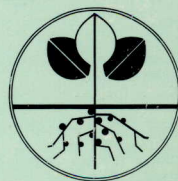


# Practical *Rhizobium*-Legume Technology Manual

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Technical Manual No. 19

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D.P. Beck  
L.A. Materon  
F. Afandi



International Center for Agricultural Research in the Dry Areas

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# **Practical *Rhizobium*-Legume Technology Manual**

**D.P. Beck  
L.A. Materon  
F. Afandi**

**Technical Manual No. 19**



**International Center for Agricultural Research in the Dry Areas  
(ICARDA) Box 5466, Aleppo, Syria**

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## Preface

Interest in the technologies appropriate for study of the legume-*Rhizobium* symbiosis has changed considerably over the last 20 years. World-wide development projects such as NifTAL have done much to transfer the fundamental methodologies for applied biological nitrogen fixation (BNF) research to scientists in the developing world, while research in industrialized countries moved increasingly toward the basic and involvement with biochemical and molecular techniques. There clearly remains considerable scope to improve N<sub>2</sub> fixation in legumes, particularly in the developing world, through application of appropriate BNF techniques. Whether research objectives are increased yields through inoculation, improved inoculants, or enhanced legume N input into cropping systems, the key to success is a step-wise strategy using a set of proven methodologies which enable adequate interpretation of research results. This allows a continuing evaluation of the research process and reorientation toward practical and achievable goals in N<sub>2</sub> fixation research.

This manual began as a collection of laboratory and field protocols which were used in our early BNF training courses at ICARDA. As our research work and that of our regional colleagues evolved, the number of procedures used grew to a point where a compilation was needed. This manual is the result of several years of methodology testing and development, and represents the majority of techniques used in BNF research at ICARDA. Although written primarily for application with the ICARDA-mandated cool-season legumes, including the annual medics, lathyrus, vetch, lentil, chickpea and faba bean, the practices and principles will apply widely to temperate legumes and rhizobia. Most of the techniques presented here have been developed by others and are therefore not original; in many instances, however, procedures have been modified for ease of use or improved results.

The manual has two major objectives. The first five sections focus on the handling and identification of rhizobia, with production of high-quality inoculants as the overall aim. The last four sections deal with field research, and present methods to determine the factors affecting inoculation response and the role of legume N<sub>2</sub> fixation in farming systems. Chapters have been designed to stand alone, entailing some repetition. Where necessary, cross-reference to supporting sections is given. We have attempted to present the material in such a manner that it is within the intellectual horizons of a reader who has completed a basic agronomic or biological science program. However, this version covers in detail most of the laboratory, greenhouse, and field procedures used in BNF work at ICARDA. We therefore hope that newcomers and experienced individuals alike will find some of the presented material of value.



## Acknowledgments

The authors would like to dedicate this manual to two outstanding scientists and pioneers in the fields of rhizobial ecology and inoculant production. They are Emeritus Professor Jim Vincent of Australia and the late Dr. Joe Burton of the U.S.A. We have been privileged to work with these outstanding individuals, and wish to acknowledge their influence on us and on our contemporary colleagues.

This manual would not exist without the assistance and advice of our supporting staff. We would therefore like to thank Ms. Monika Zaklouta, Ms. Fadwa Khanji, Mr. Bakri Abudan, Ms. Siham Kabalan, Ms. Sahar Sabouni, and Mr. Elias Khoudary. We wish particularly to thank ICARDA editors Ms. L. Sears and Ms. E. Talmage, the layout assistance of Ms. W. Meskine and Mr. H. Khairallah, and the support of Dr. S. Varma. Special thanks to Mrs. Cristina Materon for production of illustrations. Finally, we are grateful to Dr. Aart van Schoonhoven, who provided the impetus and inspiration necessary to complete the task.

# **I. Manipulation of *Rhizobium***

## A. *Rhizobium* Manipulation

### A.1. Nodule Collection and Preservation

**Nodule collection.** Collection of nodules is generally performed to supplement a strain collection with superior strains. For this purpose, nodules should be collected from healthy, green plants suspected of high  $N_2$ -fixation activity. Such plants usually have medium to large nodules with pink-red interiors which may indicate active fixation. Nodules from the tap root portion of the root system are best (Figure A.1.1).

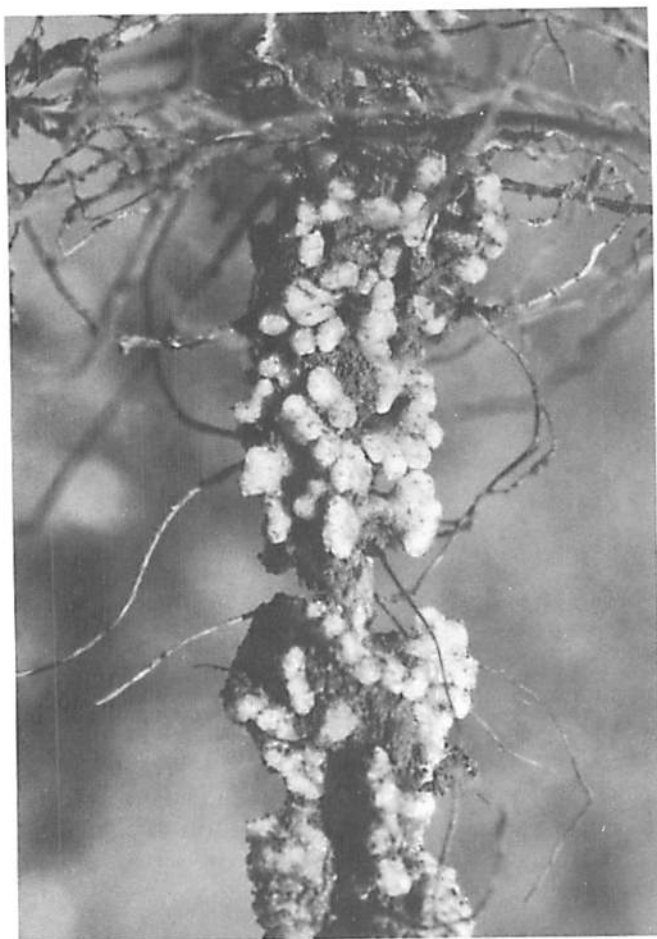


Fig. A.1.1. Healthy roots of a chickpea plant showing effective tap root nodulation.

When removing plants from the field for nodule collection, use a spade to dig approximately 15 cm to either side of the plant stalk to at least 20-cm depth. Using the spade, slowly lift out the clump of soil and roots (Figure A.1.2). Carefully remove soil from the roots with your hands, breaking soil clumps with care to avoid damaging secondary roots where many nodules often are found. Carefully place the whole plant in a plastic bag.



Fig. A.1.2. Using spade to lift nodulated root system from soil.

In the laboratory, remove plant top and carefully wash the roots under running water with a screen (or sieve) underneath to catch detached nodules (Figure A.1.3). For storage and isolation purposes, nodules should not be pulled from the root (plucked); rather, the root should be cut 1–2 mm each side of the nodule (Figure A.1.4). This keeps the nodule intact and greatly improves the chances of obtaining a clean viable culture of rhizobia. Carefully blot nodules dry with paper towel or cotton cloth before storage.

**Nodule preservation.** Fresh nodules may be stored in the refrigerator up to 48 hours. Do not freeze nodules as the formation of ice crystals within the nodule may kill the

bacteroids. Frozen nodules may, however, be used for serological typing (see Section C.3).



Fig. A.1.3. Roots being washed with tap water prior to nodule detachment.

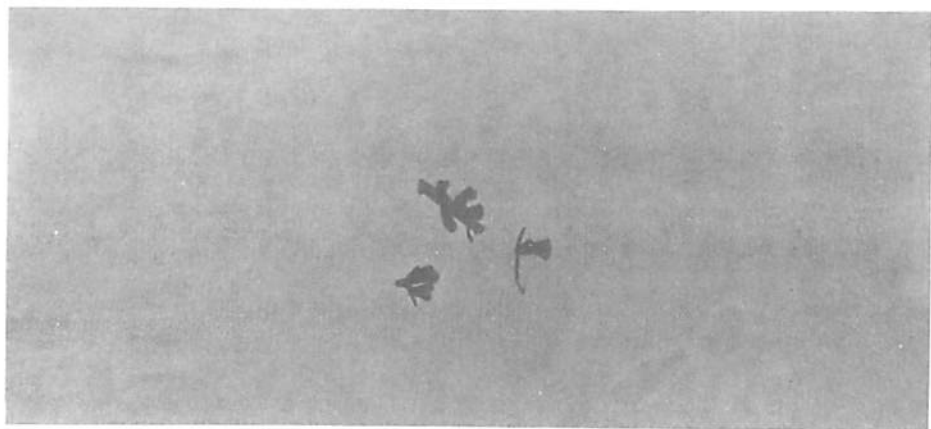


Fig. A.1.4. Detached nodules with portion of root attached.

For long-term storage, desiccation of nodules in glass vials is recommended. The type of nodule preservation vial suggested is shown in Figure A.1.5 below, and utilizes a desiccant, such as anhydrous  $\text{CaCl}_2$  or silica gel, to remove water quickly from nodules and keep them dry, preventing growth of fungi or other bacteria. Nodules preserved in this way can be kept for 6–12 months, although recovery of the *Rhizobium* during isolation after rehydration may be difficult after long-term storage, depending on the legume species and strain.

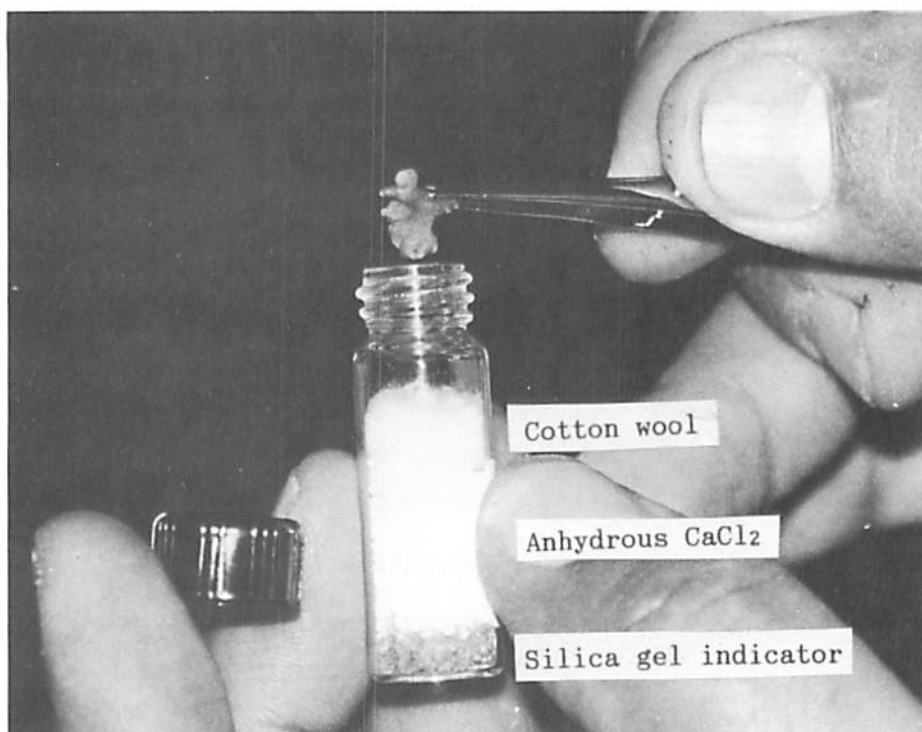


Fig. A.1.5. Nodule desiccation vial containing anhydrous calcium chloride to dehydrate nodules. Nodule rests on cotton mat to avoid direct contact with desiccant.

**Note:** *Rhizobia* are killed by oven drying of nodules; do not expect to isolate living bacteria from oven-dried nodules.

Place three to five medium-sized nodules (or two to three large nodules) on top of cotton wadding in the vial. Nodules should be free of excess water for the desiccation vial to work properly. Carefully label the vial with date, location, host legume, and, if appropriate, treatment.

## A.2. Isolation of Rhizobia from Nodules

Clean, fresh nodules, with a portion of the root still attached, are best for isolation of rhizobia. The attached root (2–3 mm on each side of nodule) can be used to hold the nodule with forceps (Figure A.2.1), reducing the possibility of damaging the nodule during sterilization and isolation.

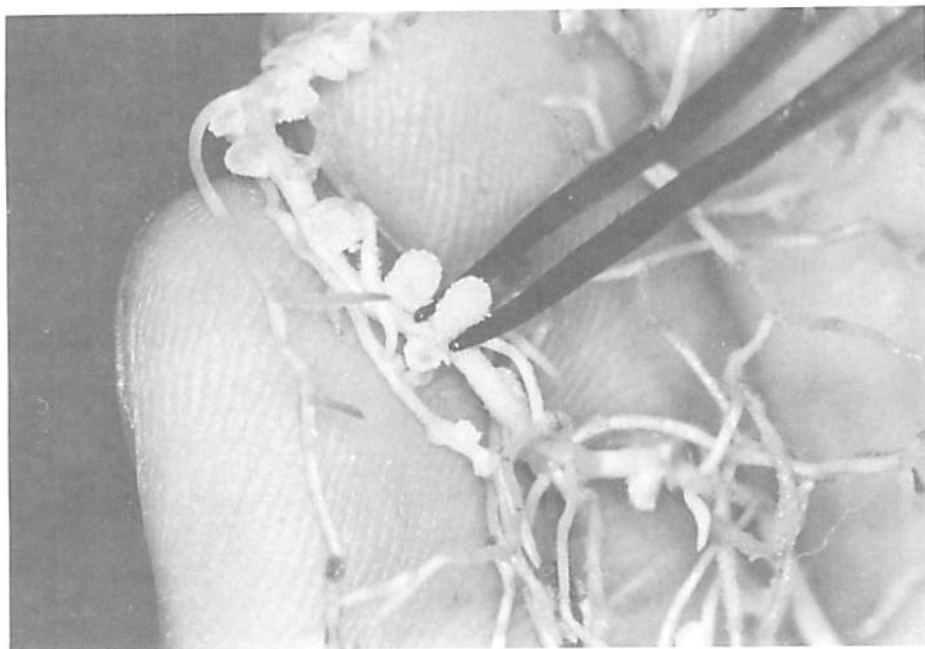


Fig. A.2.1. Removal of nodule from root using forceps to cut root on each side of the nodule.

Desiccated nodules must be rehydrated before sterilizing. Place nodules in a small container with distilled water and leave in the refrigerator overnight. Soaking nodules for one hour at room temperature is acceptable for nodules that have been in the desiccation vial for only a short time.

**Nodule sterilization.** Immerse intact nodules for 5–10 seconds in 95% alcohol (ethyl or isopropyl). Transfer immediately to a 5% solution of calcium hypochlorite (or chlorox), and soak for two or three minutes to surface sterilize the nodule. Immediately following sterilization, rinse nodules in five to seven changes of sterile distilled water, leaving the nodules in the final rinse until used for isolation of

rhizobia. Solutions for sterilization and rinsing may be kept in separate petri dishes, beakers, flasks, or vials, with nodules moved from one container to another (Figure A.2.2) using sterile forceps (forceps are quickly sterilized by dipping into alcohol and flaming); alternately, a single container may be used, and solutions changed as needed. In this method, nodules are left in the flask each time (Figure A.2.3).

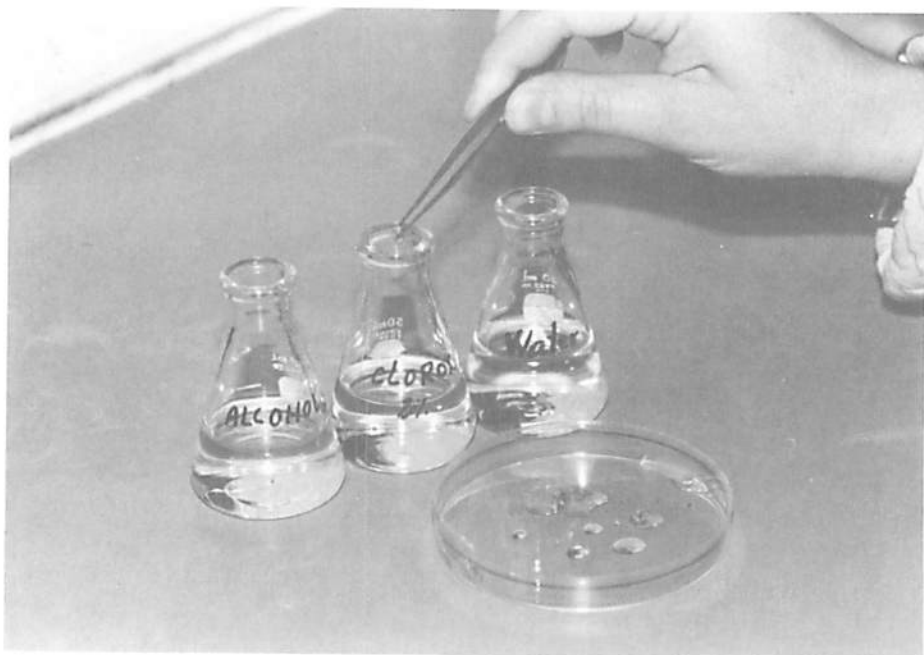


Fig. A.2.2. Sterilizing nodules by moving from one flask of sterilant to another with forceps.

An acidified mercuric chloride solution (0.1% weight/volume, sterilization time one minute) or a 3% solution of hydrogen peroxide (sterilization time three minutes) may also be used for sterilizing nodules, in place of clorox. However, mercuric chloride is toxic to humans and hydrogen peroxide is expensive, making chlorox the preferred choice. When hydrogen peroxide is used, the rinses with sterile water may be omitted.

### Isolation from Nodule: Crushed Nodule Method

Crush the sterilized nodule with a pair of blunt-tipped forceps in a large drop of





Fig. A.2.3. Nodule sterilization in test tube by changing sterilizing solutions.

sterile water in a petri dish (Figure A.2.4). Another method is to crush the nodule in a sterile test tube with a sterile glass rod, using 1-2 drops of sterile water to make a

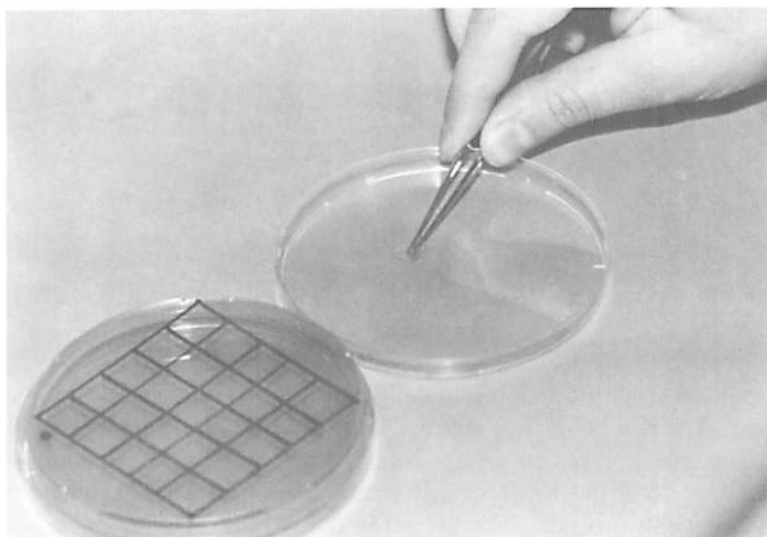


Fig. A.2.4. Crushing a nodule in petri dish cover using sterile forceps.

slurry (Figure A.2.5). Streak one sterile inoculation loop full of the suspension on yeast-mannitol agar (YMA) plates containing Congo red (see Section A.9), in the following manner:

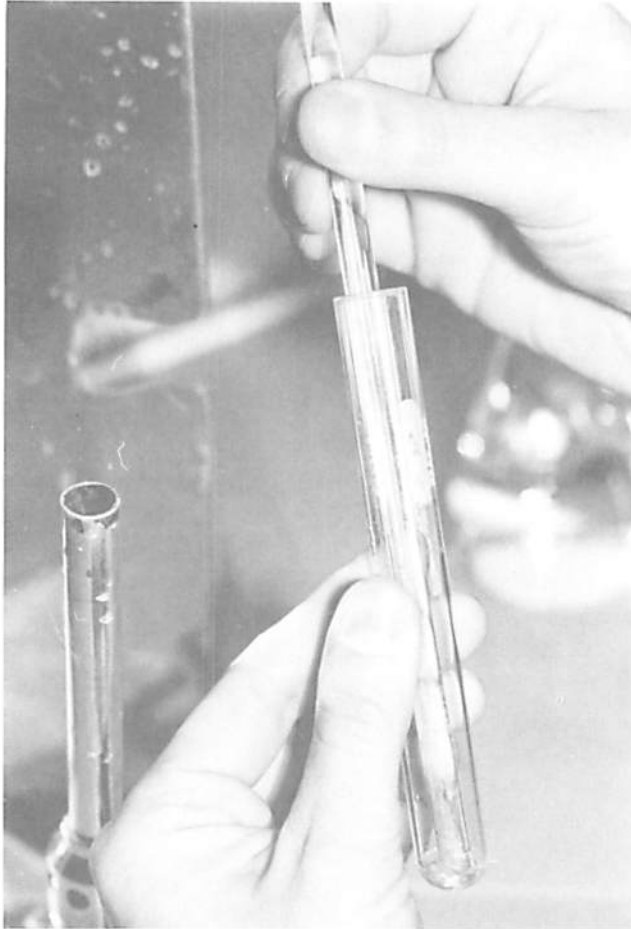


Fig. A.2.5. Crushing nodules in test tube with glass rod.

Deposit the slurry from the loop on the edge of the agar, and streak in a back and forth manner as shown below in Figure A.2.6 (1). Resterilize the loop (by flaming in Bunsen burner until it glows an orange color), touch the loop to a corner of the plate to cool it, and streak from the previously streaked area to the next quadrant of the plate (2). Repeat this procedure for areas (3) and (4); in each streaking, less of the slurry is distributed over the surface of the plate giving well-isolated individual colonies in areas (3) and (4).

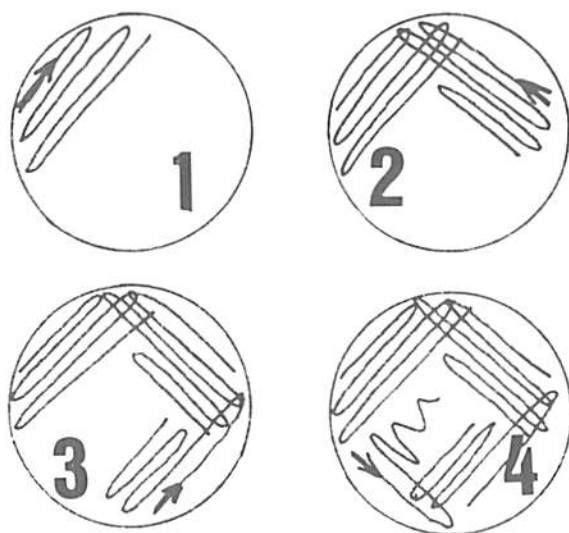


Fig. A.2.6. Method of streaking out a culture on the surface of a solid agar plate, following steps in the order of 1,2,3,4.

Note: During this procedure, the lid of the petri dish should not be set aside, but should be lifted above the dish only far enough to manipulate the inoculation loop (Figure A.2.7), and replaced each time the loop is flame sterilized.

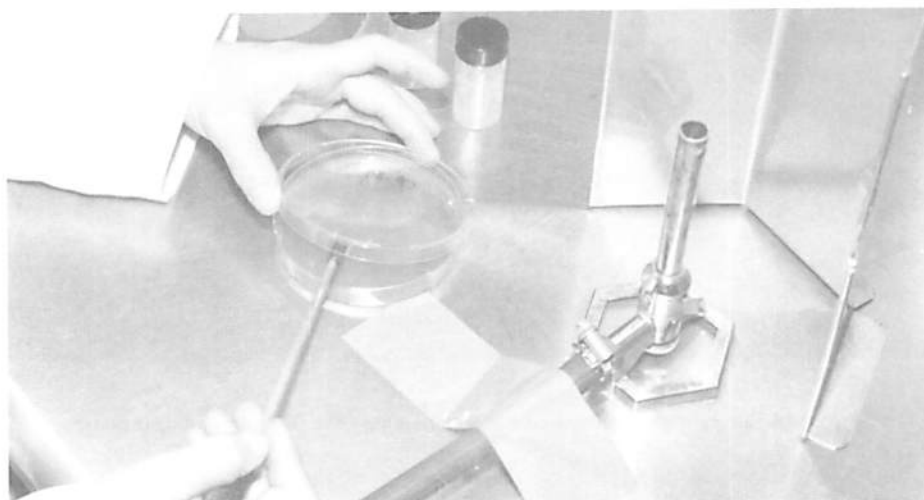


Fig. A.2.7. Plate position and angle of the loop during streaking of culture on agar plate.

### Isolation from Nodule: Needle Isolation Method

The needle method of isolation is especially useful with freshly harvested nodules of 2 mm diameter or larger. The surface-sterilized nodule is first placed on a small piece of sterile filter paper ( $2 \times 2$  cm) in a sterile petri dish. A new piece of filter paper should be used for each nodule, although the same petri dish can be used for several nodules. Firmly hold the nodule with flame-sterilized forceps (cooled briefly) on the filter paper, and carefully slice off about one-third of the nodule with a flamed, hot scalpel (or very sharp knife) (Figure A.2.8). This exposes sterile nodule tissue from which the isolation is made. Still holding the nodule with the forceps on the filter paper, insert a flame-sterilized inoculation loop (1 mm size loop) into the exposed tissue to load the loop with inoculum (Figure A.2.9). Streak directly onto YMA Congo red plate.

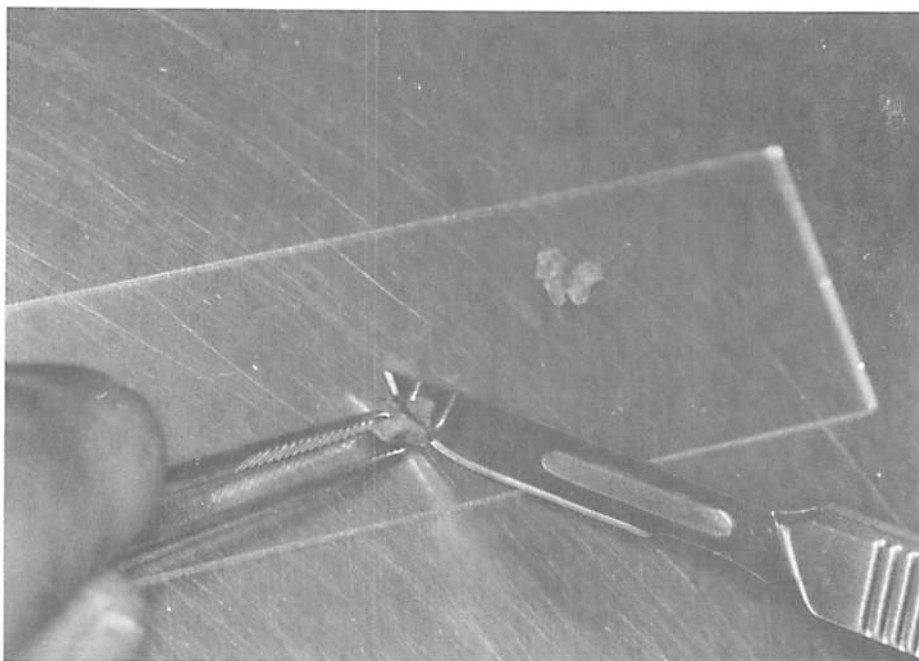


Fig. A.2.8. Slicing a nodule for needle inoculation onto the surface of an agar plate.

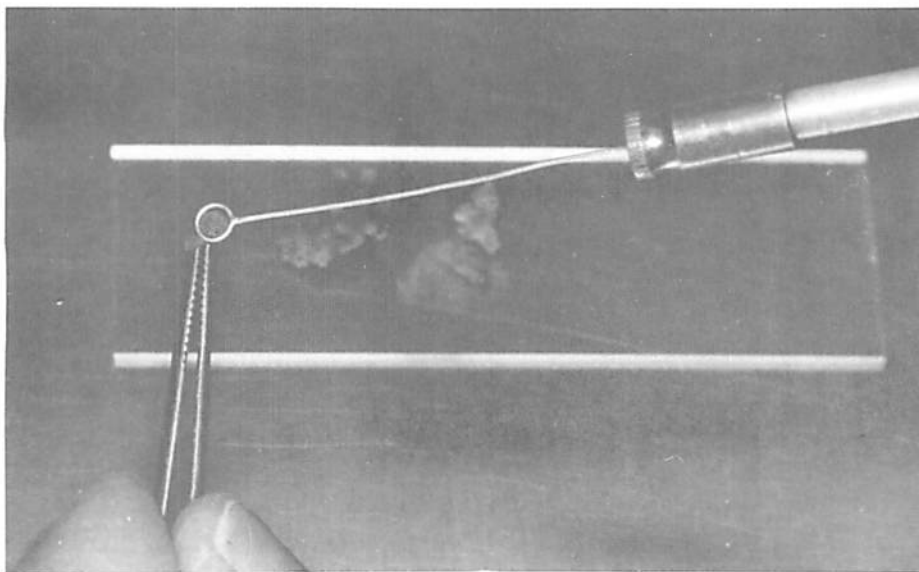


Fig. A.2.9. Loading loop with nodule contents.

The needle method requires steady hands and practice before adoption. For small nodules, the tip of the inoculation needle may be bent over and used as a hook to remove a small portion of nodule tissue, or may be used as a needle and inserted into the nodule tissue before streaking onto the agar plate. The needle should not be longer than 4–5 cm.

Keep the inoculated petri plates in an incubator at 25–28°C for several days until colonies appear. It takes 2–10 days for growth of rhizobia, depending on the strain and species. Observe colony morphology; pure *Rhizobium* generally forms white, milky, sticky, translucent, roundish colonies (Figure A.2.10).

## Isolating Rhizobia from Soils

The best way to isolate rhizobia from soil is for the rhizobia first to form nodules on a legume, which can then be used to directly isolate the rhizobia. This is because soil contains a multitude of bacterial species, and differential media to isolate rhizobia directly from soil are not reliable (Section B.3.a). It is possible to: (1) grow plants in the soil you want to isolate from; or (2) inoculate plants using the plant infection test methodology (Section B.3.b) using a dilution series of the soil. In the second method, the approximate number of rhizobia in the soil will be obtained as well as nodules to isolate rhizobia from.

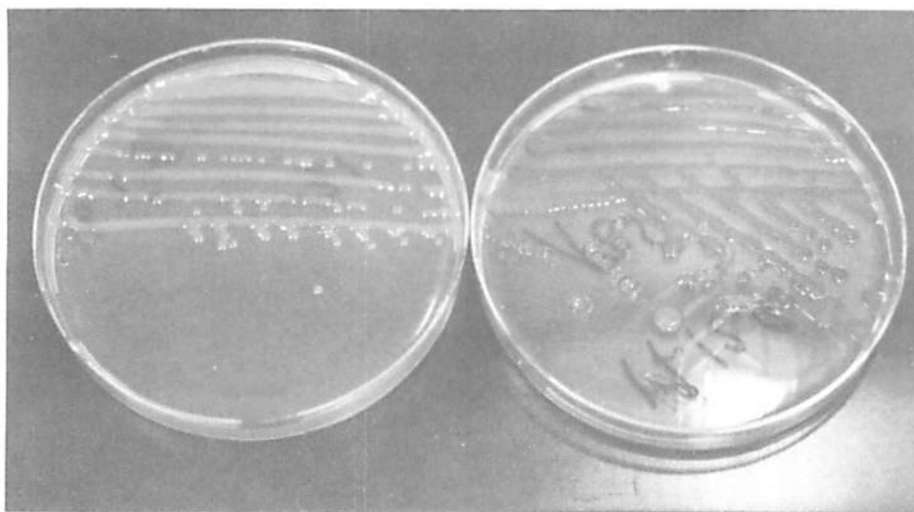


Fig. A.2.10. Rhizobial growth on YMA plate, with development of individual colonies for isolation.

### A.3. Purification of Isolates

Typically, first isolations from nodules will produce a mixture of colony types, including contaminants and perhaps more than one strain contained in the nodule from which the isolation was made. Small and large, mucoid and dry, etc., colonies may be found in a primary isolation from a single nodule (Figure A.3.1). After colonies have appeared from the first isolation, each of the types typical of *Rhizobium* spp. should be checked by Gram stain (Section A.6). If Gram negative, streak out each type of colony onto YMA containing Congo red to obtain a plate containing a pure culture of each of the types; each of these should be considered an individual culture, which must then be authenticated as rhizobia (see Section A.4). More than one colony type in a pure culture of *Rhizobium* may indicate variance in the same strain or the occupancy of two different strains in the same nodule, but it is more likely the result of contamination.

Incubate and make daily observations for the appearance of colonies typical of rhizobia. Colonies should show little or no Congo red absorption when incubated in the dark (Figure A.3.2). Check secondary isolates for purity by the Gram stain method and, if pure, transfer to screw-cap culture tube slants to form stock cultures; store at approximately 4°C in a refrigerator. The authenticity of these isolates as pure cultures of rhizobia is confirmed later by the nodulation test under aseptic controlled conditions (Section A.4).

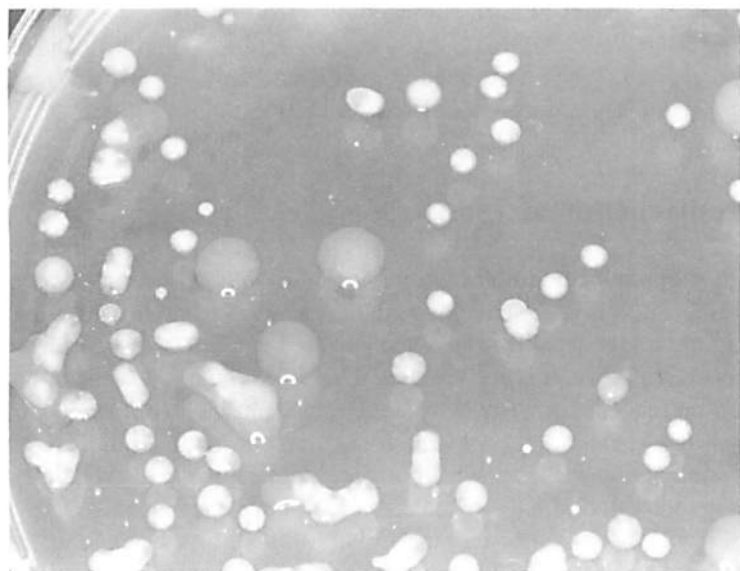


Fig. A.3.1. Mixed colony types from soil growing on YMA.

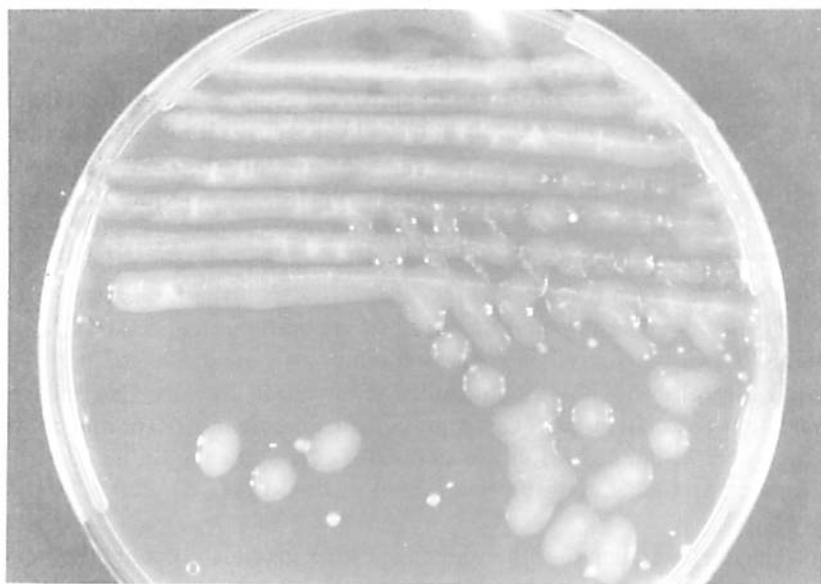


Fig. A.3.2. Some colonies appear whitish, with no Congo red absorption, while others have taken up the dye.

Keep a record of the origin of each culture (code number, date of collection, host species isolated from, *Rhizobium* species, location, date of isolation). These cultures are termed isolates until they have been authenticated and characterized as individual strains in the strain evaluation process (Section D.2).

## A.4. Authentication of Isolates

All isolates must be confirmed before being included in a collection or regarded as *Rhizobium*. Confirmation is dependent on demonstration of nodule-forming ability on a test host legume under bacteriologically controlled conditions. The only criterion required in this test is formation of nodules. The effectiveness ( $N_2$ -fixing potential) of the isolate can be recorded for future reference, but must be confirmed with further testing (Section D). Ideally, a *Rhizobium* strain is tested for its ability to produce nodules on the plant species from which it was originally isolated. Where the experimenter is interested in legume species which have specific rhizobial requirements, it may be useful to test the isolate on several species (e.g., *Medicago* spp.) or on different cultivars (e.g., with chickpea) to determine the spectrum of symbiotic reactions.

The first requirement is that the seed of the test host, the growth medium and the containers all be free from contaminating rhizobia. With some seed it is difficult to destroy all bacteria and spores that exist on the seed coat, but using a sterilant like  $HgCl_2$  (procedure described in Section B.3.b) is usually effective. The growth medium (e.g., vermiculite) should be sterilized in the growth container if possible (Figure A.4.1), as separate addition after sterilization is likely to result in some contamination.

The second need is to avoid poststerilization rhizobial contamination. Plugged tubes (described in Section B.3.b) or closed containers (Section D.2.b) are generally easy to maintain without contamination, as are any vessels which maintain a dry surface and supply water and nutrients from below the surface (as with the Leonard jar or soil core system). Results from open pot culture systems are unreliable for authentication, as contamination with air-borne rhizobia is common. An adequate number of uninoculated control units should be included in any authentication or effectiveness test to indicate if the system has been exposed to contaminants. Experiments with contaminated uninoculated controls are unreliable. The data should be discarded or considered as questionable, and the experiment repeated.

It is important to remember that this stage of testing an isolate is only the beginning. Reisolation of the strain from the nodule produced is necessary, with this pure reisolate taking the place of the original isolate in the collection.



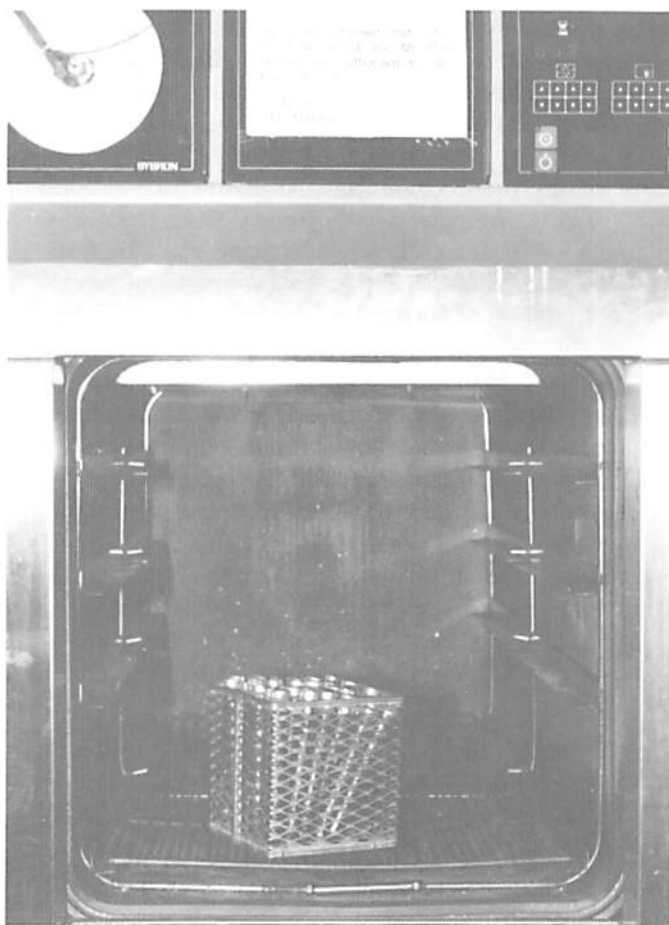


Fig. A.4.1. Vermiculite tube system prepared for sterilization by autoclaving.

## A.5. Culture Preservation

Rhizobial isolates obtained from the field or other sources must be maintained in such a way as to minimize loss of viability, genetic variation and contamination. It is suggested that a stock collection, distinct from the set of cultures utilized for everyday applications like inoculum production, be maintained separately with a view toward long-term storage. This "mother culture" collection should be maintained in duplicate (at minimum), with periodic maintenance performed as necessary to ensure that isolate properties have not been lost. As you may imagine, maintenance of a large collection (more than 100 isolates) requires considerable time and labor. It is

therefore suggested that collections be kept to a minimum size by discarding ineffective or noninfective cultures identified as such during screening. Apart from this basic collection, working cultures of those isolates utilized for general (e.g., inoculum production) and experimental purposes should be maintained separately.

Several methods of culture preservation exist. Regularly used cultures are best kept on agar slants, which are easy to produce and maintain. Long-term preservation of cultures on slants involves considerable labor, as slants must be restreaked every two to three months; in addition, cultures kept in this way without passing the isolate periodically through a nodule for authentication tend to lose viability and effectiveness. Preservation of isolates using lyophilization, or freeze-drying, requires large initial labor and specialized equipment, but has the advantage of maintaining viability over a long period (years) without losing strain properties. Cryopreservation is also an effective technology for long-term preservation of *Rhizobium* cultures, but requires special deep-freeze facilities and uninterrupted electrical power. In the following paragraphs we discuss the various methods and their applications.

**Screw-capped agar cultures.** Rhizobial cultures can be preserved in screw-capped McCartney bottles or in screw-capped test tubes of 5–10 ml size containing slants of YMA (Figure A.5.1). Tubes are filled approximately one-third full with dissolved YMA, autoclaved for 15 minutes at 120°C with caps loosely fitted, and left in the



Fig. A.5.1. Agar slants in screw-top vials. Left-hand vial contains culture with heavy growth.

autoclave without venting until pressure reaches zero. After securely tightening caps, tubes are left at a slant while the agar cools and solidifies (Figure A.5.2). The angle at which the tubes of agar are cooled is such that the top of the slant is 1 cm from the screw-threads. Before inoculation, slants should be stored under refrigeration to prevent drying out.



Fig. A.5.2. Cooling autoclaved tubes with melted YMA in a slant position.

The procedure for inoculation of agar slants is as follows:

1. Label vial, indicating date and name/number of strain.
2. Flame the inoculating loop.
3. Select a representative colony growing on a YMA plate or a portion of a streaked slant and remove it with the inoculating loop.
4. Remove cap, hold it with your finger and flame the lip of the vial briefly (Figure A.5.3).
5. With the loop make a 'snake'-shaped streak onto the surface of the YMA slant, from bottom to top (Figure A.5.4).
6. Place vial in the incubator at 26–30°C until streak becomes visible.
7. Place vial in refrigerator for storage.

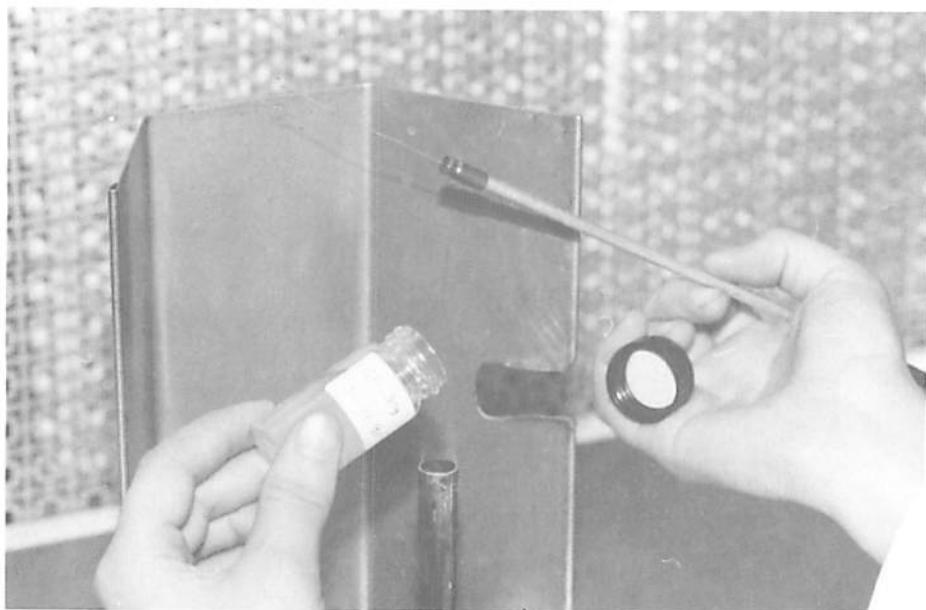


Fig. A.5.3. Flaming slant tube lip. Observe how fingers are holding the tube cap.

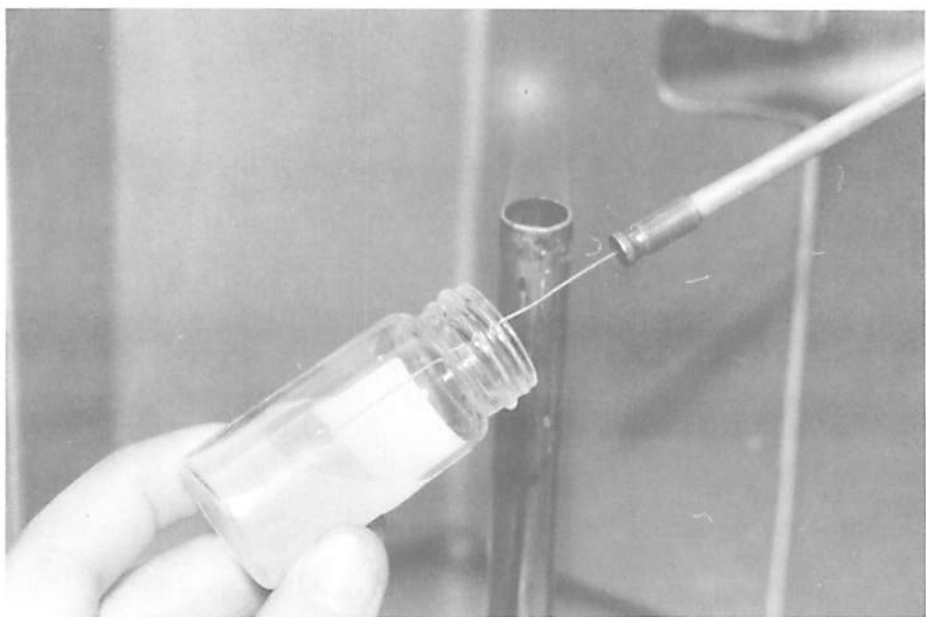


Fig. A.5.4. Streaking slant tube with wire loop.

Cultures can be stored in the refrigerator at 4–6°C for two or three months only, after which drying of agar or mutation of bacteria increase the probability of loss of the culture. The screw caps should be tightly closed to avoid drying of the agar, which is a major problem with this method of storage. The caps should have noncorrodible rubber or plastic seals; to improve the seal the joint between cap and tube can be wrapped with parafilm (Figure A.5.5). Periodic transfer of slant cultures to fresh agar slants is recommended on a regular basis, as mutation may cause isolates to lose some of their characteristics such as effectiveness. Bacterial cultures kept this way on slants continue to grow and divide, and continuously utilize food sources as well as produce wastes. After two or three months, waste products build up as the food source disappears, and bacteria begin to die and mutate. Continuous evaluation of strain characteristics as well as a periodic plant-infection test (inoculating a plant grown under sterile conditions with the strain, then reisolating the strain from the nodule formed) is recommended after the culture has been transferred several times.



Fig. A.5.5. Agar slant tube cap wrapped with parafilm.

If cultures are antibiotic-resistant mutants, or strains with properties such as acid or salt tolerance, it is also advisable that the YMA of the slant be supplemented with the corresponding concentration of the antibiotic or nutrient. Failing to do so will increase the probability of losing the specific tolerance characteristic.

**Porcelain beads.** A method for extending viability is to dry cultures on the surfaces of porcelain beads and store them at 4–6°C. This method has the advantage that viability of the cultures can be maintained up to three years without regular transfers, as dryness of the cultures and low relative humidity reduce the rhizobial growth rate to a minimum and extend culture life. However, survival of cultures using this method can be sporadic, and specific strains behave quite differently on beads. Each strain should be tested for its ability to survive on beads before it is consigned to storage with this method. The procedure for preparation and inoculation of porcelain beads is as follows:

1. Place 4 g of anhydrous silica gel desiccant into 15-ml tubes. The tubes should have rubber-lined screw caps or tight rubber bungs.
2. Place a layer of 2 cm of clean glass wool over the silica gel.
3. Add 10–15 clean, dry unglazed porcelain beads of 4–8 mm diameter (Figure A.5.6).

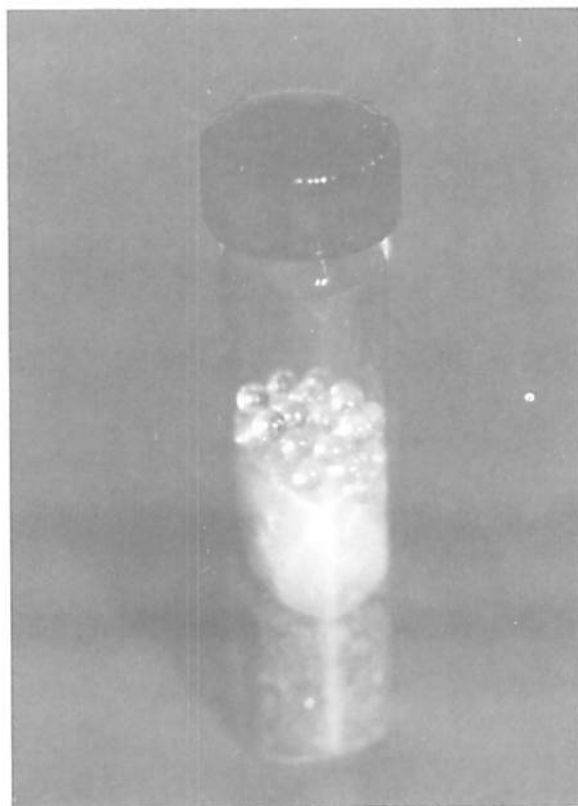


Fig. A.5.6. Tube containing beads with silica gel desiccant and indicator at the bottom.

4. Place tubes in rack, cover with aluminum foil, and heat in oven at 160°C for 90 minutes. Rubber bungs and lined caps can be sterilized separately by autoclaving.
5. Sterilize a set of cotton-plugged tubes containing 0.1 g of maltose each. Add 1 ml of  $10^9$  culture suspension to each tube. Shake to dissolve the maltose in the culture suspension.
6. Add the beads aseptically to the suspension. Shake until suspension is uniformly distributed onto the beads. Invert tubes to drain out the excess suspension onto the cotton plug.
7. Aseptically transfer the rhizobia-coated beads to the tubes containing the silica gel. Seal tightly.
8. Bacterial cells will die in the presence of moisture within this system. Silica gel absorbs any moisture present, and will aid in the detection of moisture by changing its blue color into pink if its capacity to absorb moisture is overwhelmed.

To reconstitute the culture remove beads aseptically with forceps from the tube and transfer them to an Erlenmeyer flask containing YM broth (Figure A.5.7). Shake



Fig. A.5.7. Transferring bead to flask containing broth.

thoroughly and by means of a loop, streak the inoculum onto the surface of a petri dish with YMA. Alternatively, you may allow the rhizobia to grow in the flask containing YM broth on a reciprocating shaker for two or three days before transfer to a petri plate of YMA Congo red to check for contamination.

**Lyophilization (freeze-drying).** This method of preservation requires expensive equipment, including a freeze-dryer, centrifuge, ampule constrictor, and spark tester; in addition, the glass ampules themselves are quite expensive. It is the most widely used method for long-term storage of rhizobial cultures in institutions dealing with large collections or supplying a large number of strains on request. The time and labor required for lyophilization of a number of strains is considerable, and much practice is necessary before proficiency can be expected. Maintenance of the freeze-dryer, particularly the less expensive models, is extensive.

In practice, small quantities of high-density cultures are suspended in a protective medium in glass ampules and dried by removal of water vapor during freezing under near-vacuum. Technically, the most difficult portion of the procedure is sealing of ampules while still under vacuum, using a special constricting torch. Properly prepared vacuum-sealed ampules containing freeze-dried bacteria can be kept indefinitely and require only a small storage space. Storage at low temperature and in the dark is recommended.

## **Suspending Media for Lyophilization**

The suspending medium for the culture to be freeze-dried is a buffer which provides protection during freezing, drying and reconstitution. The suspending medium should aid in maintenance of the osmotic levels and provide a final water content of about 1%. A 10% sucrose + 5% peptone suspending medium is used successfully on strains from several *Rhizobium* species at ICARDA. It is important to bear in mind that dried cells become sensitive to oxygen at relative humidities below 10%. Lack of near-vacuum conditions may be overcome by including glutamate (sodium glutamate, 0.5%) in the suspending medium as an antioxidant, but best is successful freeze drying with a positive seal against vacuum loss.

## **Preparation of Inocula for Lyophilized Ampules**

1. Suspend a fresh slant tube culture with 1 ml sucrose-peptone medium (SPM).
2. Mix thoroughly on a vortex mixer to break cell clumps; use 0.1 ml of this suspension for serial dilution and plate count to estimate the viable count before lyophilization.
3. Carefully add 0.1 ml of the bacterial suspension directly onto the cotton plug at



the bottom of the pre-sterilized ampule (Figure A.5.8). Place another cotton plug into the ampule 1 cm above the bottom.

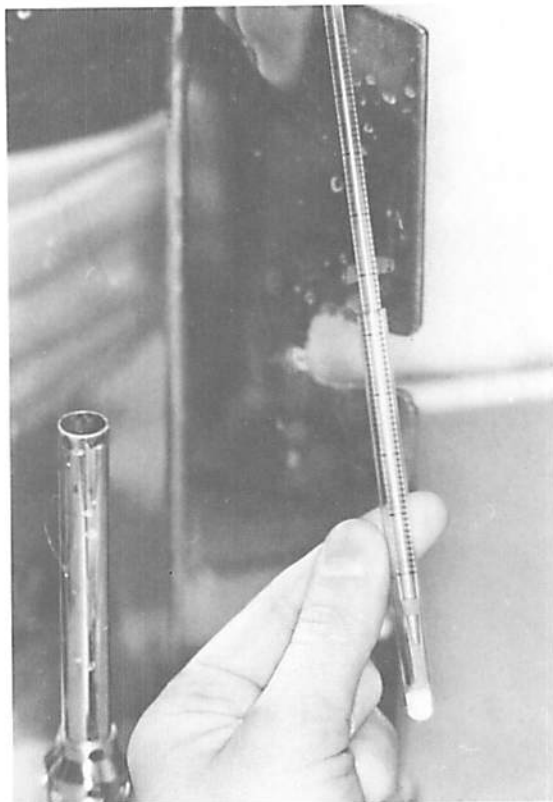


Fig. A.5.8. Addition of 0.2 ml concentrated culture suspension onto cotton swab in an ampule.

4. Place ampules in a beaker; place beaker under a bell jar.
5. Perform primary freeze-drying (see following sections).

### Preparation of the Freeze-Dryer Unit

With the freeze dryer unit utilized at ICARDA (Edwards EF4 Modulyo) the operator needs to demoisurize the oil before proceeding. This procedure may not be required for other (usually more expensive) units. Before operation of this equipment, carefully read the instruction and service manual, as mistakes may cause damage to the unit.

The process involves the following steps (see Figure A.5.9):



Fig. A.5.9. Lyophilizer machine with: 1) bell jar; 2) ampule manifold; 3) vacuum gauge; 4) temperature gauge; 5) drain valve control; 6) air admittance valve; 7) vacuum line; and 8) vacuum pump.

1. Clean and disinfect the bell jar and condenser chamber with water and then 100% alcohol.
2. Apply a small amount of high-vacuum grease to the rubber seals of the bell jar and mount it securely onto the condenser chamber.
3. Open the gas ballast valve and close the air admittance valve.
4. Turn on the main power switch of the freezing unit.
5. Wait until the chamber temperature stabilizes to  $-50^{\circ}\text{C}$  which corresponds to a vapor pressure of  $4 \times 10^{-2}$  mbar.

6. Switch on the vacuum pump and keep running it for one hour.
7. Switch off the freeze-dryer unit and the vacuum pump.
8. Open the air admittance valve to release vacuum in chamber.
9. Remove the bell jar.
10. Defrost condenser chamber using warm water.
11. Open the drain valve to release water from the condenser chamber. Close valve when finished.
12. Dry, clean, and disinfect condenser chamber and bell jar.

## Primary Freeze-Drying

Evolution of water vapor from the frozen cultures will cause the temperature to fall to a point where heat lost due to sublimation is balanced by heat gained from the surroundings. The cultures are first frozen and then dried while in the frozen state (under vacuum), thus avoiding damage to the structural integrity of the cells and a change in their solute concentration. Two steps in the freeze-drying process are necessary: the first step removes the bulk of the water, the second removes the remainder and allows sealing of the ampules under vacuum.

The procedure for the first lyophilizing step is as follows (see Figure A.5.9):

1. Place beakers containing ampules on the elevated baseplate of the freeze-dryer.
2. Apply a small amount of high-vacuum grease on the rubber seals of the bell jar and mount the bell jar onto the condenser chamber.
3. Close air admittance valve.
4. Turn on the main power switch of the freeze-dryer unit.
5. Wait until temperature stabilizes at  $-50^{\circ}\text{C}$  and corresponding vapor pressure needle indicates  $3.4 \times 10^{-2}$  mbar.
6. Switch on the vacuum pump.
7. Allow the system to run for two hours.
8. Switch off freeze-dryer unit and vacuum pump.
9. Release the air admittance valve.
10. Remove bell jar.
11. Defrost the condenser chamber using warm water ( $< 50^{\circ}\text{C}$ ).
12. Open drain valve, allow water to flow out, and close drain valve when finished.
13. Dry, clean and disinfect condenser chamber.

Primary freeze-drying will remove approximately 95% of the moisture in the cultures. At this stage, identification labels are inserted into ampules containing the frozen cultures. Culture identification number and date of lyophilization are indicated in a typewritten line on a small strip of paper (Figure A.5.10). Push the cotton plug further inside to allow for the constriction procedure.

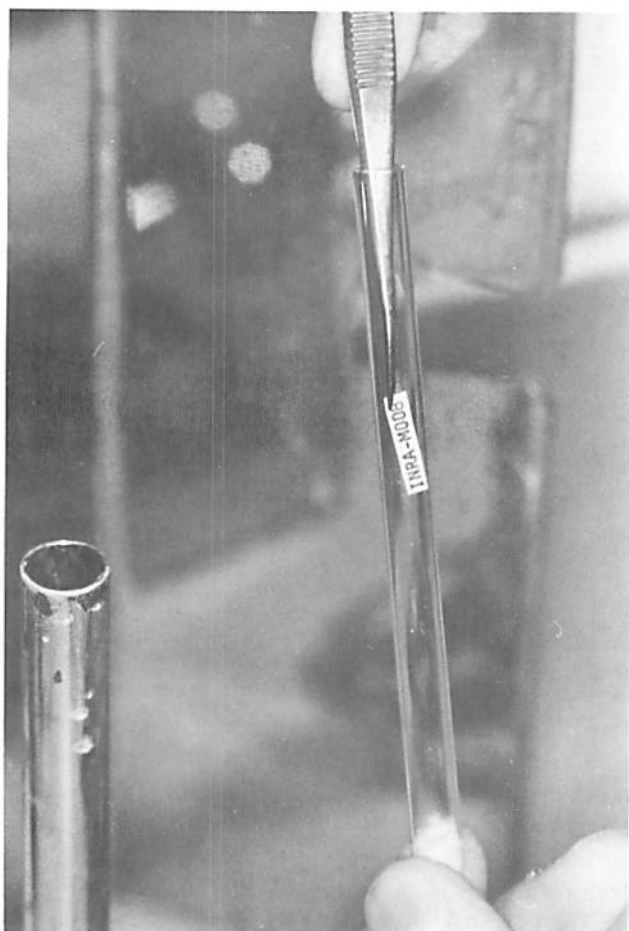


Fig. A.5.10. Insertion of labelled paper strip into ampule containing a rhizobial culture.

### Ampule Constriction

Ampules are constricted after primary freeze-drying by rotating them over the flame of a gas-air source of an Edwards ampule constrictor (model 2) or equivalent (Figure A.5.11). The place for constriction should be in the middle of the ampule tube and above the top cotton plug (Figure A.5.12). This device constricts and stretches the ampules to a narrow diameter at this point which will later be simpler to close completely leaving the portion of the ampule containing the rhizobia sealed against loss of vacuum.

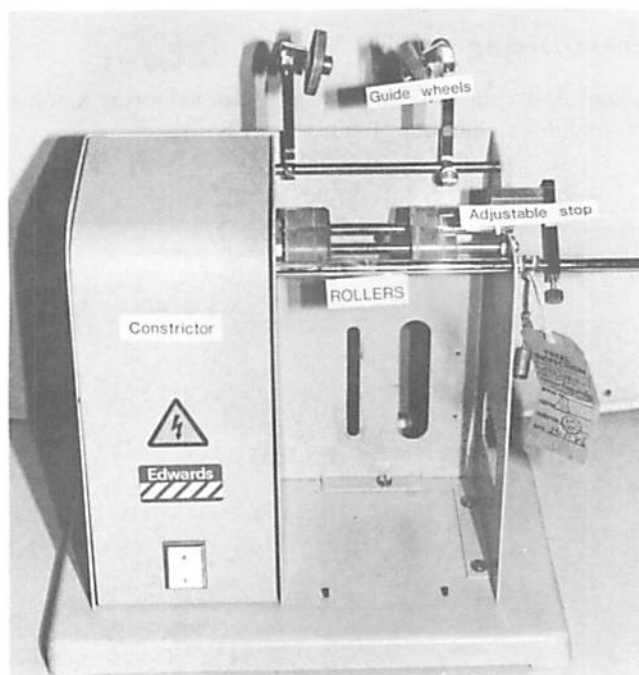


Fig. A.5.11. Ampule constrictor showing essential components.

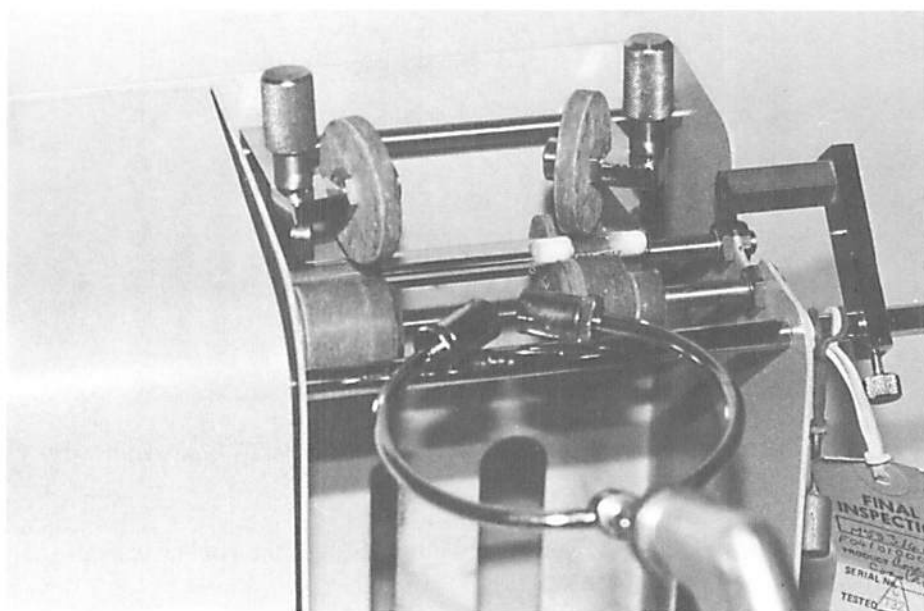


Fig. A.5.12. Ampule is constricted above cotton barrier using a double-headed torch to soften glass and stone rollers to cause constriction.

## Secondary Freeze-Drying

1. Carefully load desiccant (anhydrous  $\text{CaCl}_2$ ) into the tray assembly and place it inside the condenser chamber (Figure A.5.13).

