveness is generally measured by growing plants in a N-free medium and measuring the total N in the plants. The more sophisticated procedure of exposing plants to ^{15}N-labeled N_2 gas and measuring the incorporation of ^{15}N into the plant also may be utilized. Methods for using ^{15}N will not be described here. Direct measurements on nitrogenase activity may be achieved by using the rate of C_2H_2 reduction to C_2H_4, but this does not directly measure N_2 fixation by the plant. Methods for using C_2H_2 reduction are described in section 49-7.

In testing for effectiveness of a rhizobial strain or its host plant, plants are grown in virtually N-free material. All other nutrients required for plant growth must be supplied by the system. Conditions must be maintained that

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prevent contamination between containers or from outside sources. Subirrigation systems are used to prevent surface contaminants from being washed to the roots. Leonard (1943) developed a system and used it extensively in evaluating effectiveness of legume inoculants. The period of time plants are to be grown and the type of plant (large seeded vs. small seeded) determines the type of container to be used. For short-term growth and small-seeded legumes, culture tubes or plastic pouches may be adequate, but for large-seeded legumes, larger pots are necessary.

Effectiveness in N_2 fixation is used only in a comparative sense. Comparisons can be made between rhizobial strains or between nodulated plants and nodule-free plants supplied with N fertilizer. A fertilizer N standard should be included so that the results of experiments run at different times under similar conditions can be compared. When N fertilizer is used, the time of application and the length of the experiment must be standardized to overcome changes in relative differences. Nitrogen-fertilized plants generally will yield more than nodulated plants during the first few weeks of growth, because it takes some time for the symbiotic system to be initiated.

49-6.2 Materials

1. High-quality legume seeds (record cultivar).
2. Cultures of *Rhizobium* (record strain number).
3. Chemicals for nutrient solutions: potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), potassium sulfate (K_2SO_4), calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$), ethylenediaminetetraacetic acid disodium salt (EDTA disodium), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), potassium chloride (KCl), boric acid (H_3BO_3), manganous sulfate monohydrate ($MnSO_4 \cdot H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), and ammonium sulfate [$(NH_4)_2SO_4$].
4. Plastic specimen containers (1 liter) with lids (American Scientific Products, Div. of American Hospital Supply Corp., McGaw Park, Ill.).
5. Rolls of absorbent cotton for wicks.
6. Nitrogen-free support medium (sand or vermiculite).
7. Calcium hypochlorite [$Ca(OCl)_2$] or ethanol.

49-6.3 Procedure

All materials must be rhizobia free, but they need not be sterile. Unless contaminated by dust, vermiculite is rhizobia free when obtained from the factory, as are the chemicals, the new containers, and tap or distilled water. Used or contaminated containers require a soapy wash, followed by a rinse with a 5% solution of a commercial household bleach to kill any residual rhizobia. Follow the bleach treatment with a rinse with water to remove all traces of the bleach. Containers may be either plastic or glass, but the following method describes the use of plastic because they are more convenient.

Fig. 49-2. Cutaway of modified Leonard jars used in growing legumes for evaluation of rhizobial effectiveness.

Two 1-liter plastic containers are required for each growth unit (Fig. 49-2). One container serves to hold the plant nutrient solution, and the other holds the rooting medium. Cut a hole approximately 2 cm in diam in the center of a lid and the bottom of one of the pots. Cut a hole about 6mm in diam at the outer edge of the lid. Fit the lid onto the container holding the nutrient solution. Make a cotton wick by cutting a 20-cm long strip of absorbent cotton that is wide enough to snugly fit through the holes in the container and lid. The wick will serve to draw nutrient solution into the rooting medium. It should extend from the bottom of the nutrient solution up to approximately 6 cm into the pot containing the plants. Set the upper container on the lid of the nutrient reservoir. Snap the lid on the lower container so that it fits snugly to prevent cross-contamination of rhizobia from other pots. Fit a piece of Tygon tubing into the smaller hole of the lid for replenishment of nutrients. Place a cap over the tubing to prevent contamination. After assembling the two containers, fill the top container with potting material. Vermiculite, perlite, or moderately coarse sand perform well, because they have adequate capillaries for supplying water while remaining well aerated. Hold the cotton wick upright while adding the potting material so that the wick extends into the potting material. Fill the container to within 2 cm of the top. Prepare the N-free nutrient solution according to the

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composition provided in Table 49-1. The nutrient solution made as described in Table 49-1 is modified so the concentration of Mg is doubled and all other macronutrients are reduced to one-fourth the concentrations in the original solution (Munns, 1968). Use of Munns (1968) solution at one-half strength resulted in salt toxicity symptoms on cowpeas (*Vigna unguiculata* L.) and peanuts grown for 6 weeks. Cowpeas indicated symptoms typical of Mg deficiency.