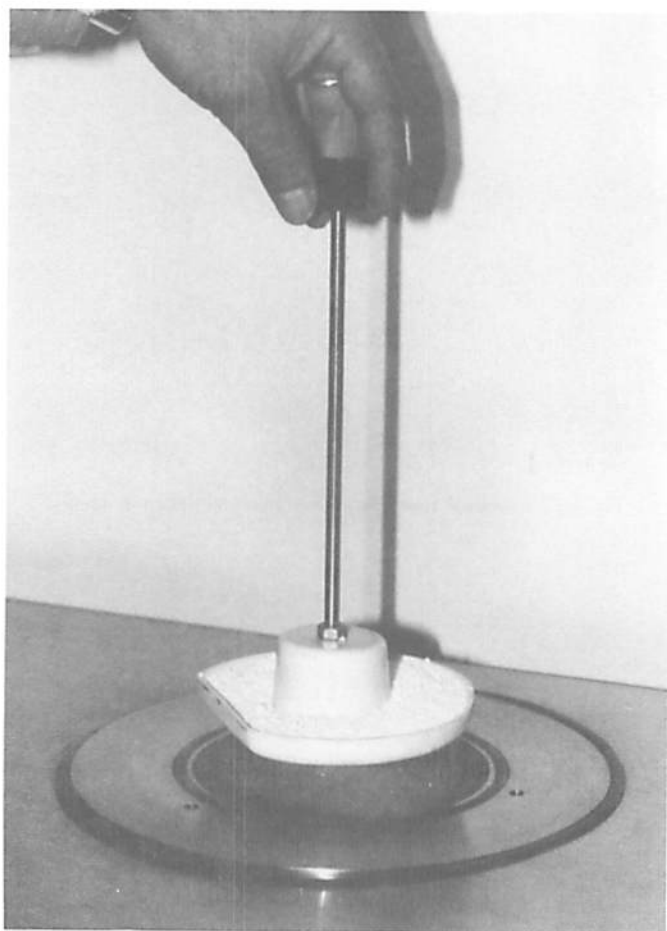


Fig. A.5.13. Loading desiccant tray assembly into lyophilizer chamber.

2. Push the open ends of the constricted ampules onto the rubber teats of the manifold assembly (Figure A.5.14).
3. Mount the manifold assembly onto the condenser chamber (Figure A.5.15).
4. Close the air admittance valve.

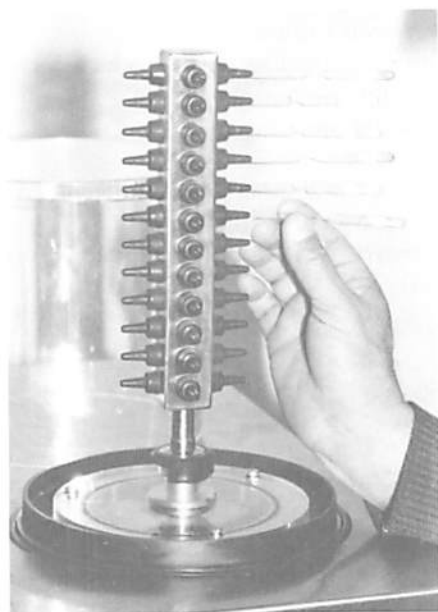


Fig. A.5.14. Inserting constricted ampules onto teats of manifold.  
It is important to ensure a good seal for each ampule.

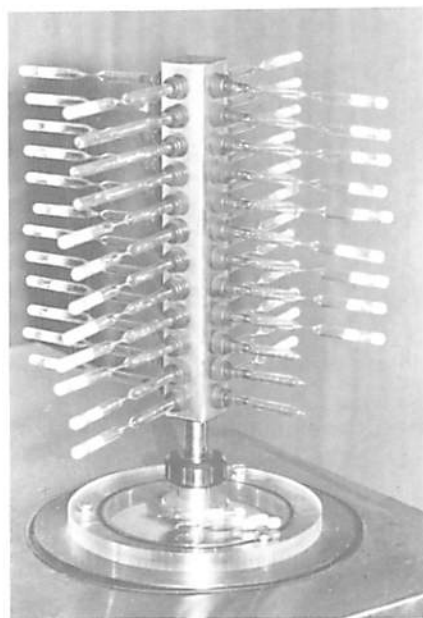


Fig. A.5.15. Manifold assembly with ampules mounted onto condenser  
chamber of lyophilizer.

5. Turn on main power switch of the freeze-dryer unit.
6. Allow the temperature to stabilize at  $-50^{\circ}\text{C}$  which corresponds to a vapor pressure of  $3.4 \times 10^{-2}$  mbar.
7. Switch on the vacuum pump.
8. Close the gas ballast valve.
9. Allow system to run for 18–20 hours until vapor pressure indicator on the lyophilizer reaches approximately  $10^{-2}$  mbar.
10. Apply heat to the constricted area of the ampule by using a special hand flame torch (Figure A.5.16).

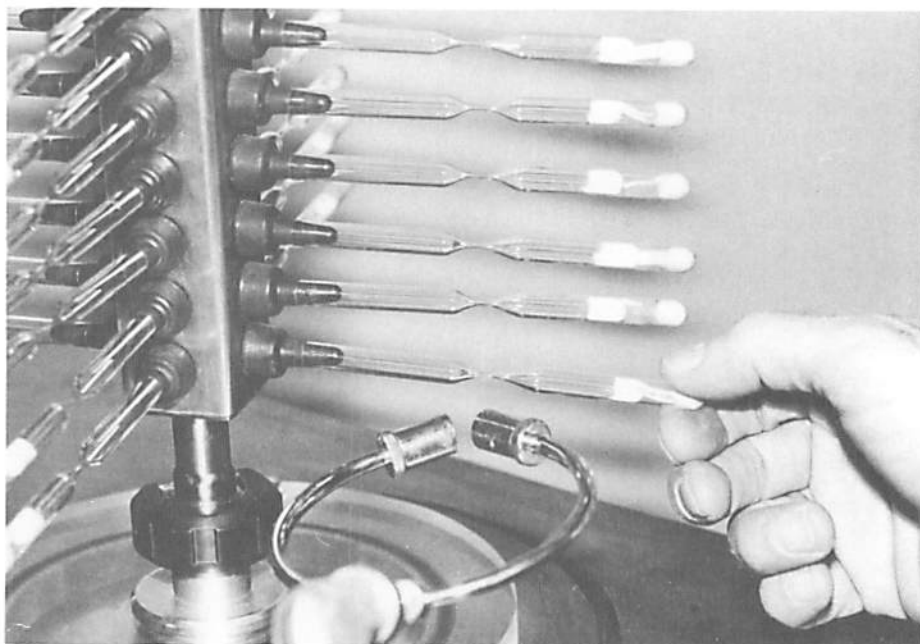


Fig. A.5.16. Heating constricted area of vial with a double-headed torch.

11. Carefully pull and twist the ampule as the glass softens (Figure A.5.17). A complete seal is necessary to ensure vacuum retention.  
**Note:** If operation fails to produce a seal and vacuum is lost (indicated by drop in pressure meter or different sound in the vacuum pump), the procedure must be stopped for at least 20–30 minutes to allow the seal for pressure (vacuum) to come back to normal.



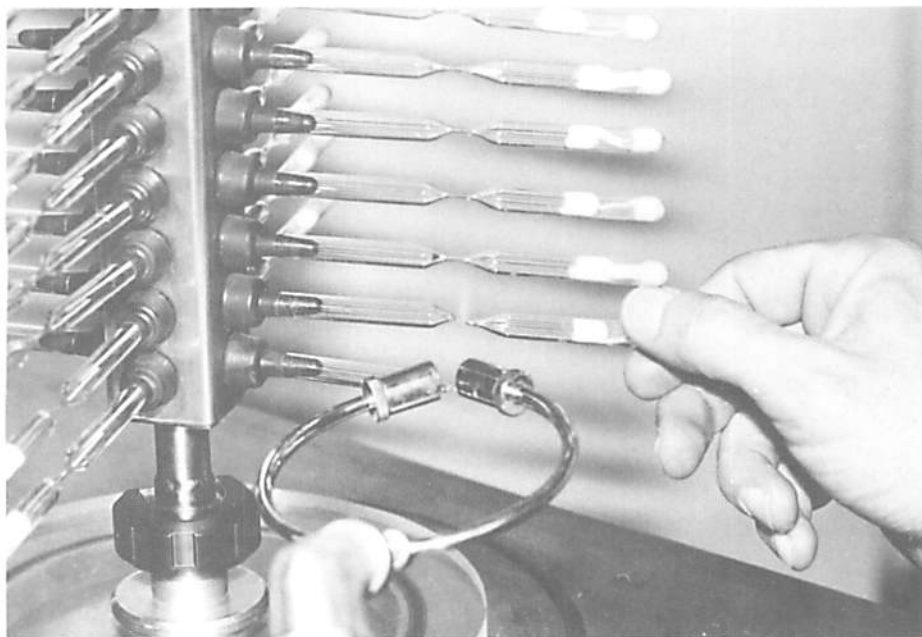


Fig. A.5.17. Twist-sealing ampule as glass softens.

12. After all ampules have been sealed, switch off vacuum pump and freeze-dryer units.
13. Open air admittance valve.
14. Remove manifold assembly, discard remaining pieces of ampules.
15. Defrost and proceed as indicated above.

### Ampule Vacuum Verification

Once the sealed ampules have been produced they should be checked for presence of vacuum using a spark tester (Edward Vacuum Spark Tester pictured in Figure A.5.18). This device consists of a transformer and high-voltage probe; the probe is touched to a finished ampule and discharged. At discharge, a properly sealed ampule will display a blue spark inside (Figure A.5.19). If no vacuum exists in the ampule, the spark will not be seen or will be a light pink. Ampules not showing a blue spark should be discarded, as lack of vacuum will cause the rhizobia to die within a short period. Special care should be taken not to overexpose the culture to the discharge. Long or repeated exposures will kill bacteria, reducing the numbers of viable cells.



Fig. A.5.18. Spark tester showing essential parts.

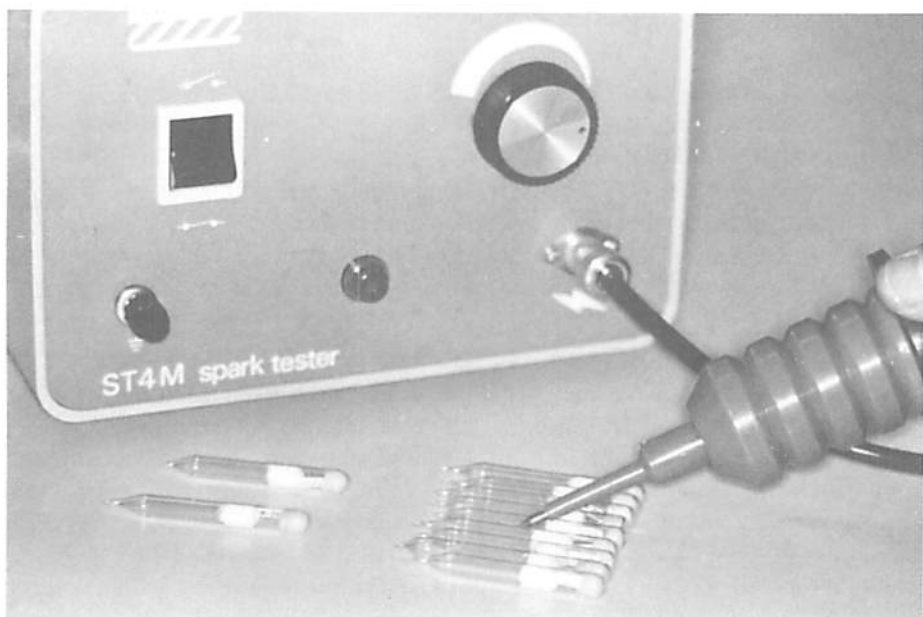


Fig. A.5.19. Testing vacuum of ampule with spark tester. Blue spark inside indicates proper vacuum.

## Storage of Ampules

The completed ampules containing freeze-dried cultures should preferably be stored under refrigeration and in a dark place. Because of their small size, hundreds of ampules can be stored in a small space.

## Culture Viability Verification

It is necessary to check culture viability after freeze-drying in order to evaluate the efficacy of your efforts, and to establish a baseline of rhizobial survival just following lyophilization. Not all strains behave the same, so all strains preserved in one run must be tested. Some strains may lose 2-3 log levels of population, while others may drop only minimally in population. Sample ampules should be reconstituted (following paragraph), and the contents diluted serially and plated out on YMA-Congo red plates for enumeration and evaluation for contamination. Contaminated batches should be discarded. The following should be recorded in a log book recording history of lyophilized cultures: strain number, date of freeze drying, date of counting (after freeze-drying), and number of rhizobia/ml.

## Reconstitution of Freeze-Dried Cultures

Use a file to make a deep scratch on the ampule, above the top cotton stopper (Figure A.5.20). Carefully, under aseptic conditions, crack it open. The contents of the ampule are rehydrated with a few drops of sterile 0.85% saline or 1.12%  $\text{CaCl}_2$  solution using a Pasteur pipette (Figure A.5.21). From the suspension, streak a loopful on a YMA plate containing Congo red. Incubate plate and check for contaminants.

## A.6. Gram Stain

The Gram stain technique is one of the most basic of microbial differentiation methods and is used to divide all bacteria into two broad categories, Gram positive (+) and Gram negative (-), on the basis of their reaction to the Gram stain process. The technique indicates fundamental differences in cell wall structure of bacteria, with those retaining a purple color (indicating absorption of crystal violet dye) called Gram positive, and those retaining a pink color (indicating absorption of safranin dye) called Gram negative. All species of *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*, commonly referred to as rhizobia, stain a pink color and are Gram negative.

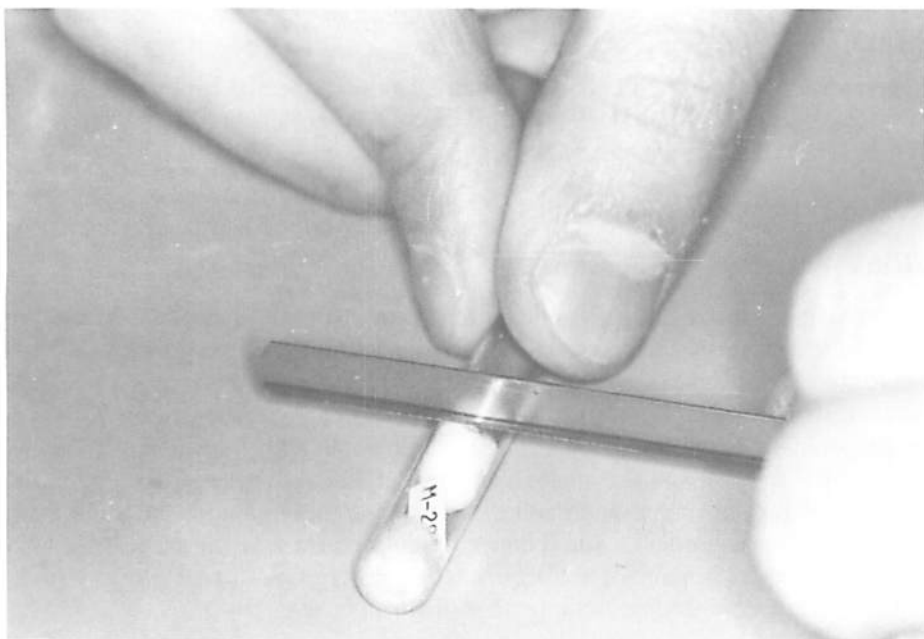


Fig. A.5.20. Scratching ampule with file prior to breaking it open.



Fig. A.5.21. Breaking open an ampule under laminar flow hood.

The reagents required are simple to make and maintain, and the only equipment required is a microscope with sufficient power to discern individual cells. The staining procedure likewise is not difficult, but it must be noted that bacteria staining as Gram (-) are not necessarily rhizobia; many other species of soil, water and airborne bacteria are also Gram (-) (e.g., *E. coli*, *Pseudomonas*).

## Gram Stain Reagents:

### 1. Crystal violet solution

|                  |        |
|------------------|--------|
| crystal violet   | 10 g   |
| ammonium oxalate | 4 g    |
| ethanol          | 100 ml |
| distilled water  | 400 ml |

Some precipitation may occur in this solution upon prolonged storage, which may affect the quality of staining. If so, warm the bottle with the cap open in a water bath at 37°C, then close and shake until the precipitate is dissolved.

### 2. Iodine solution

|                  |        |
|------------------|--------|
| iodine           | 1 g    |
| potassium iodide | 2 g    |
| ethanol          | 25 ml  |
| distilled water  | 100 ml |

This working Gram iodine solution deteriorates rapidly, especially when exposed to light and/or heat. Working solution may remain stable up to three months under normal conditions of daily use. It should be discarded when color loss becomes apparent or when adequate results are no longer obtained.

### 3. Decolorizing solution

|                 |       |
|-----------------|-------|
| ethyl alcohol   | 95 ml |
| distilled water | 5 ml  |
| or              |       |
| ethyl alcohol   | 75 ml |
| acetone         | 25 ml |

### 4. Counterstain solution

|                          |        |
|--------------------------|--------|
| 2.5% safranin in ethanol | 10 ml  |
| distilled water          | 100 ml |

## Staining Procedure:

1. Prepare a thin, evenly distributed smear of the material to be Gram stained.  
Note: In very thick smears, decolorization cannot be adequately evaluated or

controlled; very thin smears are easily over-decolorized.

- a. bacteroids from fresh nodules and bacteria growing in broth culture can be applied directly to a microscope slide with a swab or inoculating loop.
  - b. growth from bacterial colonies can be picked with an inoculating loop or needle and mixed with a drop of water or saline placed on the slide.
2. Allow the smear to completely air-dry without heat, and then heat-fix quickly passing the slide through the (low) flame of a Bunsen burner two or three times (the slide should be just hot to the touch for correct smear fixation; if exposed to excess heat, bacteria may not stain properly) (Figure A.6.1).  
**Note:** A simple staining rack can be made by suspending two glass or metal rods over a sink with drain (Figure A.6.2).
3. Cover the smear with crystal violet solution and allow the stain to act for approximately one minute.

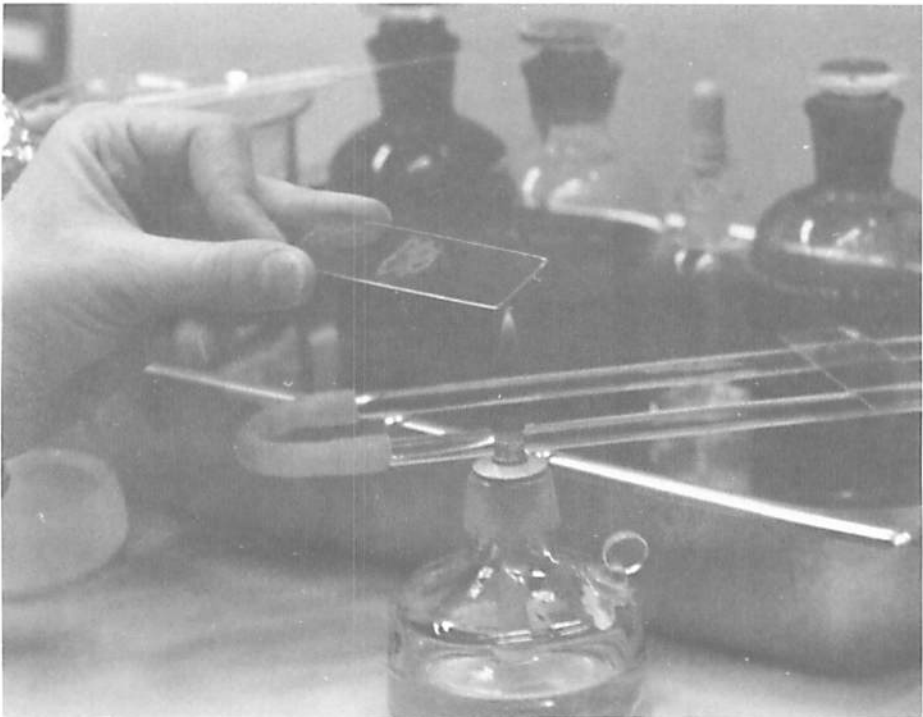


Fig. A.6.1. Heat fixing a smear over alcohol burner flame.



Fig. A.6.2. Simple staining rack holding slides over tray.

4. Remove excess stain by **briefly** rinsing the slide with tap water (Figure A.6.3).

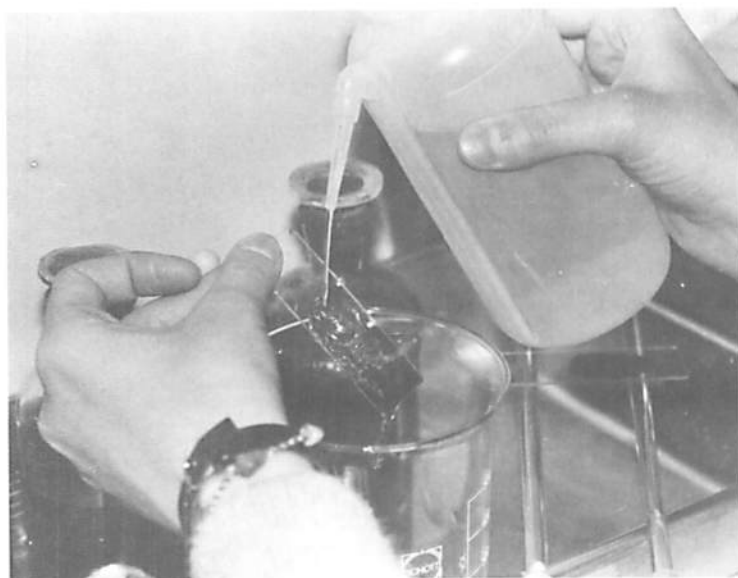


Fig. A.6.3. Rinsing the excess stain with water, taking care not to hit smear directly.

5. Shake off the excess water, flood the smear with iodine solution, and allow it to stand for one minute or longer.
6. Rinse off the iodine solution with tap water and carefully apply either decolorizer solution **just until** no more color is being washed from the smear. Quickly rinse off any remaining decolorizer with tap water.  
Note: This step is critical; do not over-decolorize.
7. Shake off any excess water and apply the safranin counterstain solution for approximately one minute.
8. Wash the slide in tap water, blot dry, and examine microscopically.

## A.7. Classification of *Rhizobium*

Under the present classification system (according to Bergey's Manual), three genera exist within the family *Rhizobiaceae*. They are *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*. To provide some understanding of why rhizobia are grouped as they are, we describe briefly the previously used rhizobia classification system, and in addition provide a method of grouping based on effectiveness.

Because of the high degree of specificity between species of legumes and certain *Rhizobium*, the previous classification system used to identify these bacteria was based solely on host (legume) specificity. Seven species (or groups of closely related strains), plus a miscellaneous group to cover a multitude of legumes, designated on the basis of preferred host, were established:

| Species                                 | Preferred host                                                                                   |
|-----------------------------------------|--------------------------------------------------------------------------------------------------|
| <i>R. leguminosarum</i>                 | <i>Pisum</i> , <i>Vicia</i> , <i>Lathyrus</i> ,<br><i>Lens</i> , <i>Cicer</i> , <i>Trifolium</i> |
| <i>R. trifolii</i>                      | <i>Trifolium</i> spp.                                                                            |
| <i>R. phaseoli</i>                      | <i>Phaseolus</i> spp.                                                                            |
| <i>R. meliloti</i>                      | <i>Medicago</i> , <i>Melilotus</i> , <i>Trigonella</i>                                           |
| <i>R. lupini</i>                        | <i>Lupinus</i> , <i>Ornithopus</i>                                                               |
| <i>R. japonicum</i>                     | <i>Glycine</i>                                                                                   |
| <i>Rhizobium</i> (cowpea miscellaneous) | <i>Vigna</i> , tree species, other (mostly tropical) species                                     |



Within each species designation, various rhizobial strains are able to infect the related group of legume. The rhizobial strains within a species were grouped together and called a 'cross-inoculation group'. Within the cross-inoculation group, the strains able to infect and bring about  $N_2$ -fixation were called effective, and those only able to infect but not fix N were ineffective.

With the newer classification system (released in the 8th edition of Bergey's Manual, 1984), the above seven distinct rhizobia species were further split into two groups based on their growth habits and physiological characteristics. **Group I** consists of species that are considered "fast" growers (generation time of two to four hours in liquid media, showing visible colonies on solid media in three to five days) and utilize a wide range of carbohydrates while producing acid metabolites. **Group II** species are "slow" growers (generation time of six to eight hours with visible colony growth after five to eight days) which utilize pentose sugars as a carbon source and produce alkali metabolites. The species *Rhizobium leguminosarum* is now divided into three 'biovars', relating to legume species nodulated.

#### Group I

| Genus            | Species              | Biovar          | Host species                                                |
|------------------|----------------------|-----------------|-------------------------------------------------------------|
| <i>Rhizobium</i> | <i>leguminosarum</i> | <i>viciae</i>   | <i>Pisum</i> , <i>Vicia</i> , <i>Lens</i> , <i>Lathyrus</i> |
| <i>Rhizobium</i> | <i>leguminosarum</i> | <i>trifolii</i> | <i>Trifolium</i>                                            |
| <i>Rhizobium</i> | <i>leguminosarum</i> | <i>phaseoli</i> | <i>Phaseolus</i>                                            |
| <i>Rhizobium</i> | <i>meliloti</i>      |                 | <i>Medicago</i> , <i>Melilotus</i> ,<br><i>Trigonella</i>   |
| <i>Rhizobium</i> | <i>loti</i>          |                 | <i>Cicer</i> , <i>Lotus</i>                                 |
| <i>Rhizobium</i> | <i>fredii</i>        |                 | <i>Glycine</i>                                              |
| <i>Rhizobium</i> | spp.                 |                 | Other species                                               |

#### Group II

|                                 |                                                                                                                                                                         |
|---------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Bradyrhizobium japonicum</i> | <i>Glycine</i> , <i>Macroptilium</i>                                                                                                                                    |
| <i>Bradyrhizobium</i> spp.      | not yet clearly classified but includes some<br><i>Cicer</i> , <i>Sesbania</i> , <i>Lupinus</i> , <i>Acacia</i> ,<br><i>Ornithopus</i> , <i>Leucaena</i> , <i>Vigna</i> |

## A.8. Sterile Technique

When handling cultures of *Rhizobium* in the laboratory, certain techniques amounting to a system must be followed if consistent results are expected. Rhizobia face a world of harsh competition, and most other bacteria and fungi are faster than rhizobia in their utilization of food sources. These other organisms are also ubiquitous, which means they are everywhere: on hands, all surfaces, in soil, air and water. These other organisms must be eliminated if pure cultures of rhizobia are to be grown. If mixed cultures of rhizobia and other organisms are introduced into a growth media, such as that used in production of inoculants, the other organisms will almost surely outcompete the rhizobia and you will end up with an inoculant containing few rhizobia. This is the reason for using a sterile technique in the laboratory: to eliminate other microorganisms from the media in which rhizobia are grown.

Basic caution and understanding of the omnipresence of contaminating microorganisms applied to laboratory work with rhizobia will lead to good results. Following are some specific points that should be followed:

1. All work with *Rhizobium* cultures should take place under a laminar-flow bench, which pumps pure filtered air (Figure A.8.1) or a clean bench (described later in this Section). A Bunsen burner (preferred) or an alcohol lamp should be lit at all times when using the clean bench.

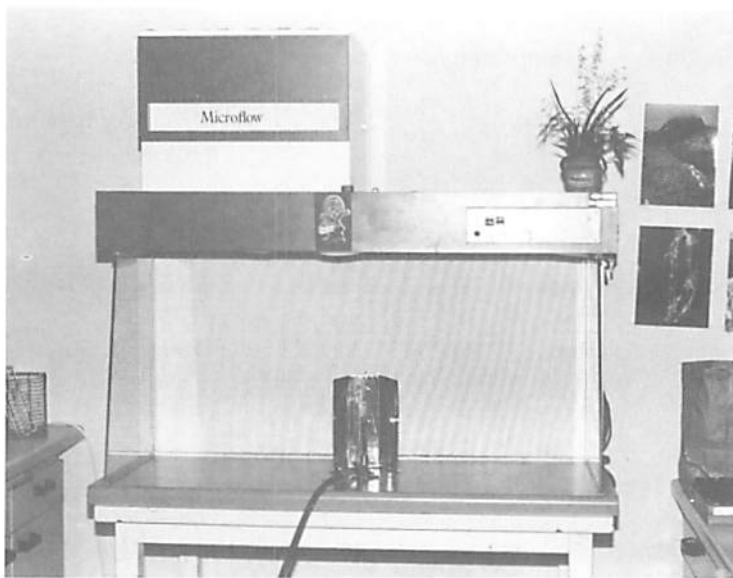


Fig. A.8.1. Laminar flow bench for aseptic handling of cultures.

2. Before beginning work, all surfaces of clean bench (or laminar hood) and the hands should be cleaned with 70% alcohol.
3. All containers, pipettes, tubes, swabs, cotton, etc., should be sterilized by autoclaving (steam sterilization at 121°C) before use. After autoclaving, these items should be closed (containers with caps or cotton stoppers) or wrapped in paper or tinfoil before sterilization to prevent contamination after removal from the autoclave.
4. All media, diluents, etc., must be sterilized by autoclaving. Sterilization time depends on the quantity or bulk of the items being autoclaved; small amounts of liquid (up to 500 ml) will require only 20 minutes at 121°C, volumes over 5 L may require sterilization of up to one hour.  
**Caution** must be taken when autoclaving media containing carbohydrates such as mannitol; excessive sterilization (heat) may alter carbohydrate composition, making it unusable by rhizobia.
5. When caps, covers, or plugs are removed to manipulate rhizobia, it should be for the minimum time possible. Upon opening a container, the mouth should be briefly flamed over the Bunsen burner to kill contaminant around the opening (Figure A.8.2).

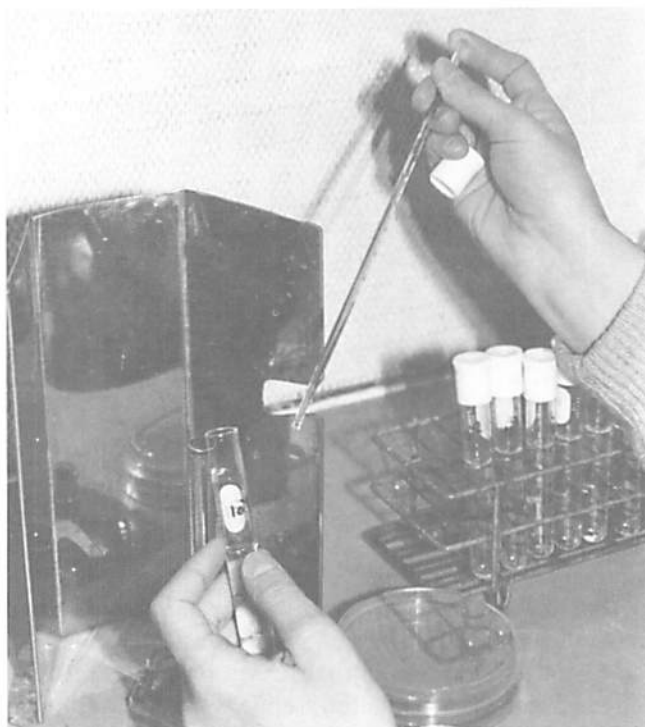


Fig. A.8.2. Flaming test tube lip over burner flame.

6. All metal utensils, such as forceps and knife, should be dipped in alcohol then set alight in the burner flame (Figure A.8.3); they should be used immediately when the flame goes out. Inoculation needles and loops should be placed in the hottest part of the burner flame until they glow an orange color (Figure A.8.4); the entire length of the wire should be flamed, not just the tip. These utensils should be used immediately after cooling slightly.
7. Before discarding used cultures, autoclave all materials containing living cultures.

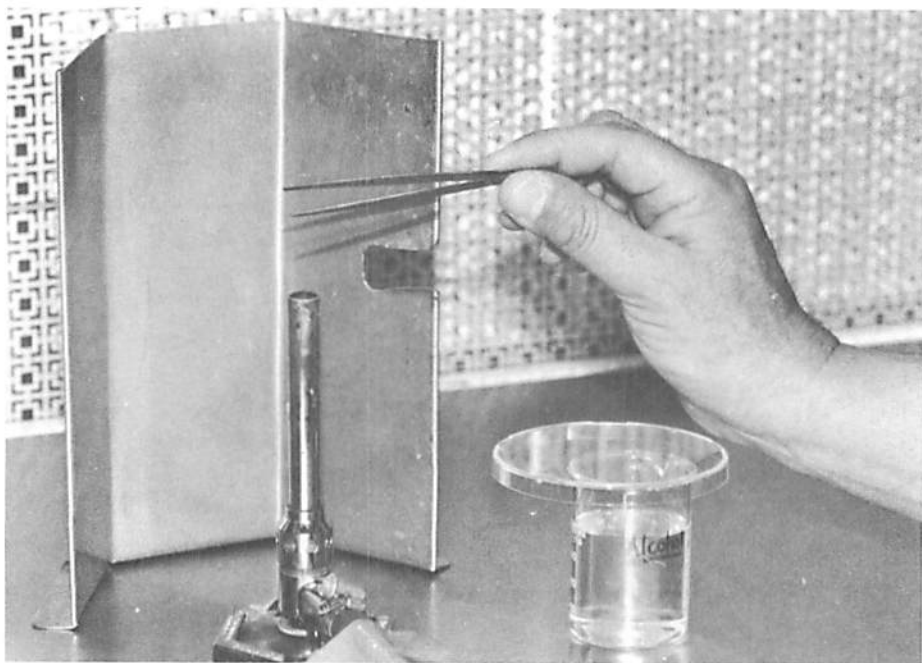


Fig. A.8.3. Flaming forceps after alcohol dip.

### Simple Transfer Chamber

A simple transfer chamber for aseptic (sterile) work can be constructed from the materials and plans following. In this design of the transfer chamber, specific attention is given to the placement and position of the Bunsen burner as this is critical to producing a sterile environment suitable for aseptic work.

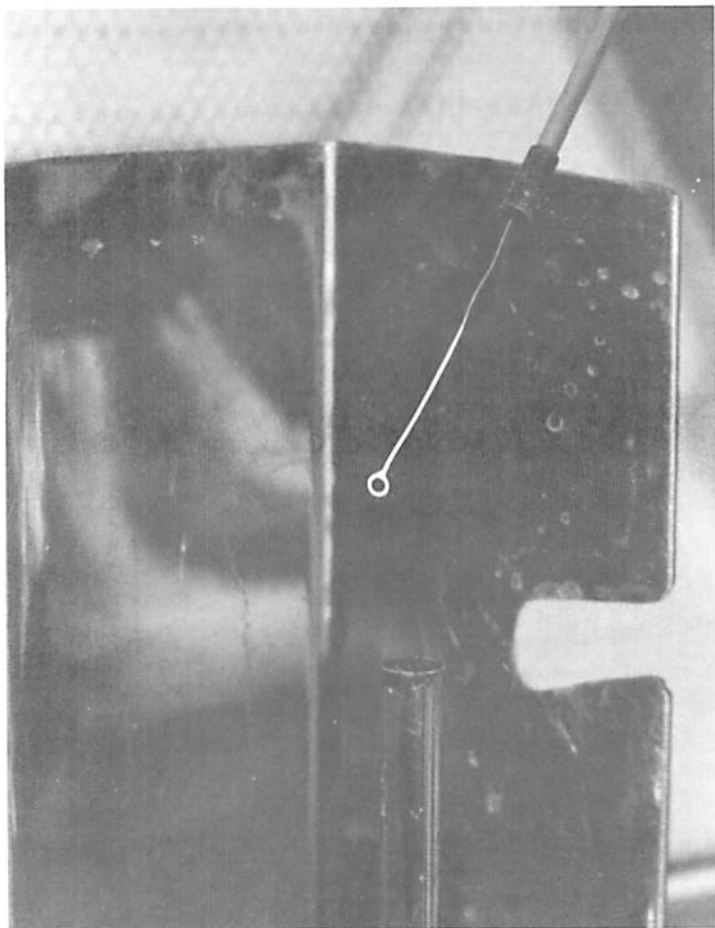
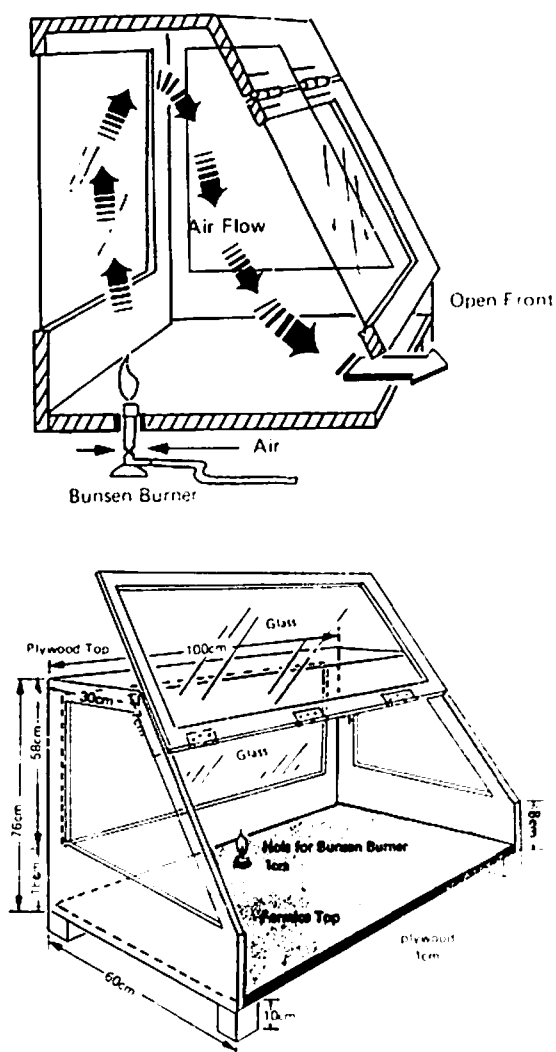


Fig. A.8.4. Flaming the length of wire loop in hottest part of burner flame.

The Bunsen burner is positioned so that approximately 3 cm of the tip of the burner protrudes into the chamber through a hole in the base (Figure A.8.5). In this position, the gas supply line and the air intake ports of the burner are left on the outside of the chamber. When the burner is lit, the flame eventually warms up the air inside the chamber and a one-way warm air current is created which exits through the open front thus preventing entry of contaminants.

A few simple steps are essential to proper functioning of the chamber: (1) open the hinged door and wipe the interior thoroughly with 70% alcohol. Allow to dry before lighting burner; (2) turn on the gas and light burner. Flame should be blue and adjusted to no more than 6 cm in height; and (3) close the hinged door and wait 20

minutes before using chamber. This allows the proper air flow to be established.



**Fig. A.8.5. Simple transfer chamber showing cross section of chamber illustrating working principle and construction drawing (NIFTAL Project).**

When work in the chamber is completed, turn off the flame and **disconnect the gas line** on the outside of the chamber. A leaky gas valve may cause the chamber to fill with gas, which would result in an explosion when the burner is lit. This is not a theoretical danger; it has happened. With correct procedures and precautions, this simple inexpensive transfer chamber will enable the user to produce consistently pure *Rhizobium* cultures.

### **Transfer Chamber Components**

1. Back; made of plywood, hardwood and glass (0.2–0.5 cm thickness)
2. Bottom; made of plywood with formica surface, includes 1.5–2.0 cm diameter hole for Bunsen burner
3. Top; made of plywood
4. Reinforcement; made of wood, serves as anchor for hinged door
5. Door; made of plate glass with hardwood frame, attached to the reinforcement with hinges.
6. Sides; made of plywood and glass
7. Wooden moldings (8); to hold glass for front window and door
8. Wooden moldings (8); to hold glass for rear window (optional)
9. Wooden moldings (16); to hold glass for side windows
10. Wooden legs (4); 10 cm high. Entire chamber sits on separate table of height to allow comfortable work.

The plywood used should be 1.5 cm thick with a smooth finish on both sides. All wooden surfaces of the chamber, inside and out, should be painted with a hard-coated, smooth oil-based epoxy type paint.

**Note:** This chamber was designed and promoted by the NifTAL Project, University of Hawaii, in its 'Applied technologies' series.

## **A.9. Media Preparation**

### **A.9.a Media for Rhizobia**

#### **Routine Complex Media**

The basic medium used in culturing of root-nodule bacteria is yeast extract-mannitol agar (YMA) or broth (YMB). Rhizobia generally grow well with supplements of complex extracts such as yeast. Mannitol is commonly used as the carbon source; however, *Bradyrhizobium* is known to grow better on other carbohydrates (galactose or arabinose). Rhizobia (fast growers) show visible growth after three days while bradyrhizobia (slow growers) show growth after seven days.

The composition of standard YMA is:

|                                      |        |
|--------------------------------------|--------|
| K <sub>2</sub> HPO <sub>4</sub>      | 0.5 g  |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O | 0.1 g  |
| NaCl                                 | 0.2 g  |
| Mannitol                             | 10.0 g |
| Yeast extract                        | 0.5 g  |
| Agar                                 | 15.0 g |
| Distilled water                      | 1 L    |

adjust pH to 7.0 if necessary

\* Do not add agar for liquid media YMB.

Some laboratories recommend the addition of 3 g of CaCO<sub>3</sub> as a neutralizing agent for acidity during long-term storage, but only to media which are used to store fast-growing (acid-producing) cultures of rhizobia.

If large quantities of media are frequently used, it is convenient to prepare stock solutions of the reagents as follows:

| Stock solution | Reactants                            | Concentration g/L | ml/L of medium |
|----------------|--------------------------------------|-------------------|----------------|
| A              | K <sub>2</sub> HPO <sub>4</sub>      | 50                | 10             |
| B              | MgSO <sub>4</sub> ·7H <sub>2</sub> O | 10                | 10             |
| C              | NaCl                                 | 20                | 10             |

The combined media mixture is sterilized by autoclaving at 120°C for 20 minutes (for 1 L; larger volumes will require more time). The pH is then corrected aseptically to 7.0 with approximately four drops of NaOH (1.0 N) into 1 L of the preparation. Mix thoroughly and recheck the pH (remove a small portion aseptically and check with pH meter).

To prevent excessive water condensation in plates it is necessary to allow the medium to cool down to about 50°C before dispensing it into sterile petri dishes. The agar solidifies at 44°C. The plates should be poured a day before they are used to allow the agar to dry out slightly and increase its water-absorption capacity. Each glass plate contains 30-40 ml of medium (25 ml in the case of plastic disposable plates). Precautions should be exercised to avoid contamination during the pouring operation. The lip of the flask or tube should be passed through the flame of a burner before



pouring. In some cases, special dispensers and sterilized syringes are also utilized to dispense sterile molten media.

For the preparation of slants (slopes) used for storage of rhizobia, molten medium (heated carefully over a burner to just below boiling to dissolve agar thoroughly - **do not boil**) is dispensed into the vials before autoclaving. Vials of 25–50 ml capacity are generally used for this purpose. Approximately half of the volume of the vial can be filled with the YMA medium. After autoclaving, screw caps are tightened and vials are left to cool on a 30–45° slanted surface (Figure A.9.1).



Fig. A.9.1. Liquid agar, automatic syringe, slants, and sterilized agar tubes cooling in a slanted position.

## Selective YMA Media

### 1. Additives

Contaminants from senescent nodules or from the working environment are likely to interfere with the normal growth of rhizobia on YMA. The incorporation of biostats to YMA medium aids in the suppression of growth of fungi and actinomycetes thus

making the medium more selective for rhizobia. The supplemented media should be used cautiously as the biostats may differentially affect different strains of rhizobia. The most commonly used biostats are:

### **Congo Red**

Besides suppressing the growth of some actinomycetes it also assists in the recognition of rhizobia from other soil bacteria. Rhizobia and bradyrhizobia absorb the dye weakly (colonies with a white or pinkish coloration) whereas many other bacteria (e.g., *Agrobacterium*) take it strongly (reddish colonies). However, the distinction is not absolute; this property is one of several sometimes shared by *Agrobacterium*, *Rhizobium*, and *Bradyrhizobium*, most commonly *R. meliloti*. Dye adsorption may also be affected by the nature of the medium and conditions of cultivation. Congo red is soluble in water and in ethanol. Stock solutions are usually prepared by adding 2.5 g of Congo red to 100 ml of sterile water. A 1-ml aliquot of this stock is added to 1 L of YMA medium before sterilization.

### **Brilliant Green (Malachite Green, Ethyl Green, Emerald Green)**

A staining constituent of bacteriological media, it excludes the growth of many microorganisms. It is recommended that this biostat not be used with a pH indicator in the YMA medium. Stock solutions of brilliant green are usually prepared by adding 3 g to 100 ml of sterile water or ethanol; 1 ml of the stock is added per liter of medium to obtain a final concentration of 30 ppm. The brilliant green can be added to the medium before autoclaving.

### **Actidione (Cycloheximide)**

This antibiotic helps in the suppression of fungal growth. A stock solution can be prepared by adding 3 g actidione to 250 ml of ethyl alcohol. It is recommended to filter-sterilize the stock solution. Alternatively, actidione may be dissolved in sterile water and used immediately. A 1-ml aliquot of the stock is added to 1 L of a cooled 50°C YMA medium. Final concentration will be 12 ppm. Extreme caution must be exercised in handling this product as it is toxic.

### **Rose Bengal**

Stock solutions are prepared by adding 3 g of rose bengal to 100 ml of sterile water. An aliquot of 1 ml is added to 1 L of cooled YMA medium. Some investigators combine actidione with rose bengal to strengthen the exclusion of contaminants able to grow in YMA. To prepare a modified rose bengal-actidione medium use stock solutions prepared for actidione and rose bengal. Add same proportions as if additives were being used separately.

## **2. pH Indicators**

Acid and alkali production under standard conditions can be used to divide root-nodule bacteria into two major groups, which correspond to their rate of growth: fast growers, acid producers (*Rhizobium leguminosarum*, *R. loti*, *R. fredii*, and *R. meliloti*); and slow growers, alkali producers (*Bradyrhizobium* spp.).

Solutions made of pH-indicator substances can be prepared as stock solutions and added to YMA medium to demonstrate relatively small changes in acidity or alkalinity of the media as indicated by changes in color of the medium by the above root-nodule bacteria. Many non-rhizobial contaminants will also be revealed in this medium by their most drastic effect in pH. Some commonly used pH indicators for rhizobial studies are:

### **Bromthymol Blue**

This indicator turns yellow at pH 6.0 and blue at pH 7.6, and is green between pH 6.0 and 7.6. Stock solutions are prepared as 0.5% bromthymol blue (BTB) in 0.016 N NaOH (pH 6.8). Add 5 ml to the medium before autoclaving. In general, bradyrhizobia will turn the green YMA medium into blue whereas rhizobia will turn it into yellow.

### **Bromcresol Purple**

This turns yellow at pH 5.2 and purple at 6.8. Stock solutions are prepared as 0.5% bromcresol purple (BCP) in 0.016 N NaOH (pH 5.5). Add 5 ml to YMA medium before autoclaving.

### **Bromcresol Green**

This turns yellow at pH 3.8 and blue-green at pH 5.4. Stock solutions are prepared as 0.3% bromcresol green (BCG) in 0.016 N NaOH (pH 4.5). Add 5 ml to YMA medium before autoclaving.

## **A.9.b Growth Medium to Screen Rhizobia for Acid Tolerance**

The composition of the defined medium and final concentrations are:

|    | Compound                             | mg/L                     |
|----|--------------------------------------|--------------------------|
| A. | Na <sub>2</sub> SO <sub>4</sub>      | 100.00                   |
|    | MgSO <sub>4</sub> ·7H <sub>2</sub> O | 200.00                   |
|    | CaCl <sub>2</sub> ·H <sub>2</sub> O  | 5.00                     |
|    | MnSO <sub>4</sub> ·4H <sub>2</sub> O | 1.11                     |
|    | FeSO <sub>4</sub> ·7H <sub>2</sub> O | 5.00                     |
|    | ZnSO <sub>4</sub> ·7H <sub>2</sub> O | 1.00                     |
|    | CuSO <sub>4</sub> ·5H <sub>2</sub> O | 0.50                     |
| B. | KH <sub>2</sub> PO <sub>4</sub>      | 3.40                     |
|    | K <sub>2</sub> HPO <sub>4</sub>      | 4.35                     |
| C. | Lysine                               | 100.00                   |
|    | Thiamine HCl                         | 1.00                     |
|    | Pantothenic acid                     | 1.00                     |
|    | Biotin                               | 2 × 10 <sup>-3</sup>     |
|    | Folic acid                           | 0.125 × 10 <sup>-3</sup> |
| D. | Agar                                 | 20.00 g/L                |
| E. | MES                                  | 30.7 mM                  |
| F. | Distilled water                      | 1 L                      |

#### Stock Solutions Needed:

1. Make a stock solution in a ratio of 100 times the concentration of the components of A (except for Na<sub>2</sub>SO<sub>4</sub> and MgSO<sub>4</sub>·7H<sub>2</sub>O) for 100 ml of distilled water. To avoid waste of chemicals, we recommend adjusting concentrations for 25 or 50 ml volumes so that an aliquot of the stock will contain the required final concentration for the final volume (1 ml for 1 L, 0.5 ml for 500 ml, etc.)
2. Make a stock solution for B in a ratio of 100 times the concentration of both components for 100 ml of distilled water. Adjust concentration and volume according to final volume required.
3. Make a stock solution for C using a ratio of 100 times the concentration of each component in 100 ml of distilled water. Because of costs, we recommend using the same ratio of concentration but in lower volumes (25 or 50 ml). Then take the necessary aliquot according to the final volume to be prepared.

#### Filtration:

1. Filter the stock solution B using a 0.45-μm pore size membrane. Membrane and holder must have been autoclaved previously.
2. Similarly, filter stock solution C using a new membrane.

### **Preparation: (Assume 1 L)**

1. Add 1 L of distilled water into a flask. Add 1 ml of stock A and 20 g of agar. Autoclave at 120°C for 15 minutes. Final pH will be approximately 5.1.
2. Add 1 ml of both stocks B and C to the above sterile solution. Temperature of the solution must range between 45 and 50°C.
3. Add 7.15 g/L (30.7 mM) of MES [2(N-morpholino) ethane sulfonic acid] to the above sterile solution and shake strongly. No need to filter MES. Temperature of the solution must range between 45 and 50°C. Final pH will be approximately 8.16.

### **Adjustment of pH**

1. Adjust pH with 0.3N HCl, 1M NaOH and 0.1M NaHCO<sub>3</sub> · HCl to decrease pH and the other two to increase it. Aseptically insert pH probe into flask to measure pH until desired pH is obtained. As the pK<sub>a</sub> of MES is 6.09, the minimum possible pH of this medium is 5.2.
2. Pour volumes of approximately 20 ml into petri dishes. Let them cool, then invert and refrigerate them.
3. We have tried pH values of 6.0, 5.5, and 5.2 with successful results in screening *R. meliloti*.

### **A.9.c. Plant Growth Media**

Legume plants produced from surface-sterilized seed can be grown under sterile conditions in glass tubes, plastic pouches, and jars of all types. These systems allow studies of rhizobia for enumeration, strain selection, and host-plant compatibility studies using a mineral solution lacking mineral nitrogen.

For any experimental system, all the necessary nutrients, with the exception of mineral or combined nitrogen, must be provided at the levels adequate to ensure that nutrition does not limit the expression of symbiotic capacity. Several elements are essential for the successful formation and function of legume nodules. Calcium, phosphorus, molybdenum, cobalt, boron, copper, and iron each have a particular role in the symbiosis. However, in formulating any nutritive solution for growing plants in the above systems, consideration must be given to growth of the entire plant and other elements, such as sulphur, potassium, chloride, and zinc, must be included.

There are many nutrient solutions used by researchers working with rhizobia. Some popular mineral solutions are those of Thornton (1930), Jensen (1942), McKnight (1949), Fåhræus (1957), Munns (1968), Harper and Gibson (1984), and Broughton and Dillworth (1970). However, they are similar in the concentration of the

component ions and in general most elements are provided in excess amounts so that any plant-nutrient disorders are likely due to toxicity rather than to deficiency. Such is the case of phosphate, which is used in excess to increase the pH-buffering capacity of the nutritive medium.

The majority of legumes nodulate well in the pH range of 6.0-8.0, but there is wide variability among species in their ability to form nodules outside this range. For example, *Medicago* species nodulate poorly below pH 6.0 and rarely nodulate below pH 5.2. Clovers are more tolerant to low pH than medics, but pH 4.7 is the lower limit for nodulation of most clover (*Trifolium*) species. Low pH also promotes the availability of Al and Mn which are known to produce toxicity problems in legumes. On the other hand, caution must be exercised that the pH of the medium is not above 8.0 as it may lower the availability of elements such as Fe, B, Zn, and phosphate.

In liquid or solid systems having nutrient solutions, legumes usually cause a drop in the pH of the solution in the rooting medium because of hydrogen ion ( $H^+$ ) excretion during cation uptake and excretion of organic acids. With the uptake of nitrate ( $NO_3^-$ ) from the medium the pH tends to rise, whereas with ammonium ion ( $NH_4^+$ ) uptake the pH falls. The use of  $NH_4NO_3$  as a source of nitrogen in control treatments will minimize the pH change. With agar medium, appropriate mixtures of  $K_2HPO_4$  and  $KH_2PO_4$  can be used as a buffer to establish and maintain the required pH for four to five weeks depending on the volume of the medium, plant species and growth conditions.

In systems in which solid rooting substrates such as sand, gravel, vermiculite, and perlite are used, it is necessary to thoroughly wash the substrate with running water overnight to bring the pH to near neutrality. Some researchers use acidified water for this purpose. Newly purchased vermiculite, for example, sometimes has a pH in excess of 9.0, depending on the source. After washing the substrate, it is advisable to rinse the material with distilled or deionized water. Some tap water may contain traces of nitrate, which if adsorbed onto the substrate will affect nodulation and plant growth of legumes under experimentation.

The formulations of the nitrogen-free mineral nutrient solutions used at ICARDA are:

1. For large-seeded legumes (food and forage legumes):  
Modified Broughton and Dillworth (1970)
2. For small-seeded legumes (pasture legumes):  
McKnight (1949).

The composition of the modified Broughton and Dillworth's nitrogen-free mineral solution used at ICARDA is:

| Stock | Compound                                   | Amount<br>g/L | Final solution<br>concentration* |
|-------|--------------------------------------------|---------------|----------------------------------|
| 1     | $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  | 294.1         | 1.00 mM                          |
| 2     | $\text{KH}_2\text{PO}_4$                   | 136.1         | 0.50 mM                          |
| 3     | $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  | 123.3         | 0.25 mM                          |
|       | $\text{K}_2\text{SO}_4$                    | 87.0          | 0.25 mM                          |
|       | $\text{MnSO}_4 \cdot \text{H}_2\text{O}$   | 0.338         | 1.00 $\mu\text{M}$               |
| 4     | $\text{H}_3\text{BO}_3$                    | 0.247         | 0.30 $\mu\text{M}$               |
|       | $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  | 0.288         | 0.50 $\mu\text{M}$               |
|       | $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  | 0.1           | 0.20 $\mu\text{M}$               |
|       | $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ | 0.048         | 0.01 $\mu\text{M}$               |
|       | $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  | 0.056         | 0.01 $\mu\text{M}$               |
| 5     | Fe citrate <sup>+</sup>                    | 5.4           | 10.00 $\mu\text{M}$              |

\* For each liter of full-strength solution add 0.5 ml from each of the five stock solutions.

+ Modified from original formulation.

The composition of McKnight's nitrogen-free mineral solution used at ICARDA for small-seeded legumes grown in vermiculite is:

| Stock<br>solution* | Compound                                           | Amount<br>g/L |
|--------------------|----------------------------------------------------|---------------|
| 1                  | $\text{CaSO}_4$                                    | 24            |
|                    | $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$          | 4             |
|                    | $\text{KH}_2\text{PO}_4$                           | 4             |
|                    | KCl                                                | 4             |
|                    | $\text{FeCl}_3$                                    | 0.20          |
| 2                  | $\text{MnSO}_4 \cdot \text{H}_2\text{O}$           | 1.54          |
|                    | $\text{H}_3\text{BO}_3$                            | 2.80          |
|                    | $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$          | 0.22          |
|                    | $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$          | 2.49          |
|                    | $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$          | 0.08          |
|                    | $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ | 0.021         |
|                    | $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$          | 0.286         |

\* Add 300 ml of stock solution 1 and 4 ml of stock solution 2 to 3.7 l. of distilled water.

When growing seed of *Medicago* or *Trifolium* species in tubes containing agar slopes we recommend the Fânhracus solution with agar for firmness.

| Compound                                  | Amount   |
|-------------------------------------------|----------|
| <b>Macroelements</b>                      |          |
| $\text{KH}_2\text{PO}_4$                  | 100 mg/L |
| $\text{NaH}_2\text{PO}_4$                 | 150 mg/L |
| $\text{CaCl}_2$                           | 100 mg/L |
| $\text{MgSO}_4 \cdot \text{H}_2\text{O}$  | 120 mg/L |
| Fe citrate                                | 5 mg/L   |
| <b>Micronutrient stock 1</b>              |          |
| $\text{H}_3\text{BO}_3$                   | 2.86 g/L |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  | 2.08 g/L |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.22 g/L |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | 0.08 g/L |
| $\text{Na}_2\text{MoO}_4$                 | 0.11 g/L |

Add 1.0 ml of stock 1 (micronutrient solution) to the 1-L preparation done with the macroelements. Shake vigorously and then add 16 g of agar. Autoclave the seedling agar preparation at 121°C for 15 minutes. Let it cool down to about 50–55°C and dispense equal volumes into tubes. Let tubes rest in an inclined position to obtain slanted surface to which seedlings then will be transferred.

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## B. Enumeration

Estimating numbers of rhizobia in soils, inoculants and laboratory cultures is an important activity in most *Rhizobium* laboratories, and is essential in inoculum production. The methodologies described apply to this range of needs, and vary in terms of accuracy, facilities and time required. Use of nephelometry (optical density measurement) for enumeration in broth media is fastest and simplest, but may be inaccurate. Plant infection tests provide a reliable estimation of infective rhizobia populations in soils and inoculants, but require six to eight weeks. Counts in YM agar plates can be performed in the time required for the rhizobia to produce a few generations and are accurate in pure or even fairly clean cultures, such as inoculants. The most useful techniques for each of the *Rhizobium* technologists' requirements have been emphasized in this section; information on alternate methodologies is presented in shorter form.

### B.1. Direct Population Counts in Liquid Culture

#### B.1.a. Microscopic Cell Counts

The size of a microbial population may be measured by counting the number of individual cells with a microscope. There are four important limitations to keep in mind if using this procedure.

1. Counting individual cells under a microscope is tedious and not practical for large numbers of samples.
2. At least  $10^6$  bacterial cells per ml must be present before a single cell can be seen in the microscope field.
3. Living cells cannot be distinguished from dead cells, so counts will likely be higher than actual number of viable cells present.
4. Only suspensions of pure cultures, such as broth cultures, can effectively be measured using this method.

In order to count the number of cells present, and extrapolate to populations in culture, a microscope slide containing a special chamber of known volume is used; the variety described here is the Petroff-Hausser type.

The Petroff-Hausser counting chamber (Figure B.1.1) is a machined glass slide with a sunken platform within a well. The platform is located exactly 0.02 mm below the slide's surface and contains a precisely etched grid of 25 squares; each of these squares is further divided into 16 squares. The grid has an area of  $1 \text{ mm}^2$  and a total volume of  $2 \times 10^{-5} \text{ ml}$ . Because the gap between the grid surface and the overlying

glass coverslip is so precise it is possible to relate the number of cells counted in the grid with the volume of suspension; from this the concentration of bacterial suspension can be calculated (with a large degree of variance). The only advantage of this method is that a population estimate can be obtained almost immediately. Depending on the age and condition of the culture suspension, it can be inaccurate. These factors make the technique of use only in special situations.

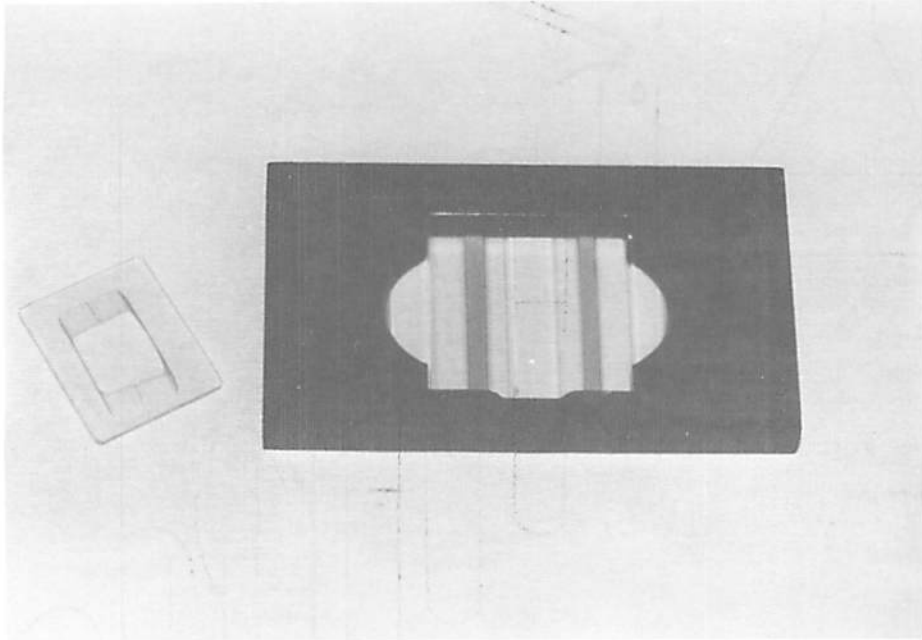


Fig. B.1.1. Petroff-Hausser counter for bacterial cell enumeration.

### B.1.b. Viable Cell Counts

A viable cell is defined as one that is able to divide and form a colony. Viable counts are therefore performed by determining the number of cells capable of forming colonies. The most widely used method utilizes a yeast mannitol (YM) agar plate surface to grow individual cells until they become visible colonies. A known volume of rhizobial suspension is placed on the surface of the plate; population of the original suspension then can be calculated from the number of colonies produced (assuming one cell produces one colony). Dilutions are usually made so that the number of colonies produced is countable (try to imagine accurately counting 1000 dots on a small piece of paper) but high enough to be statistically accurate (counting only three to four colonies per plate will induce a high coefficient of variability).