Moisten the potting mixture by pouring nutrient solution onto its surface. After this initial wetting, water and nutrients are supplied by capillary rise through the cotton wick. Fill the reservoir to within 2 cm of the top. A manifold system may be used to facilitate watering (Weaver, 1975).

Plant the seed carefully so they are all at equal depth and equally spaced from other seeds. Plant four to six seeds/pot for large-seeded legumes and 20 to 30 for small-seeded legumes. After planting, inoculate the seeds by dribbling a suspension of inoculum onto them. Use the plant nutrient solution to dilute the inoculum to the desired concentration. Cover the surface of the pot with a layer of pea-size (5 to 10 mm diam) gravel to aid in reducing loss of water by evaporation and to prevent contaminating rhizobia from reaching moist potting mix and possibly plant roots. When seedlings emerge, thin to two seedlings for large-seeded plants and approximately 10 for small-seeded plants in each pot. All pots in a trial must contain the same number of plants, otherwise interplant competition affects results. Attempt to keep plants that are healthy and that emerge over the same 24-hour period.

Grow the plants under conditions that provide temperature and light regimes suitable for good growth and nodulation of the legume. Nodulation is more sensitive to elevated temperatures than many other plant growth processes. Grow the legumes at least 1 month. Often 6 weeks or longer is required to obtain good effectiveness differentiation between strains. Removal of cotyledons after the first true leaves appear enhances the differences obtained with early harvests of large-seeded legumes. Small seeds have very little N reserves and generally nodulate more rapidly than large-seeded legumes. Consequently, they do not have to be grown as long to differentiate between strains. Dry the plant tops at 65°C immediately after harvest for dry weight and N determinations. Root and nodule measurements may also be made at this time.

49-7 ACETYLENE REDUCTION

49-7.1 Principles

The discovery by Dilworth (1966) and Schollhorn and Burris (1966) that the nitrogenase enzyme that fixes N_2 also reduces C_2H_2 to C_2H_4 resulted in development of a widely used method of indirectly estimating N_2 fixation rates. The method is frequently referred to as the *acetylene reduction method* (AR). Burris (1975) discusses in detail the early history in the develop-

ment of AR, which is not covered here. Acetylene reduction is a quantitative measure of nitrogenase activity, not N_2 fixation. For quantitative estimates of N_2 fixation, measurements of $^{15}N_2$ incorporation should be made.

Acetylene reduction has appeal as a research tool because of its sensitivity and ease of use. The C_2H_2 is reduced to C_2H_4 in the nodule material, and the C_2H_4 is released into the incubation vessel. Detection of the C_2H_4 is usually accomplished by gas chromatography using a H flame ionization detector. Nanomole quantities of C_2H_4 are readily detectable. Thus, exposure of a single small nodule to C_2H_2 in a closed system for just a few minutes results in easily detectable quantities of C_2H_4 being produced if the nodule has an active N_2-fixing system.

Two electrons are required to reduce 1 mol of C_2H_2 to C_2H_4, whereas six electrons are required to reduce 1 mol of N_2 to $2NH_3$. The theoretical conversion factor of 3 mol of C_2H_4 produced/mol of N_2 reduced is commonly used to relate AR to N fixed. This factor frequently is not very realistic. The conversion factor has been reported to range between 1.5 and 8.4 mol of C_2H_2 reduced/mol of N_2 fixed (Bergersen, 1970; Hardy et al., 1973). Reasons for this wide range may be due to differential solubility of C_2H_2 and N_2 in water; C_2H_4 is released from nodules but NH_3 is assimilated, and ATP-dependent H_2 evolution does not occur during AR but does occur during N_2 reduction (Burris, 1974).

Perhaps the main limitations to relating AR to N_2 fixation occurring in situ are due to the destructive and short-term nature of the assay system. Generally, plants are dug to obtain nodulated roots, and the roots are exposed to C_2H_2 for 30 min to 1 hour. Removal of plant tops from roots decreases AR (Mague & Burris, 1972; Mederski & Streeter, 1977), and removal of nodules from roots further diminishes AR even during short incubations (Mague & Burris, 1972; Fishbeck et al., 1973). Dinitrogen fixation by legumes occurs over most of the growing season, and the rate of N_2 fixation fluctuates during a single day (Sloger et al., 1975). To quantitatively determine cumulative N_2 over a period of time, one must obtain and integrate many measurements (Sloger et al., 1975). Generally, this is not done because of the effort required, and therefore the data obtained from a few AR analyses must be considered qualitative.

In spite of the many limitations in relating AR to the quantity of N_2 fixed, it is a very useful assay. It is particularly useful in evaluating relative effects of short-term treatments on N_2 fixation and as a screening tool in qualitatively measuring for N_2 fixation. Plants in pots can be assayed regularly to determine changes in AR during growth and development of the plant.