All viable cell count methods rely on a set of serial dilutions which are intended to bracket the expected number of cells in the culture. A visual presentation of the dilution series is given in Figure B.1.2, and will be further elaborated for each of the

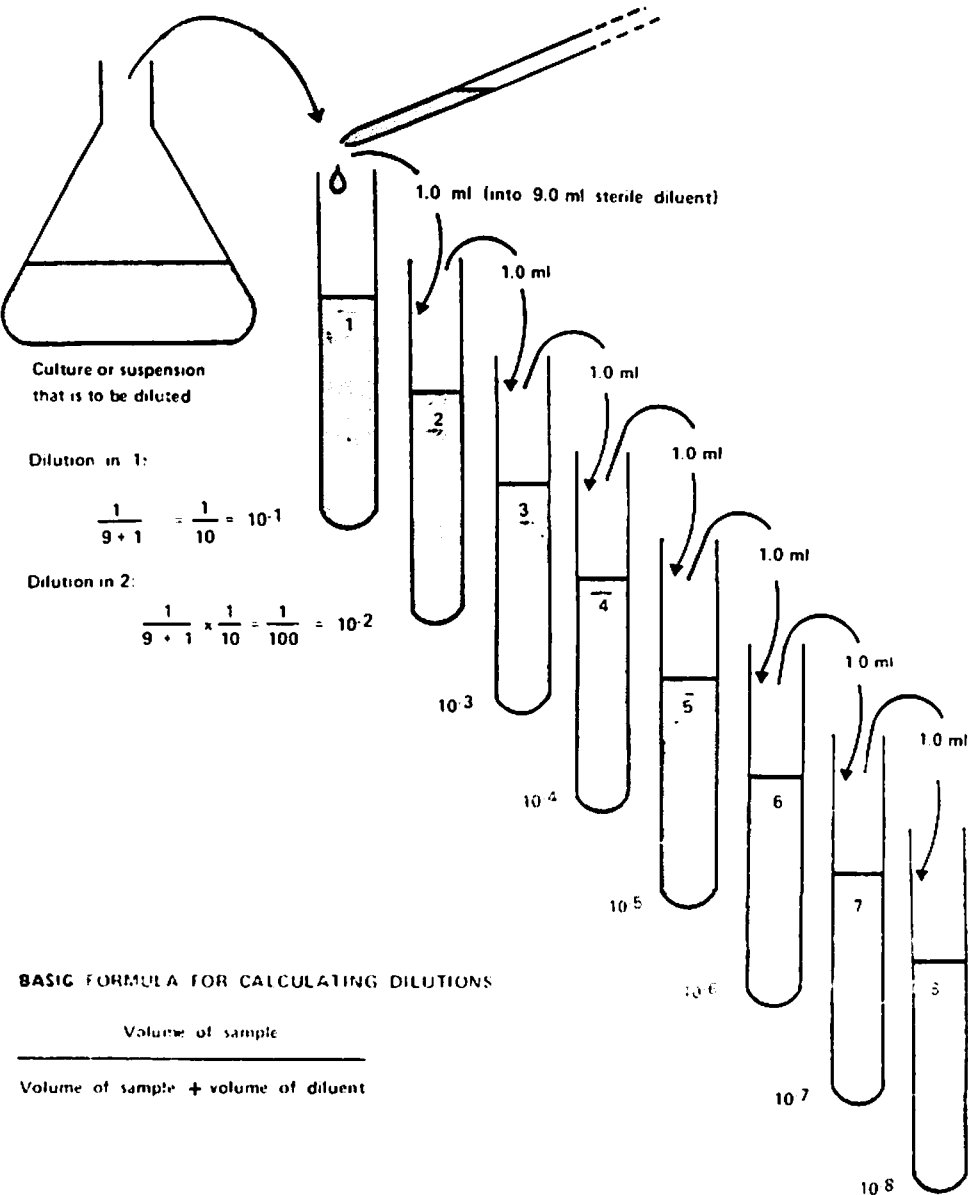


Fig. B.1.2. Ten-fold dilution series illustration.

applications. The number of cells in a pure broth culture may be estimated visually by color and opacity; a culture which is opaque and milky will usually contain  $10^8$ – $10^9$  cells per ml; a culture only slightly cloudy will contain  $10^5$ – $10^7$  cells (Figure B.1.3). Most soils contain between 0 and  $10^5$  cells per gram; inoculants up to  $10^{10}$  per gram. Dilutions are chosen to overlap on the high and low sides of expected population, with an attempt to count 50–300 colonies per plate. The number of colonies on the agar surface, multiplied by all dilutions used, gives the population in the original suspension.

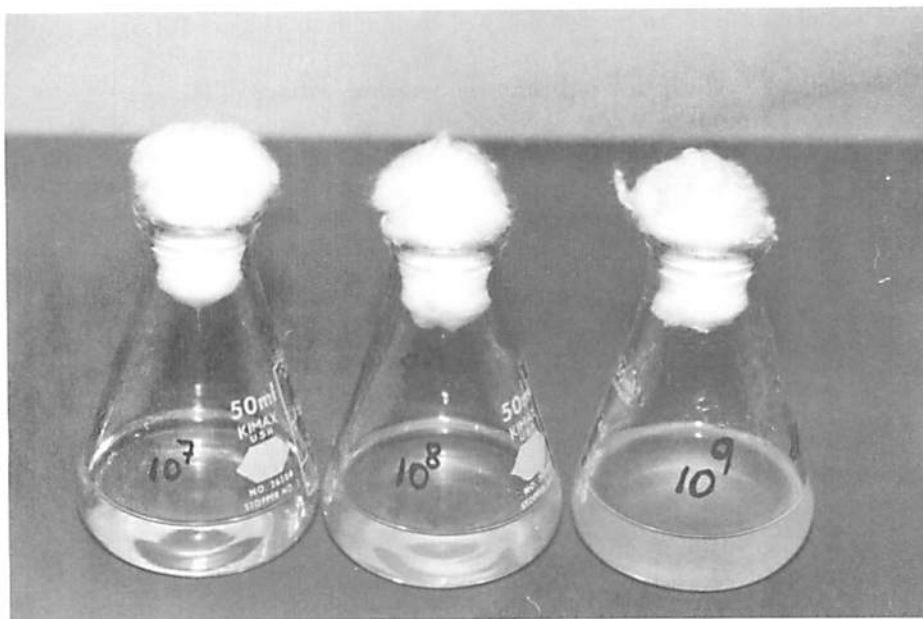


Fig. B.1.3. Visual growth densities in erlenmeyer flasks containing YM broth at  $10^7$ ,  $10^8$ , and  $10^9$  dilutions.

The most common version of this technique is the 'spread plate', but versions utilizing an "agar sandwich" ('pour plate') or counting the number of cells within one drop on the plate ('drop count') are used by some technologists. Emphasis in this handbook is placed on the spread plate method, with short explanations and calculations for the other two.

In the **spread plate** technique, a small volume of diluted culture (0.1 ml) is aseptically pipetted directly onto the surface of an agar plate containing YM media, or YM with Congo red (see Section A.9). The culture is then dispersed with a sterile glass-rod spreader evenly over the surface of the plate. This spreader can easily be made from a

straight glass rod, in the shape shown in Figure B.1.4. The rod used for spreading the culture must be dipped in alcohol, flame sterilized and cooled briefly before using on each plate. The sample is most evenly distributed over the surface of the agar if a small turntable (Figure B.1.5) is used; with this device, the spreader rests lightly on the surface of the agar after pipetting the bacterial suspension onto the center of the plate, and in gently spinning the turntable the suspension is evenly spread over the entire agar surface. If a turntable is not available the plate can be manually rotated with one hand one-third of a turn at a time while the spreader is held in the other. The petri dish cover should not be set aside while spreading, but should be held 4-5 cm over the plate surface to prevent entry of contaminants (Figure B.1.6).

With this method, colonies develop uniformly over the surface of the agar; contaminants with different colony morphologies can be easily detected, particularly if Congo red YMA plates are used. If the spreading procedure and dilution series are performed correctly, colonies will be separated and easy to count (Figure B.1.7). The following is a sample exercise to acquaint you better with the technique.

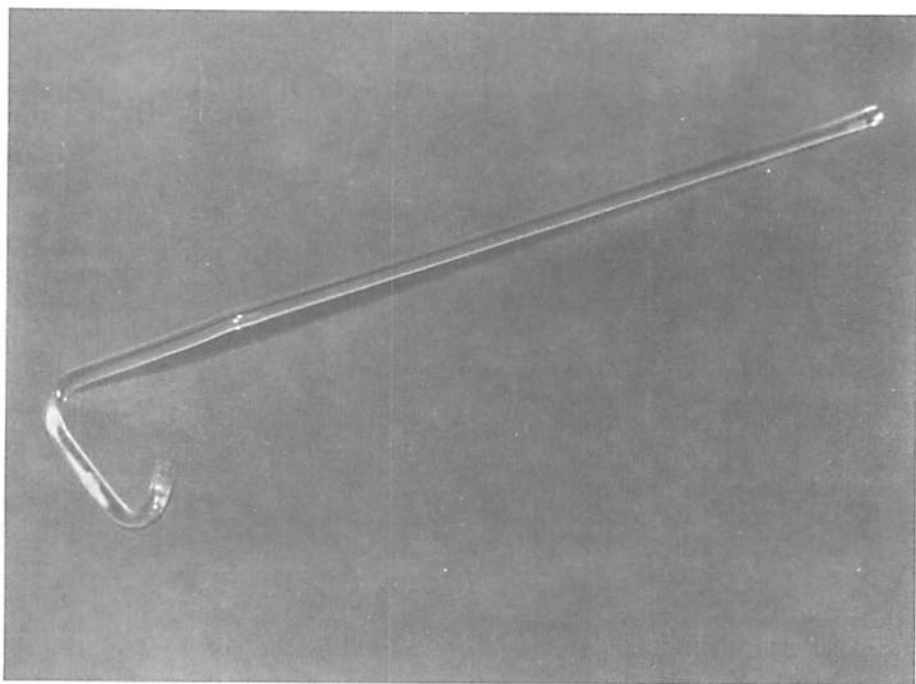


Fig. B.1.4. L-shaped glass spreader rod.

Note slight bend about 5 cm from 'L' to facilitate application to agar surface.



Fig. B.1.5. Spreading suspension on the agar surface aided by the spinning action of a turntable. Note that petri dish cover is held just over agar to minimize possibility of contamination.

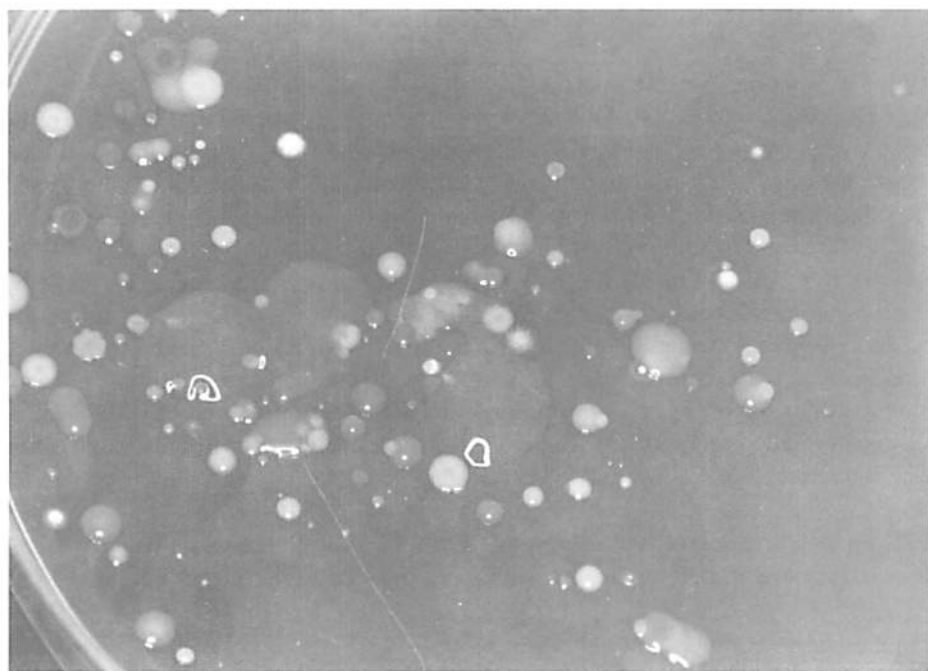
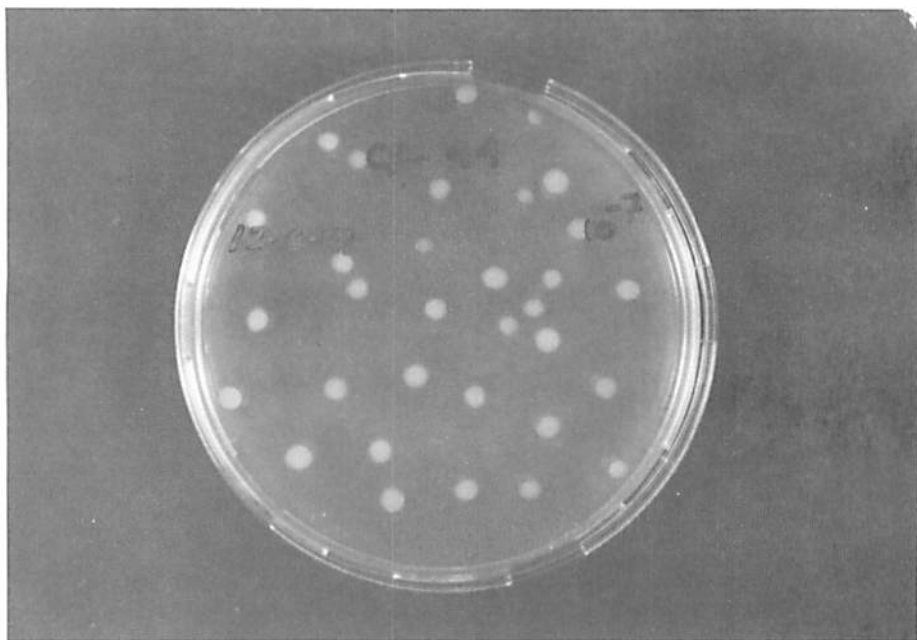


Fig. B.1.6. YMA plate showing colonies of rhizobia and contaminants.



**Fig. B.1.7. Colonies of rhizobia on the surface of a YMA plate for colony count.**

**Sample exercise.** Prepare a 10-fold dilution series up to  $10^7$  beginning with 5 g of peat inoculant, using sterile 0.01 M  $\text{CaCl}_2$  as the diluent. The steps involved in the dilution series are as follows: 5 g peat inoculant is transferred into a bottle containing 95 ml sterile diluent; this is shaken vigorously by hand for one minute, or on a mechanical shaker for 10 minutes. One ml of this suspension is pipetted into a test tube containing 9 ml sterile diluent; this tube is the  $10^3$  dilution. After mixing carefully by hand or vortex mixer (do not shake), 1 ml of this dilution is transferred to another tube containing 9 ml sterile diluent; this tube is the  $10^4$  dilution. Proceed with the dilution process until you reach the  $10^7$  step. Using three plate replications per dilution, transfer 0.1 ml of the  $10^7$  dilution onto the surfaces of each of the (usually 3) plates, and spread (Figure B.1.5). Repeat this procedure with the  $10^6$ ,  $10^5$  and  $10^4$  dilutions in succession.

**Note:** If pipettes are scarce, the same pipette can be used through the whole procedure for each sample, as long as you work back from the highest dilution to the



lowest (i.e., from the dilution containing the fewest cells to that containing the most); care must be taken to rinse the pipette well in the dilution being currently pipetted before taking out a sample.

This is an easy procedure to follow:

1. Make all dilutions using test tubes filled with sterilized diluent (usually prepared for duplicate or triplicate samples).
2. Label plates (three or four per dilution sample) with treatment, replication, date and dilution.
3. Taking the highest dilution, pipette 0.1 ml to each of the three plates.
4. Flame sterilize the glass-rod spreader before using on each of the three plates in turn.
5. Proceed with other dilutions in same manner.
6. Invert plates (so that the half holding agar is on top) and place in incubator at 25-28°C.
7. Check the plates each day for growth. When colonies are easily discernible on agar surface, count the number of colonies on plates of the dilution having 40-300 colonies visible; this will give you the most accurate count.
8. Multiply the average number of colonies from the three plates (or six plates if you used two sample replications) and the dilution series to get the population in the original material. A sample calculation where the average count is 125 colonies per plate on the  $10^5$  dilution (should be 12-13 colonies on the  $10^6$  dilution) is:

$$\frac{100 \text{ ml diluent}}{5 \text{ g inoculant}} \times \frac{10 \text{ ml}}{1 \text{ ml}} \times \frac{10 \text{ ml}}{1 \text{ ml}} \times \frac{\text{plate}}{0.1 \text{ ml}} \times \frac{125 \text{ colonies}}{\text{plate}} = 2.5 \times 10^6 \frac{\text{cells}}{\text{g inoculant}}$$

( $2 \times 10^1$  dilution) ( $10^1$ ) ( $10^1$ ) ( $10^1$ ) (125) . . . Note that all units cancel except cells per g inoculant (colonies are assumed to be formed from 1 cell); knowing what final units you want to achieve will help you in setting up the equation. Remember that you have used an increasing dilution series, so each component of the equation should increase the total.

The **pour plate** method uses the same principles of the dilution series and counting the number of colonies contained in the entire plate, but instead of growing the colonies on the agar surface, they are placed **within** the agar to reduce later contamination. A sample (usually 1 ml) of diluted culture is pipetted into an empty sterile petri dish, and approximately 20 ml of still liquid but cooled agar (44-45°C) is poured on top. The contents are then swirled gently both clockwise and counterclockwise, forward and back, left and right to thoroughly mix culture and agar. Allow the mixture to solidify completely, invert the plates and incubate at 25-28°C until individual

colonies can be seen. Colonies will appear on the surface of the medium as well as be embedded in the agar. Calculations are performed as in the spread plate method.

The most critical step in this procedure is pouring the media at the correct temperature. If too hot ( $> 45^{\circ}\text{C}$ ) the rhizobia will be killed; if too cool ( $< \text{about } 44^{\circ}\text{C}$ ) the agar will have begun to solidify and will not pour properly. About the only way to manage this is by using a water bath set at  $45^{\circ}\text{C}$ ; flasks containing the sterile molten agar media are kept in this bath until plates are poured (Figure B.1.8). Calculations are performed as in the spread plate method. If 1 ml of suspension is used to inoculate the plate, this step need not figure in the calculations; if less than 1 ml is used this will be an additional factor, to be included in the calculations as shown for the spread plate technique (more than 1 ml should not be used, as this will over-dilute the agar).



Fig. B.1.8. Flask containing media held in a water bath at  $47^{\circ}\text{C}$  (constant temperature) prior to pouring plates.

The **drop plate** technique is used when large numbers of samples must be counted, as when determining population response of several different rhizobial strains to stress in laboratory experiments. The advantage of this method is that up to six samples (e.g., three replications of two dilutions) can be placed in one petri dish, thus saving plates (Figure B.1.9). The method does, however, require extra skill (meaning more practice) and steady hands; in addition, it is necessary to calibrate the Pasteur pipettes so that all pipettes used deliver the same volume in a drop. The main disadvantage of the technique is that it can only be used with pure cultures; if a fast-growing contaminant is present, it will obscure rhizobia colonies within the drop before they can be counted.

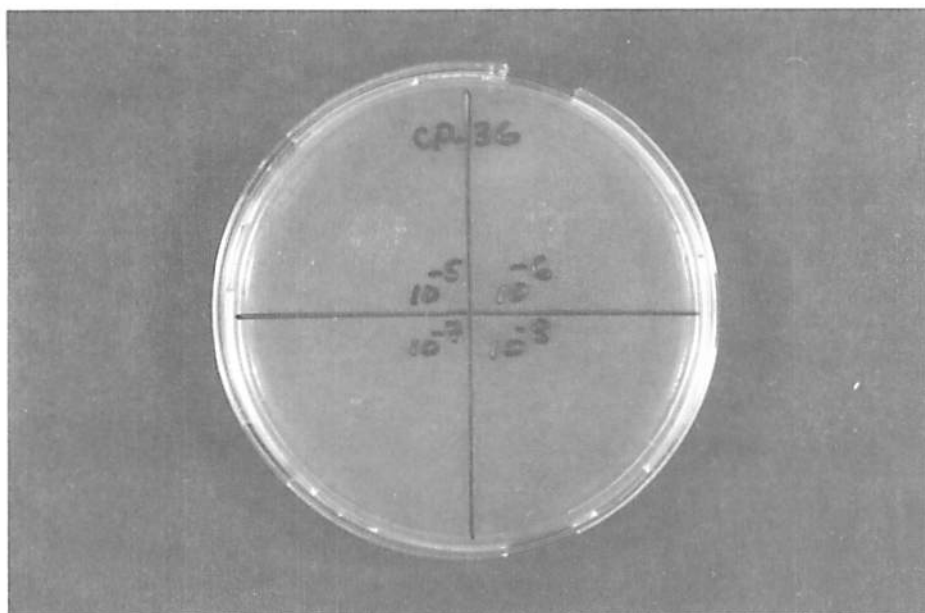


Fig. B.1.9. Drop plate with divisions showing drop area with countable colonies.

Use agar plates that are at least three days old or have been dried at 30°C overnight; a somewhat dry agar will quickly absorb individual drops, preventing the drops from running together when the plates are inverted or moved. Radially mark off six equal sectors on the outside bottom of the petri dish. Label three sectors for replications of one dilution and three for another, allowing two dilutions per plate.

As with the spread or pour plate techniques, between 20 and 200 colonies should be counted per drop to obtain statistically accurate information. Clearly, timing in colony counting is more critical with this technique. If a viewing microscope is available (such

as those used for viewing plant samples for insect or disease identification), colonies can be counted before they can be seen by the naked eye within a field of vision to allow for counting 200 colonies. If colonies become larger than a pinpoint, they will begin to grow together and numbers will be difficult to determine correctly.

An additional dilution must be taken into account when determining final population in the original sample: the volume of the drop must be known and consistent. A Pasteur pipette (Figure B.1.10) is the easiest way to deliver a drop of consistent volume, but two major factors must be taken into account: 1) the opening of the pipette must be the same in all pipettes used. Pasteur pipettes are mass manufactured and vary in opening diameter, so must be selected for uniformity; and 2) the angle of the pipette with the table top when held in the hand should remain the same for all drops delivered (Figure B.1.11). The volume of the drop will become less as the pipette is moved to the vertical position.

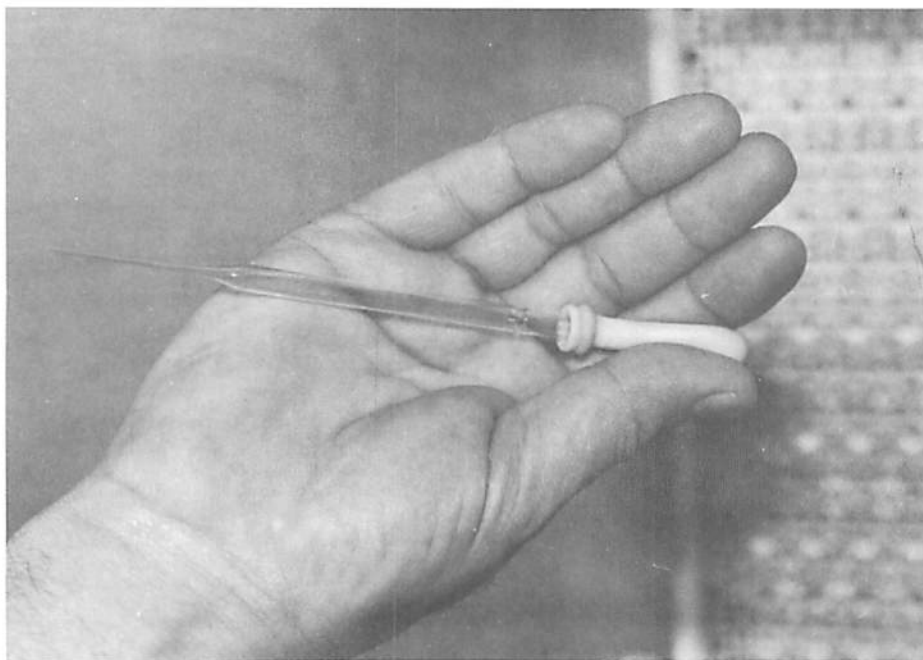


Fig. B.1.10. Pasteur pipette with rubber bulb used in applying drops to plate.

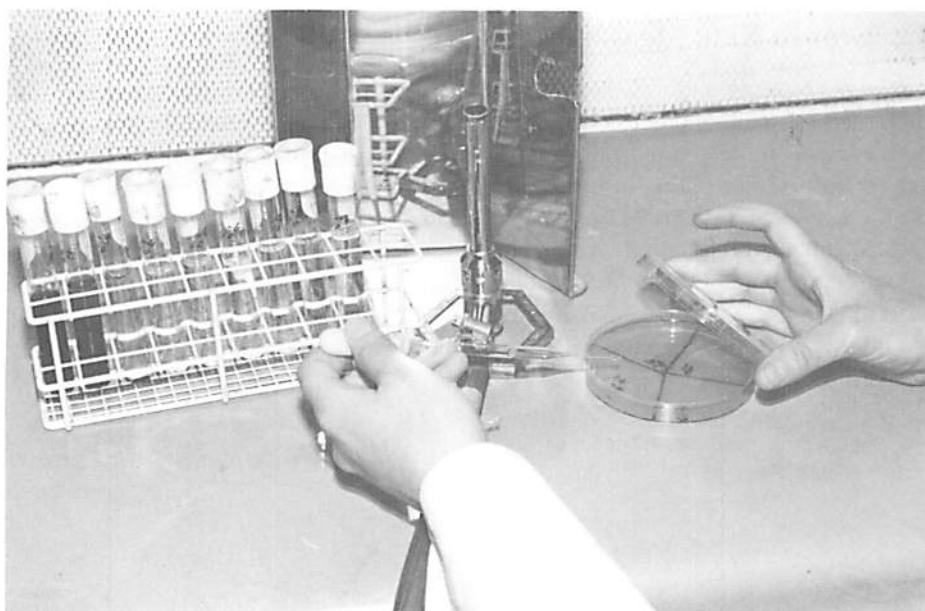


Fig. B.1.11 Correct angle for holding pasteur pipette for applying drops to agar surface

In order to use only pipettes which deliver a known value, pipettes can be calibrated with a thin wire of a standard size. Pipettes where the wire does not fit or is too loose should be used in other applications. Actual calibration of the pipettes can easily be accomplished by using a sensitive balance, with an accuracy of  $\pm 0.01$  mg. The weight of 20 drops of distilled water, divided by the number of drops, will give the volume of one drop ( $1 \text{ g} = 1 \text{ ml}$ ). The viscosity of mature cultures will generally have been reduced to that of water in the dilution series, so using water or diluent to measure drop volume (weight) will give accurate results. If a sensitive balance is not available, an estimate of how many drops are required to make 5 ml, using a burette or volumetric cylinder, will also then give you the volume of one drop. This 'drop volume' can then be used in the calculation to give the population in the original sample:

$$\frac{10 \text{ ml diluent}}{1 \text{ ml broth}} \times \frac{10 \text{ ml}}{1 \text{ ml}} \times \frac{10 \text{ ml}}{1 \text{ ml}} \times \frac{\text{drop}}{0.05 \text{ ml}} \times \frac{25 \text{ colonies}}{\text{drop}} = 5 \times 10^5 \frac{\text{cells}}{\text{ml broth}}$$

( $10^1$  dilution) ( $10^1$ ) ( $10^1$ ) ( $2 \times 10^1$ ) (25). . . Note that all units cancel except cells (which is assumed to equal colonies) per ml in original broth. The drop volume here (equal to 20 drops per ml) is approximately the delivery volume of a Pasteur pipette if held at an angle acute to the table top (see Figure B.1.11).

### B.1.c. Growth Rate Measurements

The colony-counting methods described above are particularly useful for determining growth rates of strains under controlled conditions. The most common use of a growth curve is to determine the time required for a culture to reach maximum log phase for culture harvesting in inoculum production. Figure B.1.12 shows a growth curve for a 'typical' fast-growing strain, with lag, log, stationary, and death phases. In this graph, mean viable counts have been determined every 12 hours, and transformed into  $\log_{10}$  values. Viable cells from plate counts (Y-axis) have been plotted versus time (X-axis) beginning at time-zero, resulting in a smooth curve drawn through the points. This curve will be reproducible with a strain in a given medium at a given temperature.

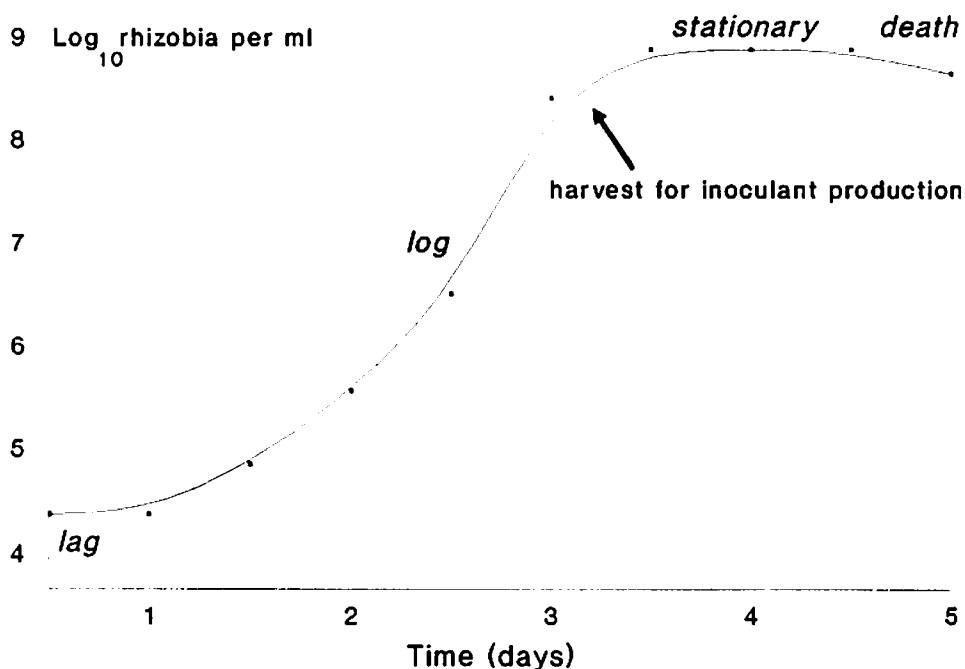


Fig. B.1.12. Growth curve typical for fast-growing rhizobia species (strains will vary), showing the lag, log, stationary and death phases. Arrow indicates optimum harvest time for inoculant production.

Temperature will affect rate of growth, so development of growth curves for specific applications (e.g., inoculum production) should be obtained under the conditions to which it will be applied. If, for example, a fermentor will run during winter months in

an unheated shed at 18°C, the applicable growth curve should not be generated in the laboratory at 25°C, as the cooler temperature will delay growth, resulting in harvesting of the fermentor before a high population is obtained.

## **B.2. Indirect Counting of Liquid Cultures**

Indirect enumeration does not imply less accuracy than direct counts; in fact the two methods described in this section have different applications and precision. Optical density measurements have the advantage of providing an answer almost immediately, and may be accurate if working with a fresh, pure culture of a strain for which a growth curve has been calibrated on the spectrophotometer. The plant-infection technique requires a long time, enough for rhizobia to infect the plant and form functioning nodules, but gives an estimation of the numbers of viable, effective rhizobia in a nonsterile sample of soil, inoculant, or broth culture.

### **B.2.a. Optical Density Measurements**

In everyday use of a limited number of strains, as in inoculant production, it is possible to characterize growth of the strains by plate counts and to correlate growth in plates with the turbidity, or optical density reading on a spectrophotometer. To do this, a growth curve must be developed using a viable cell count method (plate count), with corresponding readings taken at each sampling time on a spectrophotometer at wavelength 540 nm. This growth curve, relating optical density with population, can then be used in future applications with this particular strain in pure cultures. Optical density measurements, if calibrated beforehand in this way, are a simple, accurate and convenient estimate of cell numbers in fresh pure cultures. They require little manipulation and aseptic conditions need not be observed.

This type of measurement can only be performed on fresh pure cultures under nonstress conditions, as the technique will not distinguish between different types of bacteria or live cells from dead. In addition, some species and strains of *Rhizobium* produce large quantities of extracellular gum or exopolysaccharides (particularly in older cultures or under stress conditions, e.g., low pH), which are long-chain molecules that add to the optical density and therefore cause overestimation of the numbers of rhizobia.

A simple photometer which can read at 520–540 nm wavelength is all that is required (Figure B.2.1); the machine is adjusted to zero prior to each reading with a blank of sterile liquid medium (the medium contains suspended solids which add to the optical density of the solution; this factor is subtracted automatically if the photometer is adjusted to zero prior to reading samples). Cuvettes (special glass tubes used in a

photometer, Figure B.2.2) should be washed carefully between readings with distilled water.



Fig. B.2.1. Spectrophotometer with components labelled.



Fig. B.2.2. Cuvettes, of special optical glass, for use in spectrophotometer.



### B.2.b. Plant Infection Method

Direct counts are applicable only to pure cultures of *Rhizobium*, for any bacteria with the appearance of rhizobia on solid media (and there are many) will be counted as rhizobia. There is no selective medium that permits growth of rhizobia alone. Therefore, quantifying *Rhizobium* in mixed culture requires use of a plant infection count (also referred to here as the MPN or most probable number estimation), relying on the unique property of rhizobia to form effective nodules on legumes. Testing by plant infection is of special interest in strain authentication as it indicates only the number of bacteria capable of effectively nodulating plants and not rhizobia that have lost their infectiveness or effectiveness. The technique involves growing the plants in sterile culture in Leonard jars (Figure B.2.3), plastic pouches (Figure B.2.4), or large test tubes (Figure B.2.5) and inoculating them with a prepared dilution series from the liquid medium to be counted. The presence of nodule(s) in the various dilution series indicates that rhizobia were present in the dilution. By using the statistical tables provided, the presence/absence of nodules in the various dilutions can be transformed into an estimate of numbers of rhizobia in the original sample.

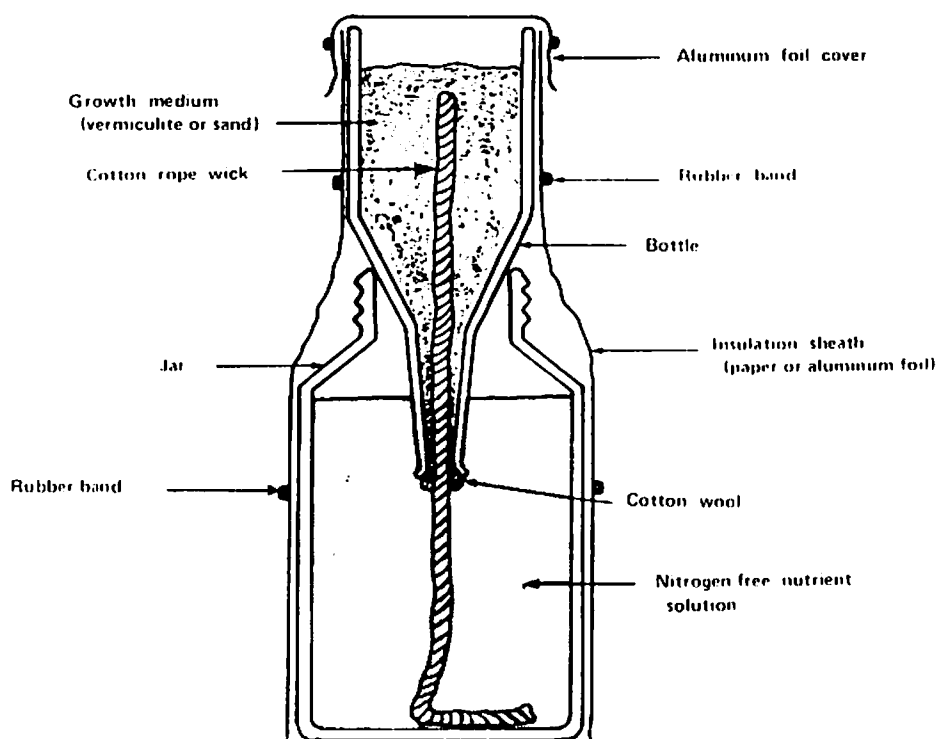


Fig. B.2.3. Leonard jar system for growing plants.

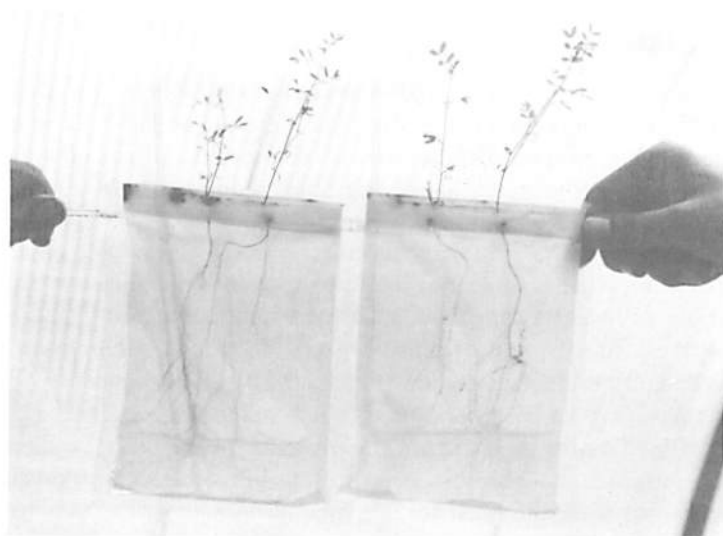


Fig. B.2.4. Plastic growth pouch with paper liner for growing plants. Nodules are clearly visible on the more vigorous plants in right-hand pouch.



Fig. B.2.5. Test tube growth system with gravel-vermiculite mixture as rooting medium.

This technique or variations of it are not the only ones available to enumerate infective rhizobia in mixed cultures, but require the least sophisticated facilities and expertise and are the most commonly used. The plant infection method, as well as other methods, are described in detail in the following section.

### B.3. Population Counts in Soils and Inoculants

Knowledge of populations of infective rhizobia in soils is extremely useful to the microbiologist, as it can be used to interpret the need for inoculation and survival of introduced rhizobia, and is the basis for various approaches to competition studies. For the inoculant producer or quality control supervisor, determination of viable rhizobia populations in inoculants is essential for a product that will be consistently effective and that will be accepted by farmers.

#### B.3.a. Differential Media

In order to enumerate rhizobia in the soil environment it is necessary to remove interference from other soil microorganisms; this, however, is a difficult task. Common soil microflora (bacteria, actinomycetes, and fungi) grow in abundance on the nutrient-rich media, making the counting of rhizobia extremely difficult. Most bacteria and fungi grow faster than rhizobia and will outcompete them in the growth media. Also, contaminants such as fungi and actinomycetes may have an antagonistic effect on rhizobial growth. We have found that adding selective agents in a basic medium can lower the contamination level enough so that estimates of *Rhizobium meliloti* populations in soil can be made, although in general all contaminants are not excluded.

Selective agents such as Congo red, brilliant green, sodium azide, and PCNB, when added to growth media, allow monitoring of particular *R. meliloti* strains in the soil, because they inhibit growth of other soil organisms. Populations can be estimated frequently, because of the simplicity and rapidity of the technique. The technique is most accurate when rhizobial numbers in the soil or substrate are high. The selective agents are useful in many ecological studies involving single or multiple strain inocula. However, the plant infection count remains a more reliable method of enumerating rhizobia in soil, particularly when they are present in low numbers.

The formulation of the medium used to successfully enumerate from soil *R. meliloti* strains ICARDA M28, M29, and M38 and others consists of:

|                                      |        |
|--------------------------------------|--------|
| K <sub>2</sub> HPO <sub>4</sub>      | 0.5 g  |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O | 0.2 g  |
| NaCl                                 | 0.1 g  |
| Mannitol                             | 10.0 g |
| Yeast extract                        | 0.5 g  |
| Agar                                 | 15.0 g |
| Distilled water                      | 1.0 L  |

Adjust the pH to 7.1–7.2, sterilize at 121°C, 15 lb pressure for 15 minutes. After the medium has cooled to 45–50°C add aliquots of the following biostats:

**For medium A:**

| Stock solution                | Stock concentration (µg/ml) | Amount to add (ml) |
|-------------------------------|-----------------------------|--------------------|
| Congo red                     | 25.0                        | 10.0               |
| Brilliant green               | 1.5                         | 1.5                |
| Sodium azide                  | 1.0                         | 0.1                |
| Pentachloronitrobenzene(PCNB) | 500                         | 5.0                |

**For medium B:**

| Stock solution                 | Stock concentration (µg/ml) | Amount to add (ml) |
|--------------------------------|-----------------------------|--------------------|
| Rose bengal                    | 25.0                        | 10.0               |
| Brilliant green                | 1.5                         | 1.5                |
| Sodium azide                   | 1.0                         | 0.1                |
| Pentachloronitrobenzene (PCNB) | 500                         | 5.0                |

After adding corresponding aliquots to basic medium, allow the plates to dry for two days. Make soil dilutions and add 0.1 ml of soil suspension corresponding to 10<sup>1</sup> (10 g soil in 90 ml sterile water), 10<sup>2</sup> and 10<sup>3</sup> dilution levels and spread uniformly onto the surface of the plate. Incubate at 26°C for three days. Count colonies that appear to be rhizobia. The assumption that bacteria are rhizobia may be supported by taking samples of some representative colonies at random for a Gram stain check (some knowledge of growth characteristics of rhizobia on the medium is necessary; Gram stain will not verify whether bacteria are rhizobia).

### **B.3.b. MPN Plant Infection Technique**

As mentioned in the previous section, use of selective media to estimate numbers of rhizobia in soil is often not accurate in distinguishing rhizobia from other soil bacteria, and will not allow the experimenter to distinguish between the different species of rhizobia. Therefore, controlled plant infection tests are usually used to estimate numbers, type, and effectiveness of soil-borne rhizobia.

The plant infection count (also called the most probable number or MPN) is commonly applied to estimate numbers of rhizobia in soil or to determine the quality of inoculants produced from nonsterile carrier materials. Basically, the method uses plants grown under sterile conditions, which are inoculated with the soil or inoculant in solution. By making a series of dilutions of this soil/water mixture, adding each dilution to several plant replicates, and then counting the proportion of plants that form nodules at each dilution, the number of rhizobia in the original sample can be calculated from statistical tables. The accuracy of the method depends on the ability of a single rhizobia cell to form a nodule on the test plant. This assumption may not be completely valid, particularly when the plant is grown on vermiculite or gravel/vermiculite mixture, as it is necessary that the growing roots encounter all rhizobia cells in the container. This leads to an underestimation of the population being counted; this underestimation will vary with species and with the moisture content of the growth system. The method does, however, provide the best estimate of the number of infective rhizobia in soil.

**Sample selection.** As soil is the most complex system to deal with, further discussion will focus on counting rhizobia in soils. The same principles, however, apply to enumerating infective rhizobia in nonsterile inoculants.

Since the size of field populations of rhizobia may vary within short distances in a field, much care should be taken in sampling so that a truly representative sample of the field soil is obtained. The area should be mentally divided into equal squares, approximately 10 for each one-fourth hectare, and a subsample taken from each square. A subsample should be roughly cylindrical in shape, 3–5 cm in diameter, and taken from the 5–20 cm depth. A presterilized coring tool (Figure B.3.1) or a long-nosed hand shovel can be used to extract the subsamples, which are lumped together in a plastic bag. To prevent cross-contamination if another area is to be sampled, a second presterilized sampling tool should be used—or the first one cleaned to remove all soil, washed with 95% alcohol, and sterilized by flaming. Because of rapid changes in the bacterial populations of extracted soils, the soil samples should be tested immediately after collection. If this is not possible, the samples should be stored without drying at 4°C but for no longer than is necessary.



Fig. B.3.1. Soil sampling coring tool in use in the field (soil auger).

In the laboratory, the lumped subsamples are thoroughly mixed on a clean, sterilized, solid surface, quartered, mixed again, and so on until a small (100–200 g) composite sample, as homogeneous as possible, is obtained. Stones should be removed, but sieving is not necessary and may remove bits of organic matter in which colonies of rhizobia reside. It is desirable that soil moisture content be determined to express population figures on a soil dry-weight basis.

### Growth Systems

**Small-seeded legumes** The system described here for growing small-seeded legumes was developed in CSIRO (Canberra) to provide good bacteriological control for small-seeded legumes without restriction due to inadequate  $\text{CO}_2$  or irradiance and to allow frequent observation of nodulation of the growing plant. The same system allows for other types of studies involving the evaluation of the symbiosis by observing

shoots and the roots (see Section D). Plants with seeds weighing 1–15 mg, including *Medicago* spp., *Trifolium* spp., *Macropitilium* spp., *Stylosanthes* spp., and *Desmodium* spp., grow well in this system.

The materials needed are: wooden racks, 50 × 50 cm (Figure B.3.2), with surface of the board perforated with holes to allow tubes (150 × 18 mm) to pass through with 1–2 mm clearance [a total of 100 holes (10 × 10) can be obtained]; a good supply of glass test tubes of 150 × 18 mm dimension; Elastrator rings; Elastrator pliers; small rubber or cork stoppers of approximately 4-mm diameter; aluminum foil; vermiculite, perlite, or gravel; 10–60 ml syringe; large plastic bags (25 l); glass petri dishes; concentrated sulfuric acid; Pasteur pipettes (5.75 inch); growth chamber or greenhouse facility (min temp 15–18°C; max 25–28°C); and chemicals for nutrient solution.

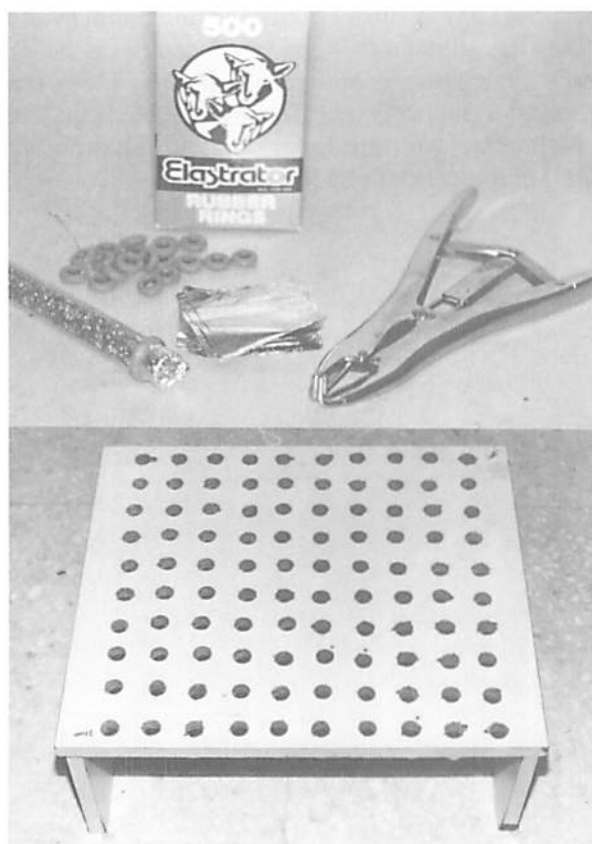


Fig. B.3.2. Main components of aseptic growth system for small-seeded legumes, including 100-hole wooden rack and Elastrator pliers.

The rooting media to fill the tubes must be thoroughly washed several times and have a pH of approximate neutrality. Vermiculite and perlite are washed thoroughly for three continuous days by changing the water at least twice a day and stirring frequently; final rinse should be with distilled or deionized water. New batches of these materials need more changes of water to bring down the pH to near neutrality. Drying is recommended in an oven at 75°C. Coarse gravel can be mixed with the micaceous media as one-fourth of the total content.

Test tubes of 150 × 18 mm are filled to the top with the rooting material. The filled tubes are moistened with 10–15 ml of a nitrogen-free nutrient solution before autoclaving; McKnight's nutrient solution is recommended (see Section A.9). Cut small circles of 45–50 mm diameter from thick aluminum foil, with sheets of foil folded to 50 mm width and interleaved with newspaper (this aids in the separation of the disks). A disk of foil is placed on the top of each tube and held in place by means of a rubber band or, preferably, a rubber elastrator ring normally used for marking and tailing lambs. The ring will hold the tube suspended in the hole of the rack (Figure B.3.3). The Elastrator ring, of stiff rubber, is easily inserted around the foil-capped tube with Elastrator pliers (Figure B.3.4, obtainable from Elastrator Pty Ltd., 414 Collins Street, Melbourne, Australia, or stock agents elsewhere). Tubes are then autoclaved at 121°C/15 lb pressure for 15 minutes.

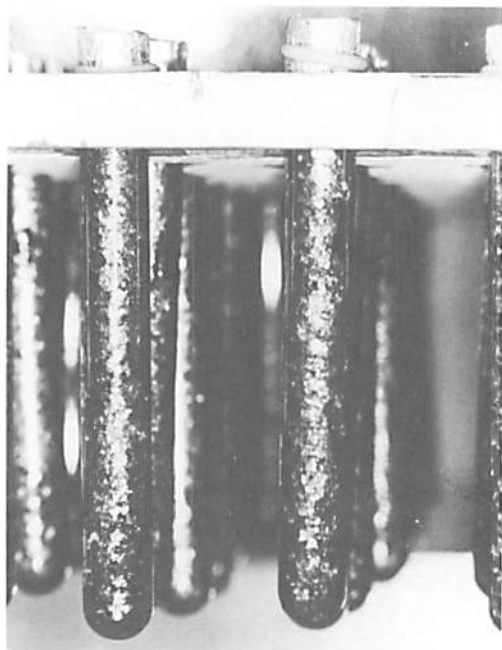


Fig. B.3.3. Tubes held suspended in a hole of wooden rack by Elastrator band.





Fig. B.3.4. Placing Elastrator ring over foil cover on tube with special pliers.

After autoclaving and cooling, tubes are prepared for planting seedlings by making a hole through the foil using a sterile needle of the same diameter as the radicle of the seedlings to be sown. The hole should be 3 mm from the edge of the tube (Figure B.3.5).

Seeds of most medics and some clover species have hard coats which make them difficult to germinate unless subjected to chemical or physical scarification. Certain species of medics, e.g., *Medicago orbicularis*, require a 45-minute exposure to concentrated sulfuric acid whereas most other medic species require a three- to four-minute exposure time to break their hard coat and render them suitable for uniform germination. After pouring off the acid carefully (**caution:** concentrated sulfuric acid is highly caustic), it is essential to flush the flask containing the acid-treated seed with abundant water and rinse them with sterile distilled water at least six times.



Fig. B.3.5. Making hole in foil cover with pointed sterile rod for placement of seedlings.

After scarification, seeds are carefully transferred to and separated from each other on the surface of a 2% water agar petri dish (Figure B.3.6). The dishes are kept inverted with a few drops of water in the cover for 36 hours at 5–8°C (cold treatment) then the dishes are placed into an incubator having a temperature of 25–26°C for 24 hours. The dishes are kept inverted so as to obtain seedlings with straight radicles (Figure B.3.7).

Seedlings of uniform appearance and of approximately 10-mm radicle length are grasped by the radicle just below the cotyledon using sterile forceps to carefully insert the radicle into the hole of a tube (Figure B.3.8). Care must be taken not to damage the radical, as this will prevent plant growth.

To improve growth and vigor of the seedlings, tubes with sown seedlings are covered with moistened paper tissues. The rack or basket of tubes should then be placed in a



Fig. B.3.6. Placing sterilized scarified medic seeds on a water agar plate for germination.

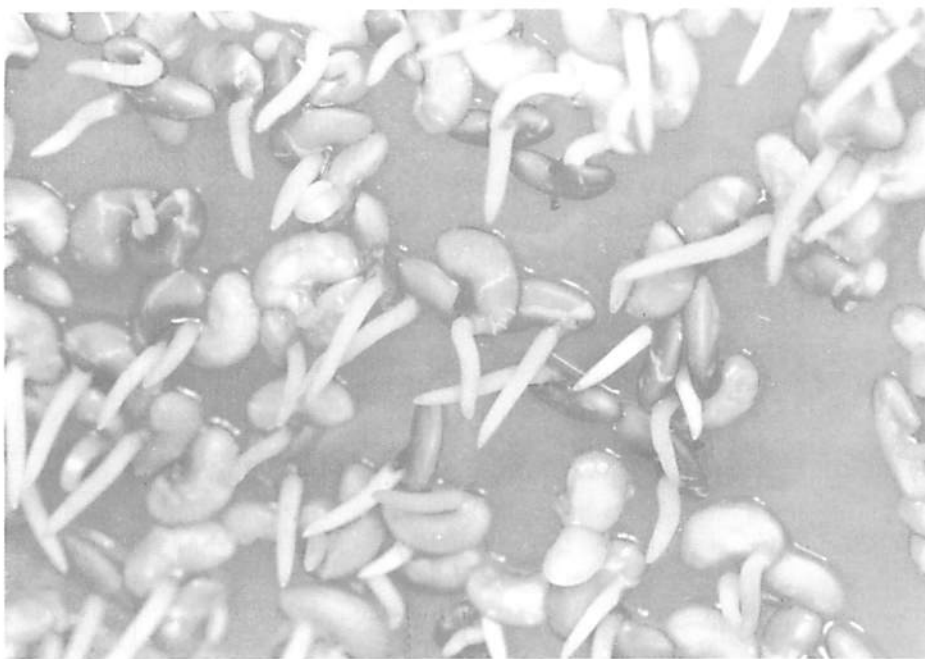


Fig. B.3.7. Medic seedlings at the proper stage for planting.

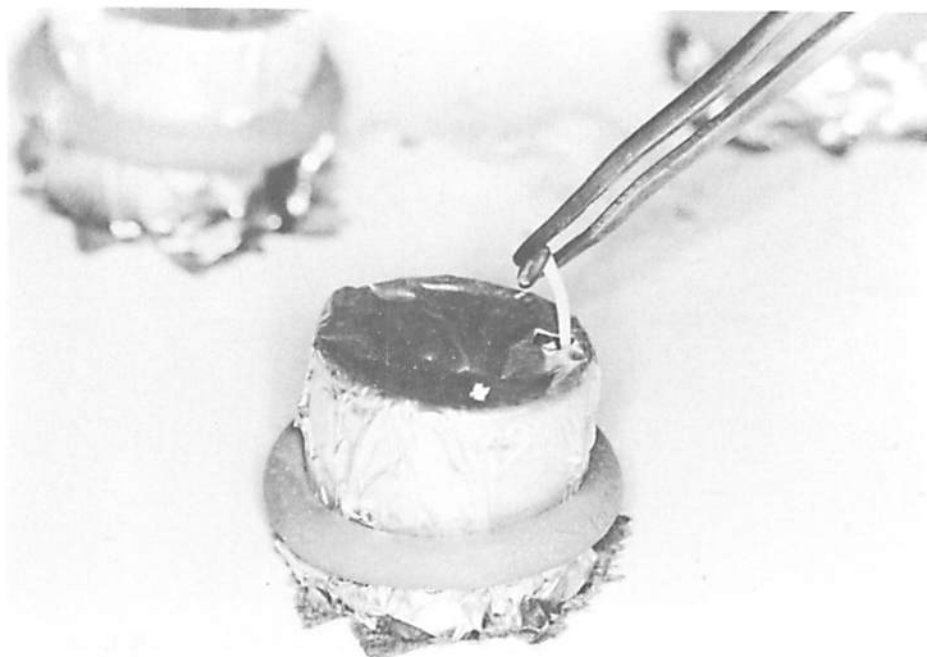


Fig. B.3.8. Carefully placing medic seedling into hole in tube cover.

large, clean, clear plastic bag containing free water (Figure B.3.9). The paper tissues are kept wet on a daily basis for a period of four to five days in an environment of 15–18°C min to 25–28°C max. Bagged material can be kept in a growth chamber with artificial lighting (Figure B.3.10) or in a greenhouse. It should be noted that sterile, distilled water is recommended for this operation. On the fourth day of the moisture treatment the bags and the paper tissues are removed from the racks or baskets containing the tubes, and tubes are placed in the wooden racks previously described (Figure B.3.11).

A sterile sharpened probe of 4 mm-diameter is used to make a hole in the edge of the foil cap diagonally opposite the seedling. The hole is covered by a small dried cork or rubber bung previously disinfected with ethanol (Figure B.3.12). The hole will serve as a watering and inoculation port. Sterile distilled water is then carefully added to each tube using a syringe inserted through the watering holes (Figure B.3.13). Watering can also be done using an automatic dispensing syringe to avoid having to continually fill a normal syringe. Water is carefully added until the seepage is seen to reach the radicle. Some space should be left for the volume of the inoculant dilution solution. The diluted inoculant can be added in the watering solution or as 0.1 ml of the dilution suspension (e.g., soil suspended in water) through the port with a pipette.

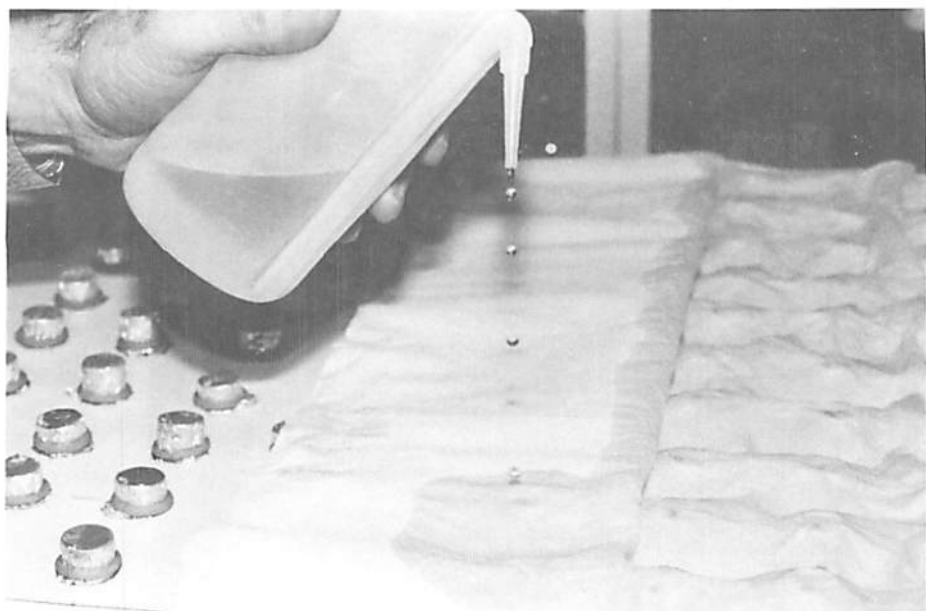


Fig. B.3.9. Wetting paper towels covering planted tubes in rack prior to covering with plastic bag for protection during early plant growth.



Fig. B.3.10. Rack containing planted tubes with plastic cover to conserve moisture and provide warm humid atmosphere.



Fig. B.3.11. Removing wet paper towelling after initial plant growth.



Fig. B.3.12. Insertion of sterile bung into watering hole in tube cover.

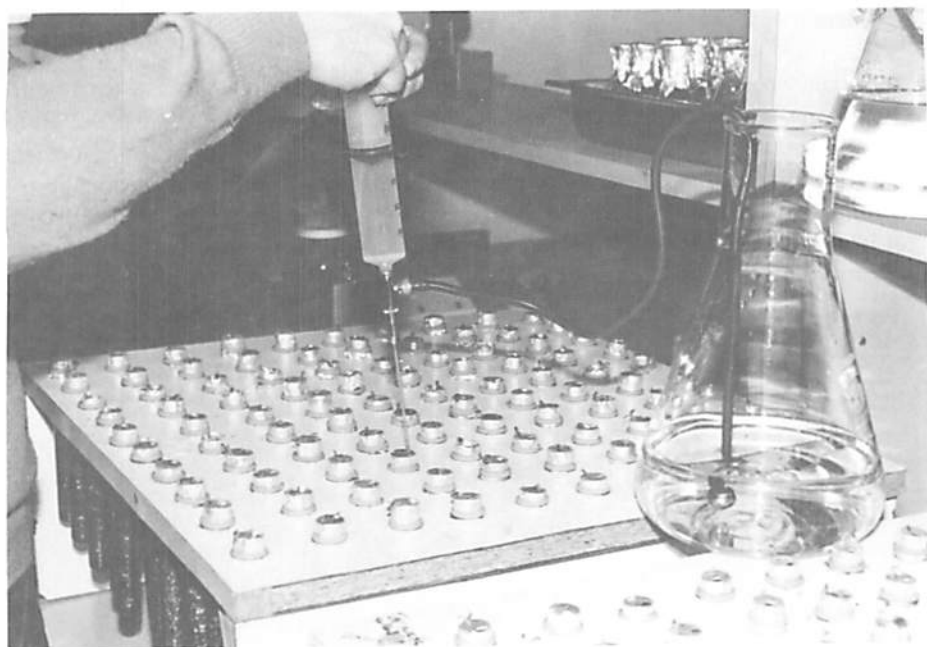


Fig. B.3.13. Watering medic tube system with a 60 ml auto-filling syringe.

Racks are then kept in a lightroom or greenhouse for 28 days after inoculation. N-free nutrient solution is added to the tubes when the water level has dropped near the lower parts of the roots. Depending on type of rooting media, temperature and relative humidity, this is generally necessary at the third week of growth. It is good practice to utilize distilled or deionized water for making all solutions, as tap water in many locations contains enough nitrate-N for small plant growth.

**Large-seeded legumes** The system for large-seeded legumes is similar to that described previously for small-seeded plants. The two main systems used are the plastic growth pouch and the large vermiculite (or gravel/vermiculite mix) filled glass tube. Both systems are effective in maintaining sterility and allow good plant growth for up to six to eight weeks. Other growth systems for evaluation of  $N_2$  fixation effectiveness are described in Section D. Plants with seeds ranging from the size of small-seeded lentil (15 g per 100 seeds) to large-seeded faba beans (3 g per seed) can be grown in the tubes. Smaller-seeded legumes, such as lentil, vetch, and lathyrus, can also be grown in growth pouches. Both systems will be described.

### Tube System

The materials needed for the tube system are: wooden racks,  $6 \times 30 \times 8$  cm (Figure B.3.14), with holes drilled to accommodate the tubes ( $150 \times 18$  mm) with 1–2 mm clearance [a total of 24 holes ( $2 \times 12$ ) can be obtained]; a good supply of test tubes of  $150 \times 18$  mm dimension; cotton wool presterilized in small packages; vermiculite, perlite, or gravel; 50–60 ml sterile syringe; petri dishes containing 2% water agar; a pair of long-handled forceps; 1-ml and 10-ml pipettes; growth chamber or greenhouse facility (min temp  $15\text{--}18^{\circ}\text{C}$ ; max  $25\text{--}28^{\circ}\text{C}$ ); and chemicals for nutrient solution.

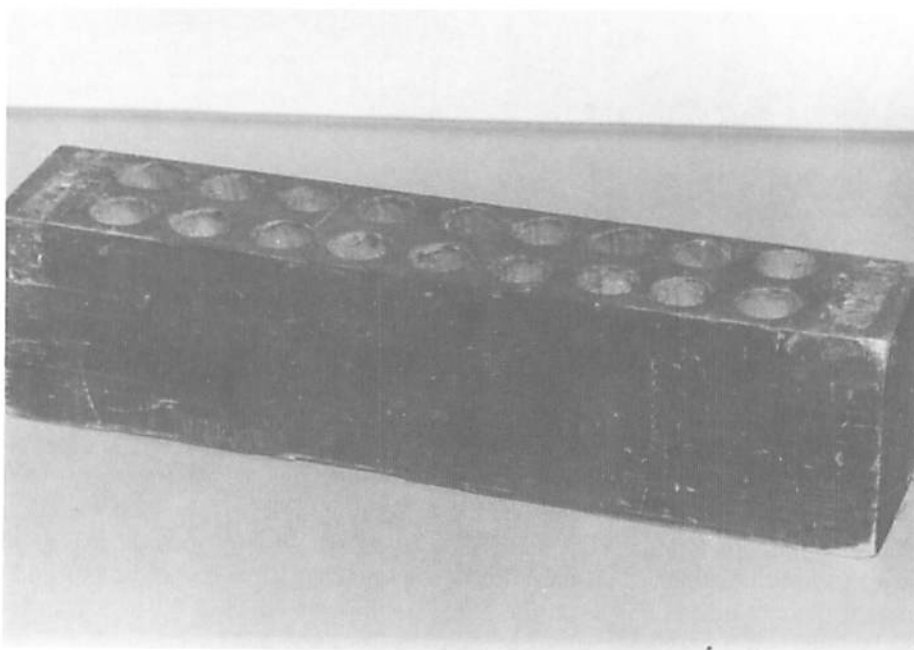


Fig. B.3.14. Wooden block with drilled 20 mm x 50 mm holes to hold 18 growth tubes for large-seeded legumes.

The rooting media to fill the tubes must be thoroughly washed several times and have a pH of approximate neutrality. Vermiculite and perlite are washed thoroughly for three continuous days by changing the water at least twice a day and stirring frequently; final rinse should be with distilled or deionized water. New batches of these materials need more changes of water to ensure cleanliness and correct pH. Drying is recommended in an oven at  $75^{\circ}\text{C}$ . Coarse gravel can be mixed with the vermiculite to make up three-fourths of the total content when vermiculite is not readily available.



Test tubes of 150 × 18 mm are filled approximately one-third full with the rooting material (Figure B.3.15). The filled tubes are moistened slightly with distilled water or nitrogen-free nutrient solution before autoclaving. A variation of the nitrogen-free nutrient solution developed by Broughton and Dilworth is recommended (see Section A.9). It is good practice to utilize distilled or deionized water for making all solutions, as tap water in many locations contains enough nitrate-N for plant growth. The racks of prepared tubes are covered loosely with aluminum foil (Figure B.3.16) and autoclaved for 15 minutes at 121°C/15 lb. pressure.



Fig. B.3.15. Filling tube system with dry gravel/vermiculite mixture prior to autoclaving.