There are several published procedures that have been used for AR. Sprent (1969) describes a method for measuring AR on detached soybean (*Glycine max* L. Merr.) nodules. Hardy et al. (1968) provides the earliest comprehensive description of using the AR method in the field and laboratory. Hardy et al. (1973) expand on their earlier description of AR in a comprehensive review article that covers precautions in using AR. Mederski and Streeter (1977) and Fishbeck et al. (1973) describe systems for AR on intact

plants grown in containers. Balandreau and Dommergues (1973) describe an in situ method for field measurement of AR using propane as an internal standard to determine soil volume sampled. Schwinghammer et al. (1970) compare AR to other methods in evaluating N_2 fixation. Burris (1972; 1974) provides a general description for using AR in estimating N_2 fixation. The following procedure is one that is used in many laboratories.

49-7.2 Materials

1. Calcium carbide (CaC_2), available from sporting goods stores.
2. Ethylene (C_2H_4) gas for standards.
3. Gas chromatograph equipped with H flame ionization detector.
4. Poropak Q column, 1.5 m long and 32 mm in diam (Waters Associates, Framingham, Mass.).
5. Widemouth canning jars, 1 pint (4.7 dl).
6. Rubber septa, 5.5 mm diam.
7. Plastic heat sealable anaerobags (Cednaco, Division of H.P.B., Inc., Hudson, Mass.).
8. Plastic syringes (50 ml) with no. 23 gauge needles.
9. Plastic syringes (1 ml) with no. 23 gauge needles.
10. Vacutainers (Becton, Dickinson and Co., BBL Microbiology Systems, Cockeysville, Md.) for storage of gas samples.
11. Nodulated roots.
12. Duct tape.
13. Automatic bag sealer for sealing plastic bags.
14. Size 000 rubber stoppers.

49-7.3 Procedure

Generate C_2H_2 by sealing with a plastic bag heat sealer 10 g of CaC_2 in a 2-liter anaerobag equipped with a gas sampling port. Size can be varied according to the amount of gas needed. Make the gas port by cutting a 2-mm slice of rubber off a size 000 rubber stopper, and seal it to the plastic bag with a 5- by 4-cm piece of duct tape. Place the rubber slice in the middle of the tape, then press the tape firmly to the bag, making certain no channels remain for gasses to escape. Remove the air from the bag by placing a no. 23 gauge needle through the sampling port, and flatten the bag by pressing on it. Generate C_2H_2 by injecting small quantities of water into the bag to react with the CaC_2. If too much water is injected, the bag will pressurize and burst. Each milliliter of water injected will generate approximately 170 ml of C_2H_2 as long as CaC_2 is present. The other products produced are $Ca(OH)_2$ and trace amounts of C_2H_4. High-purity C_2H_2 is produced by this method, because the commercially compressed C_2H_2 in cylinders is dissolved in acetone. Burris (1975) suggests that the acetone in the C_2H_2 does not pose a problem for the assay and that it may be removed if desired by passing the gas through a dry ice freezing trap or through concentrated H_2SO_4.

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A widemouth canning jar fitted with a rubber septum in the lid is used to hold the root to be assayed. The capacity of the jar should be such that O_2 depletion over the incubation time does not limit the reaction. A pint jar is usually adequate to hold a well-nodulated soybean root during a 1-hour incubation. Mague and Burris (1972) suggest that the roots and nodules should occupy <20% of the space. Tighten the lid of the jar, and remove approximately 50 ml of air with a hypodermic needle and syringe through the rubber septum. Inject 50 ml of C_2H_2 into the jar. Replacing air with a mixture of Ar and O_2 does not increase AR (Mague & Burris, 1972; Burris, 1972). Washing intact nodules on roots with water before assay does not reduce AR (van Straten & Schmidt, 1975), and blotting washed nodules dry before assay does not reduce AR (Mague & Burris, 1972). Incubate the sample for 30 min at a temperature approximating the environment the nodules were in before sampling. The rate will likely be linear for 60 to 90 min. Establish linearity over time for your assay system.

Remove a sample of gas for analyses, and store in Vacutainer tubes under pressure. Generally, a volume of gas 1.5 times the volume of the Vacutainer may be added without danger of the septums popping out. The size of the container is dependent on the quantity of gas to be injected into the gas chromatograph. Add enough sample to Vacutainers to pressurize them. The sample may be stored in vacutainers for several days without adsorption of C_2H_4. Before adding the sample to Vacutainers, test them to be certain they are evacuated. Small Vacutainers may be adequately evacuated with a 50-ml hypodermic needle and syringe to be certain they are empty. Plastic syringes have not been dependable for using as gas sample containers because of C_2H_4 loss.