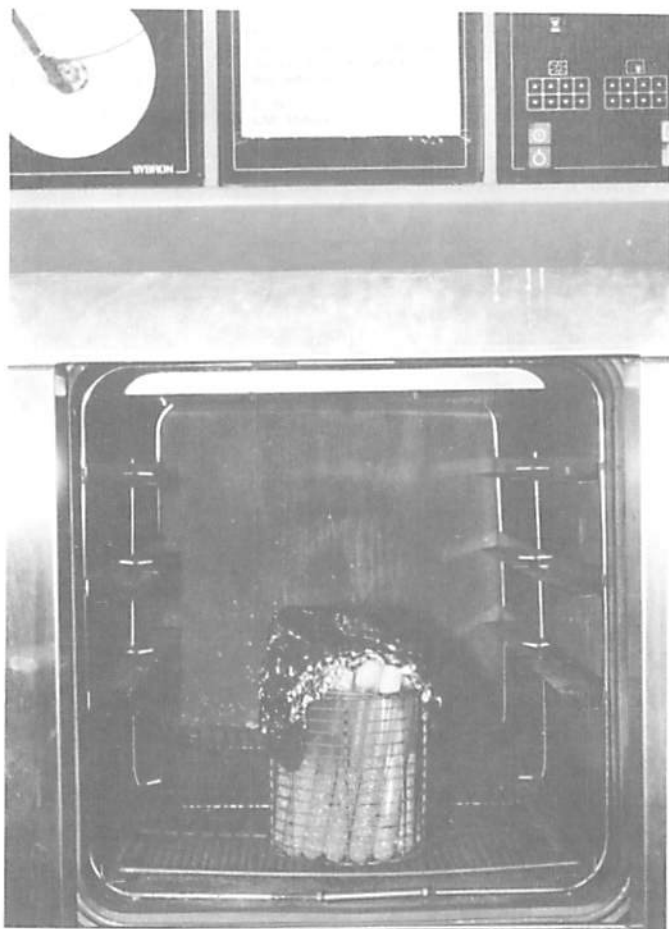


Fig. B.3.16. Prepared tubes covered loosely with foil for autoclaving.

The large-seeded legumes grown in the region do not generally require scarification to enhance germination, but seeds **must** be surface sterilized before germination to eliminate seed-borne rhizobia and other microorganisms (especially fungi) which will interfere with germination. Clean, undamaged seeds, selected for uniform size and appearance, are dipped momentarily in 95% alcohol and then immersed in 0.1%  $\text{HgCl}_2$  solution for two minutes. They are then washed thoroughly in not less than 10 changes of sterile, distilled water to remove all traces of  $\text{HgCl}_2$  (sample system shown in Figure B.3.17). (**Caution:**  $\text{HgCl}_2$  is highly poisonous, and all contact with skin must



Fig. B.3.17. Sterilization of large legume seed. Seeds are left in the flask, with sterilizing and rinsing solutions added, mixed, and poured off in turn.

be avoided.) The seeds can be left in the final change of sterile water until they are fully imbibed. The seeds are then transferred aseptically with forceps to the surface of a 2% water agar petri dish, where they are spread to give room for root growth (Figure B.3.18). The dishes are then placed in an incubator having a temperature of 25–26°C for 24 hours, after which they are placed inside a large plastic bag containing a few milliliters of water to maintain high humidity (Figure B.3.19). Seedlings with equal germination and straight radicles should be selected for planting (Figure B.3.20).

After autoclaving and cooling, tubes are prepared for planting in a clean bench or laminar flow bench. The aluminum cover is removed, and pregerminated seedlings with radicals of 1–3 cm length are transferred aseptically into the test tubes, one per tube. A pair of sterile, long, fine forceps works well to first produce a depression in which to place the seedling and then to transfer the seedling and cover it (Figures

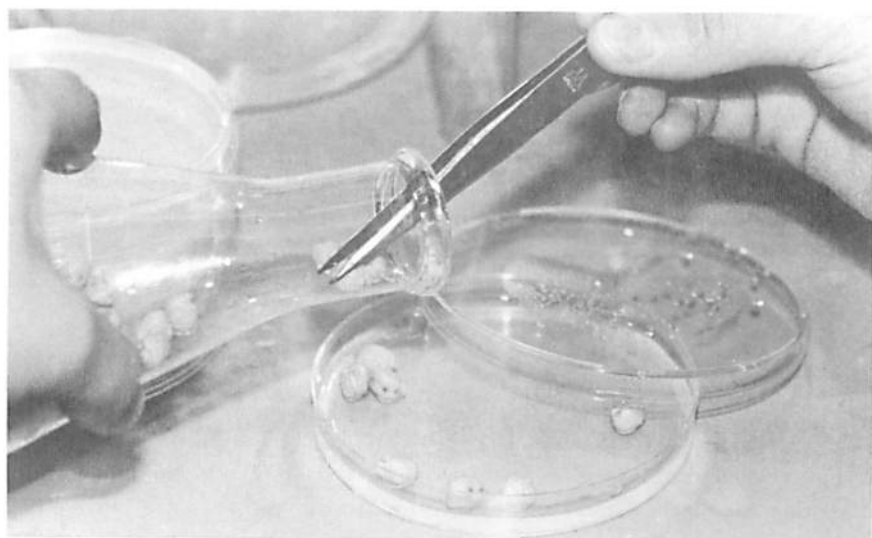


Fig. B.3.18. Placement of sterilized chickpea seed on 2% water agar for germination.



Fig. B.3.19. Plates containing germinating seed are placed inside clean plastic bag to maintain humidity.



**Fig. B.3.20.** Seedlings at proper stage for planting.  
Care should be taken to choose seedlings with uniform vigorous growth.

B.3.21 and B.3.22). The seedling is buried vertically, if possible with the radical against one wall of the tube (to allow for later viewing of the nodulation process), with the seed approximately 2 mm beneath the surface of the vermiculite. Tamp the surface of the vermiculite lightly after planting to provide a firm seed bed and uniform emergence of the seedlings (Figure B.3.23). After planting, a 1-ml aliquot of inoculant dilution is applied over each planted seed. Tubes can then be irrigated with N-free nutrient solution with a syringe (manual or automatic) so that all the media appear moist but not flooded (Figure B.3.24). The tubes are stoppered with a wad of sterilized cotton wool or a prepared reusable cotton-and-gauze stopper (Figure B.3.25) to maintain sterility and allow gas exchange for the growing plant. Racks are placed in a shaded glasshouse or growth chamber. Plants require approximately  $8-10 \times 10^3$  lux for adequate growth.

Racks are kept in a lightroom or greenhouse for four to six weeks after inoculation. Sterile N-free nutrient solution is added to the tubes when the vermiculite appears to dry out slightly (once every four to five days during early growth and more often as plants increase in size). Care must be taken that plants are not subjected to drought stress and that roots are not flooded. As the tips of the growing plants reach the cotton stopper, a piece of fresh, sterilized cotton wool is carefully (aseptically)

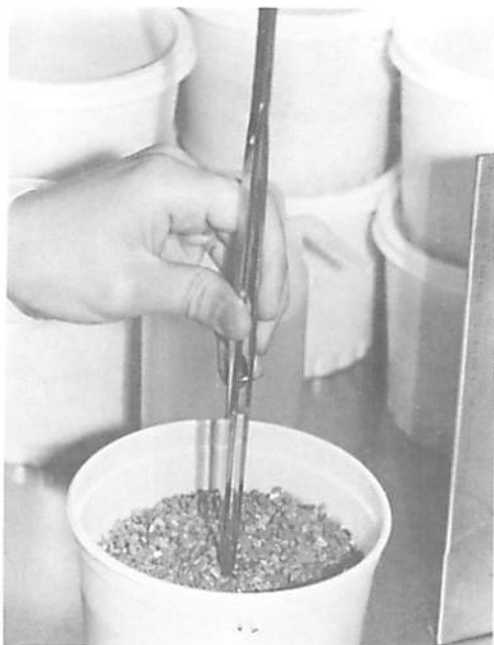


Fig. B.3.21. Large size forceps used for planting seedlings in tubes.

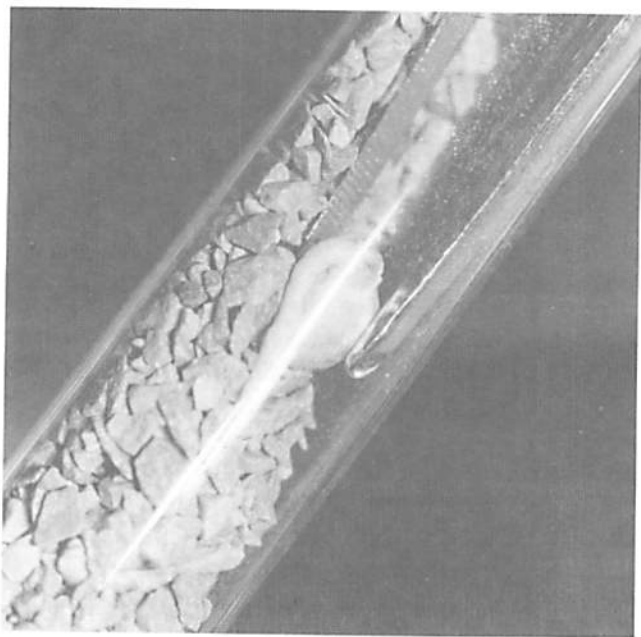


Fig. B.3.22. Planting chickpea seedling in growth tube with sterile forceps.

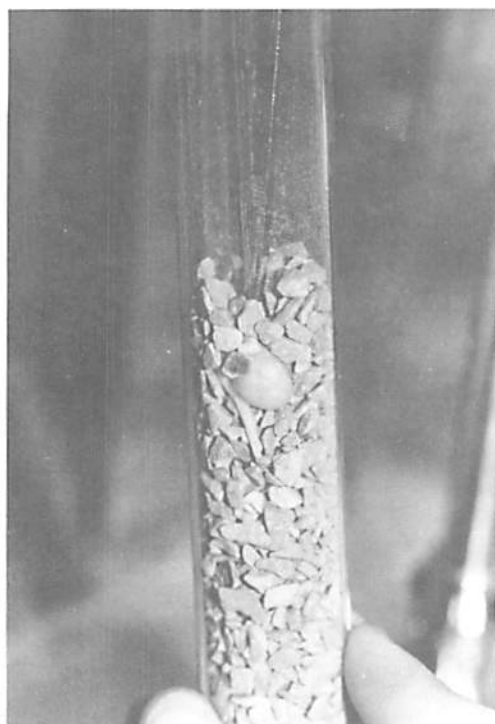


Fig. B.3.23. Tamping growth medium over seed with forceps to prevent root growth from pushing seed out of the medium.



Fig. B.3.24. Watering tube with auto-filling syringe after planting seedling.

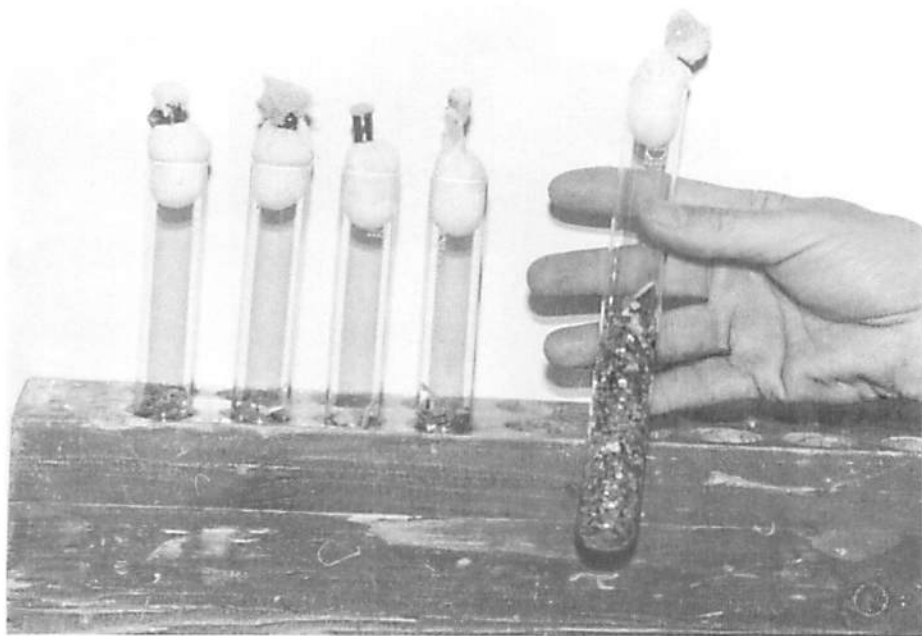


Fig. B.3.25. Planted tube with cotton-and-gauze stopper.

wrapped around the plant stem below the top set of leaves (Figure B.3.26) to allow the plant to grow while maintaining sterility in the system. While watering, care must be taken not to wet this cotton barrier. Clearly, plants cannot be grown to maturity in this system, but even large-seeded faba bean can be grown for eight weeks with daily watering during the last weeks.

### Growth Pouches

Plastic growth pouches as an alternative system for MPN determinations are useful in some situations using large-seeded legumes. They are, however, more susceptible to contamination and are not shielded against irradiated heat. Their use is therefore restricted to growth chambers or growth rooms with controlled aseptic conditions. The pouches suggested are made from polypropylene (16 × 18 cm) with paper wick liners obtainable from Scientific Products, Evanston, IL, USA. Paper liners can also be made from paper towels, as shown in Figure B.3.27. Racks to hold the pouches can be devised from many different materials; we have found better nodulation results if roots are not exposed to excessive light. One suggested type of rack is shown in Figure B.3.28.



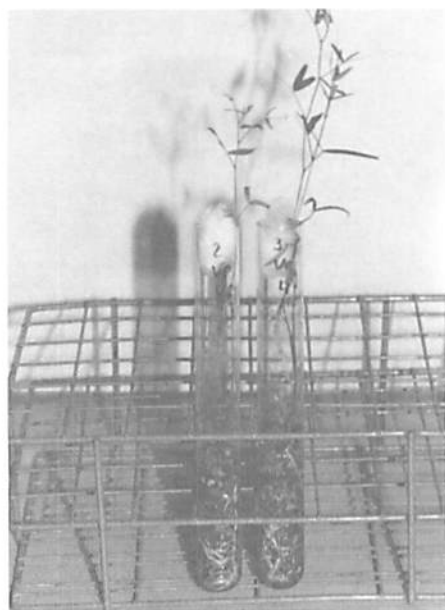


Fig. B.3.26. Plant with cotton wrapped around its stem to exclude contaminants from growth vessel.

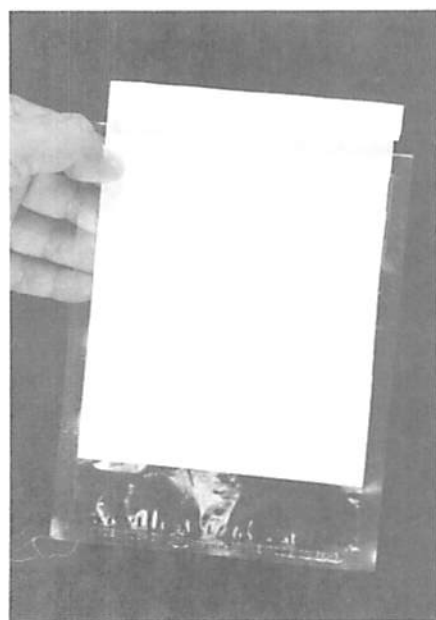
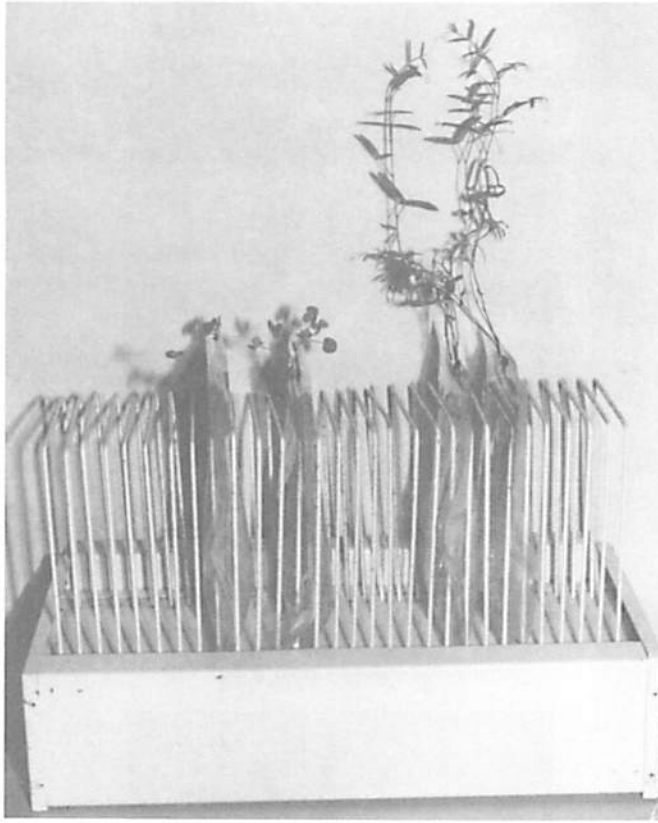


Fig. B.3.27. Growth pouch liner produced from paper towel or filter paper.  
Note fold for planting trough.



**Fig. B.3.28. Growth pouch rack made from bent wire and wood.**  
 Outside of assembly is wrapped with opaque paper to shield roots from light

Purchased growth pouches are sterile, but homemade pouches must be sterilized in the autoclave for 15 minutes after insertion of the paper liner and 30 ml N-free nutrient solution in each pouch. Purchased pouches are filled with 30 ml sterile solution before planting. Pregerminated seedlings (as described earlier) are transferred (one seed per pouch) aseptically to the trough in the liner. To prevent the growing radical from pushing the seed out of the pouch, a hole is made in the trough of the liner and the radical inserted into the hole during planting (Figure B.3.29). Holes are easily made in the trough with fine-tipped, sterile forceps when the wick is wet. Two forceps are needed: one for holding the wick and the other for making the hole.

Other growth systems (those more suitable for evaluation of the symbiotic potential of the rhizobia) are discussed in Section D.

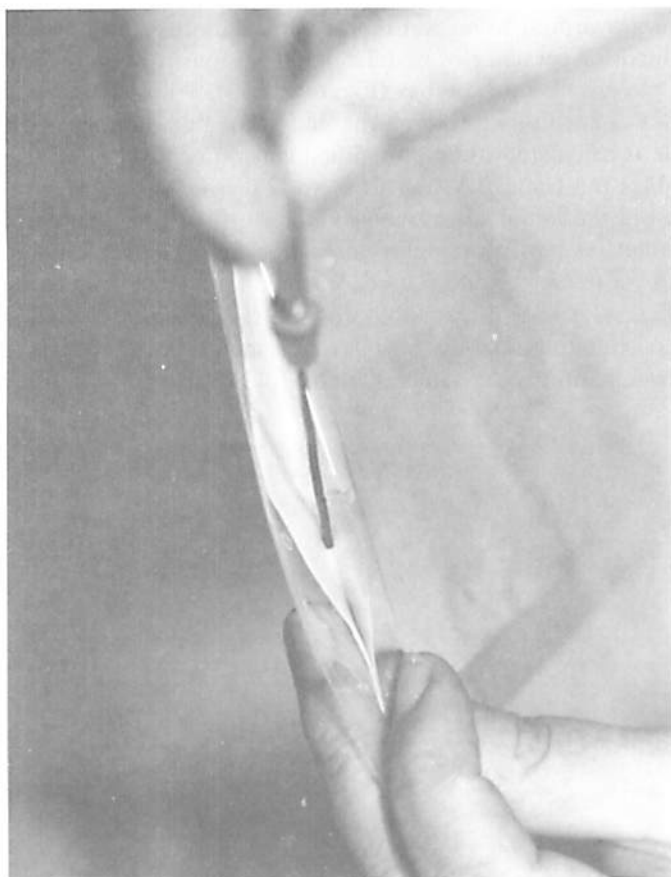


Fig. B.3.29. Making hole in liner planting trough to allow seedling root to grow toward nutrient solution.

### MPN Inoculation and Estimation

Prepare a 10-fold dilution series as described previously in Section B.1, but prepare a  $10^1$  to  $10^{10}$  dilution series. When diluting, it is good practice to suck the liquid up and down the pipette several times before transferring the subsample to the next dilution level or growth tube; this keeps the suspension in motion during sampling and reduces the risk of nonuniform distribution of rhizobia. Four plant replicates are suggested for each dilution (fewer may be used with less accuracy), with 1 ml of each of the  $10^{-1}$  to  $10^{-10}$  dilutions (plus uninoculated control to check for sterile conditions) applied to four replicate plants. A fresh pipette is used for each dilution level. Thus, 44 plants are required for each subsample tested in this example.

Test plants are grown four to six weeks after inoculation, are watered as necessary, and are examined for presence of nodules after the fourth week by observation of roots through glass walls of the tubes (Figure B.3.30). From the proportion of plants forming nodules at each dilution level, the most probable number of rhizobia in the original sample is calculated using a modified version of Fisher and Yates' (1963) tables (see Tables B.3.1 and B.3.2). This method provides for a range of 1 to  $10^5$  rhizobia per ml of the initial soil suspension, or 10 to  $10^9$  rhizobia per g soil. When counting inoculants as part of a quality control program, higher dilutions to measure up to  $10^{10}$  rhizobia per g should be used. When counting soils, up to  $10^6$  dilution should be sufficient. A five- or four-fold dilution series can be used if greater accuracy is required (e.g., counting rhizobia in soils). The method of preparation of the material for examination is the same as for 10-fold dilutions.

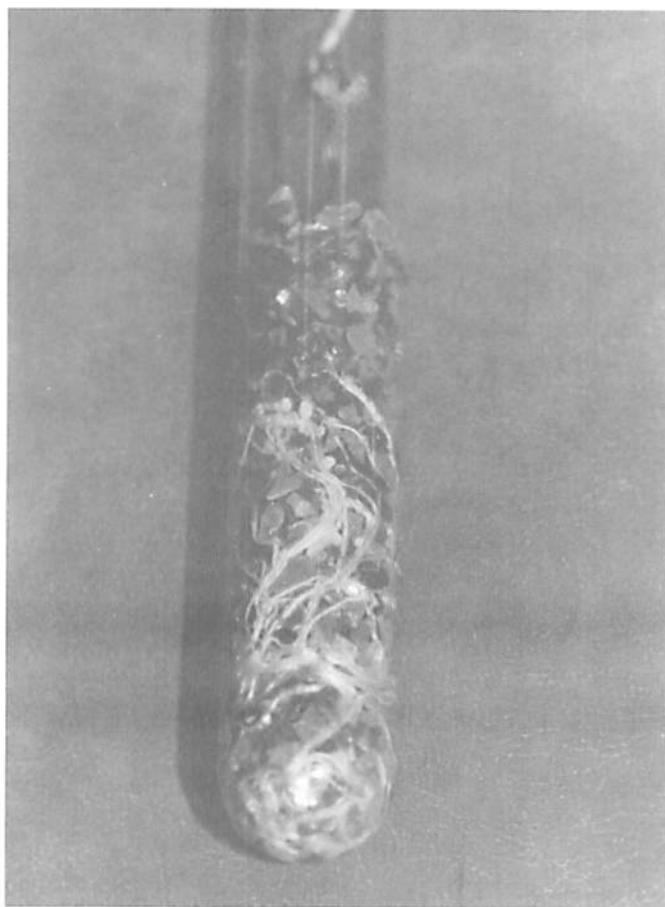


Fig. B.3.30. Roots and nodules can be observed during plant growth.

For each set, write down the dilutions used and record the nodulation, as demonstrated in Table B.3.3. The actual number of nodules on each plant does not matter for the MPN count. If four replications are used, the reading for each dilution will be 4, 3, 2, 1, or 0 nodulated units for each dilution level. The uninoculated treatment and highest dilution used should show no nodulation in each replication, an indication of the absence of contaminating rhizobia. Each series should end with a dilution in which no nodules are formed. The MPN is given for the least dilute member of a dilution series. The MPN is calculated from the most likely number (*m*) found in the MPN tables. To find this number, use the procedure in the example shown:

1. Record nodulation as shown in Table B.3.3.
2. Note number of replications (*n*) used (*n* = 4 in this example).
3. Note the number of dilution steps (*s*) used (*s* = 10 here).
4. Add the total number of nodulated (positive) units (+ = 19 here).
5. Find this number in Table B.3.1 (calculated here for 10-fold).
6. Locate the most likely number (*m*) in column for number of dilution steps (*s* = 10 here), on the same line as total number of units.
7. Note the lowest dilution (*d*) in the series (*d* = 10<sup>-1</sup> here).
8. Note the aliquot used (*v*) for inoculation (*v* = 1 ml here).
9. Calculate MPN from "*m*" according to the formula:

$$\text{MPN} = \frac{m \times d}{v} = \frac{(1 \times 10^4) \times 10^1}{1} = 1 \times 10^5 \text{ rhizobia/g}$$

Experiments with these techniques in several laboratories have shown that the results obtained are reproducible. Thus, replicate counts of a single sample are not necessary; it is preferable to count replicate samples. As mentioned earlier, rhizobial numbers will be underestimated in the vermiculite tube system, depending on experimental conditions and plant type.

A complication which usually occurs is the occurrence of 'skips', or test plants without nodules at a dilution above which most or all plants are nodulated. The MPN tables do not account for this, meaning that the experimenter must guess what the result should have been. These 'skips' result from unfavorable conditions for nodulation within the tube; e.g., failure of the plant because of poor growth conditions, failure of the rhizobia to survive or multiply, or interference from algae or saprophytic fungi. Good techniques and a well-designed growth room or greenhouse are the only solutions.

Table B.3.1.\* Number (*m*) of rhizobia estimated by the plant infection count (after Vincent, 1970).  
Ten-fold dilutions ( $\lambda = 10$ )

| Positive tubes |              |                   | Dilution step(s)  |                   |                   |
|----------------|--------------|-------------------|-------------------|-------------------|-------------------|
| <i>n</i> = 4   | <i>n</i> = 2 | <i>s</i> = 10     |                   |                   |                   |
| 40             | 20           | $7 \times 10^8$   |                   |                   |                   |
| 39             |              |                   |                   |                   |                   |
| 38             | 19           | 6.9               |                   |                   |                   |
| 37             |              | 3.4               |                   |                   |                   |
| 36             | 18           | 1.8               |                   |                   |                   |
| 35             |              | 1.0               |                   |                   |                   |
| 34             | 17           | $5.9 \times 10^7$ |                   |                   |                   |
| 33             |              | 3.1               | <i>s</i> = 8      |                   |                   |
| 32             | 16           | 1.7               | $7 \times 10^6$   |                   |                   |
| 31             |              | 1.0               |                   |                   |                   |
| 30             | 15           | $5.8 \times 10^6$ | 6.9               |                   |                   |
| 29             |              | 3.1               | 3.4               |                   |                   |
| 28             | 14           | 1.7               | 1.8               |                   |                   |
| 27             |              | 1.0               | 1.0               |                   |                   |
| 26             | 13           | $5.8 \times 10^5$ | $5.9 \times 10^5$ |                   |                   |
| 25             |              | 3.1               | 3.1               | <i>s</i> = 6      |                   |
| 24             | 12           | 1.7               | 1.7               | $7 \times 10^4$   |                   |
| 23             |              | 1.0               | 1.0               |                   |                   |
| 22             | 11           | $5.8 \times 10^4$ | $5.8 \times 10^4$ | 6.9               |                   |
| 21             |              | 3.1               | 3.1               | 3.4               |                   |
| 20             | 10           | 1.7               | 1.7               | 1.8               |                   |
| 19             |              | 1.0               | 1.0               | 1.0               |                   |
| 18             | 9            | $5.8 \times 10^3$ | $5.8 \times 10^3$ | $5.9 \times 10^3$ |                   |
| 17             |              | 3.1               | 3.1               | 3.1               | <i>s</i> = 4      |
| 16             | 8            | 1.7               | 1.7               | 1.7               | $7 \times 10^2$   |
| 15             |              | 1.0               | 1.0               | 1.0               |                   |
| 14             | 7            | $5.8 \times 10^2$ | $5.8 \times 10^2$ | $5.8 \times 10^2$ | 6.9               |
| 13             |              | 3.1               | 3.1               | 3.1               | 3.4               |
| 12             | 6            | 1.7               | 1.7               | 1.7               | 1.8               |
| 11             |              | 1.0               | 1.0               | 1.0               | 1.0               |
| 10             | 5            | $5.8 \times 10^1$ | $5.8 \times 10^1$ | $5.8 \times 10^1$ | $5.9 \times 10^1$ |
| 9              |              | 3.1               | 3.1               | 3.1               | 3.1               |
| 8              | 4            | 1.7               | 1.7               | 1.7               | 1.7               |
| 7              |              | 1.0               | 1.0               | 1.0               | 1.0               |
| 6              | 3            | $5.8 \times 1$    | $5.8 \times 1$    | $5.8 \times 1$    | $5.8 \times 1$    |
| 5              |              | 3.1               | 3.1               | 3.1               | 3.1               |
| 4              | 2            | 1.7               | 1.7               | 1.7               | 1.7               |
| 3              |              | 1.0               | 1.0               | 1.0               | 1.0               |
| 2              | 1            | 0.6               | 0.6               | 0.6               | 0.6               |
| 1              | 1            | 0.6               | 0.6               | 0.6               | 0.6               |
| 0              | 0            |                   |                   |                   |                   |

Approximate range  $10^9$   $10^7$   $10^5$   $10^3$

Factor, 95%; fiducial limits *n* = 2, 6.6; *n* = 4, 3.8

\* Calculated from Table VIII of Fisher and Yates (1963)

Table B.3.2.\* Number (m) of rhizobia estimated by the plant infection count (after Vincent, 1970).  
Four-fold dilutions (A = 4)

| Positive tubes |       |                      | Dilution step(s)     |                      |                      |
|----------------|-------|----------------------|----------------------|----------------------|----------------------|
| n = 4          | n = 2 | s = 10               |                      |                      |                      |
| 40             | 20    |                      |                      |                      |                      |
| 39             |       | $2.0 \times 10^5$    |                      |                      |                      |
| 38             | 19    | $2.0 \times 10^5$    |                      |                      |                      |
| 37             |       | 1.2                  |                      |                      |                      |
| 36             | 18    | $8.1 \times 10^4$    |                      |                      |                      |
| 35             |       | 5.5                  |                      |                      |                      |
| 34             | 17    | 3.8                  |                      |                      |                      |
| 33             |       | 2.6                  | s = 8                |                      |                      |
| 32             | 16    | 1.8                  | $1.3 \times 10^4$    |                      |                      |
| 31             |       | 1.3                  |                      |                      |                      |
| 30             | 15    | $9.1 \times 10^3$    | $1.3 \times 10^4$    |                      |                      |
| 29             |       | 6.3                  | $7.9 \times 10^3$    |                      |                      |
| 28             | 14    | 4.5                  | 5.1                  |                      |                      |
| 27             |       | 3.5                  | 3.5                  |                      |                      |
| 26             | 13    | 2.2                  | 2.4                  |                      |                      |
| 25             |       | 1.6                  | 1.7                  | s = 6                |                      |
| 24             | 12    | 1.1                  | 1.1                  | $7.9 \times 10^2$    |                      |
| 23             |       | $8.0 \times 10^2$    | $8.0 \times 10^2$    |                      |                      |
| 22             | 11    | 5.6                  | 5.6                  | $7.9 \times 10^2$    |                      |
| 21             |       | 4.0                  | 4.0                  | 5.0                  |                      |
| 20             | 10    | 2.8                  | 2.8                  | 3.2                  |                      |
| 19             |       | 2.0                  | 2.0                  | 2.2                  |                      |
| 18             | 9     | 1.4                  | 1.4                  | 1.5                  |                      |
| 17             |       | 1.0                  | 1.0                  | 1.0                  | s = 4                |
| 16             | 8     | $7.1 \times 10^1$    | $7.1 \times 10^1$    | $7.2 \times 10^1$    | $5.0 \times 10^1$    |
| 15             |       | 5.0                  | 5.0                  | 5.1                  |                      |
| 14             | 7     | 3.5                  | 3.5                  | 3.5                  | $5.0 \times 10^1$    |
| 13             |       | 2.5                  | 2.5                  | 2.5                  | 3.2                  |
| 12             | 6     | 1.8                  | 1.8                  | 1.8                  | 2.0                  |
| 11             |       | 1.3                  | 1.3                  | 1.3                  | 1.4                  |
| 10             | 5     | $8.9 \times 10^0$    | $8.9 \times 10^0$    | $8.9 \times 10^0$    | $9.6 \times 10^0$    |
| 9              |       | 6.3                  | 6.3                  | 6.3                  | 6.6                  |
| 8              | 4     | 4.5                  | 4.5                  | 4.5                  | 4.6                  |
| 7              |       | 3.2                  | 3.2                  | 3.2                  | 3.2                  |
| 6              | 3     | 2.2                  | 2.2                  | 2.2                  | 2.2                  |
| 5              |       | 1.6                  | 1.6                  | 1.6                  | 1.6                  |
| 4              | 2     | 1.1                  | 1.1                  | 1.1                  | 1.1                  |
| 3              |       | $7.2 \times 10^{-1}$ | $7.2 \times 10^{-1}$ | $7.2 \times 10^{-1}$ | $7.2 \times 10^{-1}$ |
| 2              | 1     | 4.4                  | 4.4                  | 4.4                  | 4.4                  |
| 1              |       |                      |                      |                      |                      |
| 0              | 0     | $4.4 \times 10^{-1}$ | $4.4 \times 10^{-1}$ | $4.4 \times 10^{-1}$ | $4.4 \times 10^{-1}$ |

Approximate range  $5 \times 10^5$   $3 \times 10^4$   $2 \times 10^3$   $1 \times 10^2$

Factor, 95%; fiducial limits n = 2 4.0; n = 4, 2.7

\* Calculated from Table VIII of Fisher and Yates (1963)

**Table B.3.3. Example evaluation of numbers of nodulated plants in MPN determination.**

| Dilution  | Nodulation<br>(+) or (-)<br>replications |    |     |    | Total number of<br>nodulated units |
|-----------|------------------------------------------|----|-----|----|------------------------------------|
|           | I                                        | II | III | IV |                                    |
| $10^1$    | +                                        | +  | +   | +  | 4                                  |
| $10^2$    | +                                        | +  | +   | +  | 4                                  |
| $10^3$    | +                                        | +  | +   | +  | 4                                  |
| $10^4$    | +                                        | -  | +   | +  | 3                                  |
| $10^5$    | -                                        | -  | +   | +  | 2                                  |
| $10^6$    | +                                        | -  | +   | -  | 2                                  |
| $10^7$    | -                                        | -  | -   | -  | 0                                  |
| $10^8$    | -                                        | -  | -   | -  | 0                                  |
| $10^9$    | -                                        | -  | -   | -  | 0                                  |
| $10^{10}$ | -                                        | -  | -   | -  | <u>0</u>                           |
|           |                                          |    |     |    | 19                                 |



### B.3.c. Fluorescent Enumeration Technique

Enumeration of rhizobia in soil using the fluorescent antibody technique first requires that the strain(s) being counted are known to react to a given prepared fluorescence-labelled antiserum (see Section C.5). This method is particularly applicable to evaluate the ability of a known inoculant strain to survive in soil over time, but the method is complicated, requires a well-equipped laboratory, and should be attempted only after some experience in use of the fluorescent antibody (FA) technique has been obtained. Enumeration of bacteria in soil with this technique is complicated by the colloidal and particulate nature of soil. We briefly outline the procedure below, but for those investigators interested in the technique, it is suggested that papers cited in the reference section be obtained.

Suspend 10 g soil in 95 ml of hydrolyzed gelatin extractant [1% solution of gelatin at pH 10.5, adjusted with 1 M NaOH, diluted 1:10 with 0.1 M  $(\text{NH}_4)_2\text{HPO}_4$ ] and shake for five minutes on wrist-action shaker. Remove colloidal material either by mild centrifugation ( $700 \times g$ , 5 minutes) or by flocculation with 0.7 g  $\text{Ca}(\text{OH})_2$  and  $\text{MgCO}_3$  in a 2:5 mixture (the most suitable procedure varies with soil type). Filter a known volume (1–5 ml) of supernatant through Irgalan-treated Nucleopore filters (membrane filters,  $0.45 \mu\text{m}$ , prestained with Irgalan black) and wash through with prefiltered saline. Transfer the filter to a glass slide and apply 0.5 ml of gelatine-rhodamine solution (see Bohlool and Schmidt, 1968) directly on the effective filtering area. Allow the solution to dry on the filter at  $65^\circ\text{C}$ . Apply appropriate dilution of FA (prefiltered through the membrane filter—see Section C.5 for preparation of FA and titer determination) and incubate in a moist chamber for 30 minutes. Carefully transfer the filter to a suction unit and wash through with prefiltered saline (200–300 ml saline wash is needed to provide a dark background). Mount the filter on a glass slide under mounting fluid. Count the fluorescent bacteria per field of microscope (counting several fields and taking an average) and extrapolate to numbers per gram of dry soil, taking into account the area of the microscope field, area of effective filtering surface, the volume and dilution of the soil filtered, and the moisture content of the soil.

### B.3.d. Enumeration Using ELISA Technique

Use of ELISA to determine the numbers of a specific strain of rhizobia in the presence of other rhizobia or microorganisms in soils or inoculants depends on principles similar to those described in the previous section (FA enumeration). The principles of ELISA are described in Sections C.3 and C.4, and will not be elaborated here. The basis of using this antigen-antibody reaction to count rhizobial numbers is that the reaction (production of color) will be proportional to the concentration of antigen, and hence the number of rhizobia present. This method has been described

by Nambiar and Anjaiah (1985), but because of lack of accuracy and inability to enumerate at levels below  $10^4$  rhizobia per g, has not been widely used.

## B.4. Suggested Reading

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## C. Rhizobia Strain Identification

It is useful for the *Rhizobium* worker to have a range of marker methods to use, as there is considerable variability in applicability of methods to the various rhizobial species and a wide range of uses. Some of the applications of identification methods are to:

1. Obtain evidence for likely taxonomic relationships.
2. Define strains in terms of their antigenic composition and to recognize them when used experimentally or routinely.
3. Provide markers for genetic studies and for authentication of mutants.
4. Determine authenticity and purity of a culture, including that of a noninvasive mutant; this can include quality control of inoculants.
5. Determine proportion of experimentally used strains in a mixed rhizobial population.
6. Identify strain or strains occupying a nodule.

Given the wide range of techniques available, and extensive coverage in other literature, we present here selected methodologies which we have found most useful in laboratory and field use at ICARDA. The choice of a methodology and awareness of its limitations are as important to the success of the experiment as preparation and application of the methods, and we have attempted to indicate suitable applications as well as weaknesses for each methodology presented.

### C.1. Development of Antibiotic-Resistant Mutants

#### C.1.a. Theory and Media Preparation

A major problem associated with studies of bacteria is the lack of visible variation in growth by strains of different genotypes. Strains with completely different physiological characteristics can appear morphologically identical. Use of *Rhizobium* strains in most field studies requires some way to distinguish between nodulation by the applied inoculum and that by the indigenous or resident soil bacteria. The lack of differences in obvious growth characteristics of strains in culture means that some method of distinguishing between selected strains must be used; the three methods most commonly utilized by researchers today are (a) antibiotic-resistant mutants, (b) ELISA, and (c) fluorescent antibody techniques.

Antibiotic resistance has been frequently used in rhizobial studies as a means of identifying inoculum strains, monitoring their survival in soil and inoculants and on inoculated seed, and determining strain occupancy of legume nodules. Strains

demonstrating tolerance to antibiotics have been selected by spontaneously acquired and laboratory-mutagenized mechanisms. Streptomycin, kanamycin, and spectinomycin have received the greatest attention in ecological investigations incorporating antibiotic resistance in strains of rhizobia.

The suppression of soil bacterial growth by antibiotics of certain concentrations incorporated into selective growth media indicates that the majority of soil bacteria are susceptible to several commonly used antibiotics. However, small numbers of naturally occurring mutants may be present in a given soil bacterial population. Two methodologies can be used in selecting mutants: (1) nonmutant cells are totally killed by exposure to the antibiotic in solution before plating on solid media; and (2) only mutant cells form colonies on the antibiotic-enriched plating medium. A small number of naturally occurring mutants are frequently present in rhizobial populations; mutants can be isolated with the minimum of effort even from populations in which the proportion of mutant cells is much less than 1 in  $10^6$ . We therefore suggest that the investigator avoid using dangerous mutagens (e.g., nitrosoguanidine) and rely on the spontaneous origin of mutants.

The usefulness of antibiotic-resistant markers stems from the high level of single-step resistance attainable, the stability of the trait, and the low frequency of cross resistance to other antibiotics. One question which must be considered is whether the effectiveness, as well as other characteristics of the rhizobial strains, has been affected by selecting for antibiotic resistance. In some cases strain effectiveness has been reduced during selection for resistance to some antibiotics, but selection for antibiotic resistance does not necessarily influence effectiveness. It is essential, however, to test the nodulating ability of all antibiotic-resistant mutants before using them as inoculants. In *Rhizobium*, mutants resistant to antibiotics such as streptomycin or spectinomycin that inhibit protein synthesis are less likely to have associated defects in symbiotic function than mutants selected for resistance to antibiotics that affect cell wall/membrane function. Experiments at ICARDA with streptomycin-resistant *R. meliloti* mutants have demonstrated the usefulness and stability of the markers during a period of four consecutive years. Serological and symbiotic properties are sufficiently stable to be of use in long-term ecological studies, although according to some reports antibiotic sensitivity appears less stable.

Stock solutions of antibiotics (Table C.1.1) should be prepared before starting a program of mutant selection. The advantage of preparing a stock solution is that small quantities can be diluted into larger volumes of melted agar and a standardized desired final concentration in the order of micrograms per ml (parts per million) can be obtained. The stock solution is prepared using a sterile flask and sterile solvent (usually distilled water). The solution can be sterilized by passing it through a sterile (Millipore) filter membrane with 0.2  $\mu\text{m}$  pore size (Figure C.1.1). Never sterilize the antibiotic solution by autoclaving as antibiotics will break down when exposed to heat.

**Table C.1.1. Antibiotics, concentrations and stock solutions used in the preparation of plates for the culturing of antibiotic-resistant mutants at ICARDA.**

| Antibiotic      | Concentration |        | Stock solvent | Stock/L final medium |
|-----------------|---------------|--------|---------------|----------------------|
|                 | Medium        | Stock  |               |                      |
|                 | ppm           | ppm    | ml            |                      |
| Streptomycin    | 40            | 10,000 | dist. water   | 4                    |
|                 | 60            | 10,000 | dist. water   | 6                    |
|                 | 100           | 10,000 | dist. water   | 10                   |
| Spectinomycin   | 40            | 20,000 | dist. water   | 2                    |
|                 | 60            | 20,000 | dist. water   | 3                    |
|                 | 100           | 20,000 | dist. water   | 5                    |
| Kanamycin       | 40            | 10,000 | dist. water   | 4                    |
|                 | 60            | 10,000 | dist. water   | 6                    |
|                 | 100           | 10,000 | dist. water   | 10                   |
| Chloramphenicol | 40            | 10,000 | 95% ethanol   | 4                    |
|                 | 60            | 10,000 | 95% ethanol   | 6                    |
|                 | 100           | 10,000 | 95% ethanol   | 10                   |
| Rifampicin      | 40            | 10,000 | 95% ethanol   | 4                    |
|                 | 60            | 10,000 | 95% ethanol   | 6                    |
|                 | 100           | 10,000 | 95% ethanol   | 10                   |
| Naladixic acid  | 40            | 20,000 | 1N NaOH       | 2                    |
|                 | 60            | 20,000 | 1N NaOH       | 3                    |
|                 | 100           | 20,000 | 1N NaOH       | 5                    |

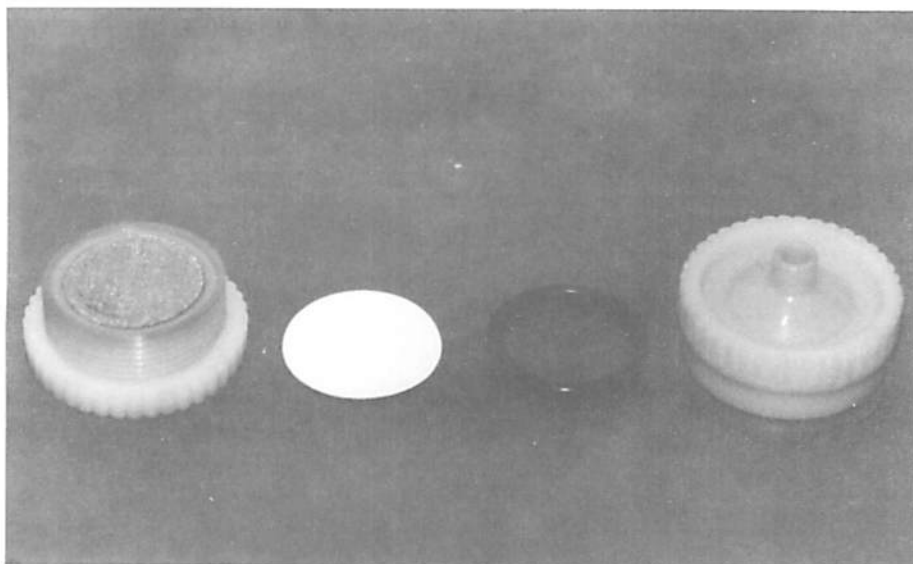


Fig. C.1.1. Filter assembly for micropore filtration.  
Completed assembly is seen in Figure C.2.1.

We occasionally do not filter the antibiotic through a membrane filter, but take great care that the transference of the antibiotic powder to the flask containing the sterile diluent is done under sterile conditions.

As an example, a concentrated stock solution of streptomycin prepared as 40 mg in 100 ml of distilled water ( $400 \mu\text{g/ml}$ ) can be diluted in 500 ml sterilized melted YMA medium to  $20 \mu\text{g/ml}$  (ppm) by adding 2.5 ml of the stock when the media has reached a temperature of  $45\text{-}50^\circ\text{C}$ . The contents are mixed thoroughly and then distributed into petri plates. Avoid vigorous shaking to reduce the formation of air bubbles. Special care should be taken to determine the exact volume of the YMA medium in the flask as some volume may be lost during autoclaving by evaporation or by rapid venting.

To calculate the different concentrations of antibiotic to be prepared the following formula may be used:  $V_i C_i = V_f C_f$  where V and C stand for volume and their concentrations at initial (i) and final (f) stages. As an example, if we wish to prepare a petri dish containing 20 ml of YMA with a concentration of 5 ppm from a concentrated stock solution containing 80 ppm:

$$\begin{aligned} V_i \times 80 &= 20 \times 5 \\ V_i &= 100/80 = 1.25 \end{aligned}$$

An alternate method to produce single plates would be to pipette 1.25 ml from the concentrated stock solution and place it inside a sterile petri dish; complete the volume with agar to 20 ml, mix clockwise, counterclockwise, backward and forward, to obtain an evenly mixed final concentration of 5 ppm antibiotic. In practice, larger numbers of plates are made (500 ml of medium will produce approximately 25 plates), and mixing of antibiotic and medium will take place in the flask before adding identical volumes of the medium to petri dishes. Care must be taken in making accurate measurement of the medium volume added to petri dishes, so that concentrations of antibiotics are consistent.

Store petri dishes containing YMA with and without antibiotic under cool conditions (4–6°C) if they are not to be used immediately. Storage will also allow the antibiotic sufficient time to diffuse thoroughly in the medium. We recommend, however, that antibiotic plates not be stored longer than two weeks before use, as antibiotics may break down with drying of the agar. Sealing plates with a thin strip of parafilm (Figure C.1.2) will prevent drying out where plates are to be kept for a longer period.

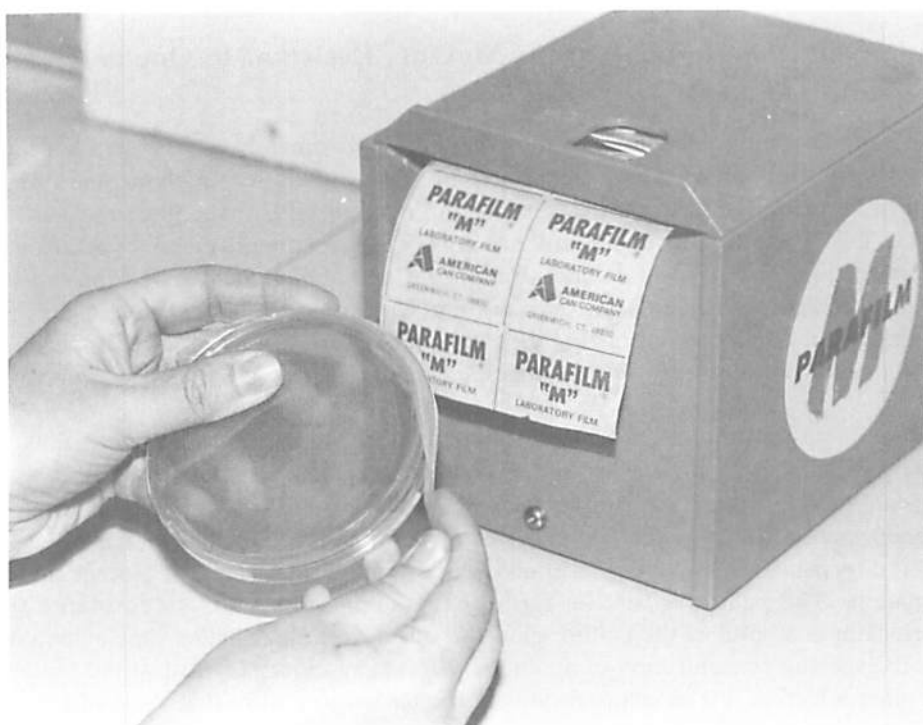


Fig. C.1.2. Sealing prepared agar plates with a thin strip of parafilm.

Working volumes of concentrated stock solutions should be prepared fresh and used at once. However, depending on the nature of the antibiotic molecule, some can be kept refrigerated providing the solution is used within a few days or, preferably, stored in deep freeze for later use. If in doubt, discard the stock solution and make another, as variations in potency of the antibiotic can give unexpected and erroneous results. According to our experience, supplementing YMA with two different antibiotics in the same plate presents no problem to rhizobial mutant growth, if the mutant strain is resistant to both antibiotics.

Commonly used antibiotics include: streptomycin, kanamycin, spectinomycin, chloramphenicol, nalidixic acid, and rifampicin. Check to be sure that the expiration date has not passed, and keep them in a dark and cool dry place (refrigerator). Whatever antibiotic marker is chosen, it should meet the following criteria: (a) resistance should not also be a characteristic of the indigenous bacteria; (b) it should be adaptable to a simple and efficient method of detection; (c) the resistant mutant should be stable, even through repeated culture or prolonged culture and storage; and (d) the mutant should not differ from the parent strain in symbiotic properties.

### **C.1.b. Selection of Spontaneous Mutants Resistant to One or Two Antibiotics**

Take a 0.1-ml aliquot of a fresh liquid culture of approximately  $10^7$  rhizobia per ml (two-day culture) and pipette it on surfaces of YMA antibiotic-supplemented dishes. Uniformly spread the bacterial suspension with a sterilized L-shape glass rod bar (Figure C.1.3); dip it in alcohol and flame it between treatments or do so occasionally within treatments. Use several concentrations of the antibiotic and start from the lowest (0 control) to the highest concentration. Use at least four replicated plates per concentration. A plate count of the original liquid culture will be required to estimate the frequency of occurrence of the spontaneous mutants in the initial suspension.

Incubate plates inverted at 26°C. If a strain is sensitive to the given antibiotic no growth will appear on the surface of the antibiotic-supplemented plate whereas abundant growth will appear on the control plate (Figure C.1.4). A spontaneously resistant mutant bacterial cell will generate a colony which will allow sufficient material for isolation and eventual transference onto a YMA slant for storage and evaluation. This culture is kept for further verification of its antibiotic resistance trait by streaking a loopful of the culture onto the surface of YMA plates supplemented with the specific concentration of antibiotic. It is always necessary to test the mutant in a plant-infection test in comparison with the parent to ensure that symbiotic properties have not changed. Some researchers recommend keeping the antibiotic-resistant mutants in slants containing a moderate level of the antibiotic to avoid back-mutation to antibiotic sensitivity. At ICARDA we preserve them in slants



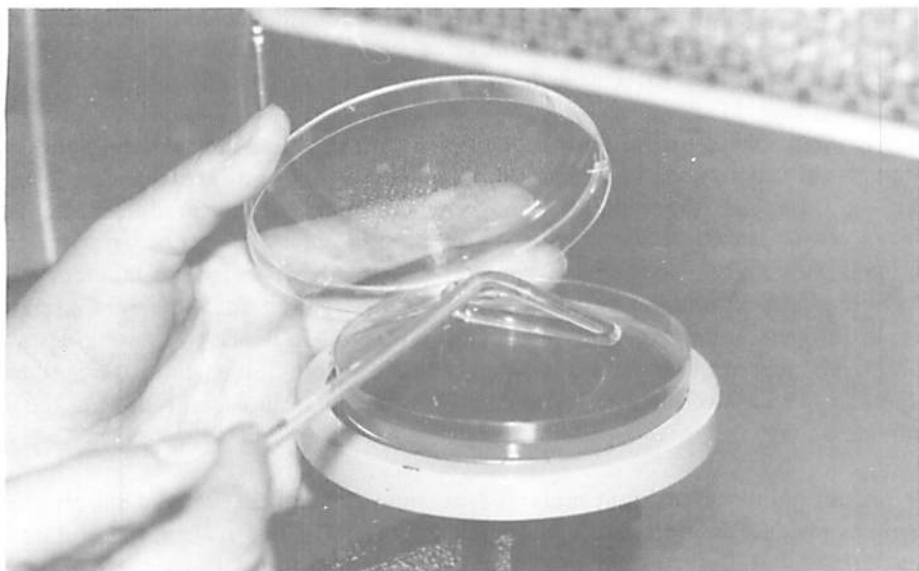


Fig. C.1.3. Using a turntable and L-shaped glass rod to allow uniform spread of a bacterial suspension on agar surface.

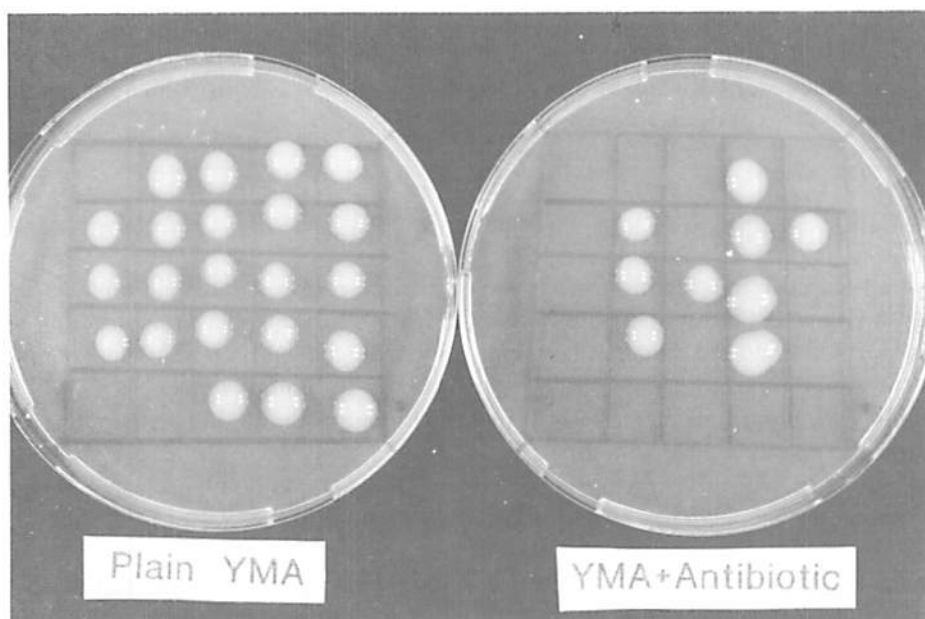


Fig. C.1.4. Differentiation of sensitive and resistant rhizobia to antibiotics.

containing normal YMA, but test periodically for antibiotic resistance and symbiotic effectiveness. In cases where the cultures have been lyophilized, the antibiotic-resistance trait and their effectiveness are not normally lost.

It may be desirable to use strains of *Rhizobium* with resistance to two antibiotics, particularly in experiments in which other singly marked strains are included. The procedure is to select the mutant using a given antibiotic (as indicated above) and then repeat the procedure using a second antibiotic. A combination of streptomycin with either kanamycin or spectinomycin works well. Double-resistance mutants are distinguishable from mutants resistant to either single antibiotic, and can be used, for example, in competition experiments using three strains in which two antibiotics are involved. Nodules can then be easily typed on plates containing each of the antibiotics for the identification of the double- and single-resistant strains (see following section on nodule typing).

Spontaneous antibiotic-resistant mutants from highly effective strains of rhizobia sometimes produce ineffective nodulation in the host. For this reason, the mutant strain should be tested for symbiotic effectiveness before using it in a comparative study. Broth cultures of both the mutant and parental strains should be grown for inoculation of legume seedlings aseptically raised in glass tubes or pouches containing N-free nutrient solution (see Section B). After nitrogen fixation is active (four to six weeks), check for nodulation morphology and plant appearance. If there are not apparent differences in nodulation, plant color, and shoot weights, it can be assumed that the mutant behaves as the parental strain. It is suggested that a comparison of the shoot weights of both treatments be done on a statistical basis, which means that from four to six replicates are necessary.

### C.1.c. Identification of Mutants from Nodules

The main purpose in selecting spontaneous antibiotic-resistant rhizobial mutants from parental strains is the capacity to differentiate from other rhizobia in media, soil, or in a nodule. The mutant strain can be identified when cultured on YMA supplemented with the antibiotic to which the strain has resistance. In "nodule typing", nodule contents are cultured on the surface of plates with and without antibiotic to determine whether the nodule was occupied by a resistant or a sensitive strain. The operation is usually done on plates having a grid pattern to allow for nodule identification. Figure C.1.5 shows a plate layout suitable for typing 24 nodules.

Nodules are surface sterilized (see Section A) and kept inside a sterile petri dish on wet filter or blotter paper to keep them turgid. The nodule is picked up with a pair of sterile blunt-tipped forceps, then gently squeezed between the tips of the forceps until nodule contents appear (Figure C.1.6). Spread this nodule squash in equal amounts in

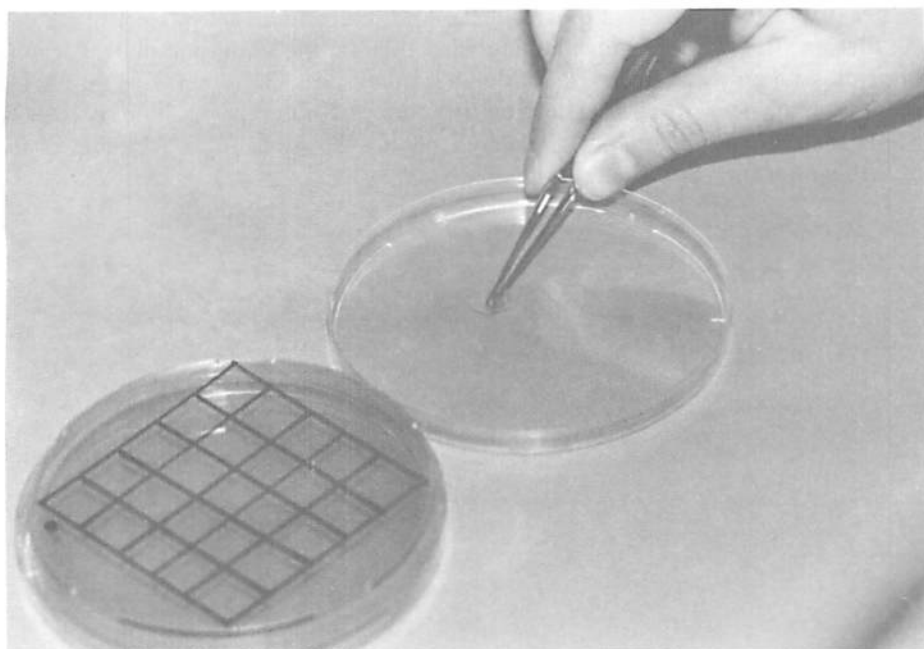


Fig. C.1.5. Grid pattern template for uniform application of nodule squashes to antibiotic plates.

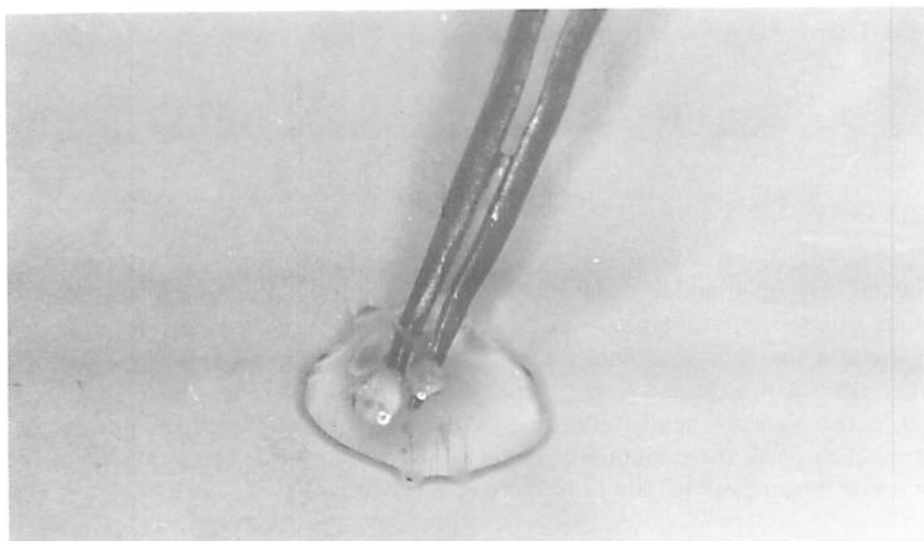


Fig. C.1.6. Squeezing nodule contents into a drop of sterile water with the tips of a sterile forceps.

the center of the corresponding square of the grid pattern on each plate (Figure C.1.7); first on the plate without antibiotic and then on the antibiotic plate. Clean forceps well with an alcohol-wetted cotton swab, then pass the forcep tips through the bunsen burner flame before repeating the procedure with another nodule. It is important that equal amounts of nodule contents are delivered to each square of the plates throughout the operation.

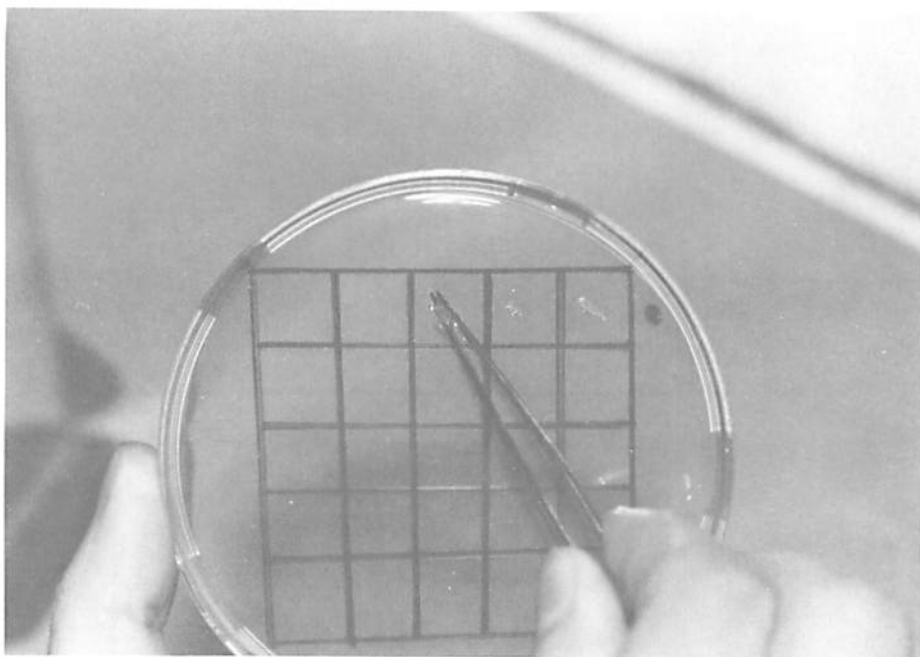


Fig. C.1.7. Application of nodule squash to grid squares on antibiotic plates.

In cases of very small nodules where not enough nodule 'squash' is available to inoculate squares on both types of plates, one could first use the plates without antibiotic as a source of inoculum for later application to the plates with antibiotic. Another alternative is to use pins to perforate the nodule and then inoculate the surface of the plates by gently scratching on the agar surface of both treatment plates (Figure C.1.8). With these methods, smaller amounts of inocula are delivered to the plate, and a longer time is required before results are visible.