Calibrate the gas chromatograph over the range of C_2H_4 concentrations expected in the samples. Ethylene concentrations between 10 and 1,000 ppm should cover the range. Include controls for measuring the background concentration of C_2H_4 in the C_2H_2. Generally, H flame ionization detectors are linear over the range of C_2H_4 concentrations encountered for nodulating legumes.

49-8 INOCULATION OF SEED AND FIELD EXPERIMENTATION

49-8.1 Principles

Practical investigations with rhizobia frequently culminate with field experiments. Usually the objective of field experimentation is to determine the effect of inoculation of some plant parameter, i.e., the percentage of nodules formed by an effective strain, nodule location, nodule mass, total N in the plant, and seed yield. Basic statistical methods and field plot designs may be used, but the investigator should include particular treatments to aid in the interpretation of results. Many of the parameters measured are quite variable, thus requiring the experimental design to provide for at least four replications.

Control treatments should be included. An uninoculated treatment indicates the effectiveness of indigenous rhizobia and, if desired, to provide nodules for characterization of their serological grouping. A second control treatment involves the application of fertilizer N to show if fixed N_2 limited yield. This treatment may not be practical for legume-grass mixtures, because use of fertilizer N can change the relative growth of the grass and the legume in the mixture.

Another aspect of field inoculation is the concern over the use of border rows to prevent cross-contamination of rhizobia between plots. If soil is not moved mechanically by some means between plots, cross-contamination is not likely. For row crops, even adjacent rows will probably not be cross-contaminated during the growing season. However, border rows may be desired for the same environmental reasons they are used in fertility and plant breeding trials and because soil movement by water or wind is often difficult to control.

Soil used in inoculation trials should be characterized as to its fertility status by standard soil tests and the population size of indigenous rhizobia determined. Such data greatly aids in interpretation of results and facilitates comparison of results from various locations. The population size of the inoculum supplied to the seeds should also be measured. This is analagous to the need to know how much fertilizer is applied when conducting fertilizer investigations. The method of inoculation must be considered for field trials. Many methods are available, but practical use depends on the type of planting equipment. The method of inoculation must provide viable bacteria to infect the plant when the root becomes susceptible. For row crops, the addition of the inoculum directly to the furrow is desirable, but for forage legumes, an adhesive used to adhere the inoculum directly to the seed may be preferred.

49-8.2 Materials

1. Legume seed.
2. Inoculant.
3. Adhesive (gum arabic or sucrose).
4. Calcium carbonate ($CaCO_3$).

49-8.3 Procedures

49-8.3.1 SMALL-SEEDED LEGUMES

Use a commercial inoculant or one prepared in the laboratory. Determine the population of rhizobia initially to properly interpret field results. Commercial inoculants are readily available and easy to use, but the population of rhizobia may vary considerably.

To prepare an inoculant in the laboratory, obtain the peat from an inoculant manufacturer to ensure suitability for survival of rhizobia. Adjust the pH to 7.0 by the addition of $CaCO_3$. Autoclave the peat, and inoculate with a culture of rhizobia to achieve an initial population in excess of 100

million/g of peat. Aseptically mix approximately equal parts, by weight, of liquid culture and air-dry peat. Peat should be moist (0.3 bar tension) after inoculation but not saturated. Store in sterile plastic bags or another type of container that provides for some aeration (Roughley, 1976), incubate for several days at room temperature, and then refrigerate at 4°C or lower until needed. Use the peat, in conjunction with an adhesive available through inoculant companies at the rate recommended on the package or at a higher rate. The adhesive greatly extends the survival of the rhizobia (Burton, 1976) and keeps the inoculum attached to the seed during broadcasting.

Dissolve the adhesive to be used for inoculation in warm water. A 40% solution of neutralized gum arabic base adhesive is recommended. Gum arabic should be supplied by an inoculant manufacturer or from a source where no preservative is used. A 25% solution of sucrose may be substituted, but it may not provide the adhesion or the protection of gum arabic. Wet the seed with the adhesive solution at a rate of approximately 20 ml/kg of seed, and mix thoroughly. More or less adhesive may be used depending on the seed size. Add the inoculum to the seed at a rate of 10 g of peat/kg of seed. Peat may be added at rates up to 10 to 30% of seed weight depending on seed size. Dry the seed by spreading it out in a cool place, or pellet it with 200 g of $CaCO_3$/kg of seed if sowing in acid soil. To pellet the seed, thoroughly mix the seed and $CaCO_3$. Screen off the excess lime after pelleting. [Do not use slaked lime. It is $Ca(OH)_2$ and will kill the seed and rhizobia.] Sow the inoculated seed as soon as possible. Do not store moistened seed, or seed viability may be reduced. The rhizobia should survive fairly well for a few days at the temperature in an air-conditioned room.

Use a plot size that is suitable to accomplish the objectives of the experiment. A randomized complete block design with five or more blocks is often suitable for field experiments. Generally, plots of 2 m by 4 m provide ample area for taking samples during the growing season without disturbing the plot area used for total forage production. A 1-m strip through the length of the plot may be used for sampling, because this area will not be under the influence of the adjacent plots. The ends (0.5 m) of the strip should not be included in the sample due to border effects. Depending on the uniformity of the experimental area, a 1-m^2 area may provide adequate sample for estimating forage production, thus leaving 2 m^2 for sampling roots for observation of nodulation.

Measurements for evaluating treatment effects include examination of roots for nodulation. Enough of the root mass of annual legumes may be easily dug with a spade to make observations on nodulation. Most of the early nodulation occurs in the top 10 cm of soil in a radius of 5 cm around the stem base. Munns et al. (1977) observed most alfalfa (*Medicago sativa* L.) nodules in an established plot to be below 10 cm. Exercise care in digging samples and cleaning soil from the roots, because nodules break from the root rather easily and may be lost. It may be necessary to soak the roots and soil in water for a few minutes to hours before cleaning the roots with a stream of water. Make measurements early in the season, because

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differences may disappear later (Thompson et al., 1974; Waggoner et al., 1979), and a record of the position of nodules in relation to the crown of the plant and the tap root should be included. Often it is adequate to simply classify the nodules as being on the upper tap root or the lower tap and lateral roots. Frequently, visual ratings of nodulation will be adequate for evaluating the treatments. Pick the nodules from the roots for weight determinations and identification of rhizobia by serological or fluorescent antibody procedures. The coefficient of variability for nodule mass and number measurements often is >50%. When using a rating system, report approximate nodule weights and numbers for each rating.

49-8.3.2 LARGE-SEEDED LEGUMES

The method of inoculant application outlined in section 49-7.3 may be modified for use with large-seeded legumes. Reduce the quantity of adhesive used by 80% due to less surface area per unit weight of large seed, and eliminate the lime coating. This method should not be used, however, if fungicides are applied, because close contact with rhizobia may be toxic to the bacteria (Ruhloff & Burton, 1951; Curley & Burton, 1975). The seed coat of peanuts is fragile and wetting and stirring to mix the inoculant can decrease seed viability.

An alternative method used to inoculate row crops is the addition of inoculum in liquid or granular form directly into the seed furrow (Weaver & Frederick, 1974; Boonkerd et al., 1978). This method protects the rhizobia from fungicides and prevents wetting the seed with the inoculant.

Use a randomized complete block design with at least five blocks for the experimental design. The size of individual plots may vary depending on the objective of the experiment. Harvest at least 2 m of row for seed yield and preferably 4 m. Provide a border row on either side of the yield row to reduce the influence of treatments on adjacent plots. Provide adequate plot size for obtaining samples during the growing season when rates of plant growth, N accumulation, C_2H_2 reduction, or nodule mass are to be measured. See section 49-7.3 for discussion on nodulation measurements. Obtain the plants from a continuous section of row rather than from throughout the plot. End trim the first few plants before obtaining a sample. Use single row plots if seed yield is the only component to be measured, if inoculation treatments are the only treatments imposed, and if small (10-20%) treatment effects are expected. Larger plots should be included for the N treatment, because border rows must be used. Attach a tube behind the furrow opener and to the reservoir designed to hold the inoculum. Add inoculum to the seed furrow by using gravity flow. The inoculum should dribble into the furrow with the seed or within 1 to 2 cm below it. The rate of inoculum application depends on the quality of the inoculant and the distribution system. Vary the rate of application by the speed of planting, the size of the orifice from the inoculant reservoir, and the concentration of rhizobia in the inoculant. Provide at least 10,000 viable rhizobia per seed by applying approximately 20 ml of liquid inoculant or ap-

proximately 1 g of granular inoculant per meter of row; more or less inoculum may be used depending on the distribution system.

Granular inoculant may be purchased from a commercial supplier or prepared in the same manner as the peat inoculant in section 49-7.3. Purchase a commercial granular inoculant for the source of peat. The pH of the inoculant will already be satisfactory. Autoclaving the inoculant will kill any rhizobia present. Air-dry the granular inoculant just before use for a few minutes at a cool temperature so that individual particles do not stick together during application.

49-9 STRAIN RECOGNITION

49-9.1 Serology

49-9.1.1 PRINCIPLES

Identification of individual strains of rhizobia by morphological differences is not possible; therefore, other methods must be utilized. Among the useful methods, one relies on the antigenic properties of bacterial cells, and another (discussed in section 49-9.2) utilizes antibiotic markers. The immune system of animals produce antibodies that are specific for particular antigens. If the antigens of two strains are different, the antibodies produced by an animal in response to antigens in their bloodstream will also be different. Because the reaction between antibodies and antigens can be observed, a means of identifying and recognizing strains or groups of rhizobial strains is provided.