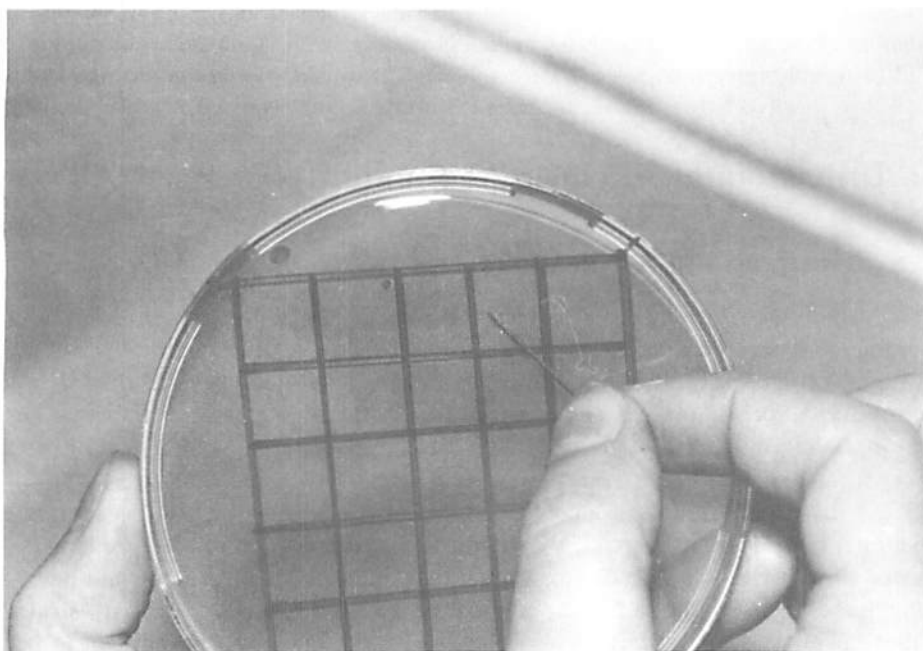


Fig. C.1.8. Application of nodule contents to antibiotic plates using the pin method.

Inspection of the plates for growth should be performed daily after inoculation; once growth is observed on the control plates, +/- growth on the antibiotic plate will indicate whether a given typed nodule contained resistant or sensitive strains. If there is growth on both treatment plates (with and without antibiotic) then it is assumed that the nodule was occupied by a resistant strain (the mutant). If growth is detected only in the plate without antibiotic then we assume that the nodule was occupied by sensitive rhizobia. Here, you can see the value of replications of plates, for confirmation of results. We recommend a minimum of two replications, or if possible more, be used.

Double or mixed infections, which are sometimes present in a proportion of nodules, can also be detected in experiments in which two single antibiotic-resistant strains are included. Growth in both plates with different antibiotics indicates that the nodule was occupied by both strains.

In the case of double-resistance mutants, growth should be detected in the plates having each of the antibiotics to which the strain is resistant. Competition experiments

having a double-resistant strain and single-resistant strains to either of the two antibiotics allow use of two or three strains in nodule identification studies; however, mixed or double infections are not detectable when using these combinations.

## **C.2. Utilization of Intrinsic Antibiotic Resistance (IAR)**

### **C.2.a. Principles and Applications**

Intrinsic antibiotic resistance differs from the induced resistance to high levels of an antibiotic (as described in the previous section) in that it uses the natural resistance of the strain(s) to given levels of various antibiotics for identification. The chief uses for IAR in *Rhizobium* studies are: (1) as a trait for comparing or characterizing strains; (2) as a predeterminant in selecting strains for antigen-antibody studies; and (3) as a marker for ecological studies. Work by several investigators, including work at ICARDA, has indicated that in many cases it is possible to distinguish between strains grown on YEM medium containing different levels of antibiotics by differential growth. However, it is a mistake to assume that different strains can always be distinguished by IAR. The larger the number of antibiotics used to compare strains, the more likely it is that differences will be discovered, but it can be difficult to be certain that IAR characteristics of two strains are identical.

At ICARDA, we have found it most useful to use six antibiotics at differing concentrations for comparison of strains (Table C.2.1), using the agar dilution method (where the antibiotic is mixed with the agar during preparation of the media). It is important that conditions and components be standardized to ensure reproducible results. Particularly important are:

- culture medium composition and pH,
- sterilization procedures,
- temperature of agar medium when antibiotic stock solution is added,
- volume of agar per petri plate,
- growth stage of test cultures,
- size of inoculum.

A critical factor is the preparation of antibiotic stock solutions. Many antibiotics are relatively unstable and can be stored only a short time; stock solutions should be used immediately, but if storage is necessary it should be at -20°C. Stock solutions must be sterilized, but most break down with autoclaving so must be filter sterilized through a 0.45- $\mu$ m filter system (Figure C.2.1). Antibiotic stocks should be added to the medium held at 45–50°C in a water bath (Figure C.2.2) or oven (use the same temperature each time), thoroughly mixed, poured, and cooled. The plates should be used as soon

as possible after preparation, preferably after plates have dried inverted for two or three days at 25°C.

**Table C.2.1. Recommended antibiotics, concentrations and solvents**

| Antibiotic      | Source    | Concentrations mg/l medium | Solvent         |
|-----------------|-----------|----------------------------|-----------------|
| Chloramphenicol | Sigma Co. | 2-10-20-30                 | 95% Ethanol     |
| Kanamycin       | "         | 10-20                      | 1 M. NaOH       |
| Nalidixic acid  | "         | 5-10-20                    | Distilled water |
| Rifampicin      | "         | 1-3-5                      | Methanol        |
| Streptomycin    | "         | 10                         | Distilled water |
| Ampicillin      | "         | 7.5-10                     | Distilled water |
| Vancomycin      | "         | 2.5-5                      | Distilled water |



**Fig. C.2.1. Filter sterilization of a stock antibiotic solution.**

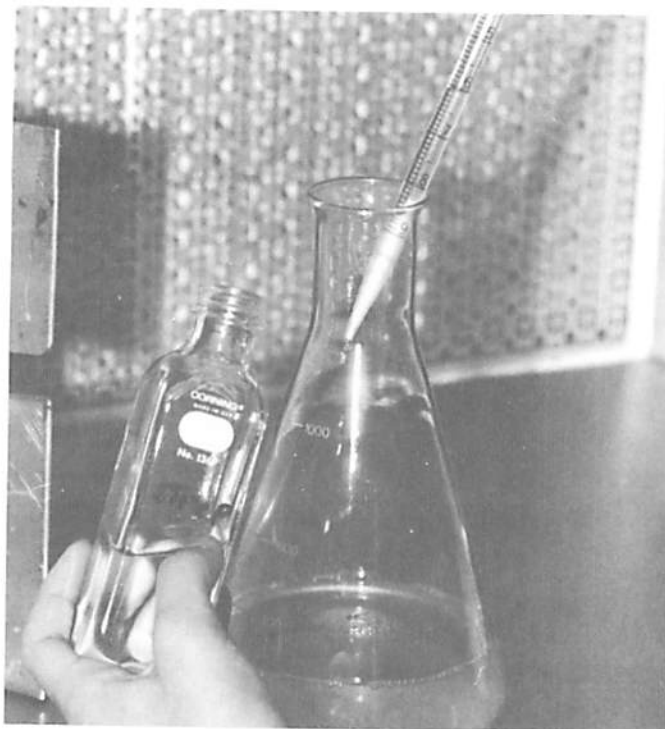
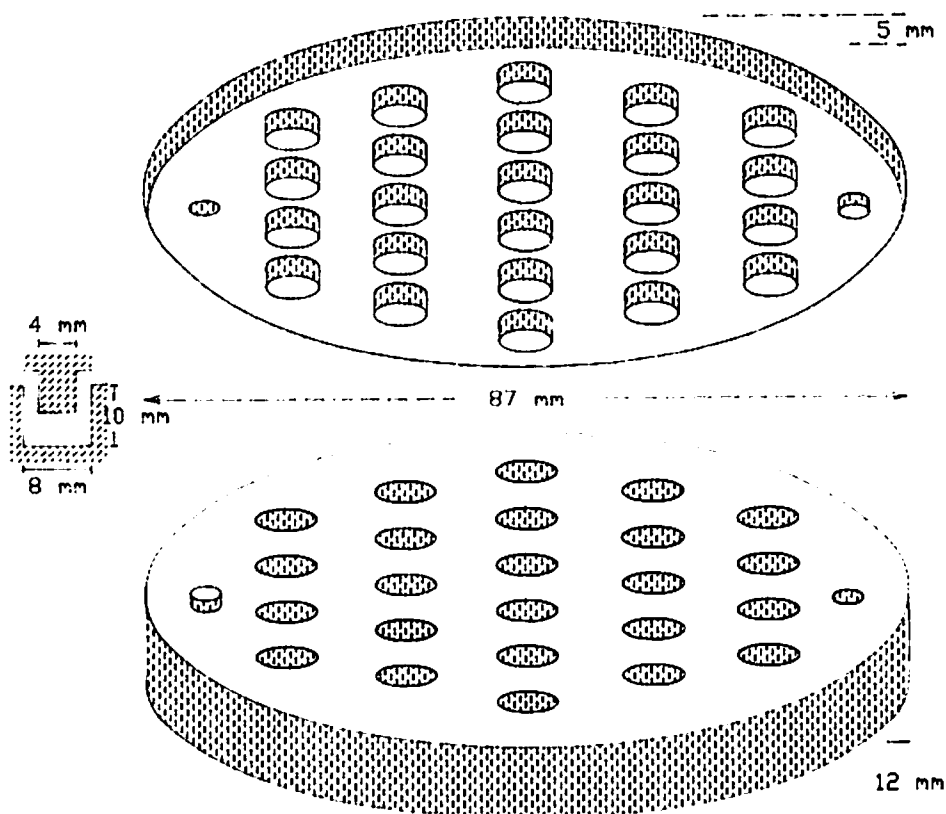


Fig. C.2.2. Adding antibiotic stock solution to warm YMA medium at 47°C just prior to pouring plates.

To obtain an inoculum of uniform size and activity for application to the plates, it is necessary to use cultures at an equivalent growth stage. For this reason, fresh cultures of approximately equal population density grown in YM broth should be used ( $10^7$ – $10^8$  cells/ml, or when growth is seen as slightly cloudy). A multiple inoculator (Figure C.2.3) will allow application of many strains to each plate while delivering equal amounts of inoculum every time. An alternative is to use a calibrated inoculation loop (1/1000 ml) for application of inoculum to the plates, although this method is much more tedious and less reproducible.

The main disadvantage of IAR is also the property that makes it valuable: the number of different tests (antibiotics/concentrations) involved. The amount of work can be reduced by using the multiple inoculator that is capable of transferring 24 samples at a time to different test media. A further problem is that when intrinsic resistance to drugs is being determined, one is looking at small differences in the concentration of that drug to distinguish sensitive and resistant strains (e.g., 2.5 and 5 mg/L). Media must, therefore, be prepared carefully and used uniformly, but even then there will always be strains that give variable results.





**Fig. C.2.3. Schematic diagram of the multiple inoculator, used to apply equal amounts of inoculum to each of 24 spots on one plate. This useful tool is easily fabricated out of brass in a metal fabrication workshop.**

## **C.2.b. Multiple-Inoculator Agar Dilution Method**

### **Requirements:**

- YEM media
- antibiotics, stored dry at 4°C in refrigerator (Table C.2.1)
- antibiotic solvents (Table C.2.1)
- 1/1000 inoculation loop or multiple inoculator (fabricated locally, see Figure C.2.3)
- water bath capable of maintaining 45–50°C

- petri dishes
- 250  $\mu$ l pipettor (optional)

**Procedure:**

1. Prepare 5 ml of 10 mg/ml stock solutions of the above antibiotics by dissolving 50 mg of each antibiotic into 5 ml of corresponding solvent.
2. From the antibiotic stock solutions prepare YEM containing the above concentrations.
3. Cool the medium in water bath (48–50°C); when the media reaches 48°C, add the antibiotic, and swirl the flask to mix the contents thoroughly.
4. Dispense exactly 25 ml of antibiotic-enriched medium into each petri dish, carefully labelling antibiotic and concentration on the plates with a marker pen (Figure C.2.4). Antibiotic media are prepared 24–72 hours before use. If to be used within 24 hours, plates must be dried, and inverted in an incubator at 35°C for at least 18 hours. There should be no excess water on the media surface at the time of inoculation.

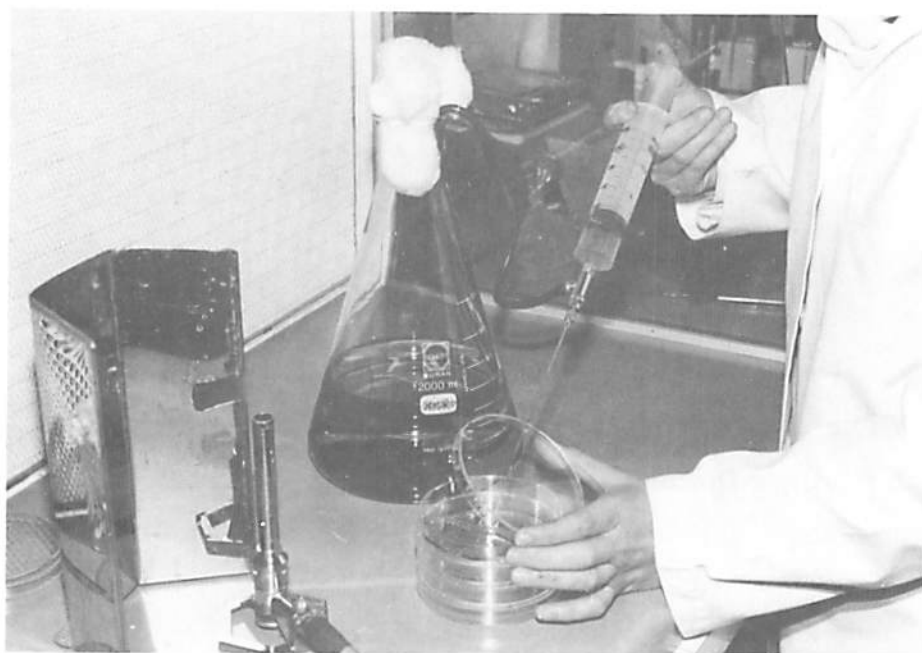


Fig. C.2.4. Using an automatic syringe to dispense equal amounts of an antibiotic-enriched medium into petri dishes.

5. Grow fresh cultures of different *Rhizobium* strains in screw-cap bottles containing 5 ml broth each, inoculating each with a loopful of viable rhizobia from a YEM agar slant.
6. Put the inoculated bottles on a shaker water bath at 26°C for 24–48 hours, depending on growth rate, until slightly cloudy.
7. Prepare a map to indicate placement and the strain identification numbers for each series of plates (Figure C.2.5). Map corresponds to inoculating pins on multiple inoculator (Figure C.2.6).
8. Transfer 250  $\mu$ l of the broth culture to the corresponding well of the multiple inoculator (Figure C.2.7).
9. Add to each well 50  $\mu$ l sterilized water. This dilution represents  $10^5$  cell/ml.

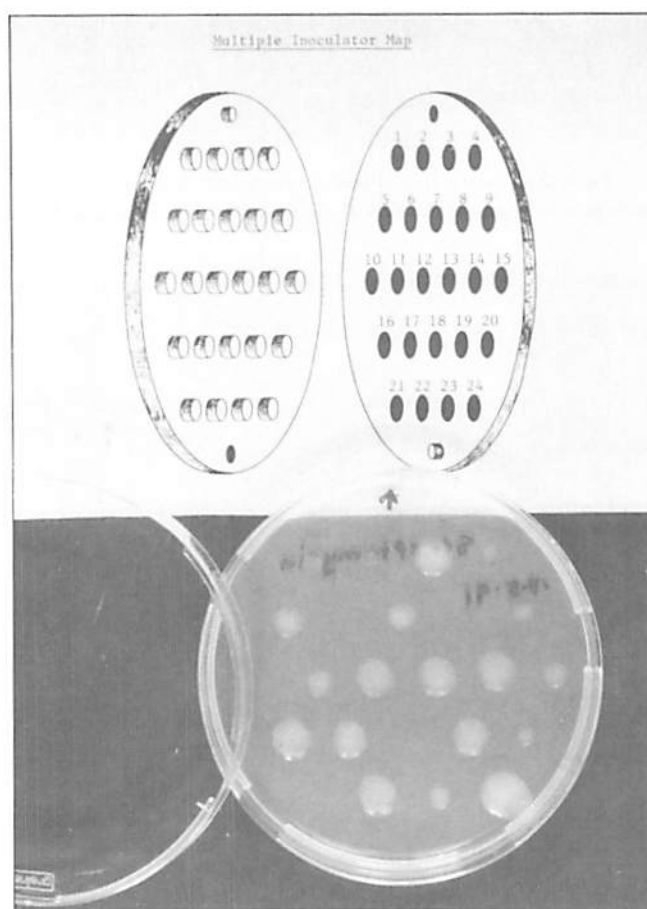


Fig. C.2.5. Differential growth of strains on antibiotic plate.  
Note that numbers on plate correspond to strain identification numbers on key-map for the multiple inoculator system.

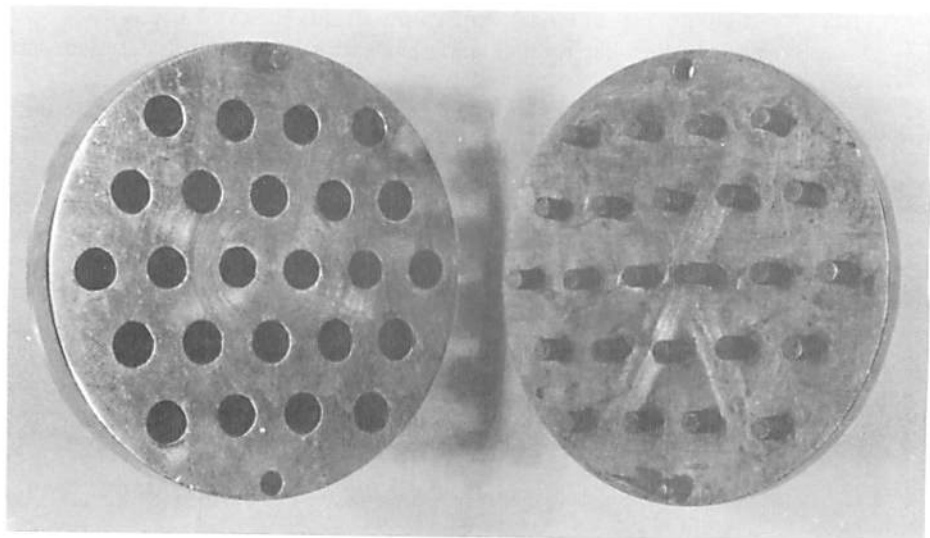


Fig. C.2.6. Orientation of inoculator should always be the same, with pins to align applicator with base used to indicate top and bottom.

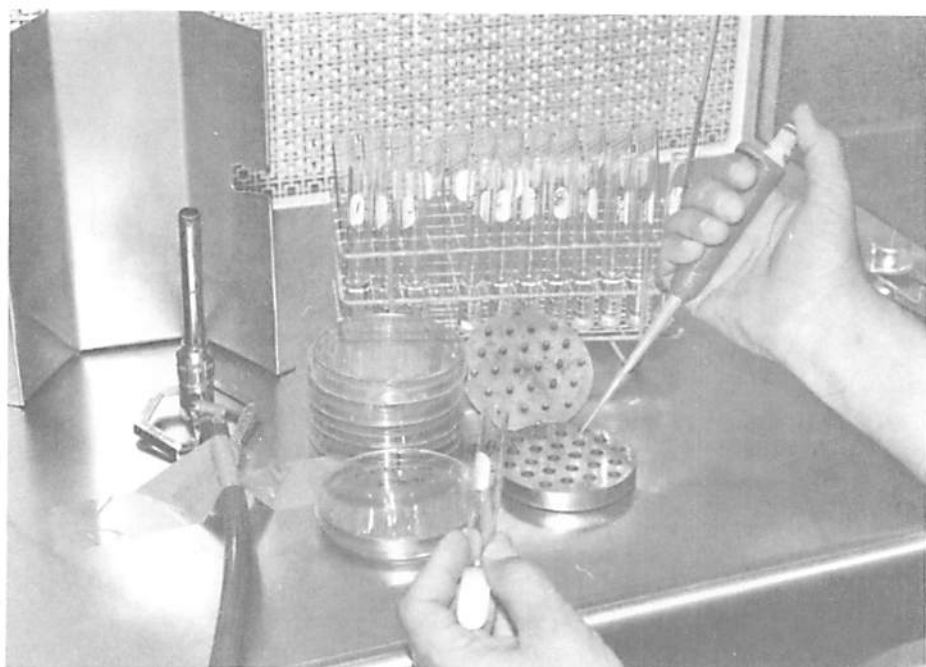


Fig. C.2.7. Transfer of broth culture to well of multiple inoculator with pipettor.

10. Put the inoculator cover on the base containing the wells (each rod fits the corresponding well, oriented by a pin on the edge).
11. Lift the cover and put it on the petri dish taking care that all rods have touched the medium (Figure C.2.8).
12. Incubate petri dishes inverted at 27°C.
13. Observe the growth of different strains of *Rhizobium* on different antibiotics media after 72 hours of incubation (for chloramphenicol, kanamycin, nalidixic acid, and vancomycin), and after seven days incubation (for rifampicin, streptomycin, spectinomycin, and ampicillin).

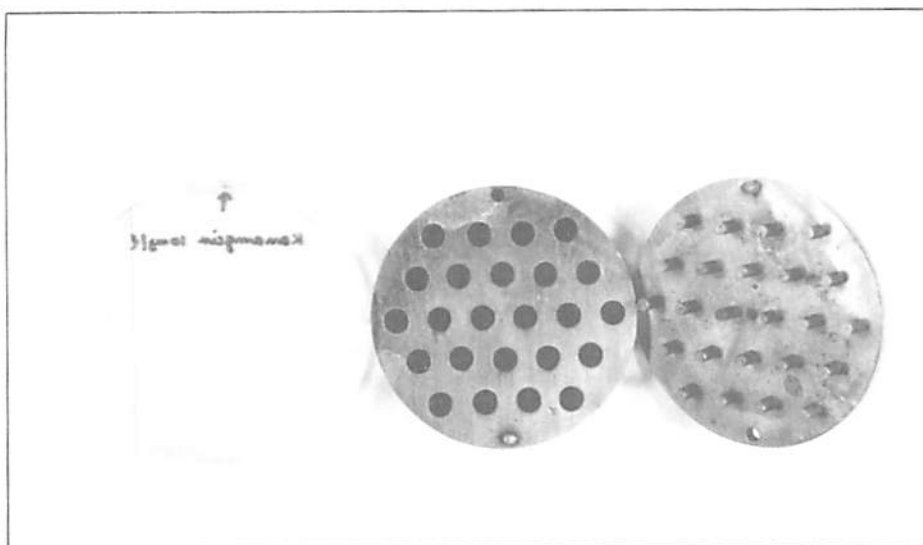


Fig. C.2.8. Note that male pin on applicator base is always at the bottom, maintaining correct orientation for all plates. A mark on the petri dish base corresponds to the male pin on the applicator handle.

We strongly recommend that at least three or four replications are conducted in each experiment, and that all experiments be conducted at least twice. Growth can be scored with a simple two-level system, simplifying statistical analysis of data: 0 = no growth; and 1 = positive growth. If growth is not clear, i.e., +/- in a particular concentration of antibiotic, using a higher concentration of antibiotic is recommended to better define resistance (see Figure C.2.5 for example). Difficulties may arise if more than one person reads the plates; at ICARDA only one person will read the growth on antibiotic plates in all experiments, which makes the scoring system more consistent. A simple table of antibiotics and concentrations *versus* strains will help to distinguish different IAR patterns.

## C.3. Serological Techniques

### C.3.a General Description

The serological reactions which are used to identify rhizobia are the result of interaction between antigens, composed of basic proteins or large molecules like polysaccharides which may be characteristic of a particular rhizobia strain, and antibodies, which are produced by an animal in response to injection of foreign protein material (antigen). Because the antibody is produced to react to a specific antigen, it can be used for identification of that particular protein-containing strain when linked with enzymes or dyes that make the reaction visible.

Several types of serological reactions are widely used in the study of rhizobia. Of these, the two which will be discussed most thoroughly here (ELISA and FA) involve antigens located at the surface of rhizobia cells. These surface somatic antigens of the rhizobia are the most strain specific, and serological examination of any collection of a species is likely to enable division into 'serogroups'. Internal antigens are widely shared within slow-growing rhizobia, but not with fast growers; likewise, fast-growing rhizobia commonly share internal antigens, but not with slow growers. For this reason, intact cells are necessary for the serological reactions described in this section. Flagellar antigens are generally widely shared between strains, so they are often deactivated by heat treatment to make the antigen-antibody reaction more specific. Specific methods have been developed which have greater sensitivity but generally involve specialized equipment and require greater experience. Precipitation in a gel-diffusion system can detect a wide range of solubilized internal or surface antigens and is probably most useful for classification work; antigen separation to speed electrophoretic diffusion can provide highly precise strain definition.

The specificity that exists between a strain's antigens and antisera makes serology a convenient means of investigating rhizobial behavior in ecological and agronomic studies. The serological methods discussed in this section — ELISA and FA — can be used for direct identification of a strain, or strains, occupying a nodule, and as a basis for enumeration of specific rhizobia in soil survival studies. These methods are not difficult to master, but attention must be given to specificity in relation to other strains and bacteria (cross reactions), sensitivity, reliability (reproducibility), antigenic stability and convenience in testing. It should be remembered that an antiserum is a complex mixture of serum proteins and antibody molecules; final concentrations of these substances can be greatly affected by antigen preparation, the immunization procedure, and the temperatures during storage and use.

The involvement of animals in production of antiserum may deter some scientists from using serological methods. Where large numbers of diverse antisera need to be prepared, it is necessary to have access to an equipped animal house and technical

help. It is possible, however, to prepare enough antisera for studies involving a few strains in fairly elaborate investigations with the simplest of facilities. Rabbits have been used almost exclusively for rhizobia antisera production, due to ease of handling and success in production of sera with good titer. The most commonly used rabbit species is the New Zealand white rabbit, preferred for its large size (making it possible to collect a greater quantity of serum) and docility (for ease of handling). It is not necessary to kill the rabbit or cause it undue suffering to produce antiserum.

### **C.3.b. Preparation of Somatic Antigens**

It is good practice to use a defined medium for growth of rhizobia in antigen preparation, as the undefined constituents of yeast extract may themselves be antigenic. In our work, we find the defined medium of Bergersen (Section A.10.a) useful for growing rhizobia for antigen preparation. It may not be necessary to use this defined medium, which supplies the necessary specific vitamins contained in yeast extract, but it may prevent problems with low antibody titer and cross-reactions.

#### **Requirements:**

- fresh slant of *Rhizobium* culture
- sterile YEM (or defined media) agar in Roux flask or Erlenmeyer flask
- filtered and sterile N-saline (1 normal) in dilution tubes (10 ml)
- sterile YM broth in dilution tubes (9 ml)
- petri dishes with YEM agar
- sterile centrifuge tubes with caps (30–50 ml cap)
- sterile pipettes (1, 5, and 10 ml)
- sterile serum bottles with rubber septum and aluminum caps
- dry and sterile Erlenmeyer flask (100 ml)
- glass rod
- vortex mixer
- high speed, refrigerated, floor model centrifuge
- incubator
- refrigerator

#### **Procedure:**

1. Aseptically add to a fresh slant containing fully grown *Rhizobium* culture about 6 ml of sterile N-saline (1 N) and suspend the bacteria thoroughly using vortex mixer (Figure C.3.1).
2. Transfer 2.5 ml of the suspension to Roux flask (Figure C.3.2) using a 5-ml sterile pipette (prepare two Roux flasks for each strain).



Fig. C.3.1. Suspending a rhizobial slant culture using a vortex mixer.

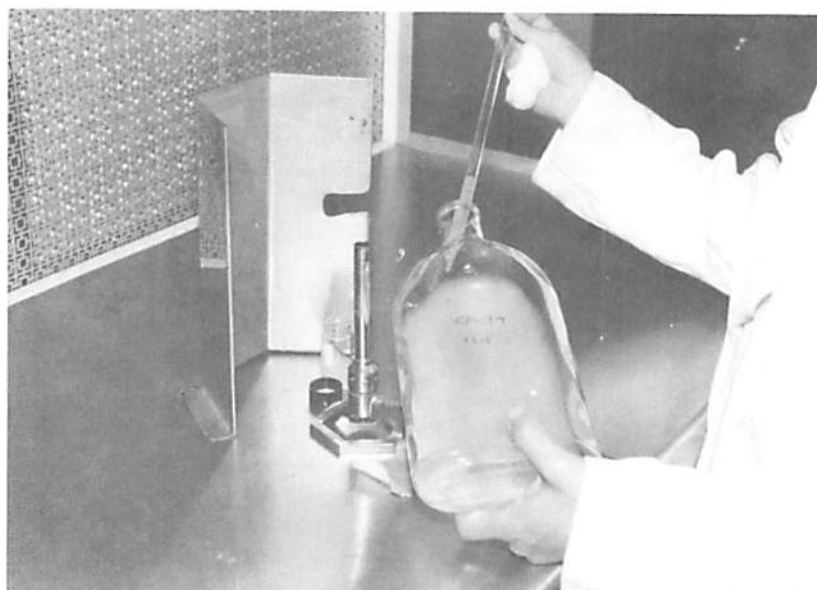


Fig. C.3.2. Transfer of the rhizobial suspension to a Roux flask containing defined agar.



3. Incubate inoculated Roux flasks at 26–28°C for three to five days for fast growers and 7–10 days for slow growers; Roux flasks must be inverted (agar surface facing down) during incubation. Daily observations of growth are recorded every day.
4. Harvest the culture in Roux flask by adding 20 ml of sterile N-saline and suspending the rhizobia colonies in the liquid using sterile glass rod (Figure C.3.3).

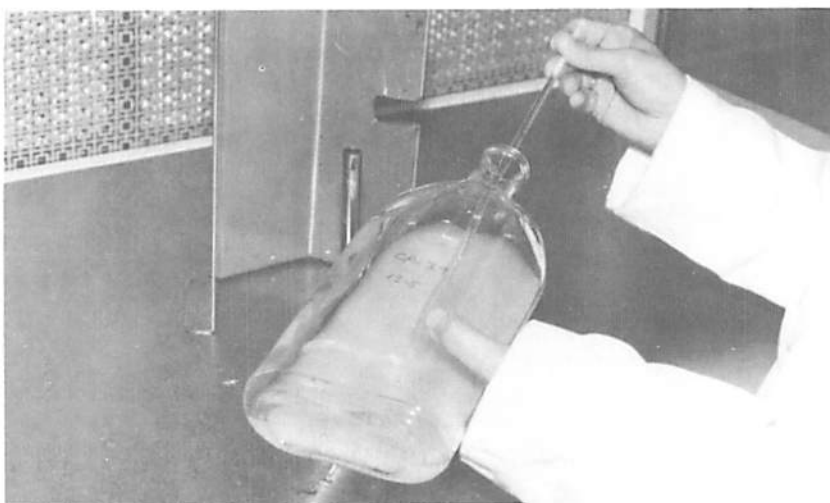


Fig. C.3.3. Harvesting of rhizobia from Roux flask in saline, using a glass rod to suspend the bacterial mat.

5. Transfer the suspension from Roux flask to a sterile Erlenmeyer flask, pouring aseptically; the two Roux flasks containing the same strain are harvested and transferred to a single 100-ml sterile Erlenmeyer.  
**Note:** If any uncharacteristic colonies (contaminants) are observed, do **not** use the culture for antigen preparation.
6. Transfer 1 ml of the suspension to sterile dilution tube containing 9 ml YM broth (save for later quality determination, including plate count and Gram stain).
7. Heat the suspension by placing Erlenmeyer flask in a boiling water bath for one hour to inactivate flagellar antigens and other proteins (Figure C.3.4).
8. Transfer the suspension to a 30–50 ml sterile centrifuge tube and centrifuge at  $10000 \times g$  for 15 minutes and 6°C (Figure C.3.5).
9. Discard supernatant (Figure C.3.6) and resuspend the precipitate in 20 ml sterile N-saline (Figure C.3.7), then recentrifuge as above. Repeat this centrifuge-washing procedure three times.

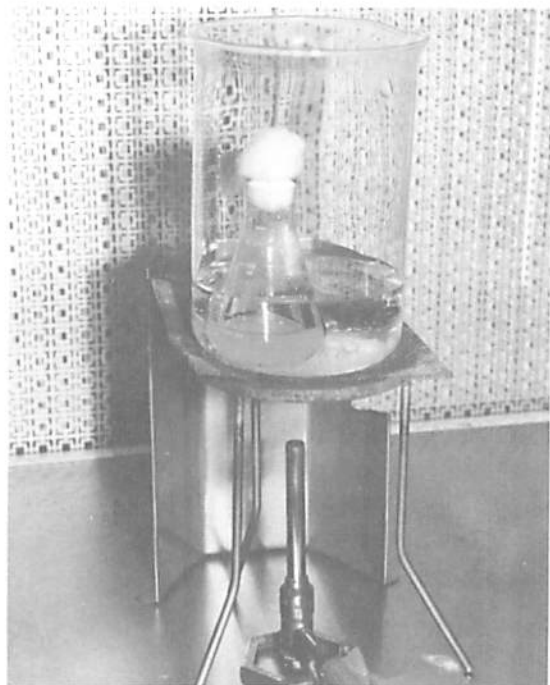


Fig. C.3.4. Heating the suspension of rhizobial cells to inactivate flagellar antigens.



Fig. C.3.5. Using a refrigerated centrifuge to obtain a pure-cell pellet.

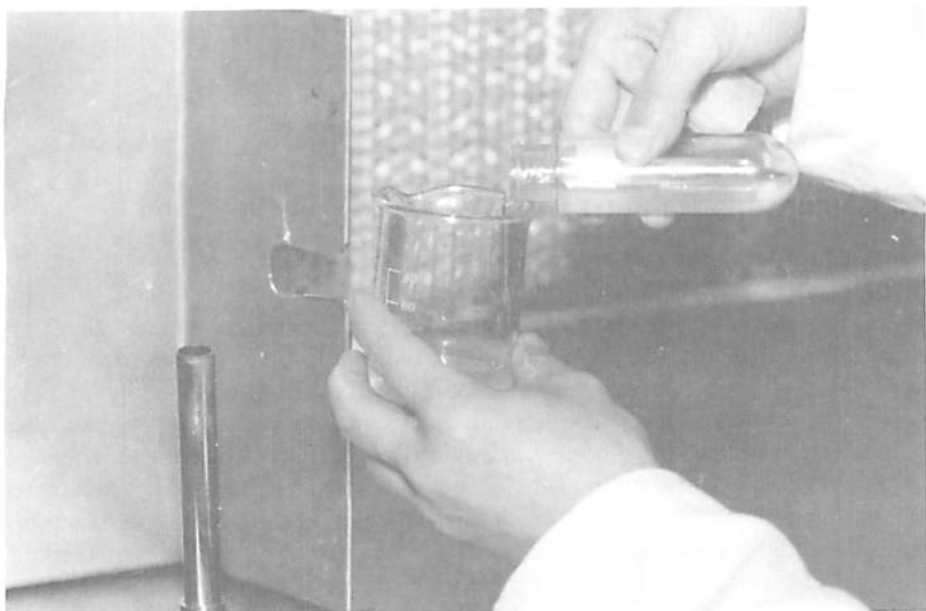


Fig. C.3.6. Discarding supernatant after centrifugation of suspension.

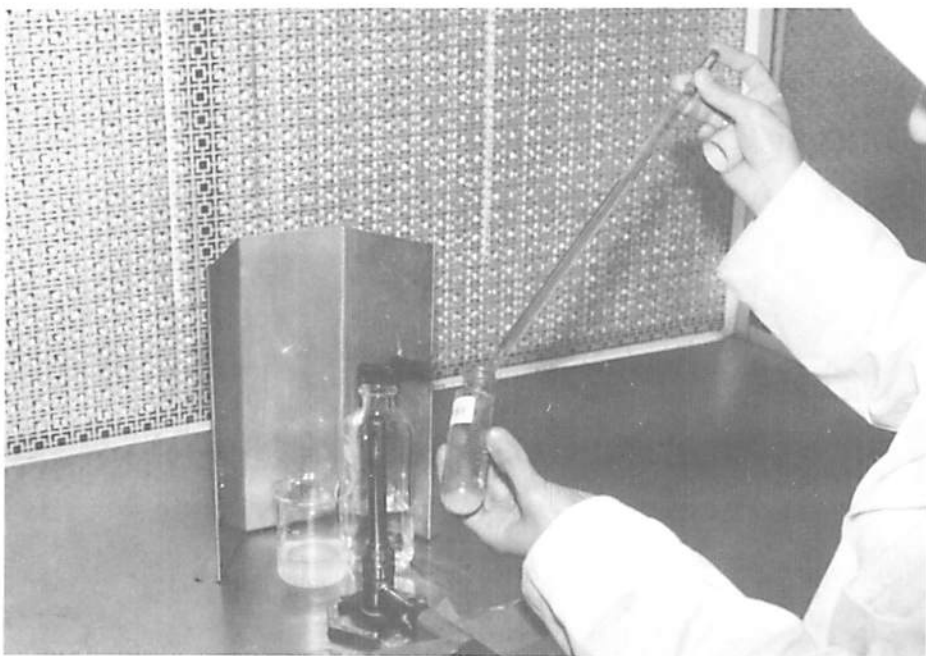


Fig. C.3.7. Suspension of pure-cell pellet in sterile N-saline.

10. Suspend the final precipitate in 20 ml sterile N-saline.
11. From this pure cell suspension, dispense 2 ml into each of 10 5-ml sterile serum bottles and store them at 4–6°C in refrigerator.

### C.3.c. Immunizing Rabbits: Injection and Blood Collection

Immunization procedures and injection schedules vary among investigators; procedures different from those given here are referred to in the reference section. Immunization procedures fall into two general types: (a) primary immunization by repeated intravenous injection of the antigen without an adjuvant, at intervals of one or more days, followed by a number of booster injections prior to bleeding; and (b) primary immunization with a single intramuscular injection of the antigen emulsified with an adjuvant, followed by one or more booster injections before bleeding. According to the literature, titers of the antisera obtained by the two methods, when recorded, were similar (the highest dilution which gives a clear serological reaction is known as the 'titer' of the reaction). We present the procedure for the first method, which we have found most effective in producing sera with high titers in work with chickpea and lentil rhizobia at ICARDA. Whenever possible, two rabbits are used with each antigen because of the variability in response by different animals.

#### Immunization and Blood Collection

Two types of injection are described in the following procedure. The intravenous injections of 0.5 to 2.0 ml are injected into the large marginal ear vein of one ear (Figure C.3.8). If necessary, both ears may be used for the injections, but we try to

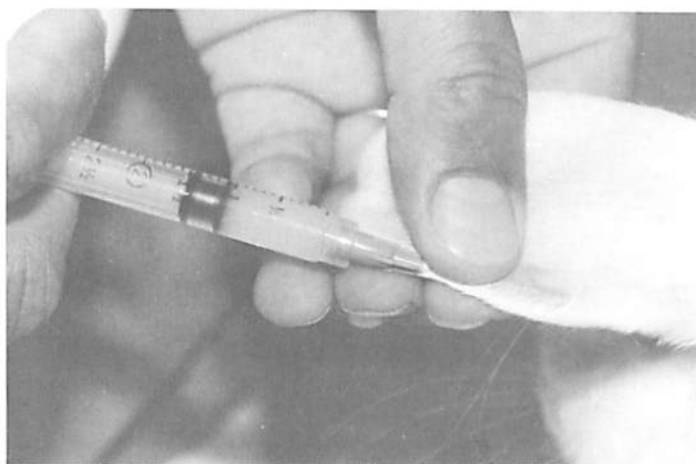


Fig. C.3.8. Injecting 2 ml of antigen into the marginal ear vein of a rabbit. Note that hair is pulled from vein area to allow unobstructed view of vein.

keep one ear free for later bleeding of the rabbit. We have found it useful to place the rabbit in a restraining cage (ref. Thomas Scientific Catalogue No. 1123-H50) while injecting or drawing blood (Figure C.3.9). The intradermal injections of 2.0 ml each are injected under the loose skin of the hindquarters (above the rear legs — Figure C.3.10). The antigen suspension prepared previously is freshly unfrozen (but kept in an ice bath at all times before use) and used to immunize young adult rabbits according to the following schedule:

| Day   | Route of injection    | ml antigen |
|-------|-----------------------|------------|
| 1     | Intravenous (IV)      | 0.5        |
| 2     | IV                    | 1.0        |
| 3     | IV                    | 1.5        |
| 4-7   | Rest                  | -          |
| 8     | IV                    | 1.5        |
| 9     | IV                    | 2.0        |
| 10    | IV + intradermal (ID) | 2 + 2      |
| 11-16 | Rest                  | -          |
| 17    | 1st bleeding          | -          |
| 24    | ID                    | 2          |
| 31    | 2nd bleeding          | -          |
| 38    | ID                    | 2          |
| 45    | 3rd bleeding          | -          |
| 52    | ID                    | 2          |
| 59    | 4th bleeding          | -          |

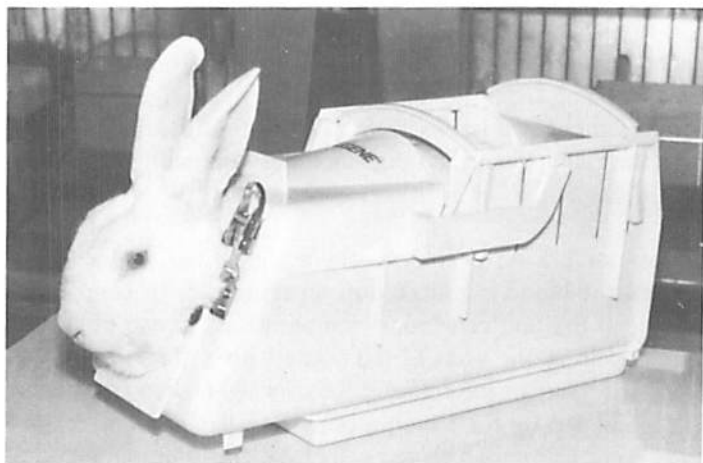


Fig. C.3.9. Commercial restraining cage to facilitate the handling of rabbits during injection and bleeding activities.

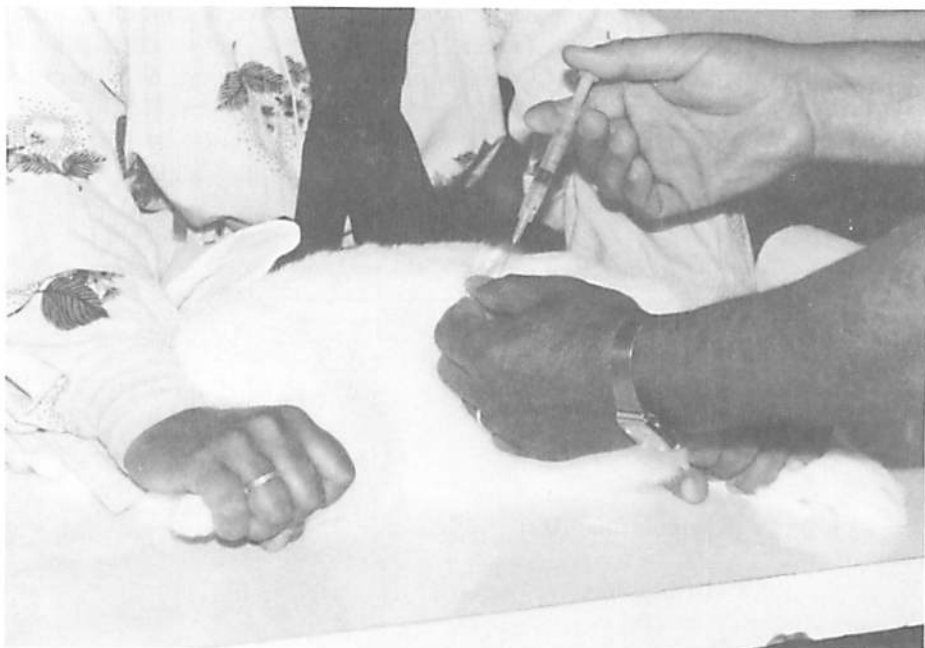


Fig. C.3.10. Intradermal injection just under the skin above the rear legs of the rabbit.

Different methods of blood collection are used by various workers; some kill the animal and collect as much blood as possible from the jugular vein into a large beaker after the titer has reached a high level (as determined by test bleeding from the ear). Others use a heart puncture to collect 50–80 ml directly from the cavities of the heart. We prefer bleeding from the marginal ear vein, taking 10–20 ml of blood on successive bleedings about two weeks apart (Figure C.3.11); this of course is much less damaging to the rabbit than the other methods, and will be the only method described here.

In order to collect blood, a cut is made in the marginal ear vein with a sharp blade (Figure C.3.12) and the blood is collected in serum bottles or centrifuge tubes. After blood collection, keep the bottles at room temperature for one hour, then carefully loosen the blood clot from the walls of the bottle with a glass rod (Figure C.3.13). Store the serum (with clot) in the refrigerator overnight, then transfer to a centrifuge tube and centrifuge at  $10^4 \times g$  for 15 minutes at  $4^\circ\text{C}$ . It may be necessary to recentrifuge the serum to remove all red cells from the supernatant sera. Transfer the supernatant (clear serum without any traces of cells) to a sterile screw-cap tube. It may not be necessary to add preservatives to serum samples if they are stored frozen

(in the deep-freeze compartment of a refrigerator). Repeated thawing and refreezing do not seem to harm the activity of the sera, but caution should be exercised to keep thawed sera chilled in ice (4–6°C) during use. Sera may be kept unfrozen at 4°C if preservatives are added. We prefer to be doubly certain, and add merthiolate (sodium ethylmercuri-thiosalicylate = Thimersol, Sigma Catalogue No. T 5125) at 0.01% ( $1/10^4$  or 1 mg/10 ml) while storing at -20°C (in freezer compartment) until ready for use.

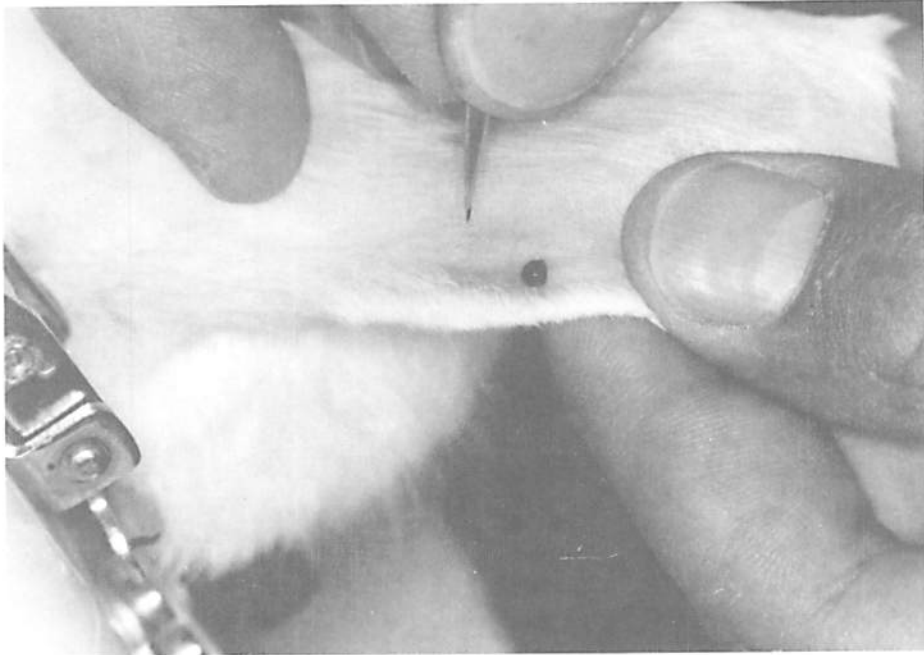


Fig. C.3.11. Using a sharp blade to cut the marginal ear vein of a rabbit for blood collection.

#### **C.3.d. Evaluation of Antisera Titer by Agglutination**

The agglutination reaction is simple to perform, and is often used for preliminary titer determination to decide when to harvest blood from the rabbit. This method, however, does not always give clear distinctions between reactions of antigenic identity and cross-reactions between closely related strains. Agglutination is based on the aggregation, or clumping together, of rhizobial cells caused by antibody molecules binding to their surfaces and cross-linking them.



Fig. C.3.12. Bleeding the rabbit through the marginal ear vein.

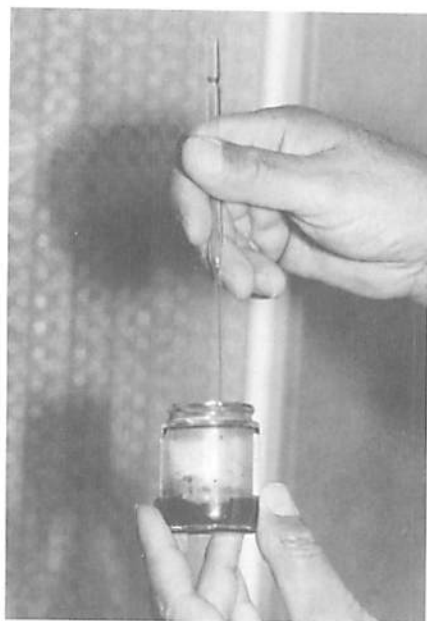


Fig. C.3.13. Carefully loosen the blood clot from walls of vial by means of a Pasteur pipette or glass rod. Avoid mixing serum (clear fluid) with the blood clot.



For greatest accuracy, agglutinations are usually performed in small tubes by mixing given amounts of rhizobia suspensions with a dilution series of an antiserum. The highest dilution that gives a clear agglutination reaction is known as the 'titer' of the reaction. Comparison of strains with a particular antiserum is made by comparing their respective titers. Strains which react similarly to a given dilution of an antiserum are considered serologically identical.

Rhizobia to be used in agglutination tests are grown on YEM agar slants (Figure C.3.14), and suspended in approximately 5 ml 0.85% saline (w/v NaCl). We recommend heating this suspension in a boiling water bath for one hour to destroy flagellar proteins which may interfere with the somatic protein reactions (Figure C.3.15). After heating, centrifuge cells for 10 minutes at 4°C, discard supernatant, and suspend cells in 2 ml saline, making sure that no clumping of cells is taking place before the agglutination. If cells are clumped at this stage, vortex mix the suspension and centrifuge at 1000 x g for five minutes; use the turbid supernatant to perform the agglutination test.

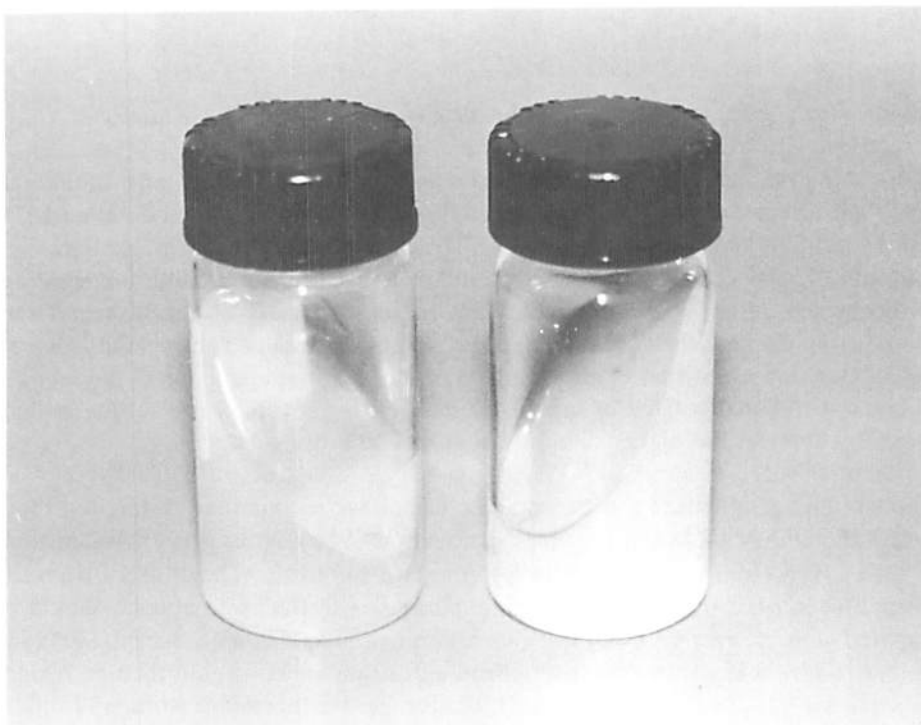


Fig. C.3.14. Rhizobial slant culture for use in agglutination tests.

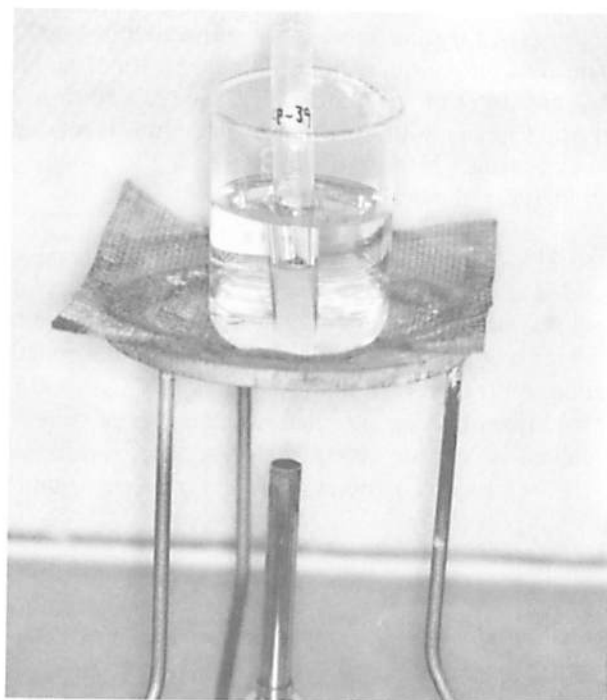


Fig. C.3.15. Heating cell suspension in boiling water bath for one hour.

The antiserum is diluted in a two-fold series with saline. Mix a constant volume of diluted antiserum with an equal volume of rhizobia cell suspension (0.5 ml each), mix tubes by swirling and place undisturbed in a rack which is kept in a water bath (Figure C.3.16). If kept at 52°C, the agglutination reaction can be determined after four hours; if kept at 37°C, the reaction may be read after 10–12 hours. Agglutination is most easily distinguished by holding tubes up against a dark background (Figure C.3.17). It is important that in all agglutination tests, an antigen control (antigen combined with equal volume of saline) is used as a negative control, so that evaluation of agglutinations at the higher dilution levels will be more clear.

Although the agglutination reaction may be useful, we recommend testing the titer of antisera by indirect ELISA if possible (see Section C.4.a); antiserum should have not less than 1/1280 titer if it is to be used to prepare conjugate. The antisera from the separate bleedings and from all rabbits immunized with the same antigen should be compared with indirect ELISA or agglutination test and, if equally acceptable, added together to give a larger volume of uniform antiserum which should then be divided into small samples (no more than 5 ml) for storage. Furthermore, storage of different lots of the same antiserum in different refrigerators may be wise, as failure of one refrigerator will not result in loss of an entire batch of antiserum (heat will cause rapid breakdown of proteins and therefore reduce the antisera titer).

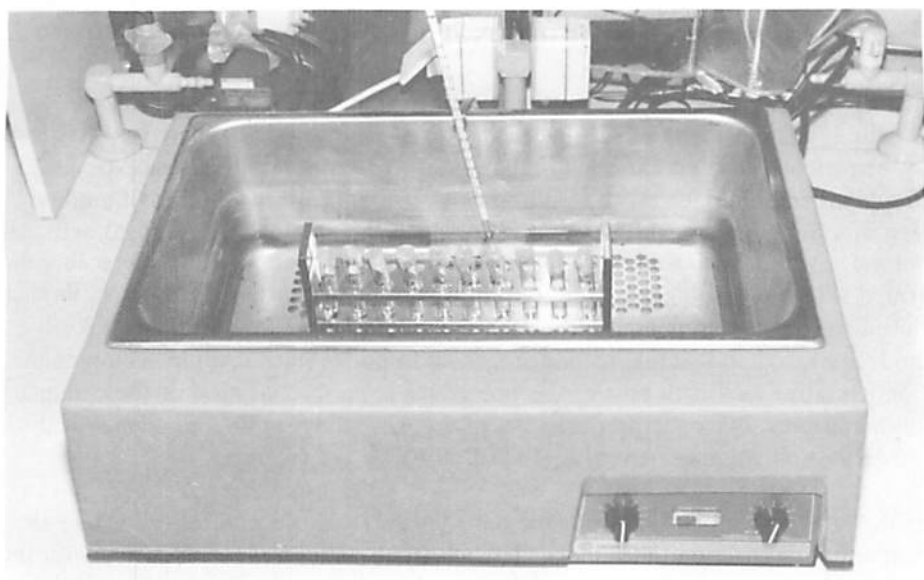


Fig. C.3.16. Rack containing mixed antiserum and cell suspension in water bath to maintain constant temperature.

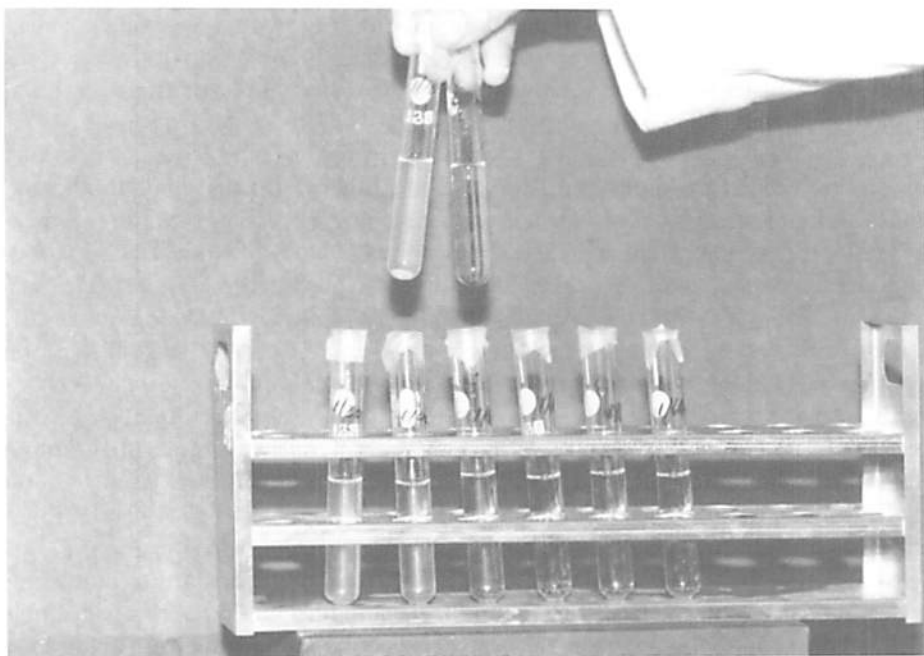


Fig. C.3.17. Holding tubes against a dark background to detect agglutination.

## C.4. Enzyme-Linked Immunosorbent Assay (ELISA)

By joining together an enzyme with the antisera produced, in a process called **conjugation**, the sensitivity of serological reactions can be greatly increased. With the use of enzyme-conjugated antibody preparations, it is possible to detect extremely small quantities of antigens with small quantities of antiserum. Binding of antibody to antigen in a **homologous** strain (strain from which the antigen is produced) activates the enzymes which form a highly colored product; no binding and therefore no color is formed with a **heterologous** strain (strain which is not reactive with the antibodies produced from the homologous strain). In addition to their sensitivity, ELISA methods are rapid and simple to perform, and do not require complex equipment. The only relatively difficult part of the procedure is the preparation of the conjugated antibody reagents, but once prepared, these are extremely stable and economical to use as only small volumes at very low concentrations are required.

In ELISA, the antigen is first adsorbed onto the surface of a plastic well where the antigen-antibody reaction takes place. The adsorbed antigen is then reacted with the enzyme linked anti-antibody. Where the antibody complex 'fits' or reacts with the antigen, an enzymatic reaction producing color takes place. The major advantages of this method are the small amounts of antisera required and the very small quantities of antigen necessary (meaning that the smallest of nodules can be tested), and the ability to perform many determinations in one plastic tray (with many wells). Thus many nodules can be tested for the strain(s) occupying the nodules with relative ease. A disadvantage of ELISA is the relatively high cost of special plastic trays (usable only once) and chemicals.

There are both direct and indirect ELISA methods. In the direct technique, the anti-rhizobia antibodies are labelled with enzyme, making it necessary to have enzyme-linked antibodies (conjugated) for each strain to be studied. This is not necessary with the indirect technique, where a second antiserum prepared in an animal species other than that used to make the anti-rhizobia antibodies is linked with the enzyme and then used to detect the attachment to anti-rhizobia antibodies produced in the rabbit. In the indirect method, it is necessary to have only one enzyme-linked type of antibody (the anti-rabbit antibody). This second antiserum used in the indirect ELISA technique can be purchased (with or without the enzyme already attached) from serological or scientific supply houses. If the second antiserum is purchased unlabelled, it can be made enzyme-linked in the same way as anti-rhizobia antibodies (Section C.4.b). The process of linking enzyme with antibody, or conjugation, is complex and requires considerable time and effort. If many strains are to be identified or 'grouped', it is recommended that the indirect method be used, as this will require much less effort than labelling each anti-rhizobia antibody individually. The direct method is, however, generally more sensitive, and should be used in ecological investigations of two or three strains.

#### **C.4.a. Indirect ELISA for Antisera Titer Determination**

The indirect ELISA involves the use of two antisera: (1) strain-specific rabbit antisera; and (2) sheep or goat anti-rabbit globulin. Anti-rabbit globulins are conjugated to alkaline phosphatase or peroxidase; these are available from commercial sources (e.g., Sigma A-8025, a preconjugated goat anti-rabbit globulin, is used at ICARDA).

In the following procedure, the indirect method of ELISA is used to determine the titer of an antisera produced in a rabbit. For this procedure it is not necessary to fractionate the antisera before testing.

##### **Requirements:**

- disposable plastic trays with 96 wells each, of special type for adsorption of proteins in ELISA tests (e.g., Fisher Catalogue no. 1424561, Immulon II plates)
- spectrophotometer with cuvettes
- goat anti-rabbit globulin; may be purchased already conjugated with alkaline phosphatase (Sigma A-8025)
- micropipettors capable of delivering 100 and 150  $\mu$ l
- drying oven or incubation chamber capable of 30–35°C
- phosphate-buffered saline
- Tween-20 (Sigma P-2287)
- p-nitrophenyl phosphate (Sigma 104-40T)
- diethanolamine (Sigma D-8885)
- antisera produced in rabbit against somatic antigen of specific rhizobia strain

##### **Reagents:**

###### **PBS: Phosphate buffered saline**

- |                                                        |         |
|--------------------------------------------------------|---------|
| - NaCl                                                 | 8.0 g   |
| - $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ | 2.7 g   |
| - $\text{NaH}_2\text{PO}_4$                            | 0.4 g   |
| - Distilled water                                      | 1000 ml |
| - pH                                                   | 7.2–7.4 |

**PBS Tween-20:** Add 0.5 ml Tween-20 to 1 l PBS, adjust pH to 7.4

**Diethanolamine buffer:** Add 97 ml diethanolamine to 800 ml distilled water, adjust pH = 9.8

**ELISA buffer substrate:** Dissolve 1 mg p-nitrophenyl phosphate (phosphate substrate) in 1 ml diethanolamine buffer

## Procedure:

1. A fresh culture on YEM agar slant is suspended in distilled water. If the titer is to be tested against differing strains for cross-reaction, suspensions of different strains (**homologous** = the strain from which the antigen was prepared, and **heterologous** or a nonreactive strain) must be adjusted to the same turbidity (using a spectrophotometer set at A600 nm with a distilled water blank, dilute with sterile saline until reading = 0.20) and then diluted 50-fold with sterile saline (0.85% NaCl). In all ELISA testing a homologous and a heterologous strain must be included as positive and negative controls, respectively. A simple map of an example ELISA plate is shown in Figure C.4.1.
2. For positive control, add 100  $\mu$ l of the homologous suspension to each of 4 wells; use four wells for each dilution of antisera.
3. For negative control, add 100  $\mu$ l of the heterologous suspension to another set of wells; use four wells for each dilution of antisera.
4. For blank controls (3 sets) add to four wells each: a) 100  $\mu$ l distilled water (water blank); b) 100  $\mu$ l of homologous suspension; and c) 100  $\mu$ l heterologous suspension. These wells will not receive rabbit antiserum.
5. Allow the plates to dry in the oven overnight at 35°C.
6. Wash the plates three times with 150  $\mu$ l PBS Tween-20 and shake off to remove excess moisture.

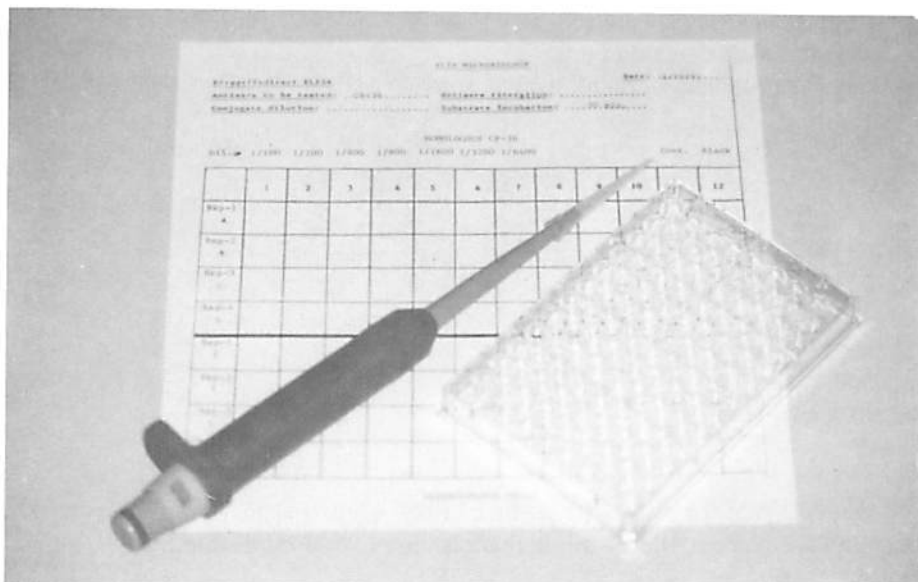


Fig. C.4.1. ELISA plate map indicating placement of controls and test wells.

7. Dilute antisera with PBS Tween-20 (Figure C.4.2).
8. Add to four duplicate wells (except blank controls) 100  $\mu$ l of serial dilutions of the whole antisera (1/100, 1/200, etc., Figure C.4.3).
9. Cover the plates with tape (Figure C.4.4) and incubate in a chamber at 30°C for four to six hours.

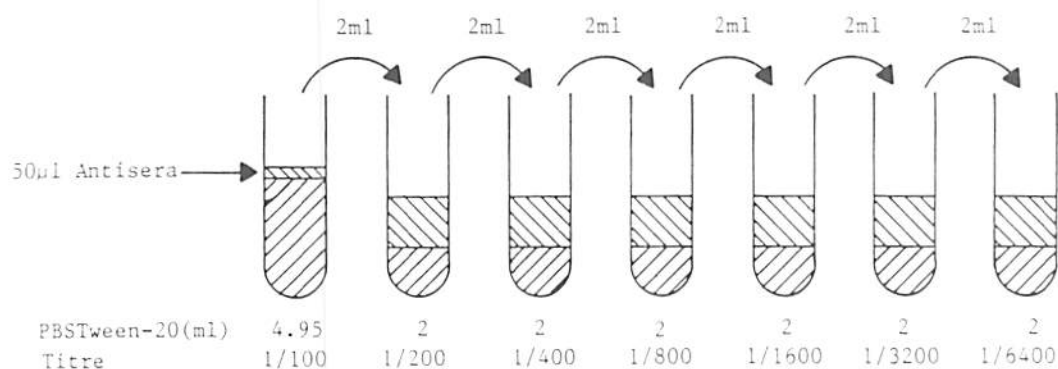


Fig. C.4.2. Dilution series for antisera using PBS Tween-20.

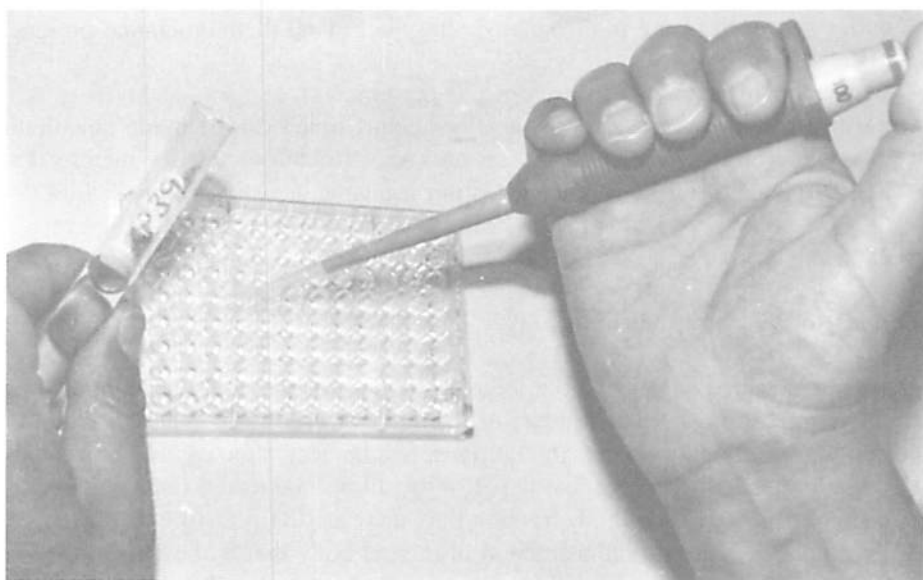


Fig. C.4.3. To each of four wells add 100  $\mu$ l of each dilution of antisera.

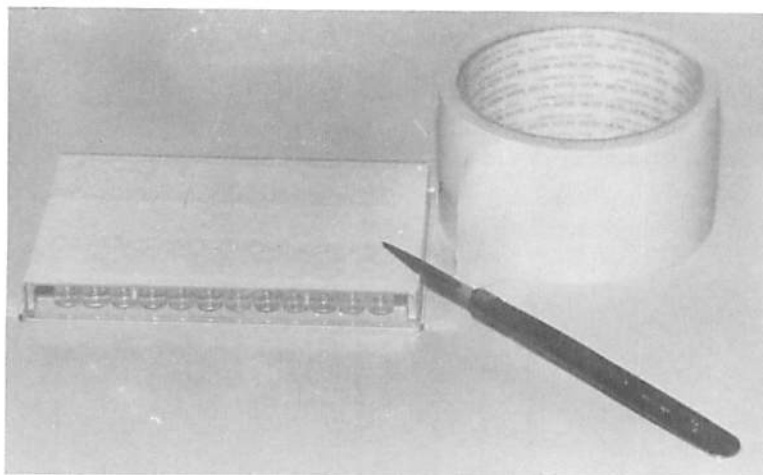


Fig. C.4.4. ELISA plate covered with tape for incubation at 30°C for 4-6 h.

10. Wash wells three times with 150  $\mu$ l PBS Tween-20.
11. Add to each well (except water blank) 100  $\mu$ l of goat anti-rabbit globulin conjugated with alkaline phosphatase and diluted 1/1000 in PBST-20. Cover the plates with tape and reincubate overnight in a chamber at 30°C.
12. Wash twice with 150  $\mu$ l PBS Tween-20 and once with PBS.
13. Shake off the plates to remove the excess liquid, then add 100  $\mu$ l ELISA buffer substrate (1 mg p-nitrophenyl phosphate/1 ml diethanolamine buffer, pH = 9.8).
14. Wait until sufficient color develops (30-60 minutes).
15. Positive results are indicated by a yellow color, which can be made quantitative by reading optical density at 405 nm on a spectrophotometer designed to read ELISA plates. If an ELISA reader is **not** available, a visible color scale of 0-4 (0 = no color, 4 = strong yellow) can be used with some success (Figure C.4.5).

#### Calculations to Determine Titer:

The titer as determined here using the indirect method to evaluate unfractionated antisera is an indication of the sensitivity of the antisera produced. Where a high titer is indicated here, the final concentrated antisera will be very effective and only small amounts will be needed in determinations. If a low titer is indicated (e.g., <1/300), concentration of the active antibody fraction may increase the titer to where the antisera is usable. It is the globulin fraction of the antibody that is most specific against the antigen, and so it will still be necessary to fractionate the globulins in the antirhizobial antisera produced in rabbits against specific strains. This process is described in the following section.



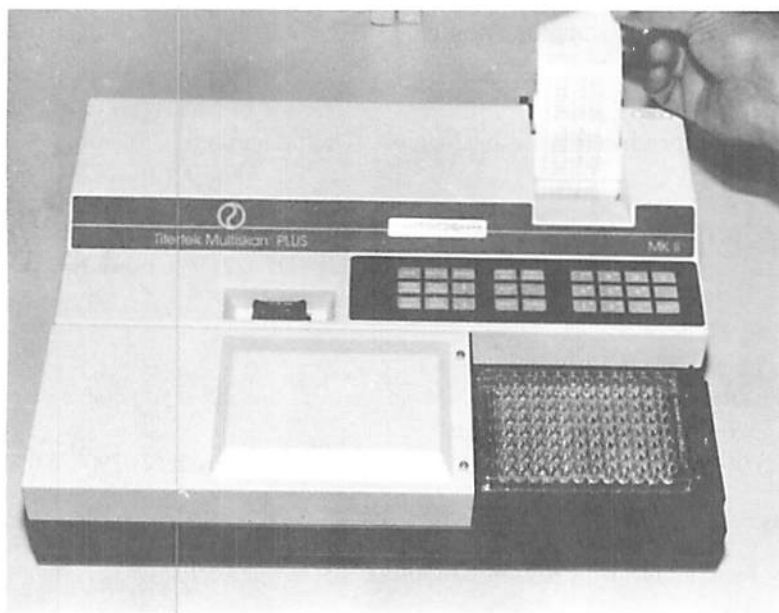


Fig. C.4.5. Reading ELISA results in an automated spectrophotometer at wavelength 405 nm.

Use of the indirect method will give a quick estimation of titer when determining whether to harvest blood from the rabbit, or it can be used for general investigation of strains. Purchase of the pre-conjugated goat anti-rabbit globulin will save considerable laboratory effort. In work at ICARDA in ecological studies with chickpea and lentil rhizobia, we have found the indirect method useful in classification and identification exercises, but we use the direct method in competition and survival studies as we have found it to give more sensitive and reliable results.

#### C.4.b. Development of ELISA Conjugate

Developing the ELISA conjugate can apply either to conjugation of the goat anti-rabbit globulin if purchased unconjugated (indirect method) or to the antisera (direct method) with alkaline phosphatase. We suggest that, in order to save considerable effort when using the indirect method, this anti-rabbit globulin be purchased pre-conjugated with the proper alkaline phosphatase. In the case where the antiserum produced in the rabbit is labelled directly, it is necessary to first isolate the globulin fraction from the antisera before the procedure of labelling (conjugation). For the ELISA technique to work, a highly purified globulin preparation is required. Two fractionation steps are necessary: (1) sodium sulfate precipitation, followed by (2) purification through a chromatography column containing DEAE cellulose.

### C.4.b.1. Purification of Globulin Fraction

#### Requirements:

- antiscrum produced in rabbits against somatic antigen of rhizobia
- solid  $\text{Na}_2\text{SO}_4$
- 1 N saline (0.85%)
- PBS, pH 7.4
- diethylaminoethyl (DEAE) cellulose beads (DE-22, Whatman 6892)
- 5-ml syringe (disposable)
- 0.5 M HCl
- 0.5 M NaOH
- magnetic stirrer
- spectrophotometer and cuvettes
- Spectrapor #2 dialysis tubing and clamps (ColeParmer N-02900-20)

#### Procedures:

##### 1. Primary Fractionation of Serum Globulins

1. To a measured volume of serum (for example, 10 ml) on a magnetic stirrer at room temperature, slowly add 14% solid  $\text{Na}_2\text{SO}_4$  (1.4 g in this example). If a magnetic stirrer is not available, constant manual stirring is necessary during addition of sodium sulfate and for approximately the five minutes following addition (Figure C.4.6).
2. Centrifuge at  $3000 \times g$  for 10 minutes and discard the supernatant.
3. Resuspend the precipitate in enough 1 N saline to give half the original volume of the serum (5 ml).
4. Slowly add 12% solid  $\text{Na}_2\text{SO}_4$  (0.6 g in this example). Centrifuge again and resuspend in saline (5 ml).
5. Repeat this precipitation and washing (steps 4 and 5) one more time.
6. Resuspend the final precipitate in saline (5 ml) and transfer to dialysis bag (Figure C.4.7). To remove all the  $\text{Na}_2\text{SO}_4$ , dialyze the precipitate at  $4^\circ\text{C}$  (in refrigerator or ice-bath) against PBS, pH 7.4. The large protein molecules cannot pass through the dialysis bag walls, while the smaller sodium sulfate molecules will move with the concentration gradient into the PBS.
7. After several (four or five) changes of PBS, determine the protein concentration of the solution inside the dialysis tubing by measuring OD at 280 and 260 nm using a crystal cuvette and spectrophotometer, as follows:

| Tube | Saline (ml) | Globulin (ml) | Dilution |
|------|-------------|---------------|----------|
| 1    | 3           | 0             | -        |
| 2    | 2.7         | 0.3           | 1:10     |
| 3    | 2.85        | 0.15          | 1:20     |



Fig. C.4.6. Manual stirring for 5 minutes during addition of sodium sulfate.



Fig. C.4.7. Resuspended precipitate in dialysis bag.

8. Adjust spectrophotometer to zero using tube 1 at 280 nm. Read OD of tubes 2 and 3. Readjust spectrophotometer to zero using tube 1 at 260 nm wave length then read OD of tubes 2 and 3. Calculate mg protein/ml for each tube using the following formula:  
$$1.4 \times (\text{OD at 280 nm}) - 0.7 \times (\text{OD at 260 nm}) = \text{mg protein/ml.}$$
9. Remember that the protein has been diluted 1:10 in tube 2 and 1:20 in tube 3; therefore multiply by appropriate dilution factor and calculate the protein concentration/ml. Take the average of tubes 2 and 3.
10. Adjust the protein concentration to 10 mg protein/ml by diluting the globulin with saline.
11. To obtain a more purified globulin solution (with higher titer), it is necessary to pass the globulin solution through a cellulose column. We have obtained good results with DEAE type DE-22, which allows protein molecules of a certain size to pass through.

## II. Preparation of DE-22 Cellulose Column

1. Place 2 g DE-22 cellulose in 30 ml 0.5 M HCl.
2. Stir at low speed for 30 minutes.
3. Pour the contents through a funnel containing Whatman No. 4 filter paper.
4. Wash with distilled water and keep on washing until pH of wash solution reaches 4 (Figure C.4.8).

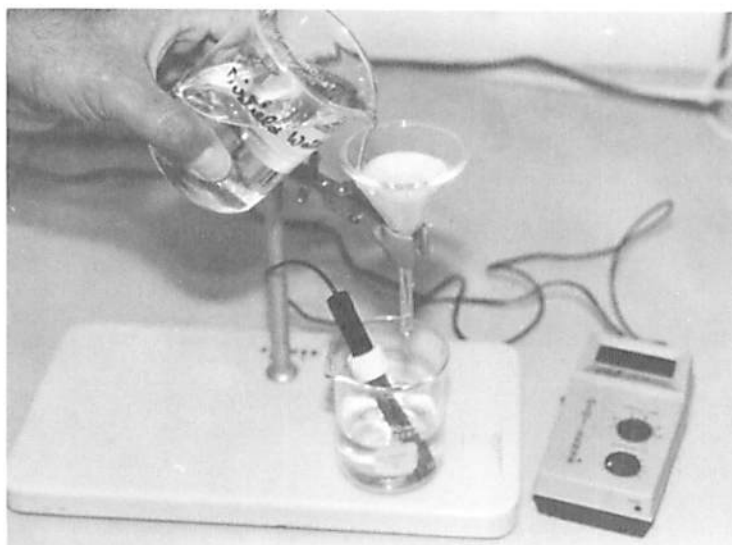


Fig. C.4.8. Washing acidified cellulose beads with distilled water until pH reaches 4.0.

5. Transfer cellulose beads to a beaker, pour off water
6. Add to the cellulose 30 ml NaOH 0.5 M
7. Leave standing for 30 minutes; decant the supernatant
8. Add another 30 ml of NaOH 0.5M, and leave standing for another 30 minutes.
9. Adjust the pH of the solution to 7 with 0.5 M HCl, then decant liquid.
10. Place beads in 125 ml PBS, cover beaker with parafilm or plastic wrap, and leave in refrigerator until use.
11. Pour the bead-PBS mixture into the column (Figure C.4.9).

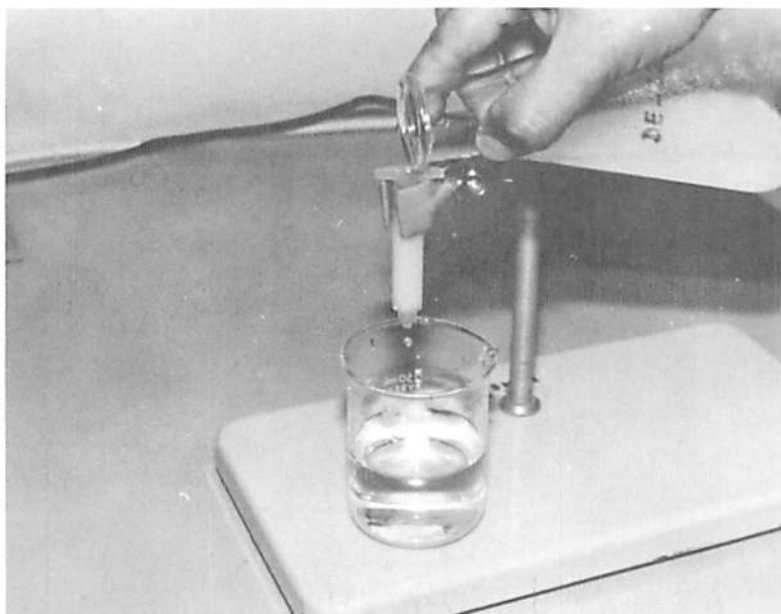


Fig. C.4.9. Pouring the cellulose bead-PBS mixture into a plastic column.

### III. Collection of Purified Globulin Fraction from Column

The cellulose within the column has to be at least 5 cm long (Figure C.4.10) and should be pre-equilibrated within the column by washing three times with PBS. After filling column with the prepared cellulose beads, outflow of solution should be adjusted with the outflow valve of the column to 10–12 drops/minute. Once the level of the final PBS wash reaches to 0.5 cm above the cellulose surface in the column, slowly add 1 ml of the dialyzed antiserum from the dialysis bag (Figure C.4.11) and at the same time start collecting separate fractions of 2 ml from the outflow (Figure C.4.12). When the antiserum has entered the cellulose (Figure C.4.13), add PBS

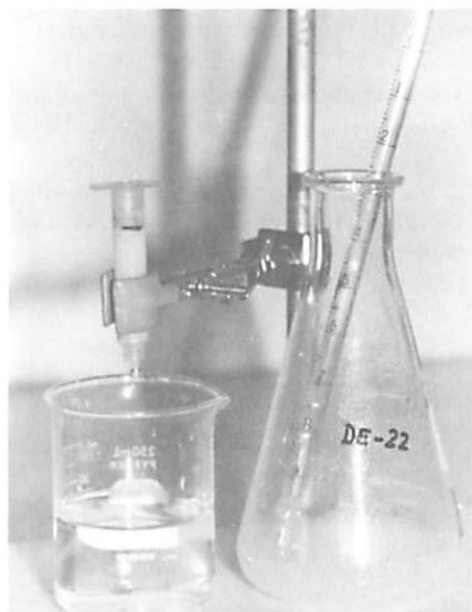


Fig. C.4.10. Equilibration of cellulose in the column with PBS.



Fig. C.4.11. Adding the dialyzed antisera to the PBS at the surface of the cellulose column.

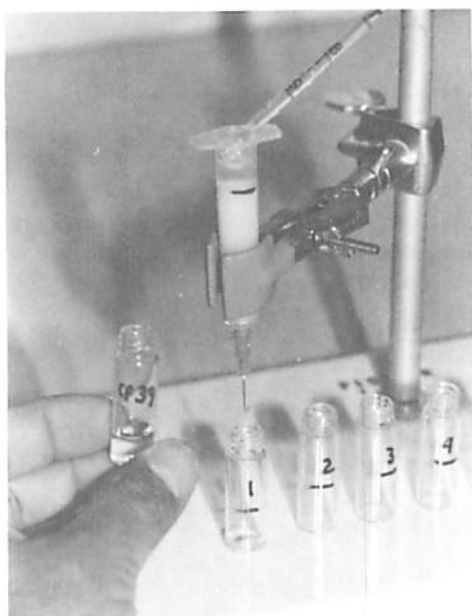


Fig. C.4.12. Collection of the first fraction from the outflow of the column as the antiserum is added.

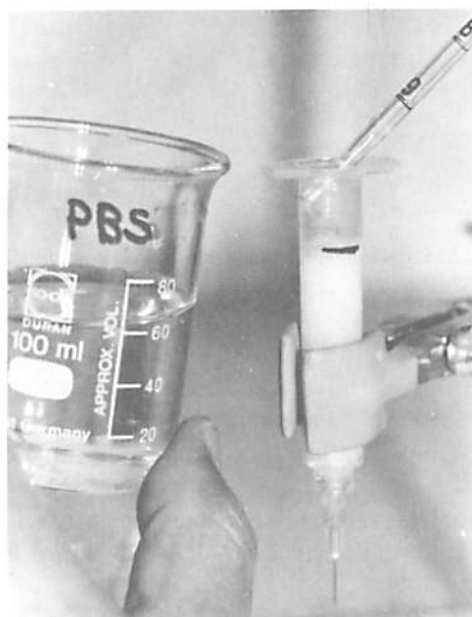


Fig. C.4.13. Adding PBS as antiserum enters the cellulose column.

carefully to fill the entire column. Collect fractions in tubes (Figure C.4.14), each tube containing 2 ml. Read OD of each fraction in a cuvette at 280 nm on a spectrophotometer blanked with PBS. Readings should be low in the first and/or second tubes, but should increase until they reach a peak, then decrease again to a value near 0. Fractions with optical density readings higher than 1.4 will be kept for dilution to a protein concentration of 1 mg/ml, but fractions with readings below 1.4 will have to be reconcentrated. A reading of 1.4 OD represents a protein concentration of 1 mg/ml, which is the desired concentration of the Ig-G (gamma globulin) protein fraction in the final product.

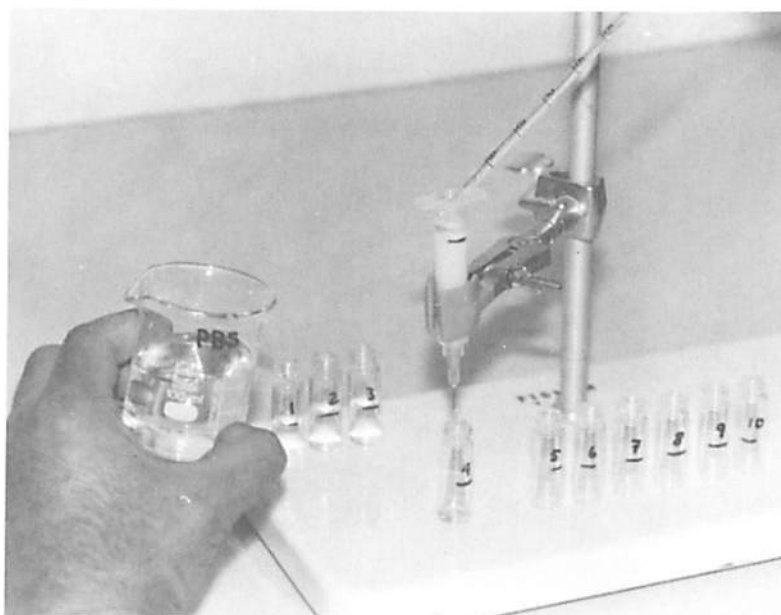


Fig. C.4.14. Collecting fractions in tubes each holding 2 ml.

If desired, you may combine all fractions with an OD reading of  $> 1.4$ , mix well and again read OD. Alternatively, each fraction can be adjusted to the proper protein concentration, and kept separately; the purest Ig-G fraction will have the highest titer and may be especially useful where low titers are a problem. To adjust the reading to 1.4 OD (concentration of 1 mg/ml) in the combined fractions with a reading  $> 1.4$ , it will be necessary to dilute with saline (PBS). Supposing a reading of 1.8 at 280 nm and a volume 4 ml, the following calculations show how much PBS should be added to obtain an OD reading of 1.4:

$$1.8 \times 4 \text{ ml} = 1.4 \times V$$



$$V = \frac{1.8 \times 4}{1.4} = 5.1$$

$$5.1 - 4 = 1.1 \text{ ml}$$

Add 1 ml PBS to the 4-ml tube, then read again at 280 nm; dilute with more PBS, drop by drop with a pasture pipette, mixing between additions, until you have a reading of 1.4.

To reconcentrate the fractions which had readings below 1.4, mix them together, measure the volume, then add 12% solid  $\text{Na}_2\text{SO}_4$  slowly on a magnetic stirrer (Figure C.4.15). Leave the solution for two hours at room temperature, then centrifuge for five minutes at  $10^4 \times g$ . Discard the supernatant and dissolve the precipitate in 1 ml PBS. Transfer to dialysis sac and dialyze five times in PBS at  $4^\circ\text{C}$  as before. Read optical density of final product in dialysis bag directly at 280 nm; there is no need to pass through the chromatography column again. If reading is above 1.4, dilute as above; if below 1.4, discard. Store the purified gamma globulin (Ig-G) of 1 mg/ml protein concentration in 2-ml amounts in the deep freeze ( $-20^\circ\text{C}$ ).



Fig. C.4.15. Reconcentration of fractions by slow mixing with sodium sulfate on a magnetic stirrer.

### C.4.b.2. Preparation of Enzyme-Labelled Antibody (Conjugation)

#### Requirements:

- purified globulin (Ig-G at 1 mg protein/ml from above steps)
- alkaline phosphatase suspension (Sigma P-5521)
- 25% glutaraldehyde (Sigma G-5882)
- Tris buffer, 0.05 M, pH 8 (Sigma T-1503)
- 3.2 M ammonium sulfate
- dialysis tubing (Spectrapor #2)
- ovalbumin (Sigma A-5378)
- sodium azide ( $\text{NaN}_3$ )
- centrifuge

#### Procedure:

1. Centrifuge 5000 units (5 mg) of alkaline phosphatase suspension plus rinsing from the bottle with 0.5 ml 3.2 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7, at 6000 x g for 20 minutes. Discard the supernatant.
2. Add 2 ml of purified globulin (with adjusted globulin concentration to 1 mg/ml) to the enzyme pellet.
3. Mix thoroughly at room temperature with vortex mixer, then dialyze at 4°C (in ice bath) with several changes of PBS (Figure C.4.16).

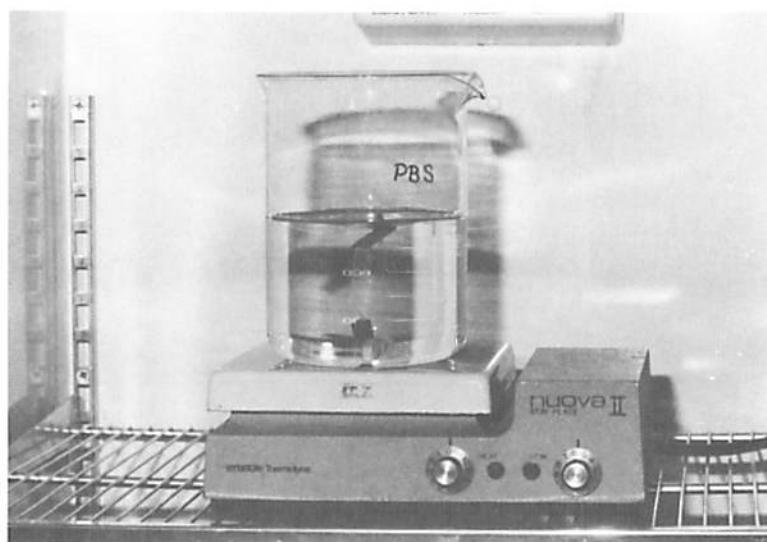


Fig. C.4.16. Dialyzing the enzyme-globulin mixture in a refrigerator at 4-6°C.

4. Add 25% glutaraldehyde to the enzyme-antibody mixture in the dialysis bag (Figure C.4.17) to give a final concentration of 0.2% glutaraldehyde (approximately 10  $\mu$ l).
5. Allow conjugation to proceed for one to two hours at room temperature, then dialyze at 4°C against several (four or five) changes of PBS to remove the glutaraldehyde.
6. Dialyze the conjugate (in the same dialysis bag) against several changes of Tris buffer, 0.05 M, pH 8.
7. Remove as much of the conjugate as possible from dialysis bag (Figure C.4.18) and add 1 ml of Tris buffer containing 1% ovalbumin and 0.02% sodium azide to the remaining contents of the dialysis bag, mix well in bag. Add this 'rinse' to the conjugate mixture (this will collect any conjugate mixture which had adhered to walls of the bag).
8. Add enough Tris buffer containing 1% ovalbumin and 0.02%  $\text{NaN}_3$  to achieve a final conjugate mixture volume of 4.0 ml. Store the concentrated stock conjugate in the refrigerator at 4°C (Figure C.4.19).



Fig. C.4.17. Adding 25% glutaraldehyde to the enzyme-antibody mixture in the dialysis bag.

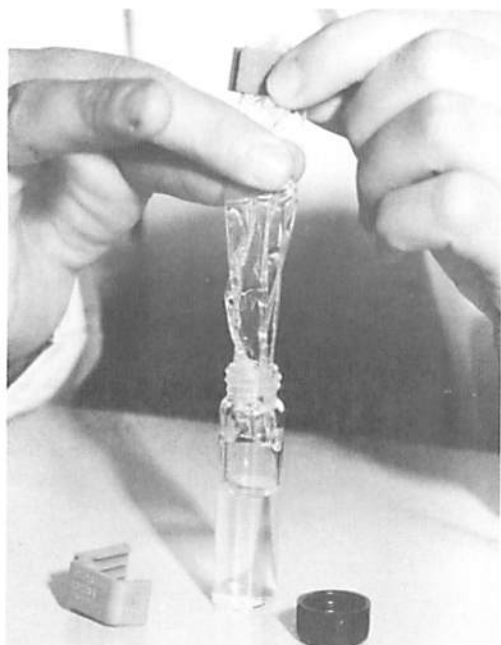


Fig. C.4.18. Removal of the conjugate from dialysis bag.

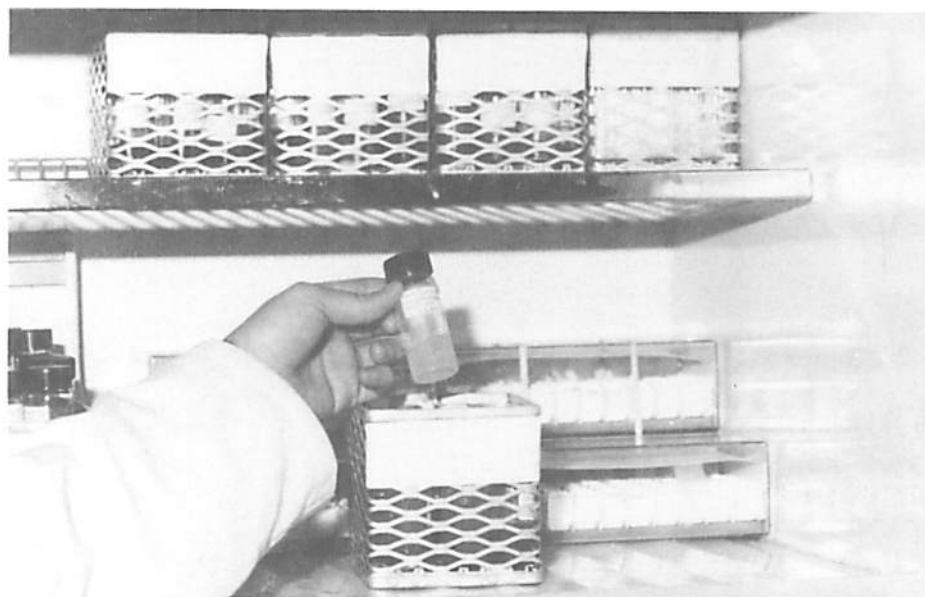


Fig. C.4.19. Concentrated stock conjugate is stored in the refrigerator at  $4-6^{\circ}\text{C}$ .

### C.4.c. Titer Estimation Using Direct ELISA

#### Requirements:

- fresh slants of rhizobia strains, homologous and heterologous
- enzyme-conjugated antiserum
- 0.85% (1 N) saline
- PBS
- PBS Tween-20
- pipettors capable of delivering 50, 100, and 150  $\mu$ l
- plastic ELISA trays, 96 wells
- ELISA buffer substrate (1 mg p-nitrophenyl phosphate in 1 ml diethanolamine buffer, pH = 8.5)

#### Procedure:

1. Rhizobia from fresh slants of homologous and heterologous strains are suspended in a few ml of 0.85% saline in small tubes.
2. Adjust the turbidity of each with saline to 0.2 optical density at A600 nm.
3. Dispense 100  $\mu$ l of antigen in each well (homologous and heterologous), using four wells per strain per dilution. Likewise, 100  $\mu$ l distilled water is used for four blank wells (Figure C.4.20).

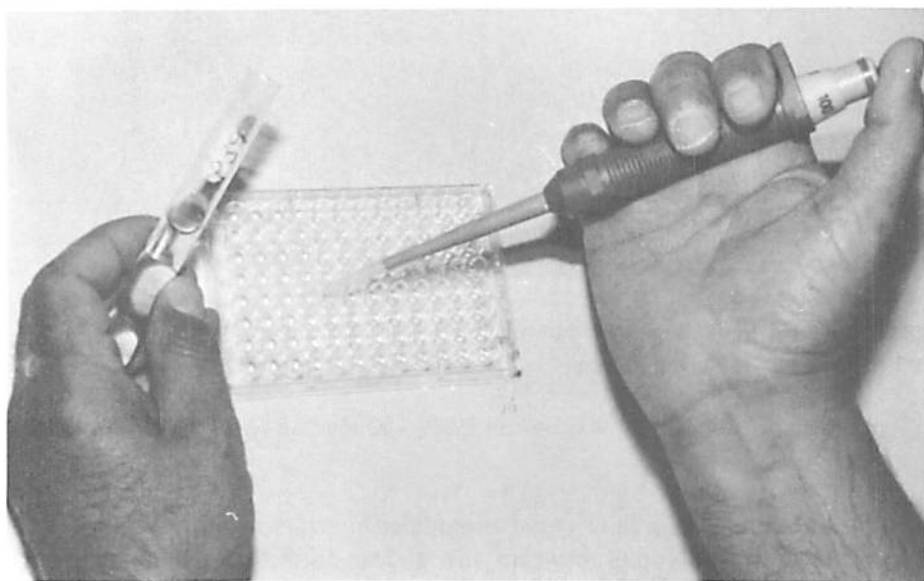


Fig. C.4.20. Distribution of 100  $\mu$ l of antigen in each well of an ELISA plate.

4. Incubate the plates at 37°C overnight.
5. Shake off the plate to remove excess moisture.
6. Wash three times with 150  $\mu$ l PBS Tween and shake off the plates to remove excess moisture (Figure C.4.21).
7. To each well add 100  $\mu$ l of specific dilution of antisera (except blanks), cover the plate with tape or enclose in a moist chamber and incubate for one to two hours at 30°C.
8. Shake off the plates to remove excess moisture and wash three times with 150  $\mu$ l PBS Tween.
9. Add to each well 100  $\mu$ l ELISA buffer substrate and incubate the plates at room temperature for 30 minutes or until sufficient yellow color develops.

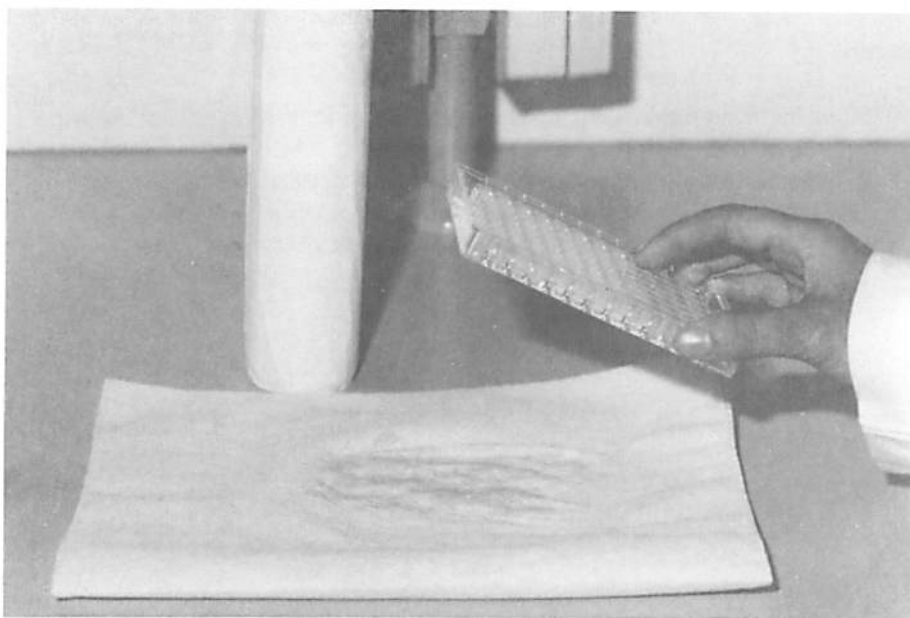


Fig. C.4.21. Removal of excess moisture from ELISA plates by shaking.

10. Read optical density at 405 nm on ELISA reader or score with color scale (see Section C.4.a).

The titer selected for use in later typing of nodules or strain identification will be the highest possible; it is important, however, that a clear distinction is made between homologous and heterologous strains. It is best to draw a graph of titer (X-axis) *versus* OD (Y-axis) and draw lines for homologous and heterologous strains. The

highest titer where standard deviations for the homologous and heterologous strains do not intersect may be used. In practice, however, we like to use a slightly lower titer than maximum to achieve clear results. Remember that the higher the titer used, the less antiserum that will be consumed.

#### C.4.d. Nodule Serotyping Using Direct ELISA

##### Requirements:

- enzyme-conjugated antiserum
- 0.85% saline
- PBS
- PBS Tween-20
- pipettors capable of delivering 50, 100, and 150  $\mu$ l
- plastic ELISA trays, 96 wells
- ELISA buffer substrate (1 mg p-nitrophenyl phosphate in 1 ml diethanolamine buffer pH = 8.5)
- spectrophotometer and cuvettes
- fresh rhizobia homologous and heterologous cultures on YEM slants
- ELISA reader (optional)
- fresh, frozen, or rehydrated dried nodules

##### Procedure:

1. Wash the nodule(s) well to remove all soil particles (Figure C.4.22).
2. Place each individual nodule in a separate test tube (Figure C.4.23)

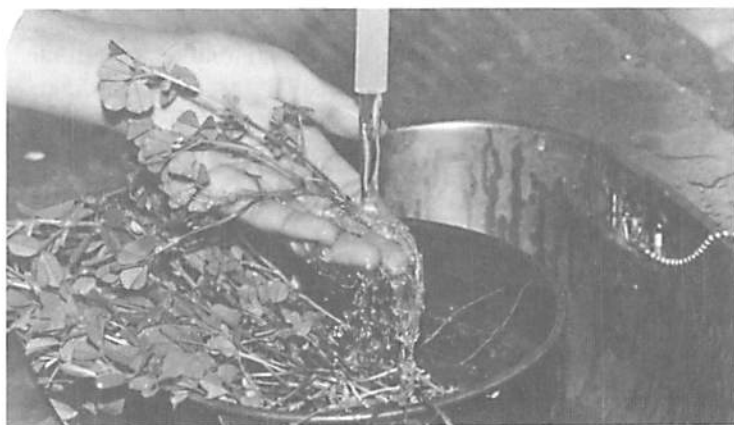


Fig. C.4.22. Removal of soil particles from nodules by washing with tap water over screen.

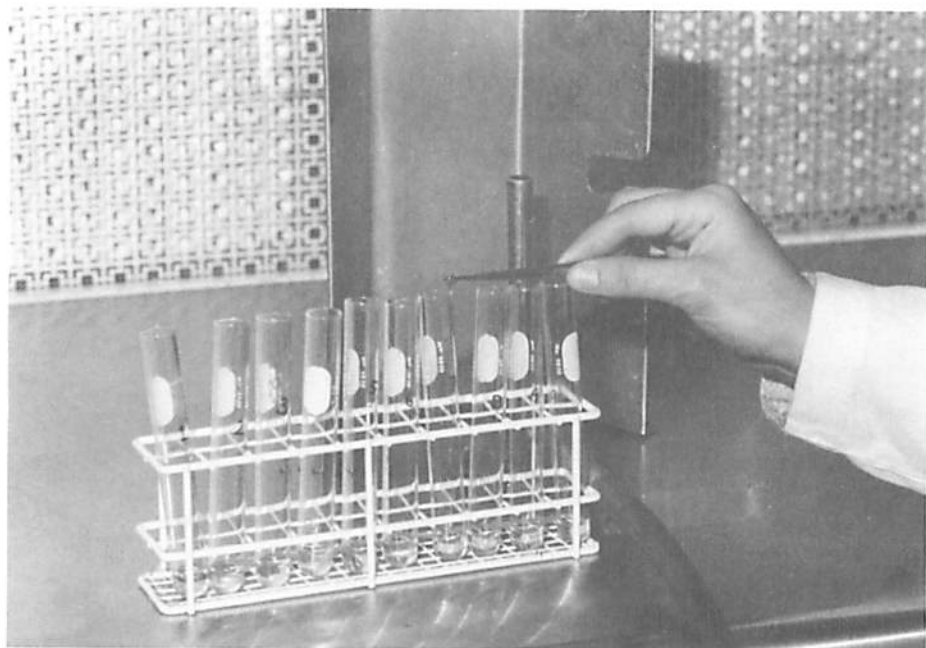
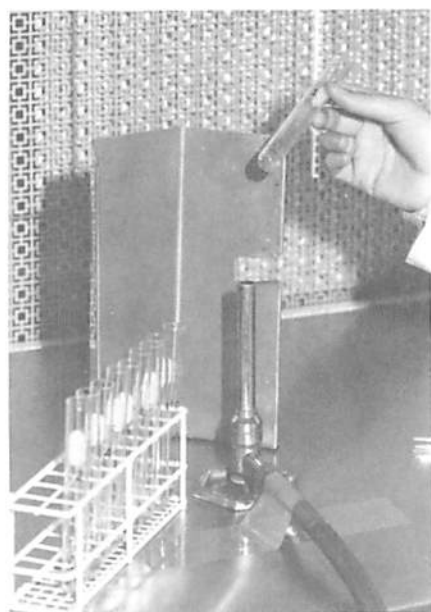


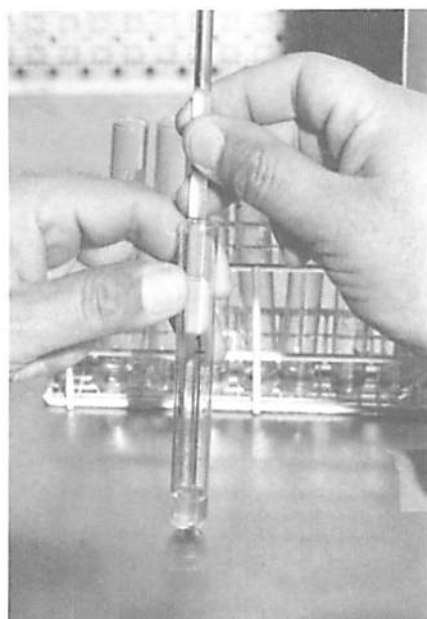
Fig. C.4.23. Individual nodules to be assayed are placed in separate test tubes.  
Care must be taken that nodule mass is approximately equal in all tubes.

3. Add 1 ml distilled water to each tube.
4. Boil the nodule briefly over Bunsen burner flame (Figure C.4.24), then crush it using sterile glass rod (Figure C.4.25).
5. Transfer the contents of the tube to spectrophotometer cuvette and adjust optical density to 0.4 at A600 nm with distilled water. Then dilute contents of tube further 1:50 with distilled water.
6. Dispense 100  $\mu$ l of each nodule solution in one well (Figure C.4.26); repeat for four replications.
7. Suspend fresh homologous and heterologous YEM slant cultures in distilled water and adjust optical density with PBS to 0.4 at A600 nm, then dilute further 1:50 with distilled water (Figure C.4.27).
8. Dispense 100  $\mu$ l of homologous and heterologous strain solutions in each of two wells of plastic trays as positive and negative controls, respectively.
9. Dispense 100  $\mu$ l distilled water in two wells for blank (Figure C.4.28).

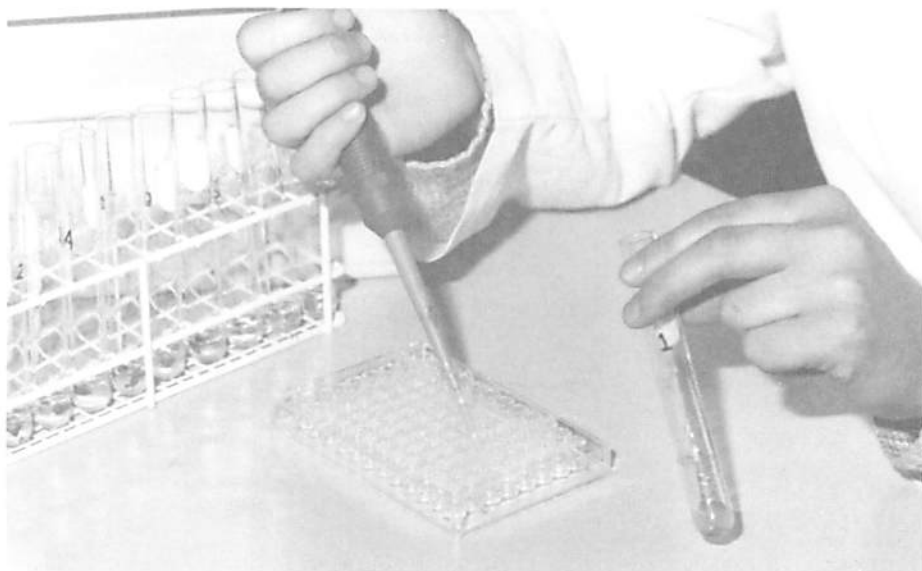




**Fig. C.4.24. Boil nodule in water briefly by holding the tube over the flame of a bunsen burner.**



**Fig. C.4.25. Crush the boiled nodules thoroughly with a glass rod prior to adjusting optical density.**



**Fig. C.4.26.** Add 100  $\mu$ l of the nodule suspension to each of four wells (replications).



**Fig. C.4.27.** Fresh homologous and heterologous YEM slant cultures are diluted in PBS to an optical density of 0.4, then diluted 1:50 in water before adding to wells as controls.

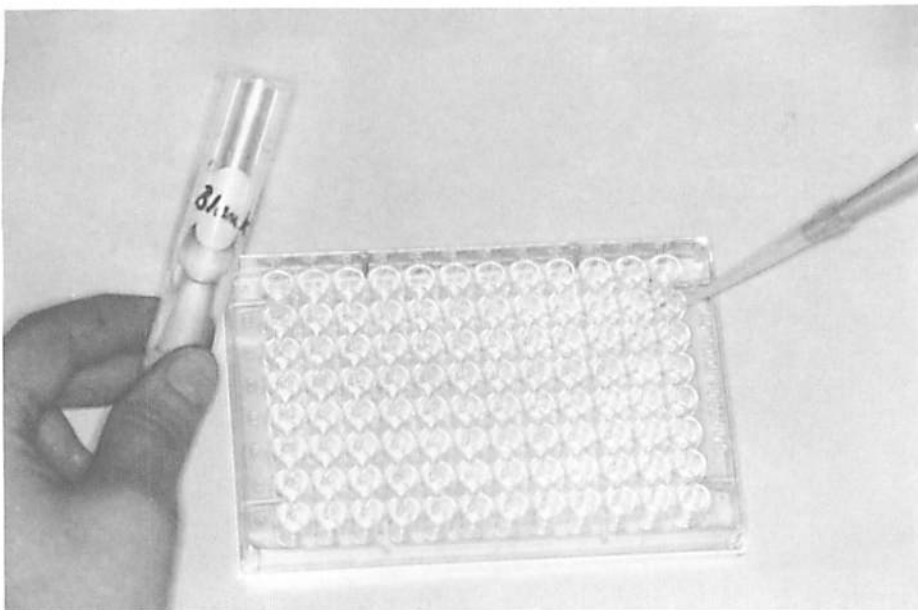


Fig. C.4.28. Each plate will have blank wells (2-water only), and homologous and heterologous controls (4 reps each).

10. Incubate the plates at 37°C overnight.
11. Shake off any remaining liquid, wash each well three times with 150 ml PBS Tween (Figure C.4.29).
12. Add 100  $\mu$ l of specific dilution of antisera (dilution predetermined to give clear reaction at highest dilution) to each well (except blanks); cover the plate with tape or enclose in moist chamber and incubate for two hours at 30°C.
13. Shake off the plates to remove excess moisture.
14. Wash each well three times with 150  $\mu$ l PBS Tween and shake off the plate to remove extra moisture.
15. Add to each well 100  $\mu$ l ELISA buffer substrate.
16. Incubate at room temperature until sufficient color is developed, usually 30–60 minutes (Figure C.4.30). Note that incubation temperature (room temperature) at this stage will affect the speed of the color development reaction; colder = slower.
17. Read OD at 405 nm on ELISA reader, or score by visual color scale.  
A typical example showing reactions is shown in Figure C.4.31.

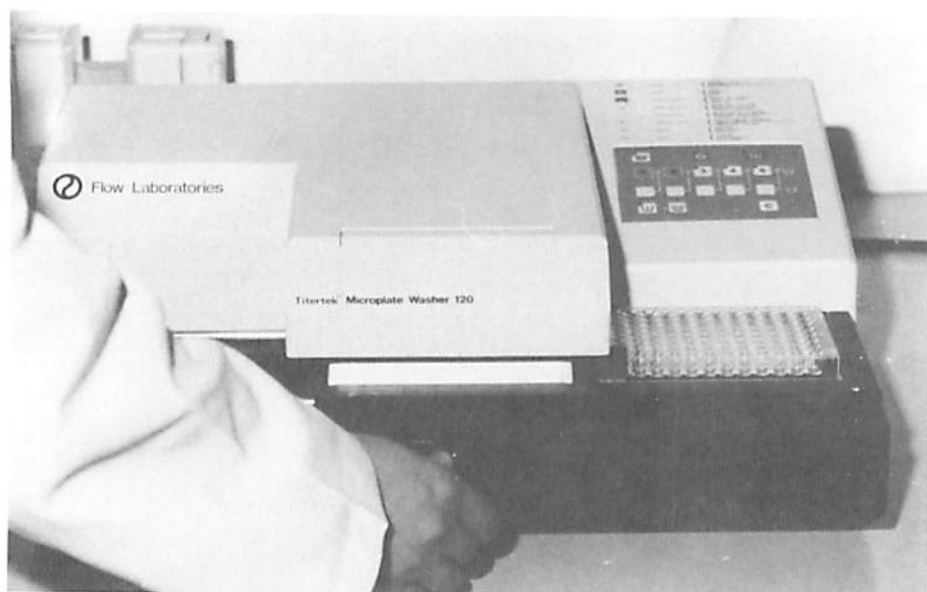


Fig. C.4.29. An automatic washer makes the multiple washing process easier, but is not necessary.

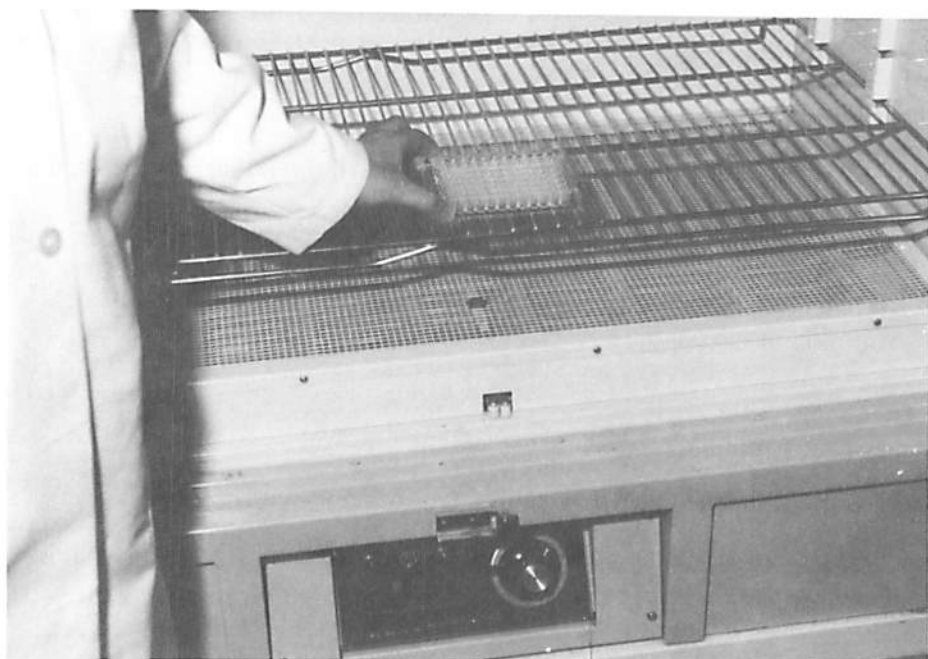


Fig. C.4.30. Incubating ELISA plates in the incubator at a standard temperature (e.g. 25°C) will standardize the reaction rate, giving a known reaction in the homologous control after a given amount of time.

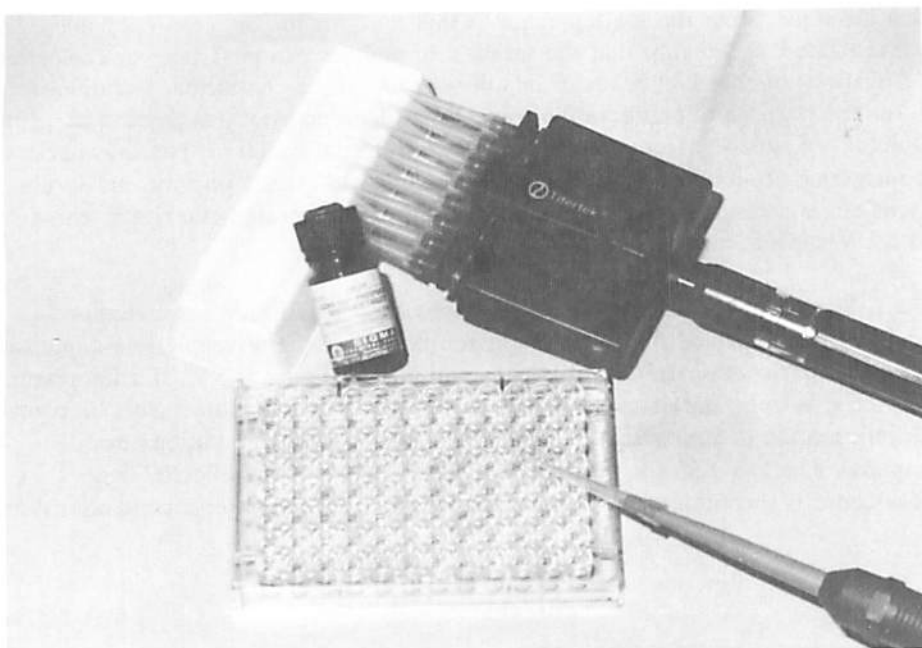


Fig. C.431. ELISA reactions produce varying yellow color which can be judged to some extent by eye.

## C.5. Fluorescent Antibody (FA) Technique

### C.5.a. Principles and Applications

In the fluorescent antibody technique, antibody molecules are covalently bonded with a fluorescent dye so that their presence, in nodules or soils, can be detected by microscopy in ultraviolet light. As with the ELISA techniques, there are direct and indirect FA methods, depending on whether the fluorochrome is attached to the antibodies that react to the antigen (**direct**) or to secondary antibodies that react with the rabbit-produced antibodies (**indirect**). In the direct technique, it is necessary to have fluorescent antibodies for each of the strains to be studied. This requirement is eliminated by using the indirect technique. By labelling the antibodies in a second antiserum produced in goat or sheep and using them to detect attachment of the antirhizobial antibodies it is necessary to have only one type of fluorescent-labelled antibody. We suggest that this second antiserum for use in the indirect technique be purchased with the fluorescent dye already attached. If purchased unlabelled, it can be made fluorescent in the same way as antibodies produced in rabbits (described in Section C.5.b.).

What is unique about the FA technique is that it allows the antigens on single cells to be investigated; this means that the smallest of nodules can be 'typed' or ecological investigations of rhizobial behavior in soil systems can be conducted. Isolation from soil for the purpose of enumeration using FA is detailed briefly in Section B.3.c.; for full details of isolation from soils see papers in reference section. The drawbacks of the technique are its cost and complexity. Practice will greatly improve antiserum production and results; major cost inputs are for a refrigerated centrifuge, chemicals, and a UV-capable microscope.

**C.5.a.1. Microscope.** The fluorescence microscope is a conventional compound microscope equipped with a special light source of suitable wavelength and intensity and the proper combination of objectives and filters (Figure C.5.1). If a fluorescent microscope is to be purchased, it is suggested that various manufacturers be contacted with information of your intended needs so that the various quotations can be compared. The HBO-50 (or 12-V 100-W quartz-halogen) for reflected light fluorescence is recommended. For transmitted-light observations, a condenser must be



Fig. C.5.1. Fluorescence microscope, with fluorescent light source, UV filters, and camera attachment

used; the phase-contrast condenser is suitable for this purpose. Special objectives are needed for use with these condensers (as described in the User's Manual for the microscope). Finally, the microscope must be equipped with the proper filter set for *Rhizobium* work, suitable for FITC work and acridine orange. Most microscope models offer an "FITC filter set" as an option. It is advisable to check with a dealer representative for your needs. Cost for the microscope setup will range from about U.S. \$7,000 to \$20,000, depending on quality and sophistication (see Figure C.5.2 for sample purchase request).

# I C A R D A

International Center for Agricultural Research in Dry Areas

INTEROFFICE MEMORANDUM

9 November, 1989

To : Dr. K. Makkouk

From : Dr. D. Beck

Subject: Purchase of fluorescent-capable microscope

The following items from a Nikon quotation represent what is needed for FA work in microbiology. This is a high quality microscope with all needed attachments, for a very reasonable price. The Nikon price list, from a Damascus distributor, is attached. Also attached is a less complete quotation from Karl Kolb for a Zeiss Epi-fluorescence standard 20 microscope. This model is less adequate for our needs. Purchasing and Supplies Dept. should obtain additional quotations along the lines of the equipment outlined below. Thanks.

| Code no. | Description                      | yen Price |
|----------|----------------------------------|-----------|
| MBA135AD | Optiphot Model X-2               | 258,830   |
| MBC11200 | Stage "3R"                       | 56,800    |
| MBB13130 | Trinocular eyepiece tube F2      | 79,200    |
| MAJ20100 | Eyepiece lenses CFWN D10X        | 22,000    |
| MDL11400 | Achromat/Aplanat condenser       | 45,100    |
| MSJ00101 | N Plan Achromat objective 10X    | 46,200    |
| MSJ00201 | N Plan Achromat objective 20X    | 51,150    |
| MSR00401 | CF N Plan Achromat obj 40X       | 176,000   |
| MSM40400 | CF Fluor objective 40X           | 148,500   |
| MSJ02901 | N Plan Achromat objective 100X   | 165,000   |
| MRL1318F | Phase contrast set(w/box)        | 221,100   |
| MRE140DB | Epi-fluorescent mercury EF-D set | 636,900   |
| MXA21044 | Eng/photo adapter                | 29,700    |
| MPC340AB | Microflex Camera                 | 375,100   |
|          | TOTAL                            | 2,314,580 |
|          | = Approx USD                     | 18,000    |

Fig. C.5.2. Sample purchase order for fluorescence microscope setup.

**Adjusting microscope for bright field.** First follow steps 1–6 to properly adjust microscope for bright (normal) field viewing as follows (Figure C.5.3):

1. Fully raise condenser with top lens swung in.
2. Focus on specimen with 10× objective.
3. While viewing, close down lamp field stop in microscope base.
4. Slightly lower condenser until stop image is in optimum focus.
5. Use the two condenser centering screws to center the image of field stop in the field of view.
6. Open up field stop until the image nearly fills the field of view, fine focus, and then open further until the image is the size of the field of view.

**Advantages:**

Evenly illuminated object field.  
Brilliant image without reflections or glare.  
Minimum heating of specimen.

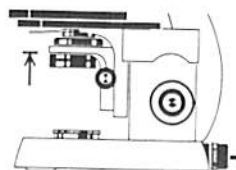
**Light-path requirements:**

Lamp field stop image on specimen.  
Objective pupil filled with light.

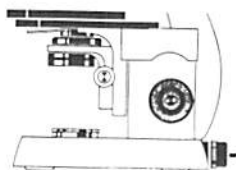
**Equipment required:**

Vertically adjustable centerable condenser.  
Illuminator with lamp condenser and iris diaphragm.

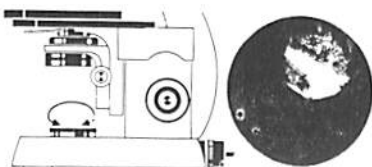
In short, a standard Opton microscope.



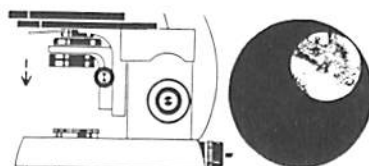
1 Fully rack up condenser with top lens swung in



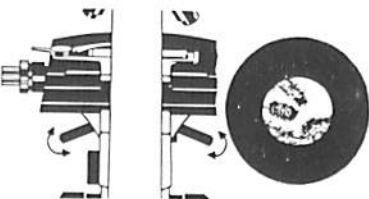
2 Focus on specimen with 6.3x or 10x objective



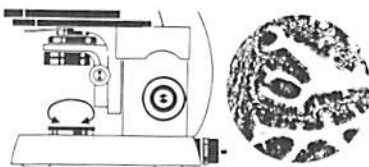
3 While viewing close down lamp field stop in microscope base



4 Slightly lower condenser until stop image is in optimum focus.



5 Use the two condenser centering screws to center image of field stop in field of view.



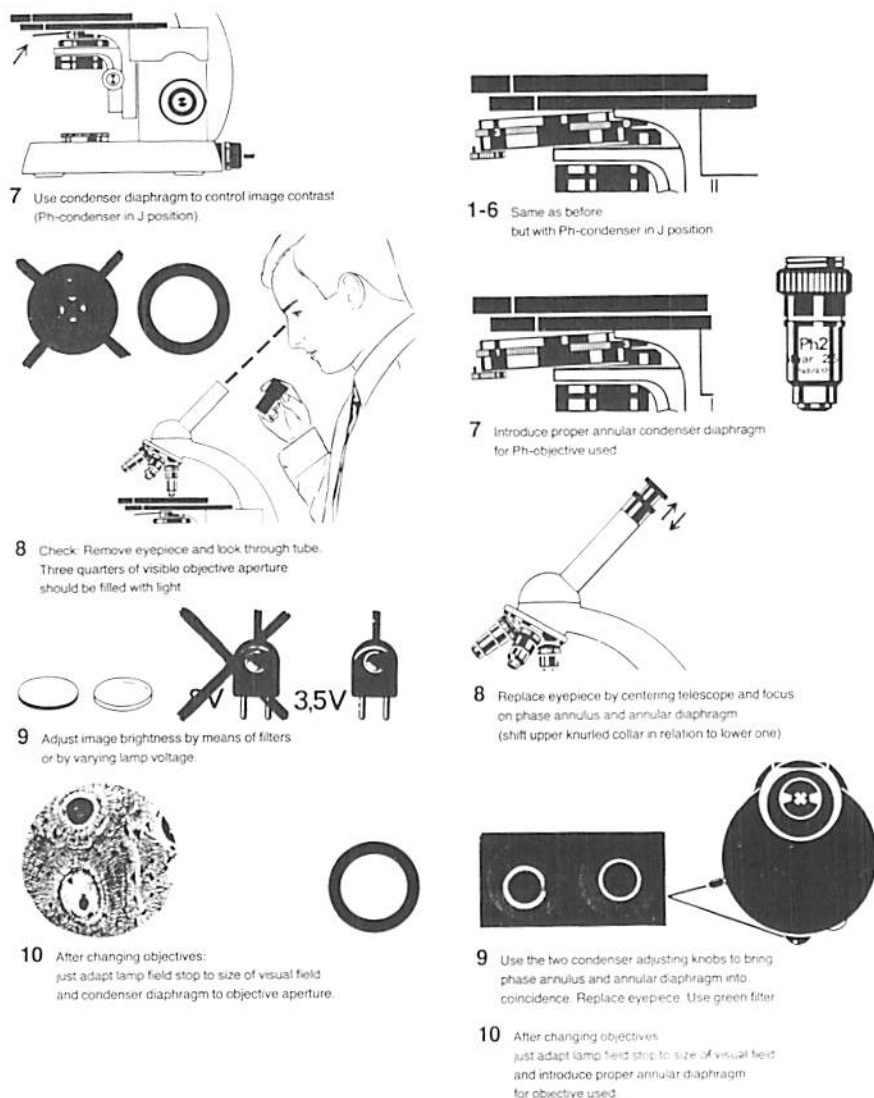
6 Open up field stop close to edge of field of view, fine focus, and open further to just clear field of view.