Three types of antigen-antibody reactions have been widely used in identifying specific strains of rhizobia: agglutination, immune diffusion, and fluorescent antibody reactions. The agglutination reaction occurs between particulate antigens and antibodies and is recognized by clumping of cells. The immune diffusion reaction occurs between soluble antigens and antibodies. It is recognized by precipitate bands forming in agar where different diffusing antigens and antibodies meet at optimal concentrations. The fluorescent antibody reaction, as commonly used, occurs between particulate antigens and antibodies conjugated with fluorescent dyes. The reaction is recognized by use of a microscope equipped with an ultraviolet light source and special filters. It has been very useful in investigating the autecology of rhizobia in soil (Schmidt, 1974) but requires relatively expensive and specialized equipment.

Immune diffusion methods require more skill than the agglutination reaction methods, and more labor and time are required. The time required for the reaction to reach completion generally exceeds 24 hours. The immune diffusion reaction makes it possible to readily distinguish between strains that have some common antigens (Dudman, 1977; Vincent, 1970).

The procedure described in the next section utilizes the agglutination reaction. The reaction occurs between insoluble antigens and polyvalent antibodies. The antibodies bridge between the particulate antigens sus-

pended in a liquid resulting in a visible precipitate in the bottom of the reaction vessel within 30 min to a few hours. Two groups of antigens may be involved: those associated with cell walls and those associated with flagellae. Cell wall antigens are likely to be less cross-reactive, but generally either antigen type is specific enough for the strains being investigated. The agglutination reaction may be utilized in such a way that hundreds of soybean nodules may be serotyped (process of identification using antiserum containing antibodies) in a single day (Means et al., 1964). The rhizobia in nodules may be serotyped directly, or if inadequate amounts of bacterial tissue are present, they may be cultured before being serotyped. Using microagglutination trays, Parker and Grove (1970) were able to serotype small nodules directly.

49-9.1.2 MATERIALS

1. Antiserum.
2. Nodules.
3. Dilution tubes containing 0.85% sodium chloride (NaCl).
4. Microagglutination trays or test tubes.
5. Pasteur pipettes.
6. Water bath.

49-9.1.3 PROCEDURE

Details of antiserum production have been provided by Vincent (1941, 1970). Utilize the serum of the blood as the antiserum without separation of the gamma globulin fraction. Determine the agglutination titer of the antiserum as described by Vincent (1941, 1970). Pool all homologous antisera (antisera against the same rhizobia strain) from different rabbits, and divide into 1-ml vials for cold storage. Keep one vial stored at 4°C, and store the remaining tightly capped vials in a standard freezer at about −20° or in the freezer compartment of a refrigerator. The antisera should remain useful for years when stored in the frozen state. Repeated freezing and thawing of antisera may reduce the titer. Refrigerate the working vial of antiserum when it is not being used. The titer should remain high for several days at refrigeration temperatures.

Prepare the antigen for reaction by diluting it with 0.85% NaCl to a cell concentration of approximately 3×10^8/ml. The suspension should be very cloudy but not opaque. The antigen may be a culture of rhizobia from an agar slant, a broth, or a nodule. Directly serotype nodules from soybeans and cowpeas. Place a single washed nodule in a 5-ml flat bottom vial, and add 2 ml of 0.85% NaCl. Crush and macerate the nodule with a flat-bottom rod. Glass rods may be used, but there is the danger of breaking while in use. Dilute the antigen to the desired concentration by adding 0.85% NaCl. Heat the antigens for 30 min at 100°C in a hot water bath or in an autoclave to destroy the flagellar antigens. The somatic antigens are generally the least cross-reactive.

Dispense the antigens into the reaction vessels. Microtiter plates are

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convenient, and because of the small wells (0.25 ml), they allow relatively small nodules to be serotyped (Damirgi et al., 1967). Add 0.2 ml of the antigen to each well. The number of wells filled should be one more than the number of antisera to be used to provide for a control to determine if the antigen autoagglutinates. Add approximately 0.05 ml of antiserum diluted 1:20 with 0.85% NaCl to each well to receive antisera, and add 0.05 ml of 0.85% NaCl to the control well. The test is qualitative; therefore, the quantities of antigen and antiserum do not have to be measured accurately. Small test tubes of 1-ml capacity may be used by doubling the quantity of antigen and antiserum suggested for microtiter plates. Test tubes of 10-ml capacity may require 2 to 4 ml of antigen and 0.5 to 1 ml of the diluted antiserum.