**Fig. C.5.3. Adjusting fluorescence microscope for bright (normal) field.**



**Adjusting for phase contrast.** To properly adjust the microscope for phase contrast viewing, first adjust bright field viewing as above in steps 1-6. Then proceed with the following steps (Figure C.5.4):

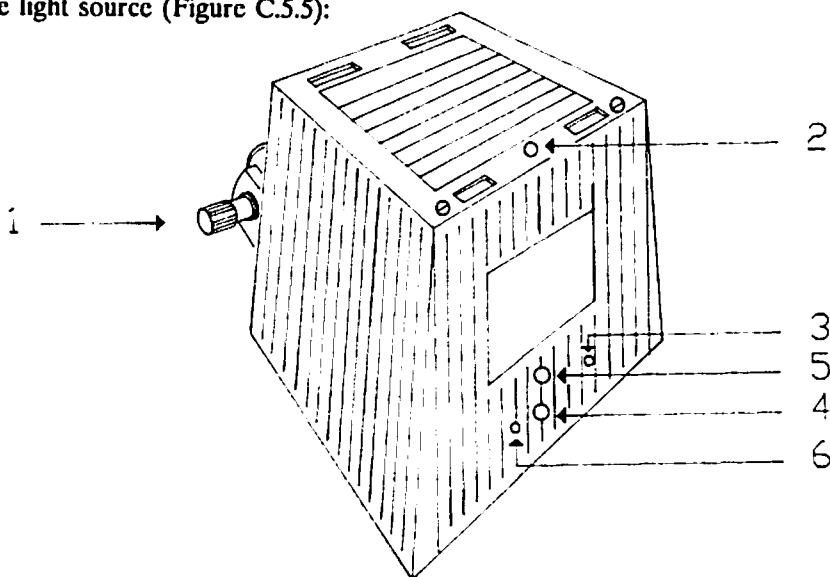
### Modifications for phase contrast (Ph)



**Fig. C.5.4. Adjusting microscope for phase contrast viewing.**

1. Use the condenser diaphragm to control the image contrast (phase condenser in 'J' position).
2. To check, remove eyepiece and look through the viewing tube. Three-quarters of the visible objective aperture should be filled with light.
3. Adjust image brightness by means of filters or by varying lamp voltage.
4. After changing objective, adjust lamp field stop to size of visual field and condenser diaphragm to objective aperture.

**Focussing and centering high-pressure mercury light source (HBO-50 or HBO-100).**  
For optimum light yield of the light source, use the proper lamp condenser suitable for the light source (Figure C.5.5):



**Fig. C.5.5. Condenser for high pressure mercury lamp of fluorescence microscope, with adjusting components: (1) knob for collector adjustment; (2) vertical lamp adjustment; (3) lateral lamp adjustment; (4) focussing of mirror image; (5) vertical adjustment of mirror image; and (6) lateral adjustment of mirror image.**

1. Open the aperture diaphragm of the reflected light microscope.
2. Take objective out of the vertical illuminator.
3. Place a sheet of white paper on the specimen stage.
4. Check the focus of the light source image; if necessary, adjust with knob (1), adjust vertically with screw (2) and laterally with screw (3). Focus the mirror image with screw (4). If the mirror image is not parallel to lamp filament (Figure C.5.6), carefully correct it vertically with screw (5) and laterally with screw (6).

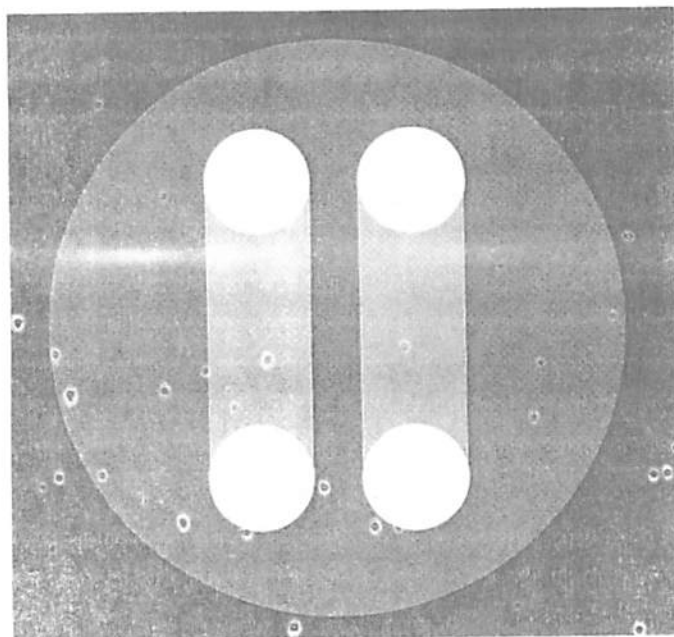


Fig. C.5.6. Correction of lamp filament to obtain proper alignment of the mirror image.

### C.5.b. Immunofluorescent Indirect Staining for Titer Determination

The titer of an antiserum may be determined by using indirect staining. The titer is satisfactory if it is not less than 1:1280, indicating that the rabbit can be bled to obtain 30–50 ml of blood. Antisera with a titer of no less than 1:1280 should be used to prepare fluorescent antibodies.

#### Requirements:

- 12-place multitest slides (Flow Laboratories, Catalogue No. 60-412-05)
- Goat antiserum anti-rabbit globulin, FITC conjugated (Sigma, F-0382)
- Glycerol (Merck, 4093)
- FITC working solution: add 50  $\mu$ l of goat anti-rabbit globulin (FITC conjugated) to a vial containing 1 ml PBS, then add 4 ml glycerol; dispense 0.1 ml of this solution in each of 50 1-ml tubes, cover with parafilm and store at -20°C in freezer compartment of refrigerator.
- Phosphate buffered saline
- Mounting fluid
- Pipettor capable of delivering 50  $\mu$ l
- Fluorescent microscope with oil immersion 60 $\times$  or 100 $\times$  objective (see Section C.5.a)

## Reagents:

### Phosphate buffered saline (PBS)

|                                                             |         |
|-------------------------------------------------------------|---------|
| Sodium chloride                                             | 8 g     |
| Di-sodium hydrogen phosphate $\cdot 12 \text{ H}_2\text{O}$ | 2.7 g   |
| Sodium di-hydrogen phosphate $\cdot 2 \text{ H}_2\text{O}$  | 0.4 g   |
| Distilled water                                             | 1000 ml |
| pH                                                          | 7.2-7.4 |

### Mounting fluid:

|                                                             |        |
|-------------------------------------------------------------|--------|
| Di-sodium hydrogen phosphate $\cdot 12 \text{ H}_2\text{O}$ | 3.2 g  |
| Sodium di-hydrogen phosphate $\cdot 2 \text{ H}_2\text{O}$  | 0.15 g |
| Glycerin                                                    | 50 ml  |
| Distilled water                                             | 100 ml |
| pH                                                          | 7.6    |

## Procedure:

1. Transfer two loopfuls of a fresh culture of homologous strain of *Rhizobium* to a vial containing 2 ml sterilized and distilled water (Figure C.5.7); mix well with a vortex mixer.

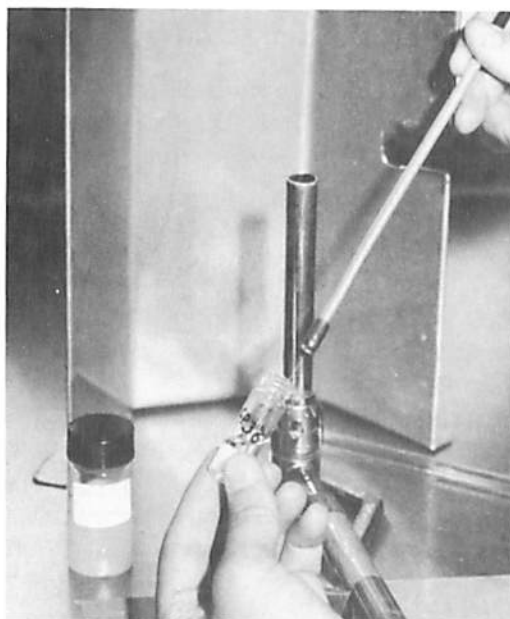


Fig. C.5.7. Transfer 2 loopfuls of culture into vial containing 2 ml sterile water.

2. Make two-fold serial dilutions of homologous antisera starting from 1:10 to a final dilution of 1:10240 (illustrated in Figure C.5.8):

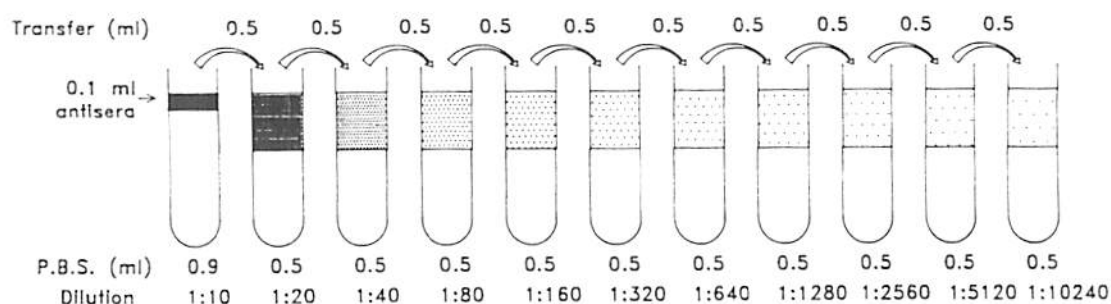


Fig. C.5.8. Two-fold dilution series of homologous antisera.

3. In each section (well) of a multitest slide add 50  $\mu$ l of homologous antigen (Figure C.5.9). Allow to dry at room temperature or use hair dryer at low setting to speed drying.

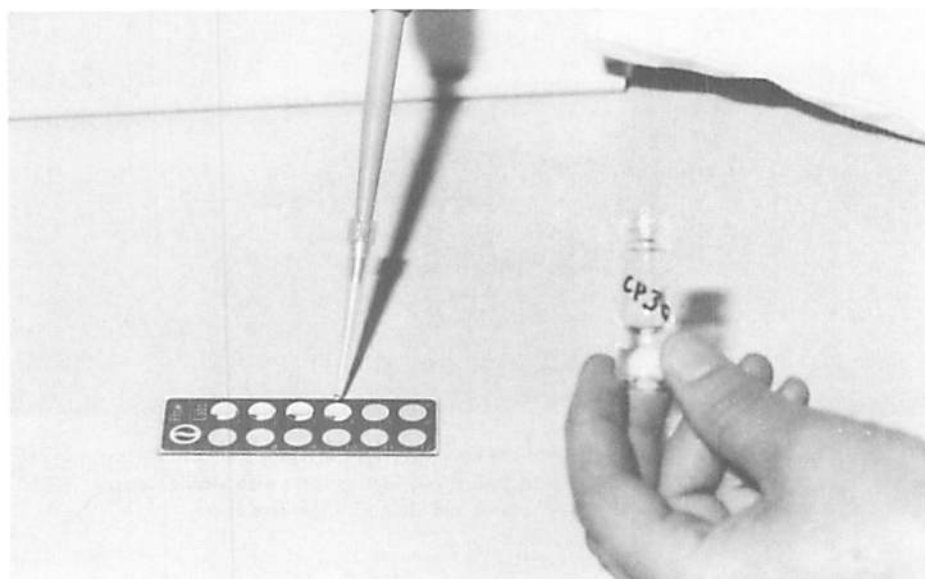


Fig. C.5.9. Add 50  $\mu$ l of homologous antigen to a marked section of the multitest slide.

4. Heat-fix slides by passing them rapidly over a bunsen flame.
5. Cover each smear (within a section) with 50  $\mu$ l of a particular dilution of antisera, so as to have a slide with 11 different dilutions of the same antiserum. Leave well number 12 free from antisera for use as a control (Figure C.5.10).

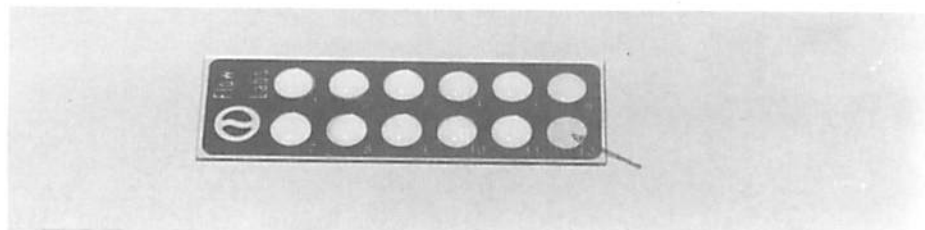


Fig. C.5.10. A blank well (number 12) is left clean as a control.

6. Allow to react in a moisture-saturated chamber (slides resting on a moistened piece of filter paper, with an inverted petri dish over the slides) for 20 minutes (Figure C.5.11).

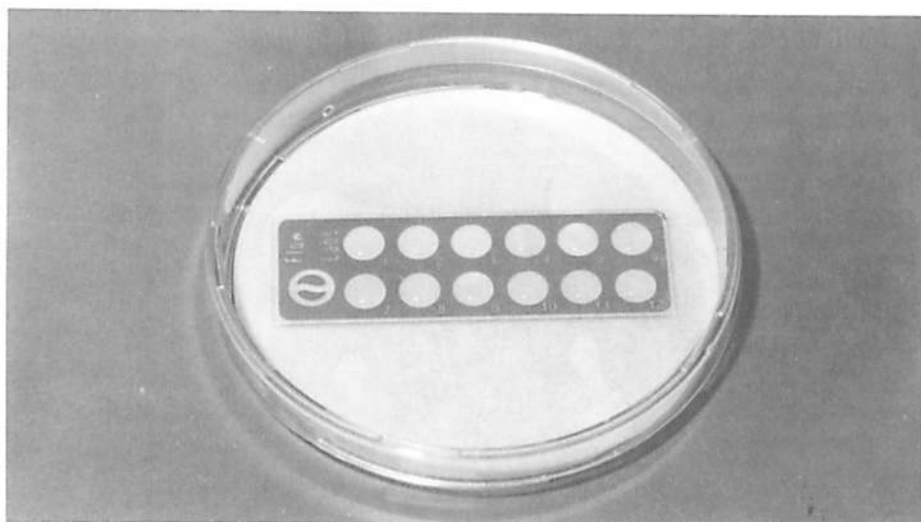
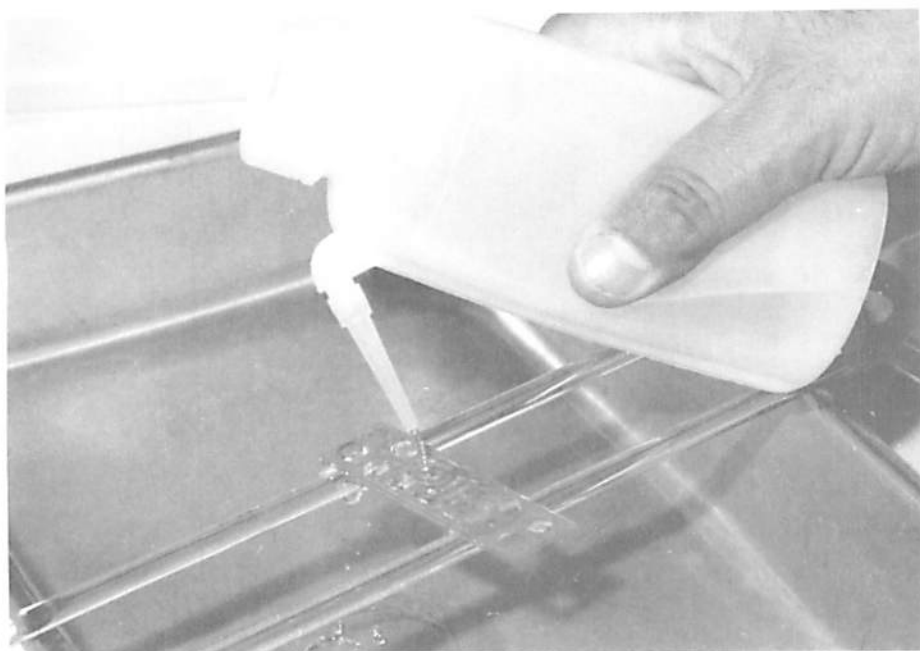


Fig. C.5.11. A moisture-saturated chamber is created by cutting several layers of paper towel to fit inside a petri dish cover, wetting them with sterile water, and covering the paper towels and slide with the dish cover.

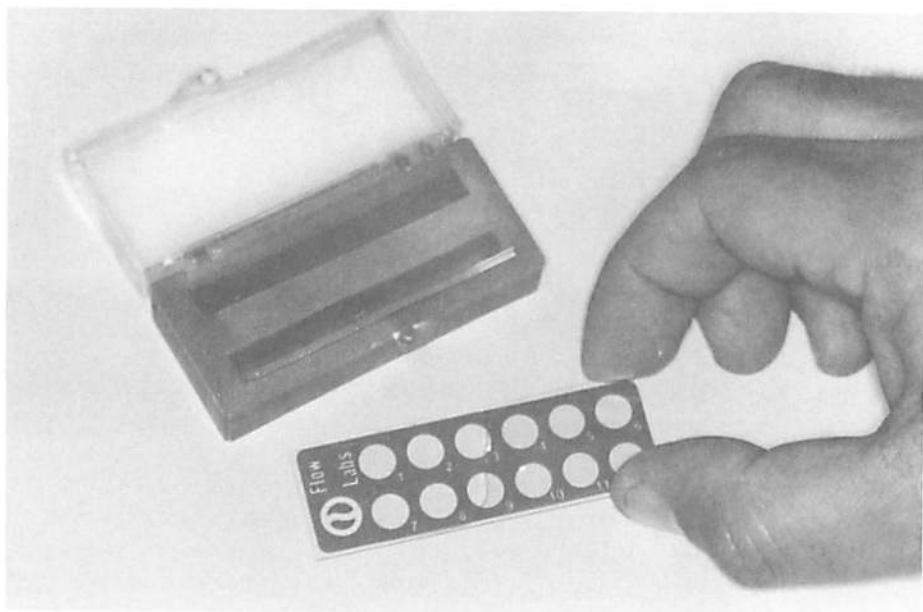
7. Remove excess antibodies by washing in PBS for 15 minutes (5 minutes for each of three separate washes), taking care to avoid dislodging the smears by hitting them directly (Figure C.5.12).



**Fig. C.5.12. Care should be taken during the washing process not to hit the smears directly with PBS.**

8. To the tube containing 0.1 ml of FITC working solution, add 1.9 ml PBS. Shake each slide to eliminate excess PBS, then add 50  $\mu$ l of dilute FITC working solution to each of the twelve smears.
9. Allow to react in a moisture-saturated chamber for 30 minutes at room temperature.
10. Remove excess FA by washing the slide in PBS for 15 minutes (5 minutes for each of three separate washes).
11. Shake each slide to eliminate excess PBS.
12. Add mounting fluid, approximately two drops per slide for 12 smears; place long coverslip over the smears, taking care to exclude air bubbles (Figure C.5.13).
13. Observe the preparations under UV microscope, using oil immersion objective at 40 $\times$ , 60 $\times$ , or 100 $\times$ .

To ensure the validity of the results, use a combination of epifluorescence and phase-contrast equipment to assess the presence of bacterial cells (first observe cells under phase contrast before switching to same-field viewing with fluorescence). Care must



**Fig. C.5.13. Coverslips should be lowered carefully from one end to the other to avoid trapping air bubbles.**

be taken to have the field of cells being observed exposed to the fluorescent light for only short periods of time, as fluorescence of cells will decrease rapidly with exposure to the UV light. Scoring for fluorescence should therefore be done immediately upon viewing a field with UV light; hence the wisdom in first choosing a field of cells using phase contrast viewing.

The intensity of the fluorescence will decrease with the higher dilutions of the specific antiserum. Grade each smear for the intensity of the fluorescence using the following scale:

- 3 + Bright yellow-green
- 2 + Yellow-green
- 1 + Dull-green
- 0 No fluorescence

An antiserum has a correct titer when the intensity of the fluorescence at dilution 1:1280 is 3 + or at least 2 +. Such an antiserum will be suitable to prepare fluorescent antibodies for direct FA, or for general use with the indirect method.



### **C.5.c. Development of Fluorescent Antibodies**

As in ELISA, it is necessary to separate the portion of the antiserum which is most reactive to the antigen (again the Ig-G fraction) before conjugating with the fluorescent dye. The procedure is not altogether different from conjugation in ELISA, and practice at one procedure will help with the other.

#### **Requirements:**

- antiserum developed in rabbit against somatic antigen of rhizobia strain
- physiological saline (0.85%)
- ammonium sulfate, 3.9 M
- Spectrapor #2 dialysis tubing
- PBS
- sodium bicarbonate 1M
- sodium carbonate 1M
- saturated barium chloride solution (about 40 g per 100 ml water)
- spectrophotometer with cuvettes
- fluorescein isothionate (FITC) (Sigma F7250)
- chromatography column, 330 × 22 mm, glass (Thomas Scientific Catalogue No. 2726-D78)
- Sephadex G-25 beads (Sigma G-25-50)
- merthiolate (sodium ethylmercurithiosalicylate, Sigma T5125)
- polyethylene glycol (PEG 600) (Sigma P-3390)
- glycerol (Sigma G-5516)
- refrigerated centrifuge capable of  $10^4 \times g$
- magnetic stirrer
- Millipore filter system and 0.45  $\mu\text{m}$  filters

#### **Procedures:**

##### **I. Fractionation of Serum Globulin**

1. To a measured amount of serum (i.e., 10 ml) add an equal volume of 0.85% filtered saline (10 ml).
2. Add slowly the same volume of 3.9 M ammonium sulfate (3.9 M = 51.25 g/100 ml distilled water) dropwise with constant stirring (i.e., add 20 ml ammonium sulfate, about 400 drops) (Figure C.5.14).
3. Allow the cloudy mixture to stand overnight (or for at least two hours) at 4°C (in refrigerator).
4. Centrifuge the mixture at  $10^4 \times g$  for 15 minutes, in a refrigerated centrifuge at 4°C.
5. Decant the supernatant fluid (Figure C.5.15) and dissolve the precipitate in distilled water to give the original volume of the serum (10 ml).

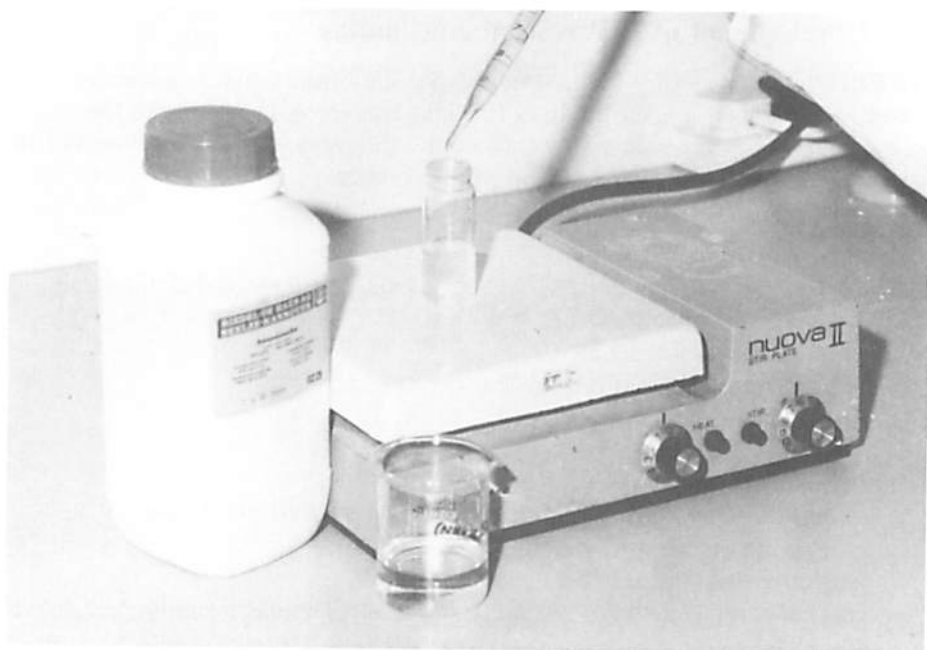


Fig. C.5.14. Adding ammonium sulfate to serum mixture on a magnetic stirring plate.

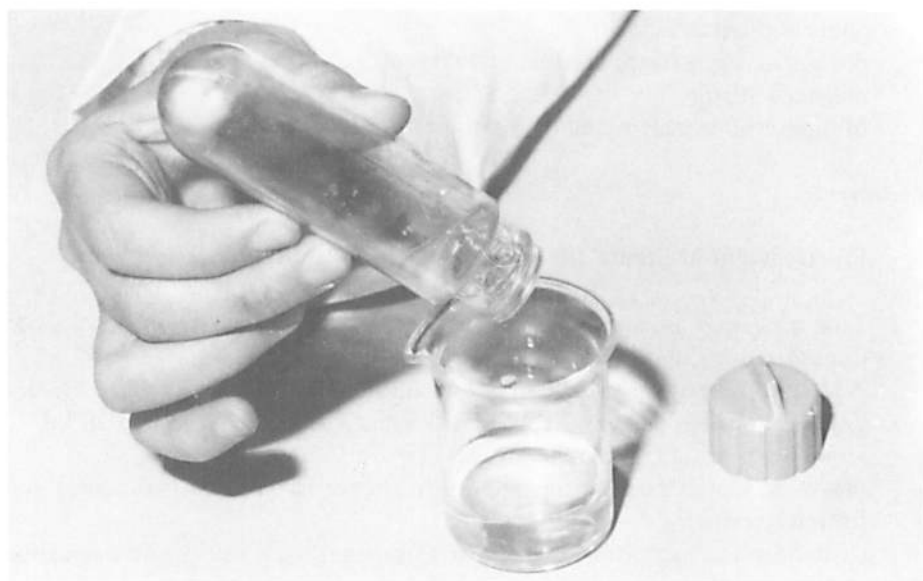


Fig. C.5.15. Decanting supernatant fluid after precipitation.

6. Repeat ammonium sulfate precipitation by adding 10 ml ammonium sulfate (same volume as volume of the serum) dropwise with constant stirring but without incubation, then centrifuge at  $10^4 \times g$  for 15 minutes at  $4^\circ\text{C}$ . Three precipitations and centrifugations are usually sufficient to render the globulin completely white and free of hemoglobin (Figure C.5.16).
7. Dissolve the final precipitate in a minimum volume of distilled water (3 ml) and dialyze (using dialysis bag, Spectrapor #2) at  $4^\circ\text{C}$  against PBS (Figure C.5.17) using several changes of PBS until sulfate is no longer detectable in the dialysate. Use saturated barium chloride to detect presence of ammonium sulfate; addition of a few drops of barium chloride to the PBS dialyzate will turn the solution cloudy in the presence of sulfate (Figure C.5.18). Before storage, determine protein concentration of globulin mixture (next step below). Keep globulin frozen at  $-20^\circ\text{C}$  after dialysis and protein concentration determination.

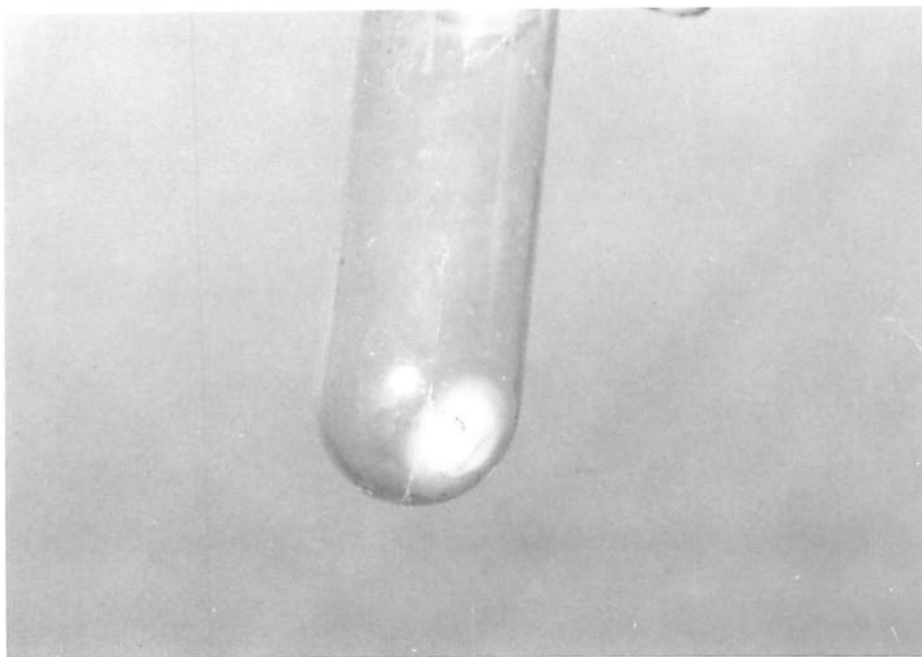
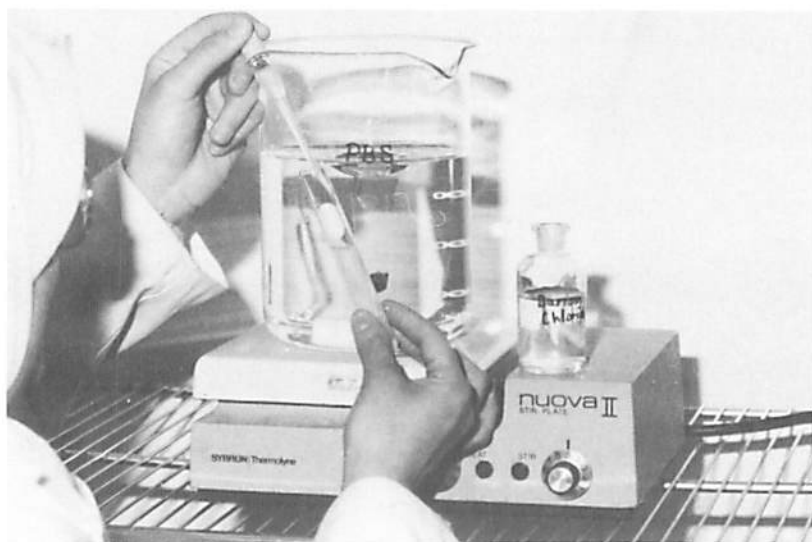


Fig. C.5.16. Globulin devoid of hemoglobin.



**Fig. C.5.17.** Add globulin precipitate to a dialysis bag.  
Dialyze at 4°C in refrigerator against PBS.



**Fig. C.5.18.** Addition of barium chloride to the PBS dialyzate (in a separate tube)  
turns the solution cloudy if any sulfate is present.

## II. Preparation of Fluorescent Antibodies (FA)

1. Determine the protein concentration of the solution inside the dialysis tubing by measuring OD at 280 and 260 nm using a crystal cuvette and UV spectrophotometer, as follows:

| Tube | Saline (ml) | Globulin (ml) | Dilution |
|------|-------------|---------------|----------|
| 1    | 3           | 0             | -        |
| 2    | 2.7         | 0.3           | 1:10     |
| 3    | 2.85        | 0.15          | 1:20     |

Adjust spectrophotometer to zero using tube 1 at 280 nm. Read OD of tubes 2 and 3. Readjust spectrophotometer to zero using tube 1 at 260 nm wave length then read OD of tubes 2 and 3. Calculate mg protein/ml for each tube using the following formula:

$$1.4 \times (\text{OD at 280 nm}) - 0.7 \times (\text{OD at 260 nm}) = \text{mg protein/ml.}$$

Remember that the protein has been diluted 1:10 in tube 2 and 1:20 in tube 3; therefore you must multiply by the appropriate dilution factor and calculate the protein concentration/ml. Take the averages of tubes 2 and 3.

2. Prepare sodium carbonate - sodium bicarbonate buffer as follows:  
Put 5 ml of 1 M sodium carbonate (dissolve 28.6 g  $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ /100 ml distilled water) in a beaker; then adjust pH 9.2-9.5 by slowly adding 25-30 ml 1 M sodium bicarbonate (dissolve 8.4 g  $\text{NaHCO}_3$ /100 ml distilled water).
3. Dissolve fluorescein isothiocyanate (FITC, Sigma F-7250) in sodium carbonate - sodium bicarbonate buffer pH 9.2-9.5 (Figure C.5.19) to have a concentration of 10 mg FITC/ml buffer (recommended to make 2 ml FITC solution; this will require 20 mg FITC. Any remaining solution can be stored at 4°C as a stock for several months).

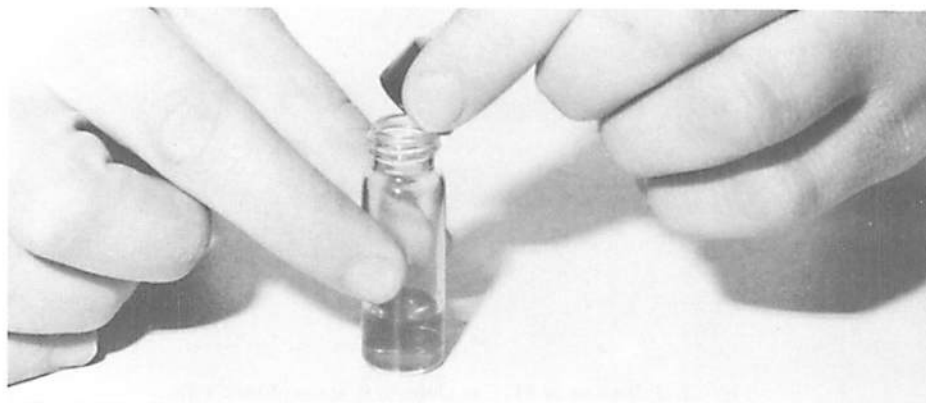


Fig. C.5.19. FITC being dissolved in sodium carbonate/sodium bicarbonate buffer.

4. To the globulin solution (1 mg protein/ml, prepared previously) add an equal volume of distilled water, then adjust pH to 9.5 using 10% sodium carbonate (Figure C.5.20). **Do not raise pH above 9.5.**
5. Add FITC solution to provide 0.04 mg FITC/mg protein.
6. **Conjugation** (binding of FITC to globulin) is accomplished in a small vial covered with aluminum foil with constant stirring on a magnetic stirrer for four hours at room temperature (Figure C.5.21).

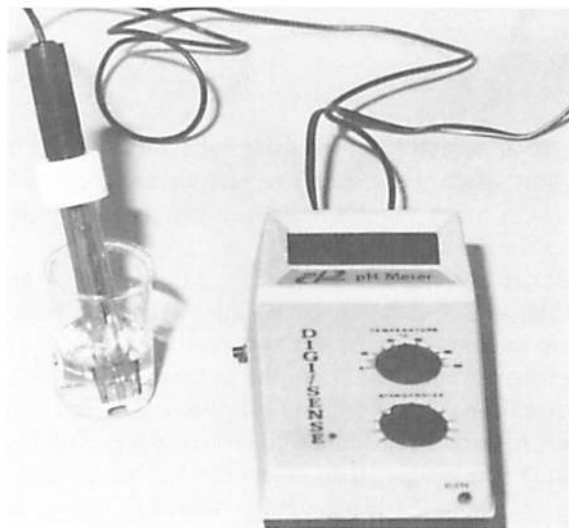


Fig. C.5.20. The pH of the globulin solution is adjusted to 9.5 using 10% sodium carbonate solution.



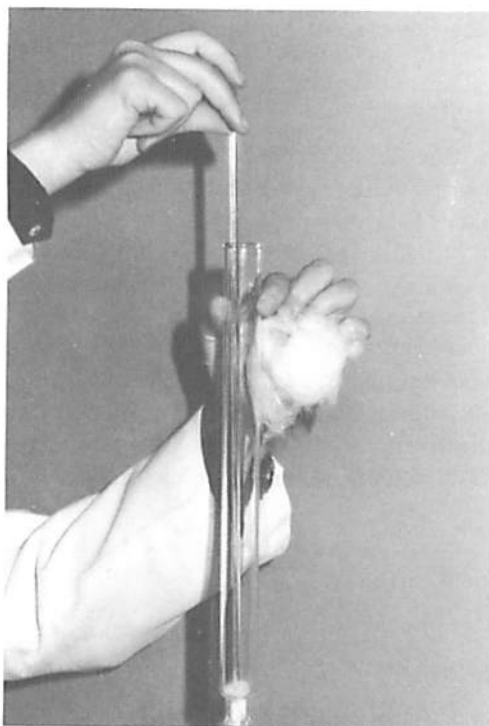
Fig. C.5.21. Binding of FITC to globulin is accomplished with continuous stirring at room temperature. Vial on magnetic stirrer contains small stirring bar.

### III. Purification of the Fluorescent Antibodies

1. Prepare chromatography column as follows:  
Put 10 g Sephadex (Sigma, G-25-50) in a 1-L flask, then add 100 ml of PBS. Allow Sephadex to settle, then remove fine particles by decanting the supernatant (Figure C.5.22); repeat until the supernatant liquid is clear (bed volume of Sephadex is 5 ml/g dry gel when swollen in PBS). Add PBS with merthiolate  $1/10^4$  to cover gel and leave at room temperature for three hours to allow the Sephadex particles to swell. Alternatively, the slurry may be heat-treated at  $90^{\circ}\text{C}$  for one hour for faster Sephadex preparation. Plug a glass chromatography column approximately  $22 \times 300$  mm at bottom with a small piece of glass wool and close the outflow (Figure C.5.23). The volume of the packed Sephadex column should be three to five times the volume of the unpurified conjugate (generally 3.5 ml, so approximately 15 ml of Sephadex is needed; each 10 ml column volume is enough to purify 3 ml of conjugate).



Fig. C.5.22. Once the Sephadex has settled, the fine particles are removed by decanting the supernatant.



**Fig. C.5.23.** The bottom of the chromatography column is packed with a small piece of glass wool to prevent loss of Sephadex.

2. Equilibrate the column by passing at least three column volumes of PBS w/merthiolate through it (Figure C.5.24) or constantly for one hour using a constant flow peristaltic pump (Figure C.5.25). Allow the PBS liquid level to settle almost to the top of the bed, then add the conjugate using a pipette (Figure C.5.26). Permit the conjugate to penetrate the Sephadex only until the entire amount of conjugate has just penetrated above column bed (Figure C.5.27). Elute the conjugate through the column with 200–250 ml PBS.
3. Collect the first eluted yellow band (this is the conjugated FITC fraction) in a small vial until no color is seen in the eluted buffer (Figure C.5.28). The unconjugated FITC fraction is seen as a slower-moving diffused yellow band; this second yellow band is not usable (unconjugated FITC), and must be discarded.
4. Continue adding PBS to the column until no more color is seen in order to clear the column; then close the bottom of the column and add PBS to cover the surface of Sephadex; cover the column with a rubber stopper (Figure C.5.29) and keep it at 4–6°C. The column can be reused many times if stored properly.





Fig. C.5.24. The column is equilibrated by adding 3 column volumes of PBS with merthiolate.

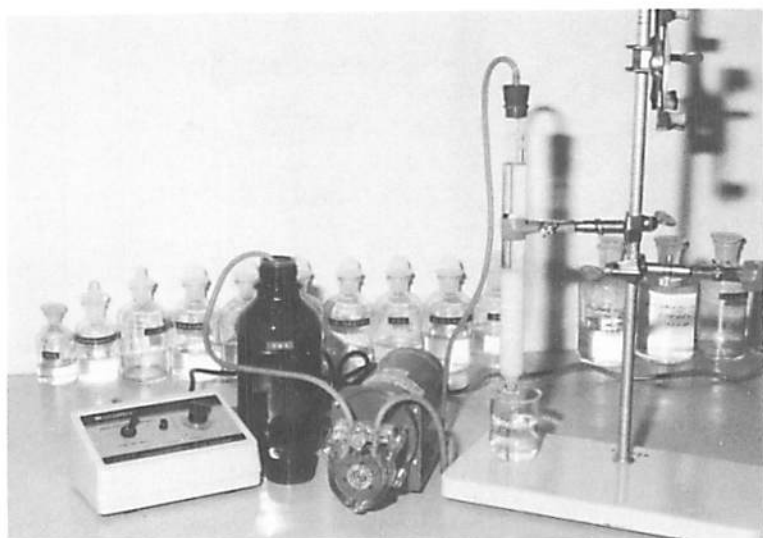


Fig. C.5.25. A peristaltic pump of constant flow can be adjusted for slow addition of PBS, making column equilibration less labor intensive.

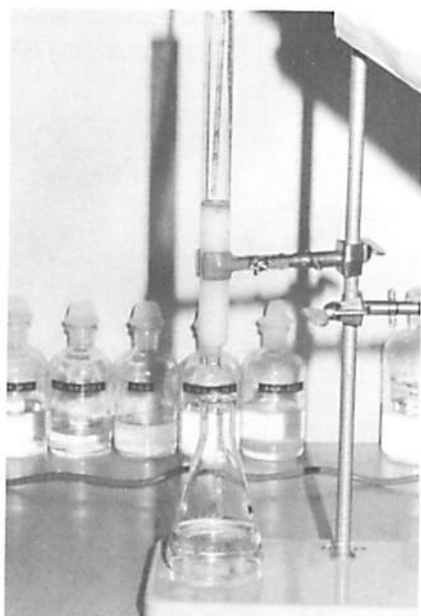
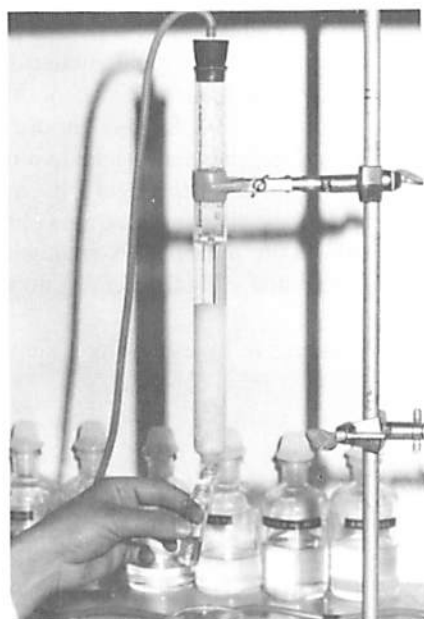


Fig. C.5.26. The conjugate is added to the column with a pipette as the last of the final PBS wash reaches the Sephadex surface.



Fig. C.5.27. As the last of the conjugate penetrates the Sephadex surface, PBS is added.



**Fig. C.5.28. Collect the first eluted yellow band in a small vial; this is the conjugated FITC fraction that you've worked so hard to get!**



**Fig. C.5.29. Cover the column with a rubber stopper after use and store in the refrigerator.**

5. The collected material (first eluted band) is diluted with PBS if less than the original volume. If greater than the original volume (usually the case), it must be reconcentrated by dialyzing it against PBS containing 5% polyethylene glycol 600 (PEG 600, Sigma P-3390), for 4–12 hours at 4°C (Figure C.5.30), or until FA solution in the bag reaches the original volume (of approximately 3 ml). Filter this solution by passing it through 0.45- $\mu$ m Millipore membrane (Figure C.5.31) then add to the filtrate an equal volume of pure glycerol (Sigma, G-5516). Distribute the purified FA conjugate in sealable tubes (0.1 ml FA conjugate in each 1-ml tube - Figure C.5.32) and store the tubes in the deep freeze at -20°C.
6. Test the quality of FA conjugate by using direct staining with fluorescent antibodies.

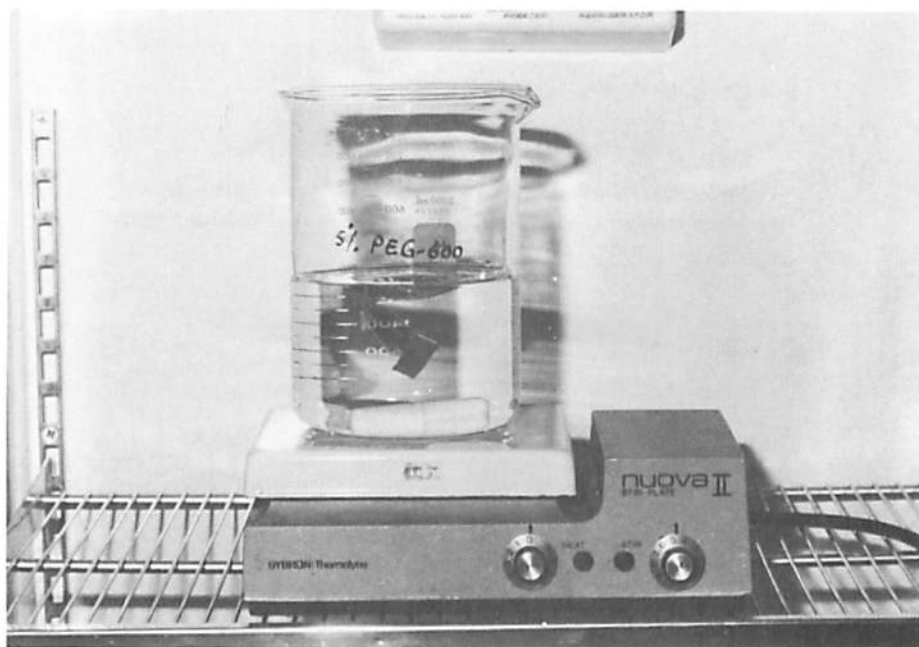


Fig. C.5.30. Reconcentration of the conjugated FITC by dialyzing it against PEG at 4°C (in icebath or refrigerator).

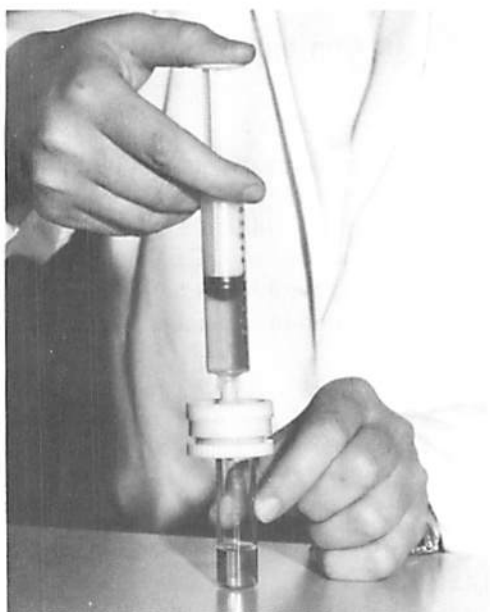


Fig. C.5.31. Filtering final solution using a  $0.45\ \mu\text{m}$  Millipore membrane.



Fig. C.5.32. Distribution of purified FA conjugate in tubes for further storage in freezer at  $-20^{\circ}\text{C}$ .

### C.5.d. Titer Estimation in Conjugate by Direct FA

#### Requirements:

- fluorescent antibodies against somatic antigen of homologous rhizobia strain
- fresh culture of homologous strain on YEM slant
- pipettor capable of delivering 50  $\mu$ l liquid
- 12 multi-test slide (Flow Laboratories, Catalogue No. 60-412-05)
- long coverslips with same dimensions as multi-test slide
- fluorescent microscope with oil-immersion 60 $\times$  or 100 $\times$  objective
- mounting fluid
- PBS

#### Composition of Mounting Fluid

- |                                                         |        |
|---------------------------------------------------------|--------|
| - Distilled water                                       | 100 ml |
| - Glycerin                                              | 50 ml  |
| - Na <sub>2</sub> HPO <sub>4</sub> , 12H <sub>2</sub> O | 3.2 g  |
| - NaH <sub>2</sub> PO <sub>4</sub> , 2H <sub>2</sub> O  | 0.15 g |
| - pH                                                    | 7.6    |

#### Procedure:

1. Prepare the antigen by transferring one large (1/100) loopful of fresh homologous culture strain from a YMA slant ( $10^9$  cells/ml) to a vial containing 1 ml distilled water (this represents  $10^7$  cells/ml).
2. To each place on the slide, add 50  $\mu$ l of homologous antigen [prepare six smears per antigen (strain) per slide] (Figure C.5.33).

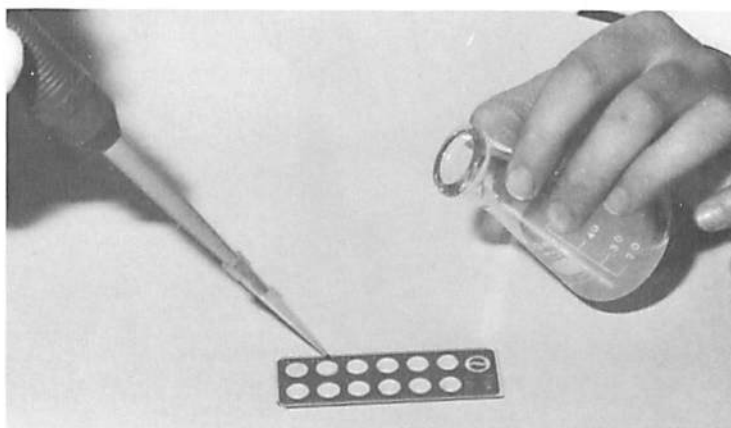


Fig. C.5.33. Six smears of each homologous antigen are added to each slide.

3. Allow the antigen to dry at room temperature or use hair dryer.
4. Heat-fix the cells by passing them rapidly over a burner flame (Figure C.5.34).
5. Prepare different dilutions of fluorescent antibodies as follows in Figure C.5.35.

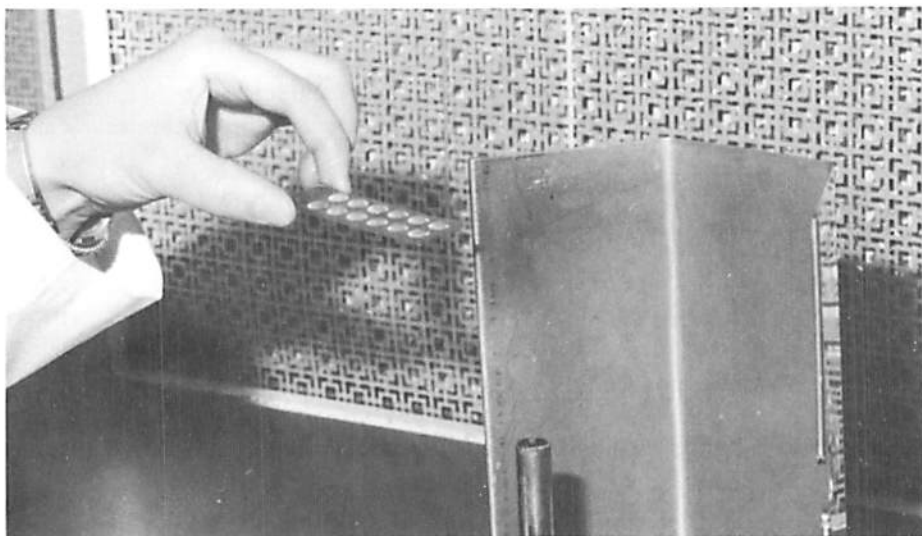


Fig. C.5.34. The antigen smears are heat-fixed by rapidly passing them over a bunsen burner flame.

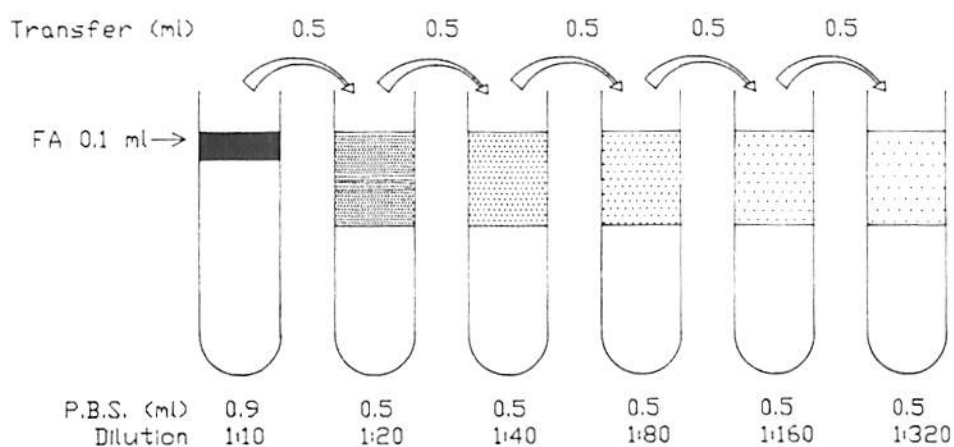


Fig. C.5.35. Dilution series for direct FA.

6. Add to each smear 50  $\mu$ l of each dilution of fluorescent antibodies preparation.
7. Allow to react in a moisture-saturated chamber at 25°C for 30 minutes.
8. Wash the slide and rinse it in PBS five minutes for each of three times taking care to avoid dislodging the smears by hitting them directly with the washing solution.
9. Add to each slide sufficient mounting fluid.
10. Cover the slide with coverslip, trying to avoid including air bubbles.
11. Examine the preparation under UV microscope and record the results as follows:

3+ Bright yellow-green  
 2+ Yellow-green  
 1+ Dull-green  
 0 No fluorescence.

See instructions in Section C.5.a for microscope use.

The antiserum is of good quality if the intensity of fluorescence at dilution 1:80 is 3+ or at least 2+. A higher titer is better, as it will allow you to further dilute the antiserum and therefore decrease the quantity used in each determination.

### **C.5.e. Nodule Serotyping by Using FA Technique**

#### **Requirements:**

- nodules for serotyping
- prepared FITC-conjugated antibodies prepared against strain(s)
- 20% calcium hypochlorite (bleach or chlorox)
- microscope slides and wax glass marker
- coverglass
- ethanol
- PBS
- mounting fluid
- UV microscope with oil-immersion 60x or 100 $\times$  objective

#### **Procedure:**

1. Select the maximum feasible number of nodules for serotyping (5–10 per plant or treatment **minimum**, more if possible).
2. Put each nodule in a tube containing enough distilled water to just immerse nodule.



3. Crush the nodules in the tubes as follows (Figure C.5.36):
  - a. Immerse glass rod in calcium hypochlorite ( $\text{CaOCl}_2$ ) for at least 10 seconds, rinse in distilled water.
  - b. Dip rod in 95% ethanol; dry it by passing it over the flame.
  - c. Squash the nodule with the sterile rod inside a small tube containing 1 ml distilled water (quantity of distilled water depends on nodule size; should be only enough to immerse nodule).
  - d. After crushing nodule, again immerse the glass rod in  $\text{CaOCl}_2$  for 10 seconds to destroy the antigen components which are on the glass rod.
  - e. Repeat steps a-d for other nodules.

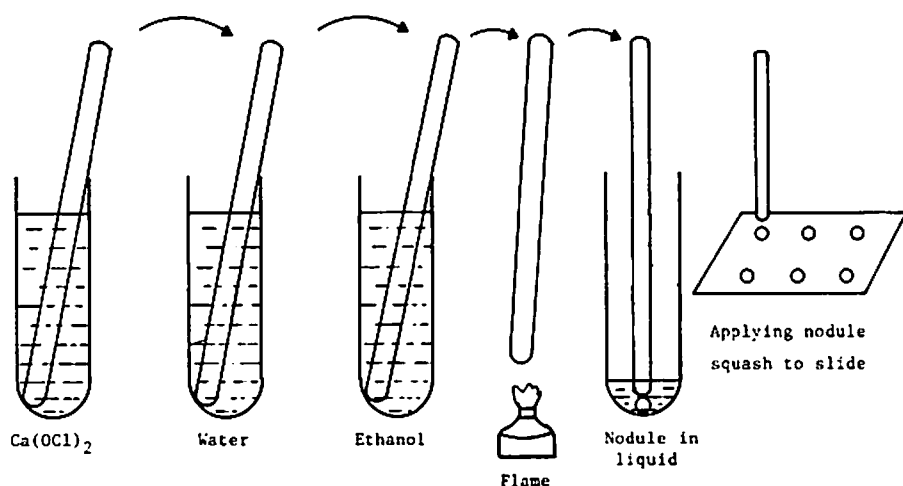
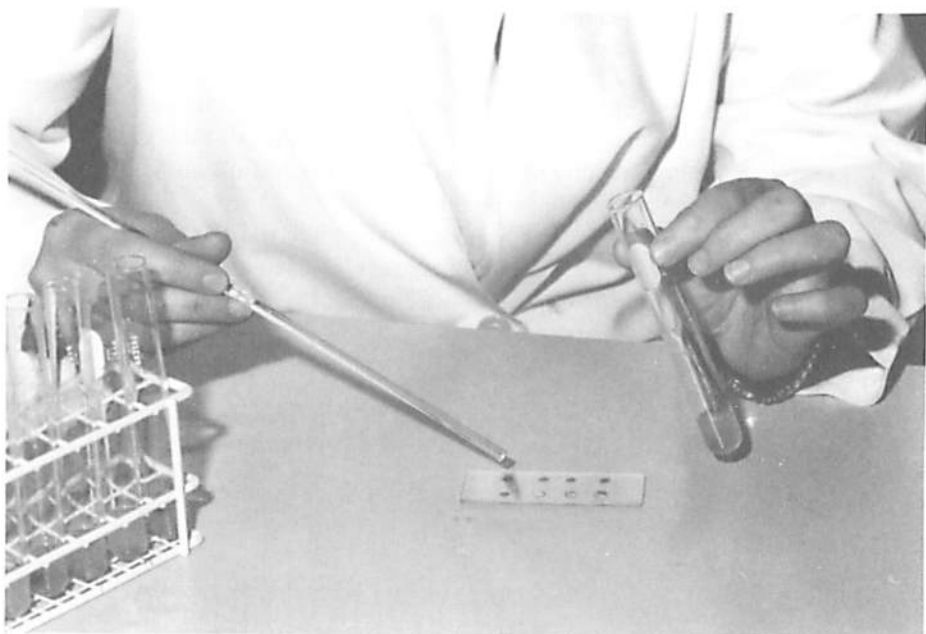
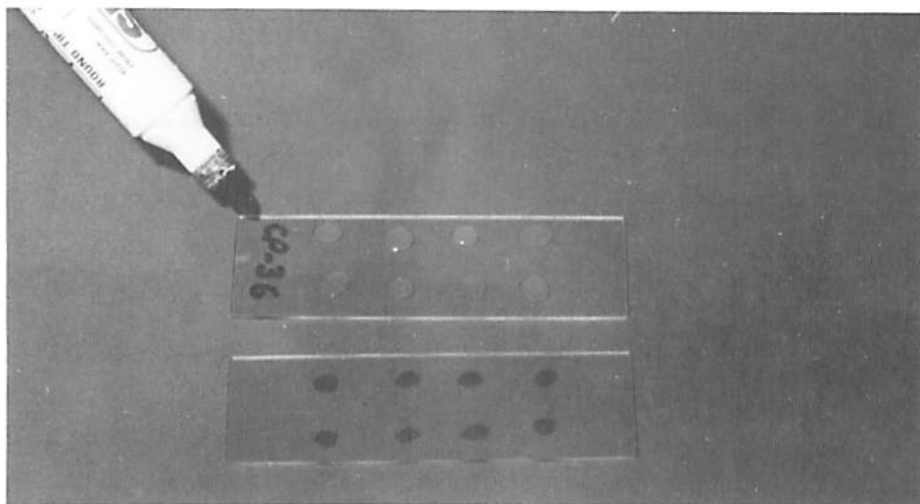


Fig. C.5.36. Crushing nodule in tube containing 1 ml of distilled water.

4. Deposit a small drop of the squashed nodule as a smear on microscope slide (Figure C.5.37).
5. Using a glass crayon or marker, divide and code each microscope slide and individual smears (Figure C.5.38).
6. Make two smears of  $50\ \mu\text{l}$  each of diluted homologous antigen (as in Section C.5.d above) to be used as control.
7. Air dry, then heat-fix the smears (pass slide quickly through flame).
8. Add to each smear  $50\ \mu\text{l}$  of the highest usable dilution (determined above, Section C.5.d) of fluorescent antibodies, allow to react in a moisture-saturated chamber at room temperature for 30 minutes.
9. Rinse the slide in PBS three times, five minutes each, taking care not to rinse off smears.



**Fig. C.5.37.** Using a glass rod to place a small drop of the squashed nodule 'soup' is placed on a microscope slide.



**Fig. C.5.38.** The microscope slide should be clearly labelled with a marker pen to indicate nodule identification numbers.

10. Add mounting fluid to the slide and place a long coverslip over the smears, avoiding bubbles.
11. Observe the preparation under UV microscope, rate from 0 to + + +, depending on the brightness of fluorescence (Figure C.5.39).
12. Compare the results obtained from nodules' smears, with homologous as + + +.



Fig. C.5.39. Observing prepared slide under a UV-capable microscope.

## C.6. Other Identification Methodologies

### C.6.a. Nodule/Colony Type

In general, nodules of legume species grown in the region do not display sufficient differences to be able to determine which rhizobia formed the nodules; the same is true for growth of rhizobia on plated media.

The shape of nodules produced by annual legumes varies from the coralloid to the elongated type (Figure C.6.1). Prolific tap nodulation (Figure C.6.2) is an indication of success of early colonization by the inoculant strain under ideal conditions and no moisture stress. Where ineffective but infective rhizobia are present in the soil, pink to reddish pigmentation inside the nodule indicates that it is functional in fixing nitrogen and was therefore formed by the introduced strain; abundance of small and whitish nodules will indicate infection by ineffective native rhizobia, and failure of inoculation (Figure C.6.3).

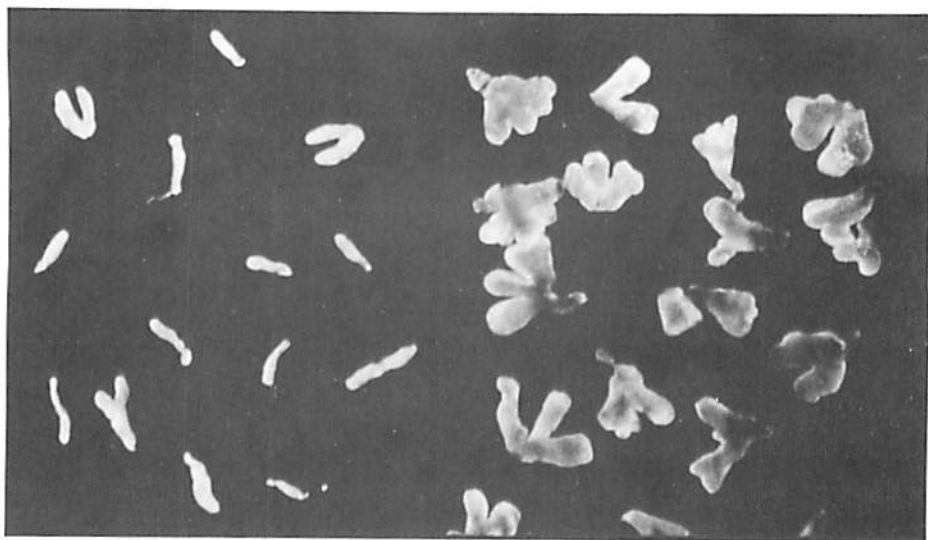
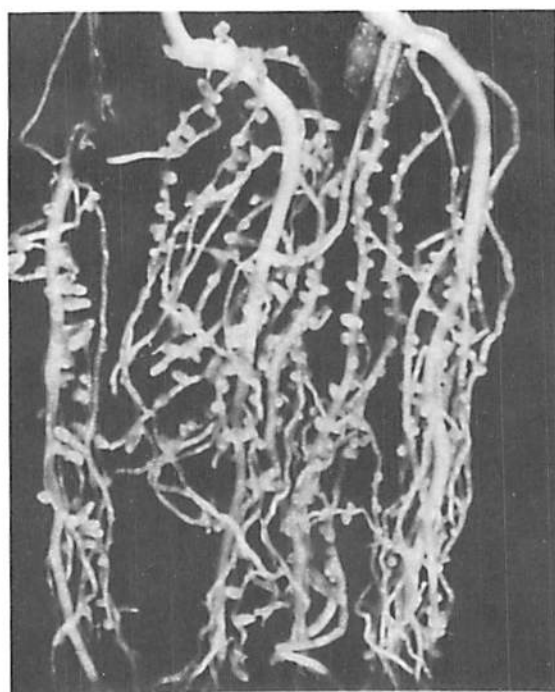


Fig. C.6.1. Effective coralloid (right) and ineffective ovoid nodules (left) from a medic plant.