Incubate the samples in a water bath at temperatures between 37 and 55°C. The warmer the temperatures, the faster the reaction. At the higher temperature, the reaction may be complete in 30 min to 2 hours, but at the lower temperatures, 4 to 12 hours may be required. Cover the microtiter plates with a lid so that the wells do not dry out during incubation.

Observe the trays after 30 min of incubation at 55°C and again after 2 hours to be certain the reactions are complete. Make the first observation at 2 hours when using lower incubation temperatures. Use a source of light that provides diffuse illumination for observing the results. Positive (+) reactions appear as a compact granular or diffuse fiber of agglutinated cells covering the bottom of the wells (Fig. 49-3). The edges of the film may be either folded or somewhat ragged, and the supernatant may be clear. Negative (−) reactions are exemplified by turbid solutions and absence of a film in the bottom of the wells. There may be a compact accumulation of some cells in the center of the well, but they should be easily resuspended by gentle tapping of the tray. Weak reactions (+ −) may be due to weakly cross-reactive strains of rhizobia, improper concentrations of antigen and antibody, or a "weak" antigen preparation.

In case of cross-reactions between strains of rhizobia, the antiserum may be reacted with the heterologous strain (the strain the antiserum was not prepared against) to remove common antibodies. To accomplish this, dilute the antiserum approximately 1:20 using 0.85% NaCl, and combine with a heavy cell suspension of the heterologous rhizobia. The concentration of the cells in the diluted antiserum should be in excess of 1×10^9 ml. React the serum and cells overnight in a refrigerator, and centrifuge to remove the cells. Repeat the procedure if the antiserum still reacts with the heterologous strain. Make certain the serum is capable of reacting with the homologous strain (the strain the antiserum was prepared against).

Isolate and culture rhizobia from small nodules and peanut nodules (section 49-3) for preparation of enough antigen for testing by the agglutination reaction. There may be adequate antigen in small soybean nodules and large clover and alfalfa nodules for filling three microagglutination wells for testing with two antisera and having a control, but enough antigen is not available to test against several antisera. Peanut nodules are large enough for serotyping, but the bacteroids agglutinate poorly perhaps

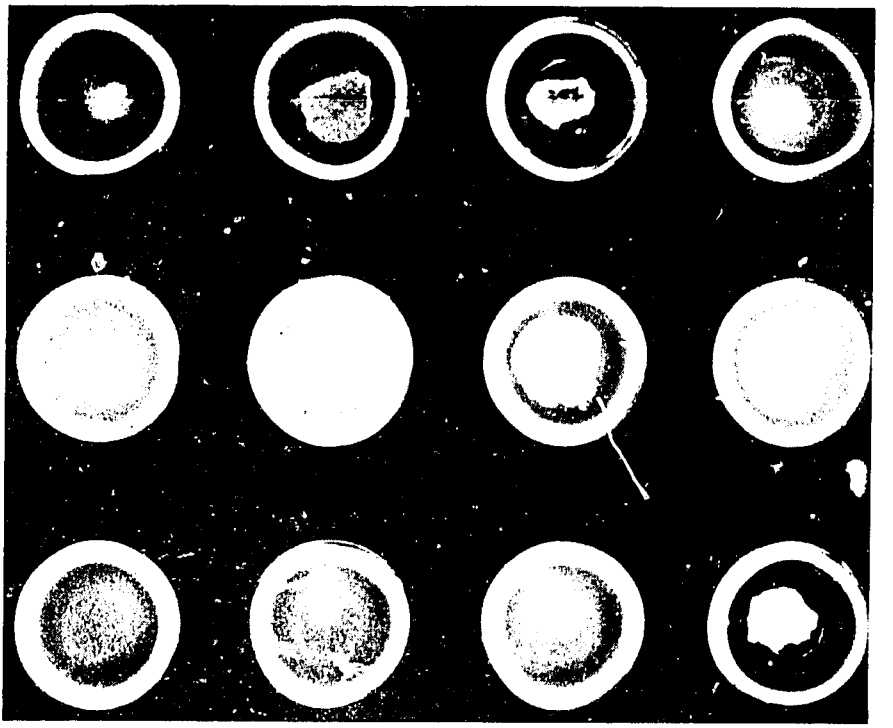

Fig. 49-3. Examples of agglutination reactions. Each row contains antigen from a different soybean nodule squash. Wells in the first column (*left*) received 1 drop of saline, but each well in the other columns received 1 drop of antiserum. A different antiserum was used for each column. Reactions in the first row (*left* to *right*) −, + +, + + +, −, second row −, −, +, -, and third row −, −, −, + + +.

because they lack complete cell walls (Jansen van Rensburg et al., 1973). They do react well with fluorescent antibody (Van der Merve & Strijdom, 1973).

49-9.2 Antibiotic Markers

49-9.2.1 PRINCIPLES

Antibiotic resistance also provides markers for identification and recognition of strains. Antibiotic markers on strains are relatively easy and inexpensive to obtain compared with development or purchase of antiserum. Fast-growing rhizobia with antibiotic resistance inoculated into soil have been isolated and enumerated by use of plate counts (Danso & Alexander, 1974). Antibiotic resistance also provides a method of identifying rhizobia that may be closely related serologically. See section 49-9.1.1 for discussion on serology.

Strains of rhizobia differ in their natural resistance to various antibiotics. Pinto et al. (1974) were able to successfully use differences in natural resistance to identify strains competing for nodule formation.

Generally, however, resistance to higher concentrations must be utilized if antibiotic resistance is to be useful as a qualitative tool (Schwinghammer, 1967). Spontaneous mutations at low frequencies of approximately 1 cell in 10 million cells in culture lead to mutants that are resistant to several times greater concentrations of the antibiotic than tolerated by the other cells. Mutagens should not be used to obtain mutants, because symbiotic and saprophytic properties of the rhizobia are more likely to be altered than when spontaneous mutants are used. Because of the high populations reached in laboratory culture, a few spontaneous mutants resistant to some antibiotics are present in most cultures. The mutants may be isolated by adding an antibiotic to a plating medium and inoculating it with a large population of cells (100 million). The colonies that form are antibiotic-resistant mutants.

After isolation, test the mutants on host plants to be certain that they have maintained their infectivity and effectiveness (section 49-8). Antibiotics that have been successfully used in the past are streptomycin, spectinomycin, rifampicin, chloramphenicol, kanamycin (Schwinghammer, 1964, 1967; Schwinghammer & Dudman, 1973, Johnston & Beringer, 1975; Kuykendall & Weber, 1978). Mutants capable of growing in the presence of neomycin and viomycin frequently were ineffective (Schwinghammer, 1967). Streptomycin is the antibiotic of choice because it inhibits the growth of most soil bacteria and when used in conjunction with cycloheximide, an inhibitor of fungi, provides a selective plating medium (Danso & Alexander, 1974). It has been used successfully by many investigators in the past and provides a stable marker (Brockwell et al., 1977). When more than one antibiotic marker is needed, antibiotics should be chosen that are not linked. Resistance to spectinomycin was not linked with resistance to streptomycin. Spectonomycin was a stable marker for several species of *Rhizobium,* and generally the mutants maintained effectiveness and antigenic specificity (Schwinghammer & Dudman, 1973).

Identification of rhizobia in small nodules is more rapidly achieved by using antibiotic markers than by isolating the rhizobia and culturing for identification using serological methods (Herridge & Roughley, 1975). Also, mixed nodule infections are readily revealed (Johnston & Beringer, 1975; Pinto et al., 1974; Labandera & Vincent, 1975).

Whenever antibiotic-resistant mutants are used, they must be checked to determine if they are stable and if they have the desired symbiotic properties. When possible, they should also be checked for saprophytic competance and competitiveness in nodule formation if results using them are to represent the parent strains.

49-9.2.2 MATERIALS

1. Culture of rhizobia.
2. Streptomycin sulfate.
3. Petri plates.
4. Yeast extract-mannitol.

5. Yeast extract-mannitol agar (YMA).
6. Bacteriological filters and apparatus for sterilization of the antibiotic.

49-9.2.3 PROCEDURE

Prepare a stock solution of antibiotic by dissolving 1 g of streptomycin sulfate in 100 ml of water. Sterilize the solution by filtration, and keep it refrigerated when not in use. Add 5 ml of the streptomycin solution to 250 ml of melted YMA. This provides a concentration of approximately 200 μg of streptomycin/ml. Pour 10 petri plates, and allow them to sit 1 day before use so that the surface will be dry.

Pipette onto each plate 0.1 ml of a suspension of rhizobia containing approximately 1 billion cells. The rhizobia may be from a 2- or 3-week-old agar slant or from the logarithmic phase of growth in shake culture. After colonies appear, they should be restreaked on streptomycin plates to make certain they are pure and resistant to streptomycin. If no colonies appear, repeat the procedure using reduced concentrations of antibiotic. If a second marker on the same strain is needed, repeat the procedure using the streptomycin-resistant mutant and yeast extract-mannitol containing spectinomycin. Alternatively, antigenic composition may be used as the second or third marker. After mutants are obtained, culture them and inoculate the host legume to compare their effectiveness (section 49-6) with the parent strain and to determine the stability of the marker.

Pick nodules from the host, and thoroughly wash and surface-sterilize them (section 49-3). Growth on both plates indicates the marker is stable. Growth on only the plate without streptomycin indicates either the marker was not stable or the nodule was formed by another strain.

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