Fig. C.6.2. Chickpea roots showing tap nodulation indicates successful early infection of seedling.



L. esculenta P-1  
X-Chilean

Fig. C.6.3. Lentil root system nodulated with ineffective rhizobia.  
Internal nodule tissue is white; externally nodules appear normal.

The shape of colonies formed by *R. meliloti* on standard yeast mannitol agar medium occasionally varies according to strain. This type of obvious colony variation leads the researcher to check more regularly to ensure that the different colony is in fact rhizobia and not some other bacteria (authentication). In such a case: (1) colonies may have an irregular border and unusual general appearance although size is similar to normal colonies; (2) some strains present an internal ring which is more visible if grown in YMA supplemented with Congo red. These are often found from nodule isolations of *Medicago polymorpha* and *M. orbicularis*; and (3) colony types of smaller size are occasionally present.

Normal colonies of *R. meliloti* have a size of 3–5 mm after three days and do not generally produce gum as does *R. trifolii* (clover rhizobia). They are able to slightly absorb Congo red, giving the colonies a rather pinkish coloration not evident in other type of rhizobia.

In general, *R. meliloti* takes three days to grow in liquid culture. However, some strains are known to grow faster or slower than the average. For instance, the highly effective strain ICARDA M3 takes 24 hours to grow, and ICARDA strains M15 and M53 take six days to grow in broth culture.

### C.6.b. DNA Fingerprinting

The characterization and comparison of rhizobia isolates by comparing restriction enzyme digests of their chromosomal or plasmid DNA (fingerprints) is highly discriminating for different strains and also allows similarities between them to be readily determined. These techniques are not difficult, but require a sophisticated laboratory set-up with electrophoresis equipment, refrigerated centrifuge, and relatively expensive chemicals and enzymes. Success is highly dependent on the selection of appropriate restriction enzymes and on the method of determining the similarities between the fingerprints of different strains. Ideally, a single electrophoretic gel will yield easily analyzable data which can be compared with data from other gels. This approach is best discussed in the paper by Forbes *et al.*, (1991), listed in the 'suggested reading' section.

## C.7. Suggested Reading

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## **D. Rhizobia Strain Selection Using Controlled Environments**

### **D.1. Selection Criteria and Experimental Design**

The selection of superior rhizobial strains is the basis for the practice of inoculation of legumes. The most important factor in selecting a strain is its ability to form effective nitrogen-fixing nodules on the legume host plant, preferably with a wide range of cultivars under various conditions. Effectiveness, measured by total nitrogen production and/or plant dry matter production, is still the single most important criterion for strain selection, although competitiveness and survival in soil also should be considered. Early nodulation over a range of soil temperature and pH, ability to grow well in broth culture and peat (or other inoculant carrier), and the ability to nodulate and fix nitrogen in the presence of high levels of soil nitrate are other important criteria. In addition, specificity of the rhizobial strain and the host genotype also should be considered. With all of these factors to consider, selection of rhizobial strains for an inoculation program is clearly not a simple matter.

When looking to improve N<sub>2</sub> fixation, three variables must be considered: the host legume, the environment, and the rhizobia. Usually, the host and environment are fixed, and the burden for improvement lies with strain selection to fit the host and environment. Depending on the sophistication of the laboratory and the time available for strain evaluation, there are three levels of testing for strain selection (in order of decreasing importance):

- effectiveness in nitrogen fixation
- competitive ability, including the ability to nodulate and form an efficient symbiosis in the presence of a less effective native rhizobial population and ability to survive and multiply in soils in the absence of the host legume
- growth characteristics, such as tolerance to heat and drying, pesticide resistance, growth and survival ability in peat (or other carrier) for inoculant production.

Experiments conducted at ICARDA with hundreds of strains have shown that strains of each inoculation group perform differently on different cultivars of chickpea, faba bean, and on the various species of medics and clovers. The strain-host specificity found in these studies indicates that host genotype must be considered when screening rhizobia.

To best evaluate strains, a two-step approach is suggested. First, effectiveness is evaluated in greenhouse trials, using aseptic growth systems adapted to plant type and sophistication of facilities. Growth systems are described in Section D.2. Second, the

competitive ability and effectiveness are evaluated in field studies or in intact soil cores in the greenhouse. The final and most important test for any strain is ability to increase yields or crop N in field trials.

### **D.1.a. Choice of Strains for Testing**

In choosing prospective inoculant strains, it is suggested that strains be obtained from field environments similar to those being considered for inoculation studies, as it is likely that environmental adaptation will be linked to strain competitiveness and persistence. If possible, strains should be taken from nodules of the host under consideration (specific species in the case of medics, cultivars for the food and forage legumes), because of the possibility of host-strain interactions.

Collection of nodules from the field for isolation of superior rhizobia is described in Section A; nodules from plants of superior vigor and nodulation are selected with the prospect that the rhizobia are of high effectiveness. We have found it useful to use our need-for-inoculation screening experiments (Section G) in selection of strains for further screening. Careful observation at flowering during this preliminary testing, or perhaps at the authentication stage, may indicate superior strains for further testing.

Because of the difficulties encountered in screening (and maintaining) a large number of isolates, it is suggested that strains for screening be carefully chosen. Much effort can be saved by building on the previous efforts of other investigators; within the region, many national institutions have active biological nitrogen fixation (BNF) programs, and sharing of superior *Rhizobium* germplasm is encouraged. Several international institutions have active BNF programs, and maintain a collection of strains already selected for effective N<sub>2</sub> fixation and competitiveness. ICARDA will supply, on request, superior strains (often already identifiable using serology or antibiotic methods) for legumes discussed in this manual suited to an experimenter's needs. In addition, the following institutions can provide superior rhizobia strains for some other legume species:

Dr. P. Somasegaran  
NifTAL Project  
1000 Holomua Rd.  
Paia, Hawaii 96779  
USA

tropical and temperate species

Mr. J. Brockwell  
Microbiology Section  
CSIRO Division of Plant Industry  
Canberra, ACT 2600  
Australia

clovers, medics, temperate species

Mr. R. Griffin  
USDA - CCNFL  
Building 001, HH-19, BARC-W  
Beltsville, MD. 20705  
USA

temperate species, soybean

Dr. Judy Kipe-Nolt  
CIAT  
AA 67-13  
Cali  
Colombia

*Phaseolus*, tropical forages

### D.1.b. Tolerance to Stress Conditions

In screening for strain tolerance to stress conditions, it must be kept firmly in mind that the rhizobia is only one component of the  $N_2$ -fixing system; the other components which will be affected by the adverse conditions are the host and the partnership, or symbiosis. With all types of stresses, the rhizobia are generally the most tolerant, followed by the host plant; it is the combination of the two, the symbiosis, that is most susceptible to harsh conditions. This makes sense, as the processes necessary for  $N_2$  fixation, including infection, nodule formation and nodule function, are the results of genetic interaction between the two symbiotic partners.

Because of the complexity (and sensitivity) of the symbiosis, testing the rhizobia for the particular stress is only the first stage of screening. A large number of strains can be easily tested in broth culture or on plated media containing high levels of salts ( $> 0.5$ – $1.0\%$  NaCl) or extremes in pH (pH  $< 4.5$  or  $> 8.2$ ), or at extremes of temperature ( $35$ – $40^\circ\text{C}$ ); in this way a collection can be narrowed down to a few strains for further testing (Figure D.1.1).

The following and most important phase of the stress screening, however, must be the combination of rhizobia and host plant, to test for ability to nodulate and fix nitrogen under the stress conditions. Providing an artificial environment requires imagination and adaptable resources. High temperatures can be simulated in a lighted growth chamber, or in a greenhouse during summer months. A hydroponic system, with the growth solution containing high levels of salts or pH extremes, can be set up for salinity or pH screening. Drought can be provided with a strict watering regime in sand or a soil/sand mixture, or in hydroponic culture using polyethylene glycol (PEG 6000) to simulate water stress. Some methods are given in detail in the reference section. Final testing of promising strains should be conducted in field studies under actual stress conditions.

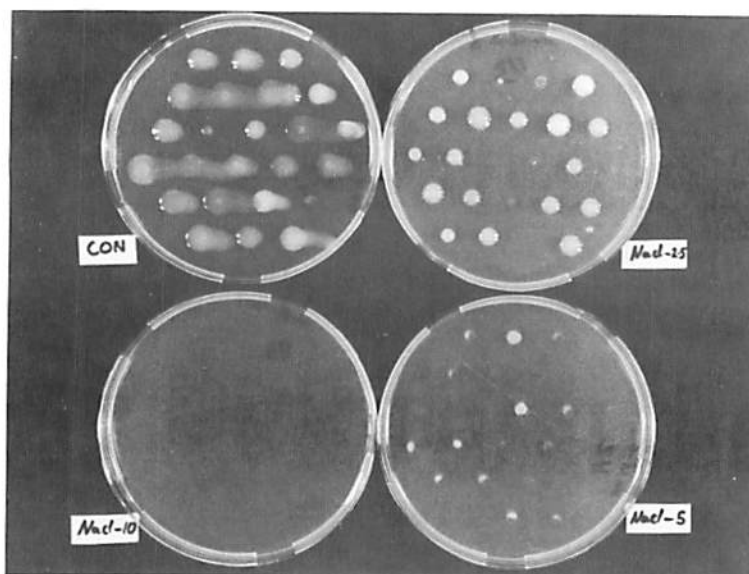


Fig. D.1.1. Growth of strains on plates of YM agar with increasing levels of NaCl concentration.

### D.1.c. Infectiveness and Effectiveness

The ability of rhizobia to produce infections on the legume root and form nodules is called infectiveness. This property is restricted to specific groups of rhizobia and the hosts where the infections are induced. Section A.7 describes the species and biovars of rhizobia and the legume groups they infect and nodulate. Infectiveness **does not** imply effectiveness in  $N_2$  fixation.

As previously stated in this chapter, symbiotic effectiveness is an important parameter in the selection of rhizobial strains for inoculants. It is the primary concern of anyone who is attempting to increase legume yields through nitrogen fixation. The term 'symbiotic effectiveness' provides an indication of a nodulated plant's ability to fix nitrogen. To measure effectiveness in quantitative terms, growth and/or nitrogen fixation of the nodulated plants is compared with the growth of plants receiving sufficient combined nitrogen or with the growth and nitrogen fixation of plants nodulated by a known superior inoculant strain.

Effectiveness of nodules can be broadly gauged by the degree of pink or red coloration of the tissue inside each nodule. As a general rule, nodules having white or green tissue pigmentation are considered inactive in nitrogen fixation. Ineffective nodulation caused by an incompatible or nonspecific rhizobial strain poses a problem to the plant as it demands photosynthate and renders little or no nitrogen fixation.

Usually the symbiotic capacity of isolates is compared with that for plants inoculated with 'standard' highly effective strains and those treated with mineral nitrogen as controls. The symbiotic effectiveness (S.E.) can be expressed as:

$$\text{S.E.} = \frac{\text{Dry mass or N content of plant inoculated with test strain}}{\text{Dry mass or N content of plant inoculated with standard strain}} \times 100$$

or, if a nitrogen-fed control plant is used:

$$\text{S.E.} = \frac{\text{Dry mass or total N of the plant inoculated with isolate}}{\text{Dry mass or total N of the N-supplied control plant}} \times 100$$

Nitrogen controls provide a clear measure of the potential growth of the legume species under given experimental conditions. Calculating the growth of the inoculated plants as a percentage of that made by the controls provides an indication of 'absolute' symbiotic effectiveness (SE), and allows comparison of strains from experiments where different environmental conditions may have been encountered. These determinations of symbiotic effectiveness are usually made 6–10 weeks after planting, after a period of active nitrogen fixation in the treatments has occurred. From comparisons with N controls, four classes of effectiveness are defined: high SE, moderate SE, low SE, or ineffective.

Both infectiveness and symbiotic effectiveness are the result of the legume host interacting with the invading rhizobial strain. The two sources of variation are therefore the rhizobial strain and the legume host. In most instances, the microbiologist must confront the situation of locating superior strains of *Rhizobium* for plant genotypes or ecotypes which have been already been selected, leaving only variability of strains for exploitation.

Occasionally the term 'efficiency' is used instead of 'effectiveness'. Efficiency is more properly expressed as a measure, e.g., mg N<sub>2</sub> fixed per g nodule mass, or per mg carbon molecules utilized by the nodules fixing N<sub>2</sub>. Although 'highly effective' associations are usually 'efficient', the two terms have a different basis of expression. We prefer to use the term effectiveness in describing the symbiotic potential of a strain or rhizobial population.

## D.2. Evaluation of N<sub>2</sub>-Fixing Effectiveness in Aseptic Culture

The concept of testing rhizobial strains under bacteriologically controlled (aseptic)

conditions for effectiveness is based on several important premises. First, all plant growth factors are controlled, with provision of adequate light, moisture, temperature and nutrients (except N) for efficient photosynthesis and plant growth. Second, no nitrogen is available in the system except that contained in the seed cotyledons. Finally, and probably most important, the system must be free from contamination by rhizobia other than those applied as inocula; this means that the system must be sterile before planting, and sterility must be maintained throughout the duration of the experiment.

### D.2.a. Plant Growth and Maintenance of Aseptic Systems

It is important that all plants experience similar growth conditions from the beginning and then throughout the experiment. Seeds should be selected for uniformity (if possible from a batch of similarly grown plants), as quantity of nitrogen contained in cotyledons will affect early plant growth and onset of fixation. Light (radiation) should be evenly distributed and artificial lighting should provide at minimum  $10^4$  lux ( $100 \mu\text{E m}^{-2} \text{sec}^{-1}$ ). A serious problem in many growth chambers is insufficient light, which produces elongated (etiolated) plants (Figure D.2.1) with low levels of photosynthesis and hence decreased  $\text{N}_2$  fixation. During later growth stages, larger plants will require more water, so it is important that watering should be periodically adjusted to provide near field capacity at all times without periods of drought or flooding. For most legumes, temperatures in the growth environment should be maintained between  $15^\circ$  and  $30^\circ\text{C}$ ; above  $30^\circ\text{C}$  nodulation and nodule function are impaired. Accuracy of results will increase with number of replications; we recommend that four replications be used where possible, with a minimum of three in all experiments.



Fig. D.2.1. Etiolated plants grown in a chamber with insufficient light.

## D.2.b. Leonard Jar Technique

Because of the widespread presence of contaminating rhizobia, open pots generally cannot be used to evaluate rhizobia strains. Small-seeded legumes can be grown in the aseptic tube system described previously for use in MPN evaluation or authentication (Section B.3.b.), as the plants can produce adequate growth to differentiate strain properties. The large-seeded legumes, however, cannot be grown to sufficient size in tubes or growth pouches to evaluate the relative effectiveness of strains.

A widely used vessel for good microbiological control and differentiation, especially with large-seeded legumes, is the modified Leonard jar. This system provides protection from surface contamination with the use of a dry inert surface mulch and watering from below. Details of the Leonard jar vary according to the kind and size of cheap containers available. The principal features (Figure D.2.2) and procedure for planting and maintenance are as follows:

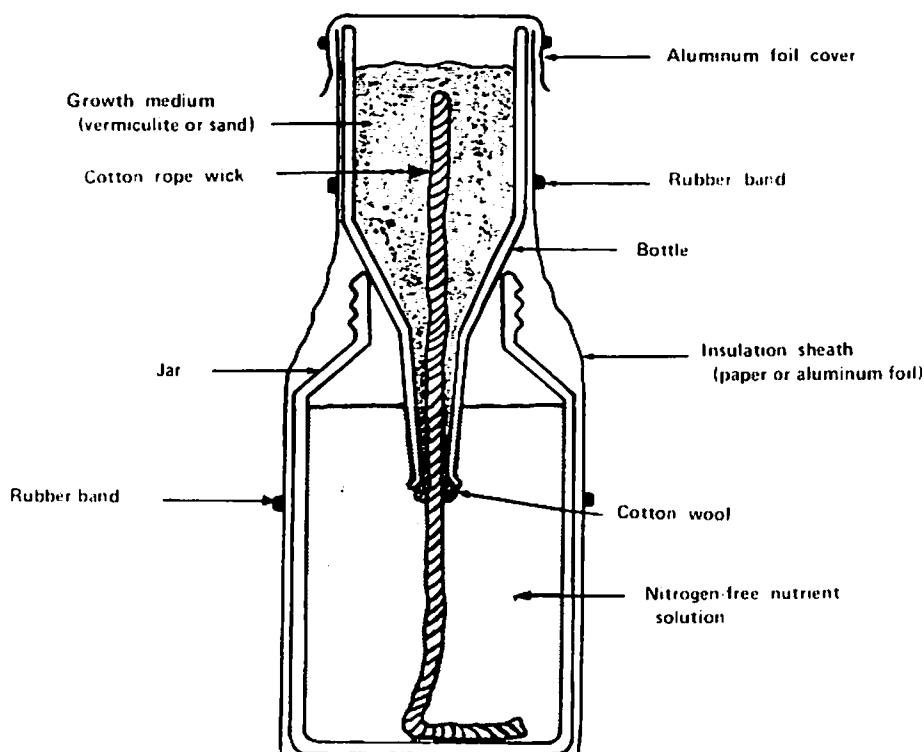


Fig. D.2.2. The components of a Leonard jar, including nutrient solution reservoir, growth vessel, wick, and growth medium.

1. The top half of the unit consists of a bottle (round beer or spirits bottle of 700–1000 ml capacity) that has been cut near the bottom to provide a level flat-ground finish. Amber (brown) bottles are best as they protect roots from light.
2. The lower half, which provides the reservoir for N-free nutrient solution, consists of a jar of such dimensions that the inverted bottle sits snugly on its rim and the neck of the bottle comes to within 2–4 cm of the bottom of the jar. When using a 1-L bottle for the top half, it may be possible that the cut-off bottom half of the bottle is large enough to be used as the reservoir (Figure D.2.3).

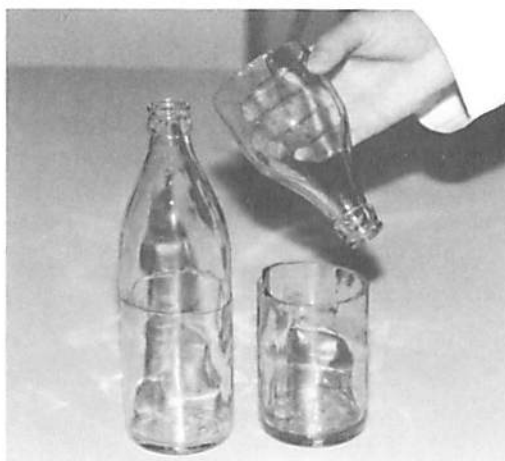


Fig. D.2.3. A 1 liter glass bottle cut in half; the bottom is used for reservoir and top as growth vessel for the plant.

3. A wick is provided to help the capillary rise of moisture from the reservoir to the top of the growth vessel. Strands of hemp, absorbent cotton wool or lamp wick may be used, but all materials should be well washed with detergent to remove oils that may resist wetting. The wick is secured in the bottle neck with a wad of cotton wool.
4. Add moderately coarse, well-washed river sand to the bottle units to within 5 cm of the top. In doing this keep the wick near the center of the vessel and have it reaching almost to the surface.
5. Prepare the N-free nutrient solution (Section A.9) and moisten the jar from the top until liquid begins to drain into the reservoir. Fill the reservoir with the nutrient solution to within 2 cm of the junction of the two parts.
6. Cover the top of the growth vessel with a sheet of fitted aluminum foil (or a glass petri dish cover), and the whole unit with moisture-proof paper secured with rubber bands or heat-resistant tape (Figure D.2.4).



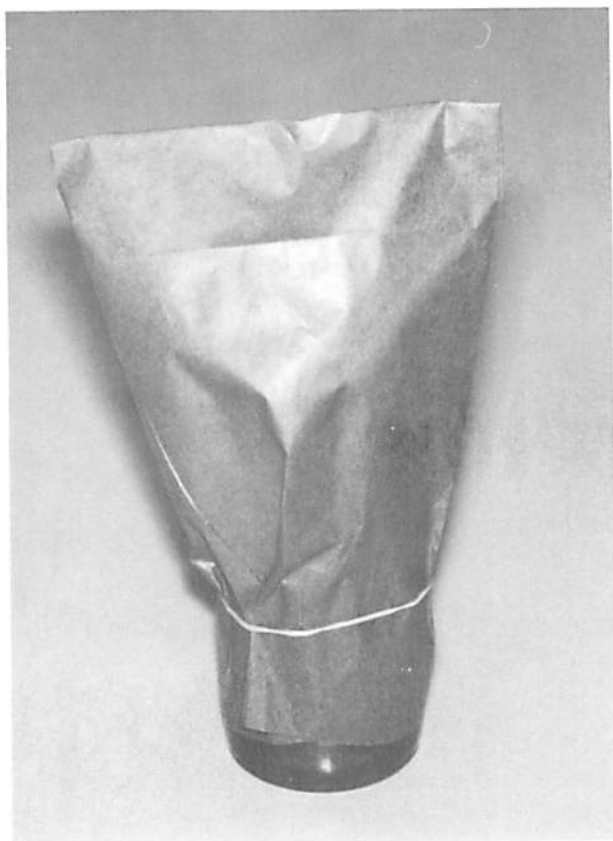


Fig. D.2.4. A prepared Leonard jar unit ready for autoclaving.

7. Autoclave whole units at 121°C for two hours. Do not vent autoclave after sterilization, but let it come down to zero pressure before opening the door. Remove jars to a clean place and keep covering intact until planting. Bottles must be completely cool before planting.
8. Sterilize sufficient gravel (3–4 mm diameter size) to cover the sand in the vessels to a depth of 2 cm, by dry heating in oven to 200°C for one hour.
9. Sterilize and pregerminate the seeds as described in Section D.2.d.
10. Place the assembly in a laminar flow or clean bench, and remove foil cover. Using sterile forceps, make a hole in the sand to accept the radicle and seed (Figure D.2.5). Dip forceps in alcohol, flame and cool, then transfer one germinated seedling to each bottle. If the treatment is to be inoculated, inoculate the seed in sand before covering with 1 ml of diluted broth culture containing approximately  $10^8$  rhizobia/ml. Cover seed with sand using forceps and tamp the sand in place over the seed to provide a firm seed bed.



Fig. D.2.5. Planting seedling in jar with forceps.

11. Cover the vessel opening with half a petri dish or clear plastic film to protect against contamination, and set the units in greenhouse or growth chamber (Figure D.2.6).
12. As soon as plants are established, and before their development is restricted by the petri dish, cover the sand with the dry-sterilized gravel to a depth of 2 cm over the entire surface. Inoculation can be carried out just prior to this step, directly onto the surface of the sand at the base of the plant, if it has not previously been done. The units are now left open.
13. Uninoculated and nitrate controls are provided as described in Section D.2.c.
14. The N-free nutrient solution in the reservoir may be depleted during growth of the legume, depending on plant size and growth conditions. If necessary, new (sterile) solution can be poured into the reservoir (with precautions to make the transfer as sterile as possible) by separating the two containers sufficiently for the reservoir to be filled.

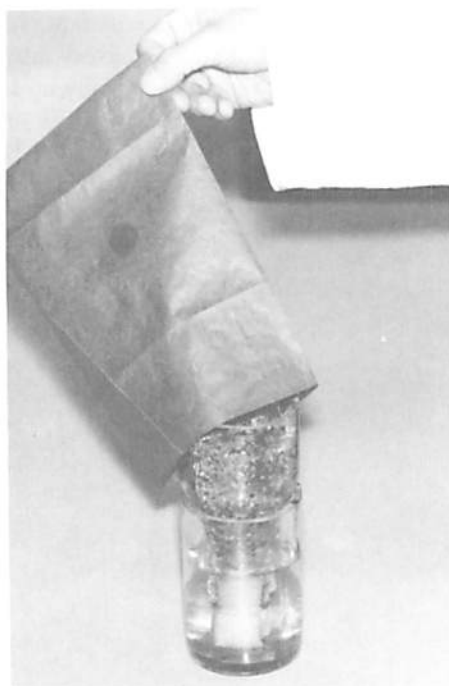


Fig. D.2.6. Complete planted Leonard jar unit.

### D.2.c. Alternate Growth Systems

As mentioned previously, small-seeded legumes like medic can be evaluated effectively in the tube growth systems described previously in Section B.3.b. Also, growth pouches (described in Section B.3.b) are suitable for evaluation of rhizobial effectiveness in small-seeded legumes, such as the medics and clovers (Figure D.2.7).

Several options, however, exist for the larger-seeded legumes, where adequate volume must be available to enable unrestricted root growth for a large plant (up to flowering stage). In devising a system, the main considerations are:

1. **Ease of preparation.** Leonard jars function well and require little maintenance after planting, but a large initial effort in preparation of the jars makes it impractical to conduct large experiments.
2. **Maintenance of sterility.** The system must be sterile initially and also must be completely closed to invasion by contaminating rhizobia during plant growth. Rhizobia can be found in nonsterile water, and even in the air of most growth environments ('riding' airborne dust particles). Addition of water or N-free

nutrient solution is a common source of contamination, so all solutions added should be sterile and the method of water delivery must be aseptic. All handling of plants should be conducted aseptically up to harvest of the experiments.

3. **Good plant growth.** Plant roots should not be exposed to flooding or drying, and should have adequate space to grow (approximately 0.5 L minimum).

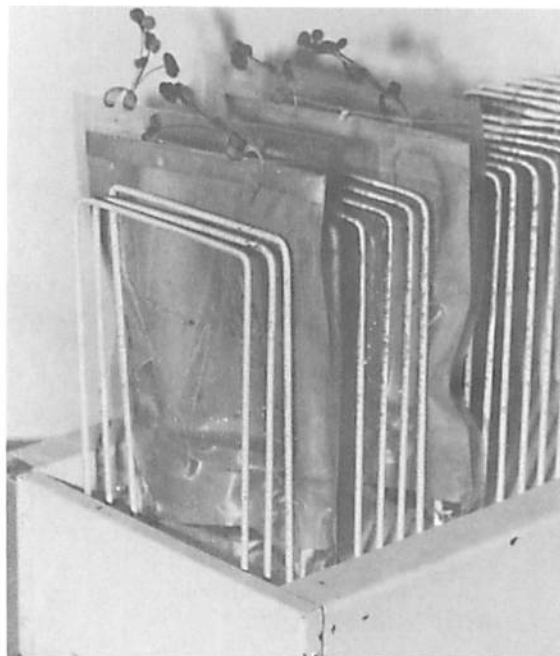


Fig. D.2.7. Medic plants in plastic growth pouch.

At ICARDA, we have developed a slightly sophisticated system using locally available materials, which allows screening of a large number of treatments with savings of labor both during preparation and maintenance of experiments. We first describe the system, then discuss how it can be modified to fit local needs and conditions.

The vessels used are disposable 1-L plastic yoghurt (labneh) containers (cups) with a tight-fitting lid (Figure D.2.8), purchased in quantity locally at approximately U.S. \$0.05 each. Holes are made in the bottom of the cups for drainage with a punch or hot metal rod; four to six holes of 5 mm diameter equally spaced are sufficient to allow good drainage (Figure D.2.9). Similarly, a hole of approximately 15 mm diameter is made in the center of the lid, through which the plant will grow (Figure D.2.10).



Fig. D.2.8. Plastic disposable yoghurt container (cup) with tight-fitting lid.

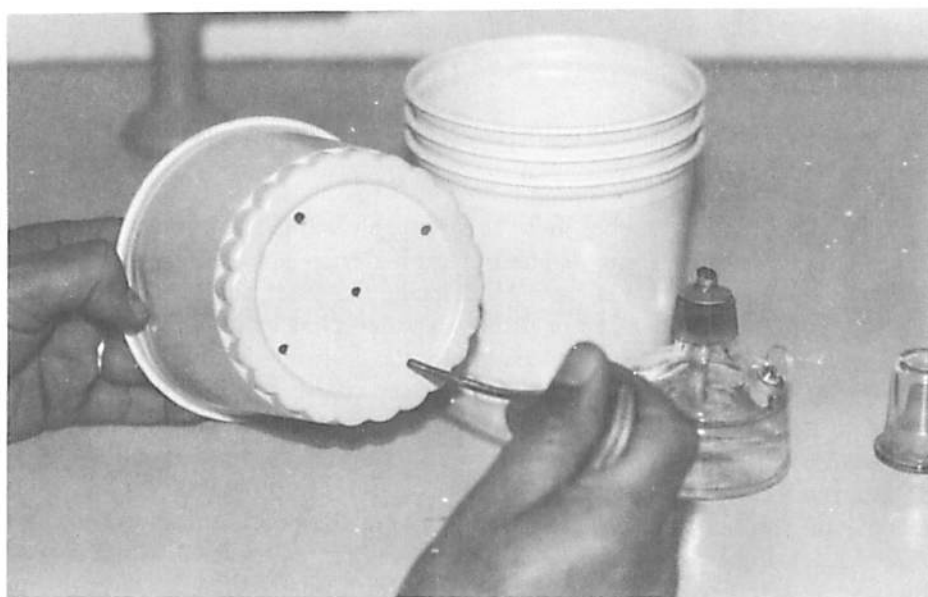


Fig. D.2.9. Using hot metal rod to make drain holes in bottom of cups.



Fig. D.2.10. Using a punch to make plant growth hole in cup lid.

The rooting media to fill the cups must be thoroughly washed, as discussed previously. Vermiculite and gravel (3–4 mm diameter, inert material—not  $\text{CaCO}_3$  based) are washed thoroughly at least three times by changing the water and stirring frequently; final rinse should be with distilled or deionized water. New batches of these materials (especially vermiculite) need more changes of water to bring down the pH to near neutrality. For cost savings, we recommend that the coarse gravel (3–4 mm diameter) be mixed with the vermiculite to make up four-fifths of the total content; plants grow well in this gravel-vermiculite mixture. This substrate may be reused indefinitely, with occasional replacement of vermiculite as it breaks down into small pieces and is lost. After cleaning, the substrate is placed (still moist) in cloth bags for autoclaving (Figure D.2.11). Each bag is autoclaved for two or three hours at  $121^\circ\text{C}$ , three times on three successive days. The bags containing the mixture are then dried for 36 hours at  $60^\circ\text{C}$  in a forced-air oven, and left in the oven for 24 hours to cool just prior to filling the cups.



Fig. D.2.11. Filling cloth bags with moist washed substrate (vermiculite and gravel) prior to autoclaving.

Just prior to filling, the insides of cups and lids are wiped with an alcohol-soaked cotton mat to sterilize them (Figure D.2.12). In a clean area, the sterile gravel mixture is rapidly transferred to each cup, so that the cup is full to within 25 mm of the top (Figure D.2.13). The lid, with a piece of tape covering the plant hole, is put in place and the system is ready for planting. Planting and system maintenance are described in the next section.



Fig. D.2.12. Sterilizing the inside of the cups and lids with an alcohol soaked cotton mat.



Fig. D.2.13. Transfer of sterilized gravel-vermiculite medium to sterile cup.

The principle for supplying water and nutrients to the system is quite different from that in the Leonard jar, where sterile nutrient solution is wicked up by capillary action from a reservoir into the sand substrate. Here, liquid is pumped from a reservoir of N-free nutrient solution and delivered to each cup by means of a water pump and spaghetti-tube distribution system (Figures D.2.14, D.2.15 and D.2.16); spaghetti tubing, volume regulators and distribution tubes are those used in drip irrigation systems. With irrigation, the gravel substrate is charged with water and nutrients while drain holes in the bottom of the cup allow excess water to drain freely away. It is **very important** that water draining from cups cannot flow on the bench surface from one cup to another, as in this way different treatments will contaminate one another; the best method is to use a drilled steel or wire mesh bench surface, suspended so that all drain water moves directly away from cups (Figure D.2.17). Alternatively, suspending each line of cups on blocks above the bench-top is adequate. When watering, sufficient solution is applied so that all cups begin to drip solution; this indicates that all cups have been supplied with enough solution to fully wet the gravel mixture. Excess solution drains away, preventing flooding and providing solution equally to all treatments. Cups are watered when gravel in the treatments dries; this is every two to three days during early plant growth, and perhaps daily with large plants during the final week before harvest.





Fig. D.2.14 N-free nutrient solution reservoir with clear tubing volume gauge.

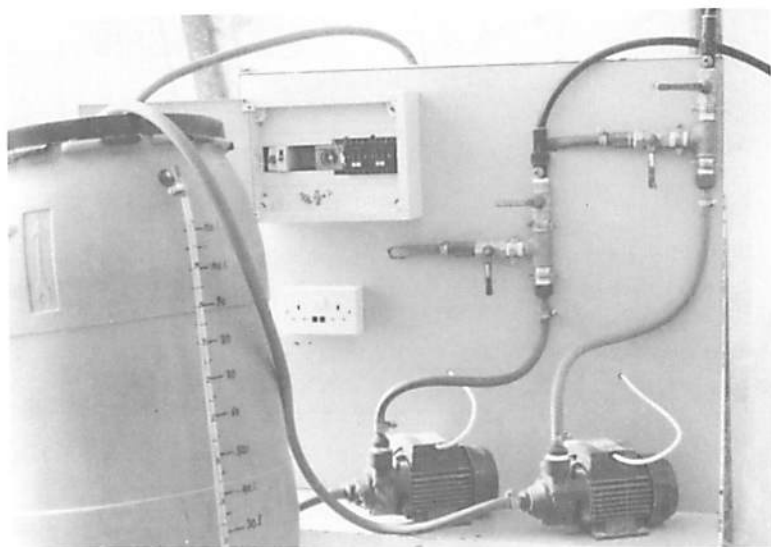


Fig. D.2.15. Pump and valve system for delivering N-free solution to tables containing cups. An automatic timer in the system can activate the pump to run at a fixed time each day.



Fig. D.2.16. Spaghetti-tube tipped with 18 gauge needle for nutrient solution distribution to cups.



Fig. D.2.17. Drilled steel table surface allowing water to drain away from cups and prevent contamination.

N-free nutrient solution in the reservoir must be free from contaminating rhizobia. At ICARDA, an ultraviolet light sterilization unit is used to sterilize the water as it is transferred to the reservoir (Figure D.2.18), but if tap water is free from rhizobia, this step may be omitted. Likewise, tap water may be used instead of distilled or deionized water for making the N-free nutrient solution in the reservoir, if the tap water is relatively free from nitrates ( $< 5$  ppm nitrate-N); N in the nutrient solution will allow non-nodulated plants to grow and will confound results. At ICARDA, tap water from local wells contains between 9 and 15 ppm N, so water is deionized with a reverse-osmosis and deionization unit (Figure D.2.19) before preparing the nutrient solution. The nitrogen-free nutrient solution given in Section A.9.c is recommended, diluted to one-quarter strength if to be used on a daily basis. Use of a full strength nutrient solution during early plant growth may interfere with growth.

This system is complex to set up, but operation requires little labor and a large quantity of evaluations may be done on an indefinite basis. Modification of the cup system is possible, however, to enable operators with varying resources to adapt to local needs and conditions. The automatic watering system can be replaced by hand watering using a large (50–60 ml) syringe (or auto-fill syringe, Figure D.2.20) and eliminating the drain holes. Nutrient solution concentration should be doubled in this case, and pots watered about once per week with care taken not to flood the system or allow the gravel mixture to dry out. Flooding will restrict root (and plant) growth



Fig. D.2.18. Ultraviolet light unit to sterilize deionized water flowing into reservoir.

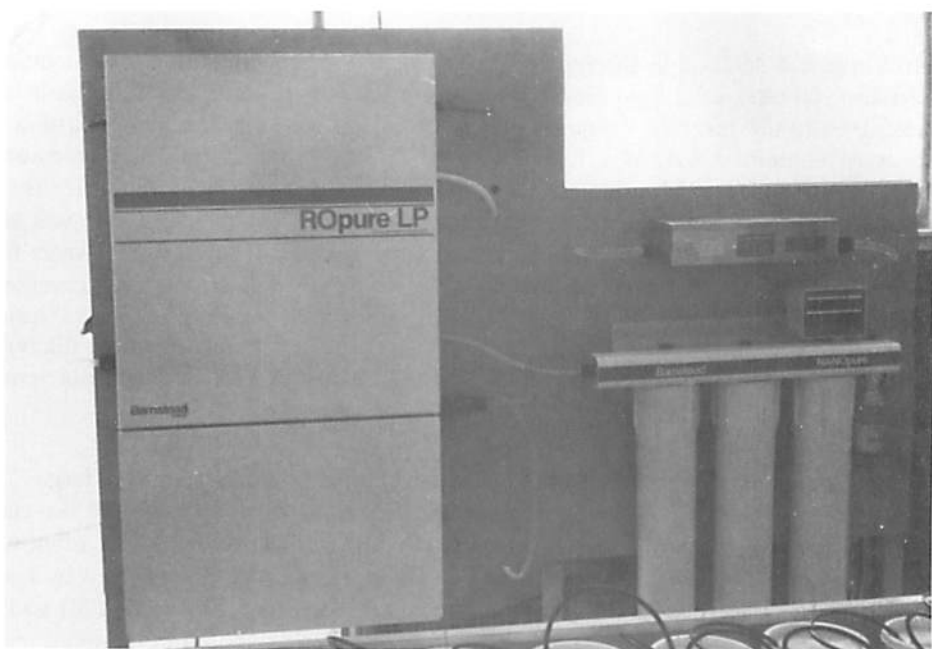


Fig. D.2.19. Reverse osmosis and deionization unit for purifying well water.  
Purified water is passed through the UV sterilizer as a final step.

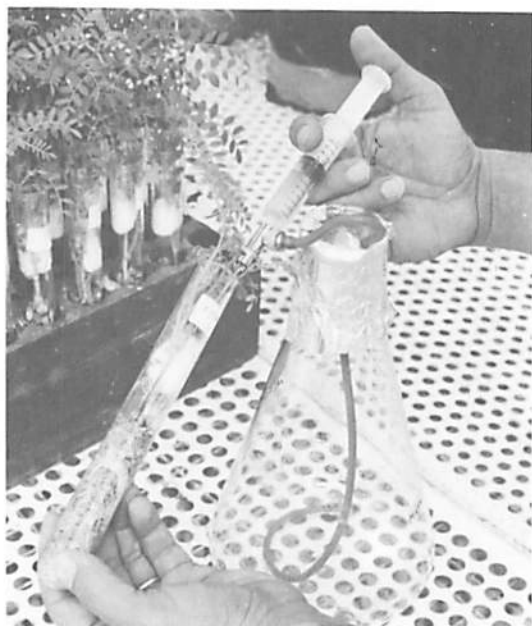


Fig. D.2.20. Auto-fill syringe can also be used to manually water closed cup system or to apply +N treatments to individual cups.

and inhibit nodulation and nodule function; insufficient water (drought) will decrease photosynthesis and plant growth, reducing differences between treatments. Smaller plants are more susceptible to flooding; larger plants more susceptible to drought. Increasing the proportion of vermiculite in this closed system will increase the water-holding and nutrient-supplying capacities of the substrate.

#### **D.2.d. Planting and Inoculation of Seeds, Trial Maintenance**

These strain-evaluation trials are based on the condition that seed of the test host, the substrate, and the containers be completely free of rhizobia, and that the system be maintained in this 'aseptic' condition throughout plant growth. We reiterate that it is therefore necessary to use aseptic techniques in all manipulations, and that solutions as well as all instruments used are sterilized.

Seed scarification, surface sterilization, and planting procedures for the small-seeded legumes are described thoroughly in Section B.3.b. Small-seeded legumes can be grown successfully for strain evaluation in the tube system previously described.

For strain evaluation, the large-seeded legumes should be grown in Leonard jars or the modified cup system described; regardless of the system, seeds must be surface sterilized before germination to eliminate seedborne rhizobia and other microorganisms (especially fungi) which will interfere with germination. Clean, undamaged seeds, selected for uniformity, are dipped momentarily in 95% alcohol and then immersed in 0.1%  $\text{HgCl}_2$  solution for two minutes. They are then washed thoroughly in not less than 10 changes of sterile, distilled water to remove all traces of  $\text{HgCl}_2$  (see figures in Section B.3.b). (Caution:  $\text{HgCl}_2$  is highly poisonous, and all contact with skin, rhizobia, or the growing plant must be avoided.) If any of the  $\text{HgCl}_2$  is left on the seed, the seedling will develop abnormally. The seeds can be left in the final change of sterile water until they are fully imbibed. The seeds are then transferred aseptically with forceps to the surface of a 2% water agar petri dish, where they are spread to give room for root growth. The dishes are then placed into an incubator having a temperature of 25–26°C for 24 hours, after which they are placed inside a large plastic bag containing a few ml water to maintain high humidity. The dishes may be kept inverted so as to obtain seedlings with straight radicles, but this is not necessary.

A pair of sterile, fine forceps works well to first produce a depression in which to place the seedling with radicle undamaged and then to transfer the seedling and cover it. The seedling is buried vertically, if possible with the radicle in the center of the vessel or just below the hole in the cover (for cup system), with the seed approximately 5 mm beneath the surface of the gravel or vermiculite (Figure D.2.21). Tamp the surface of the media lightly after planting to provide a firm seed bed and

uniform emergence of the seedlings. After planting, a 1-ml aliquot of inoculant (usually a 1:100 dilution of a  $10^9$  broth culture to  $10^7$  rhizobia/ml) is applied over each planted seed (Figure D.2.22). This can be done in the greenhouse after arrangement of the pots in a randomized design.

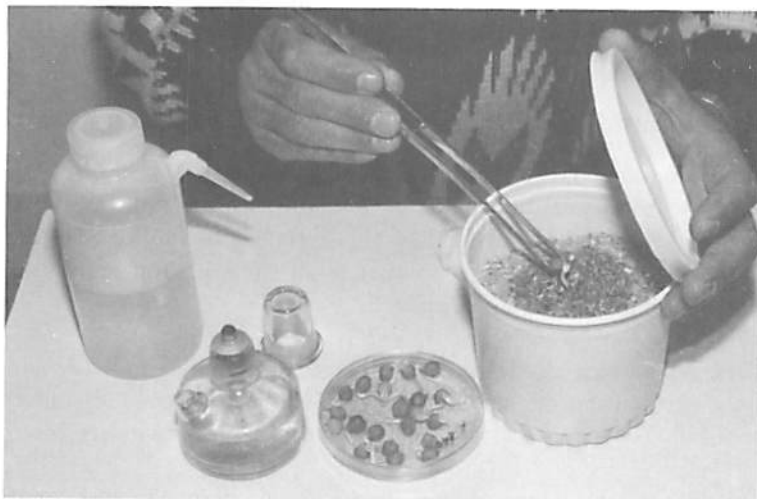


Fig. D.2.21. Planting sterile seedling in gravel of cup system.



Fig. D.2.22. Inoculating planted seedling with a suspension of rhizobia in the greenhouse.

Considerable care must be taken during the early days of plant growth (with the cup system), in that the emerging plant must be guided at the critical time through the hole in the cover (Figure D.2.23). If the growing plant tip is damaged during this operation, or if the growing plant misses the hole and begins to elongate in the cup, plant growth will be retarded and the damaged plant will not be comparable with those having normal growth.



Fig. D.2.23 Guiding emerging seedling through the hole in the cup lid with forceps, taking care to use sterile technique and to avoid damaging growing plant tip.

Vessels are kept in a lightroom or greenhouse for 8–10 weeks after inoculation. As the tips of the growing plants reach the cotton stopper, or hole, a piece of fresh, sterilized cotton wool is carefully (aseptically) wrapped around the plant stem below the top set of leaves (Figure D.2.24) to allow the plant to grow while maintaining sterility in the system. Sterile N-free nutrient solution is added to vessels when the vermiculite appears to dry out slightly (once every four or five days during early growth and more often—daily—as plants increase in size). Care must be taken that plants are not subjected to drought stress and that roots are not flooded. If watering is done through the central hole, care must be taken not to wet the cotton barrier. If the reservoir and central watering system described previously are used, nutrient solution should be added until liquid begins to drain from all pots. This will remove accumulated salts from the system as well as ensure that all pots are maintained at 'field capacity'.



**Fig. D.2.24** Wrapping plant stem with cotton wool to allow plant growth and prevent entrance of contaminating rhizobia.

Generally, plants cannot be grown to maturity in the cup system, but even large-seeded faba bean can be grown for 8–10 weeks with daily watering during the last weeks. Closed assemblies like those described above generally restrict contamination with rhizobia, as long as all manipulations (including watering of plants) are conducted in an aseptic manner.

### **D.2.e. Evaluation of Experiments**

In the nitrogen-free system, total plant nitrogen (as determined by Kjeldahl analysis of plant tissue) is the best measure of rhizobial strain effectiveness, as all N in the plant has been derived from fixation. However, both plant color and plant dry weight are usually significantly correlated with total nitrogen, and because analysis of a large number of plant samples for N is often difficult, these parameters are often used. Developing an arbitrary scale of plant color (e.g., 1–5, with 1 indicating yellow plant, 5 deep green color) allows rapid evaluation of relative effectiveness in greenhouse trials with plants grown under N-free conditions (Figure D.2.25).



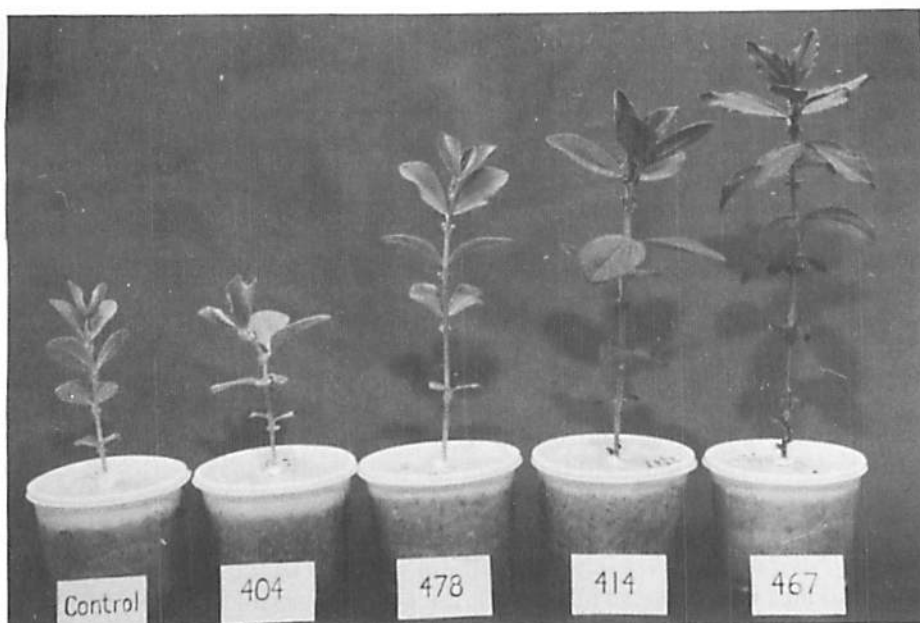


Fig. D.2.25. Symbiotic effectiveness may be ranked on a visual (1-5) scale by plant color.

Adding a treatment with sufficient nitrogen for maximum plant growth allows more accurate relative evaluation of strain effectiveness; the strain can be given a relative effectiveness indicated as a percentage of the nitrogen control. Total N production or dry matter can be used, with strain effectiveness indicated as:

$$\text{strain effectiveness} = \frac{\text{yield strain treatment} - \text{yield uninoculated control}}{\text{yield of N control} - \text{yld uninoculated control}} \times 100$$

Using this method which uses the +/- controls to evaluate strain performance, strains can be compared from one experiment to another. If a nitrogen control is not included, comparison between different experiments is not possible, as environmental factors (e.g., temperature and moisture availability) will vary from experiment to experiment and will not be taken into account. From our work, the quantities of N necessary for maximum plant growth (at 25°C for 6-10 weeks of growth) are:

lentil, 80 ppm N added as 20 ppm at weeks 3,5,6,7 (8-week growth)  
 chickpea, 100 ppm added as 25 ppm at weeks 3,5,7,9 (10-week growth)  
 faba bean, 125 ppm added as 25 ppm at weeks 4,6,7,8,9 (10-week growth)  
 medics/clovers, 70 ppm added per tube at planting.

Inclusion of uninoculated controls will indicate the sterility of the system and provide a measurement of seed N contribution to plant growth. Where nodules form on uninoculated plants the system has been contaminated with rhizobia. In this situation, it is not known whether nodules on plants inoculated with various strains have been formed by the inoculant strain or by the contaminating strain(s), and the results from the trial cannot be accepted. Where uninoculated controls are free from nodules, sterile conditions have been maintained and the trial is valid.

From comparisons with controls, classes of effectiveness can be defined. Symbiotic effectiveness is high if the isolate produces plant yield equal to or greater than N-fertilized plants, moderate SE if slightly less than N controls, and low SE if yield is near but greater than the uninoculated control. Isolates are ineffective if they produce yields similar to uninoculated controls. Each isolate treatment plant dry weight value is compared with those of N controls and the LSD at  $P = 0.05$  level is used to delineate isolates significantly different from the N controls.

With the medics, effectiveness ratings correspond to four categories: category 0 = no nodulation (NN); category 1 = less than 50% of the mass of the nitrogen control (this category indicates ineffective nodulation, I); category 2 (partially effective, PE) = strains equivalent to 50–75% of the mass of the nitrogen control; and category 3 (effective, E) = strains giving 75–100% of maximum plant growth. Uninoculated plants are used as baseline for the zero response level with symbiotic effectiveness indicated as a proportion of growth in nitrogen controls (70 ppm N per tube) or that due to a previously known superior strain of *R. meliloti*.

### D.3. Verification of $N_2$ -Fixing Potential in Nonsterile Soils

Within the region, most soils will contain rhizobia capable of nodulating the legumes that are grown. The exceptions are for those legumes which are highly specific in their rhizobial requirements and have not previously been grown at the site. Common examples are *Medicago* spp. which are not endemic, soybean, and chickpea. Knowledge of the characteristics of rhizobial populations in soils is helpful for the experimenter to assess what influence they may have on procedures and results.

The properties of rhizobia most likely to affect field experiments are the size of the population in the soil and its symbiotic characteristics (e.g., competitiveness, effectiveness, infectiveness). These factors will influence success of inoculation, and will therefore determine the inoculant producer's strategy toward strain selection (and whether there exists a need for inoculation).

### D.3.a. Soil Selection and Collection

Since the size of field populations of rhizobia may vary within short distances in a *field*, much care should be taken in sampling so that a truly representative sample of the field soil is obtained. The area should be mentally divided into equal squares, approximately 10 for each one-fourth hectare, and a subsample taken from each square. A subsample should be roughly cylindrical in shape, 3–5 cm in diameter and be taken from the 5–20 cm depth. A presterilized coring implement (Figure D.3.1) or a long-nosed trowel can be used to extract the subsamples, which are lumped together in a plastic bag. To prevent cross-contamination if another area is to be sampled, a second presterilized sampling tool should be used—or the first one cleaned to remove all soil, washed with 95% alcohol, and sterilized by flaming. Because of rapid changes in the bacterial populations of extracted soils, the soil samples should be tested immediately after collection. If this is not possible, the samples should be stored without drying at 4°C but for no longer than is necessary.

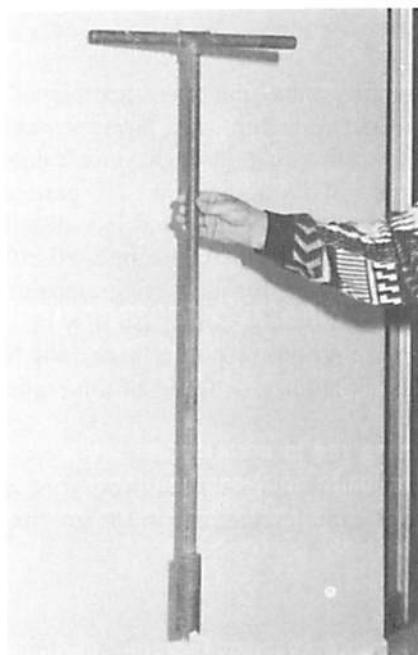


Fig. D.3.1. Boring tool for the extraction of soil samples.

In the laboratory, the lumped subsamples are thoroughly mixed on a clean, sterilized, solid surface; quartered, mixed again, and so on until a small (100–200 g) composite sample, as homogeneous as possible, is obtained. Stones should be removed, but

sieving is not necessary and may remove bits of organic matter in which colonies of rhizobia reside.

### **D.3.b. Intact Soil Core Methodology**

The soil core for use in "pot" experiments is a physically and microbiologically intact sample of a field soil. If it is not exposed to excessive heat or drying following collection, it is a truly representative piece of the field. All conditions equal those found in the field: nutrient content, soil structure, and microbial populations.

The cylinder which forms the container of the soil core can be of any available material; we have found heavy-duty PVC plastic pipe, such as is used for drainage (pressure-type), to work the best. The pipe is sharpened on one end (outer edge) to allow easier penetration into the soil. For large-seeded legumes, the 6 inch (15 cm) inside diameter is best; smaller pipe of 4 inch (10 cm) diameter can be used with small-seeded legumes if desired. The smaller the core diameter, the easier it is to insert into the soil.

These soil cores may be used for many purposes, for they allow on-station testing of soils from field sites chosen for evaluation (i.e., farmers' fields), eliminating problems often encountered in on-site testing (e.g., drought, insect damage, and harvest difficulties). For microbiology (BNF) studies, they are particularly appropriate, and are used to investigate both need-to-inoculate and inoculum response, with treatments as suggested in Sections F and G. They may also be used effectively for rhizobia competition studies, if a methodology for identifying inoculum strains from native population strains is known (Section C). Cores also may be effectively used in plant nutrition studies to determine nutrient response (especially N, as in disturbed soil samples, commonly used in pot studies, a "flush" of mineralized N is made plant-available).

The following instructions show step-by-step the process of collecting cores from the field, and preparing them for experimental use in the greenhouse.

#### **Field Soil Core Collection**

1. Sites for collection should be chosen carefully, so that they represent a region similar in farming practice, soil type, etc. Choose representative locations in the field site chosen, with at least four widely separated sampling locations per site if possible. For example, collect 10 cores from each of four sites in a field for a trial requiring 40 cores. Collect one or two cores more than are required.
2. Clear the surface 1 cm only of rock, weeds, and debris; use the sharp edge of a shovel, hoe, or spade (Figure D.3.2). Care should be taken not to disrupt the soil structure.



Fig. D.3.2. Clear the soil surface of rock, plants and debris before inserting cores.

3. Place the pipe sections in a line, with the sharpened edge down (Figure D.3.3). Pipe sections should be separated by only 3–5 cm.
4. The necessary tools (pictured in Figure D.3.4) include: (a) shovel; (b) sledge hammer; (c) impact cap with handle; (d) pipe disk (metal or plastic disk of diameter to fit snugly inside pipe section, with handle); (e) box for core transport; and (f) removal shovel (hand shovel to slip underneath core to facilitate removal).
5. Using impact cap and heavy sledge hammer, pound the cylinder to within 2–3 cm of soil surface (Figure D.3.5). Hammer blows must be evenly placed to push pipe section into soil vertically; if section enters soil at an angle, it will usually break.
6. After a line of pipe sections have been pounded into the soil, dig a trench using a mattock or pick along one side of the line of cores for easy removal (Figure D.3.6).
7. Place the pipe disk in each core before removing it from the ground (Figure D.3.7). This will prevent loss of surface soil when the core is inverted.
8. If the soil is dry and loose (such as in sandy soil) use the removal shovel, worked into place at the bottom of the core, to prevent soil from falling out as the core is removed (Figure D.3.8).



Fig. D.3.3. Outer edge of pipe section is sharpened to prevent soil compaction.



Fig. D.3.4. Tools for soil core collection including: A) shovel;  
B) sledge hammer; C) impact cap with handle; D) pipe disk;  
E) removal shovel, and F) box for core transport.



**Fig. D.3.5. Pounding core cylinder into soil using an impact cap and a heavy sledge hammer. Two persons are required as cap must be held tightly on core so that the cylinder enters the soil vertically.**



**Fig. D.3.6. Digging trench along the line of inserted soil cores for easy removal of cylinders.**



Fig. D.3.7. Placing the pipe disk over the surface soil in each core before removing from the ground.



Fig. D.3.8. Removing soil core from moist soil by breaking core off into trench. In sandy or dry soil, the removal shovel (shown) is slipped carefully under the bottom of the core (to prevent soil loss from core) and the whole assembly is removed from the ground.



9. Carefully break the core from the ground into the trench and, holding the pipe disk firmly in place, invert the core so that the bottom is facing upwards (Figure D.3.9). Trim away excess soil at the base of the core to form a flat surface (the removal shovel works well for this).



Fig. D.3.9. Trimming loose soil from bottom of soil core so that completed assembly will sit flat on table top.

10. Holding the core inverted, apply a tight-fitting plastic bag over the bottom of the core, and work the bag down to cover core (Figure D.3.10). It is necessary that this bag fit as tightly as possible to prevent soil loss from core when it is placed upright.
11. Tape is applied tightly around the bottom rim of core over the bag to minimize soil loss from bottom of core (Figure D.3.11). Holes in bottom of bag (made before going to the field with a paper punch) will allow drainage when watering cores later. Turn cores upright.
12. Carefully label each core on tape for date of collection, site, and any other necessary details (Figure D.3.12). Close top of plastic bag and transport cores to greenhouse or screenhouse, using a box strong enough to be carried with cores inside. Prevent cores from sitting in the sun for any length of time, as heat will kill soil bacteria (including rhizobia).



Fig. D.3.10. Applying a tight-fitting plastic bag over the bottom of the core.



Fig. D.3.11. Duct tape is applied tightly around bottom of core to prevent loss of soil when core is set upright.



Fig. D.3.12. Labelling core with location and date of collection.

### D.3.c. Core Preparation, Planting, Inoculation, and Maintenance

Soil-core experiments may be conducted in a greenhouse or screenhouse with temperatures controlled between 15 and 30°C if possible. Once the following preparations are complete, the core is a closed system with limited possibility of contamination by rhizobia, so experiments may be conducted in nonsterile environments where light is sufficient and temperature moderated from extremes.

1. After transfer of cores to greenhouse or screenhouse, fold down plastic bag top to leave core open. Completely randomize cores from a particular field on a flat surface (bench top or floor). It is **very important** that water draining from cores cannot flow on the bench surface from one core to another, as in this way different treatments will contaminate one another. It is best to use a drilled steel or wire mesh surface, suspended so that all drain water moves directly away from cores (Figure D.3.13). Alternatively, suspending each core on blocks above the bench top or floor is adequate.
2. Flood the core with clean, sterile, distilled water sufficient to cause drainage from holes in bottom.
3. Let water contents of the cores equilibrate for two or three days to achieve the field capacity water content.



Fig. D.3.13. Drilled steel table surface to prevent cross-contamination between pots or cores.

4. Weigh the core to determine the initial field capacity weight (Figure D.3.14). This weight, plus later additions to the core assembly, will be the weight near which the individual assembly will be maintained during plant growth. In this way, differences due to plant growth and variable water uptake in different treatments will be minimized.
5. Add any nutrient treatments (e.g., nitrogen treatment) in solution before planting, including basal applications of phosphate or potash if needed (see calculations later in this Section for amounts).
6. Surface sterilize and pregerminate seeds as given in Section D.2.d. Using a glass rod or stick, make holes in soil surface and plant evenly spaced seedlings near center of pot (eight medic, five lentil, three chickpea/vetch/lathyrus, two faba bean). Inoculated treatments are given 1 ml of a  $10^7$  fresh rhizobial suspension (fresh  $10^9$  broth culture diluted 1:100 in sterile tap water), pipetted directly onto root at planting (Figure D.3.15). Cover seeds carefully with soil. Add sterile water to soil surface as needed for early seedling growth.
7. When plants reach 2–4 cm height, thin to: six medic, four lentil, two chickpea/lathyrus/vetch, one faba bean plant per core, carefully leaving plants of equal vigor for further growth (Figure D.3.16).

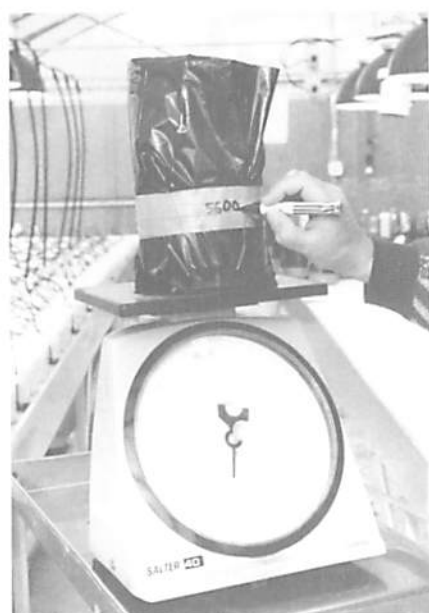


Fig. D.3.14. Weighing soil core after water equilibration to determine field capacity weight.



Fig. D.3.15. Inoculating directly onto the seedling root with a liquid suspension of rhizobia.



Fig. D.3.16 Thinning legume plants for uniformity.

8. Install the irrigation tube (2 mm PVC, 5 cm diameter  $\times$  25 cm length, with small holes drilled on one side on bottom 5 cm length) against one side of the core, with holes toward center of the core (Figure D.3.17).



Fig. D.3.17. Irrigation tube for even distribution of water in soil core.  
Note that drainage holes are placed only on side of tube pointing toward core center, to prevent direct water loss down the side of the core.

9. Gently pound the tube into the soil to a depth of about 8 cm (Figure D.3.18).

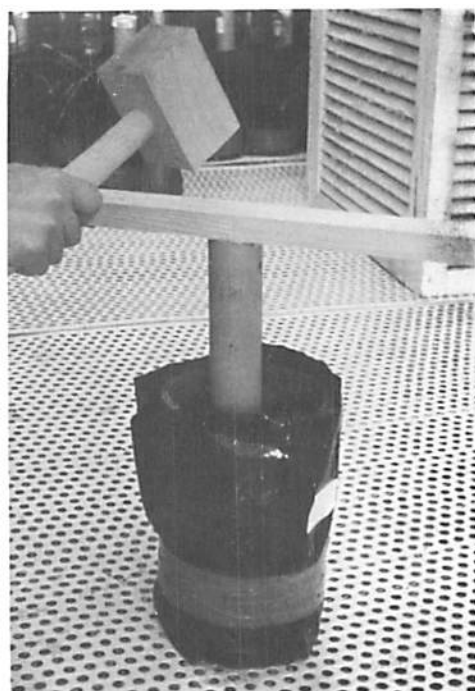


Fig. D.3.18 Insertion of the irrigation tube into core soil using wooden mallet. Tube is inserted to a depth of approximately 8 cm.

10. Add gravel (which has been heat-sterilized at 200°C - see Section D.2.b.) to the soil surface to prevent contamination by rhizobia from the air. Entire surface should be covered by at least 1 cm of gravel (Figure D.3.19).
11. Cover irrigation tube with aluminum foil cap, or other suitable cover (Figure D.3.20).
12. Label core with treatment, location where collected, field capacity weight (which now includes weights of gravel and irrigation tube with previous field capacity weight), planting date, and replication number (Figure D.3.21).
13. During active plant growth, add 50 ml distilled, sterilized water to each core daily, or as needed. Once per week (or more if necessary), the core should be weighed and water added up to the field capacity weight (Figure D.3.22). Always add water through the irrigation tube, not on the gravel surface. Any water applied to the surface of the gravel (including splash from other pots) will wash contaminants into the soil core.



Fig. D3.19. Covering surface of core with heat-sterilized gravel to prevent surface contamination.

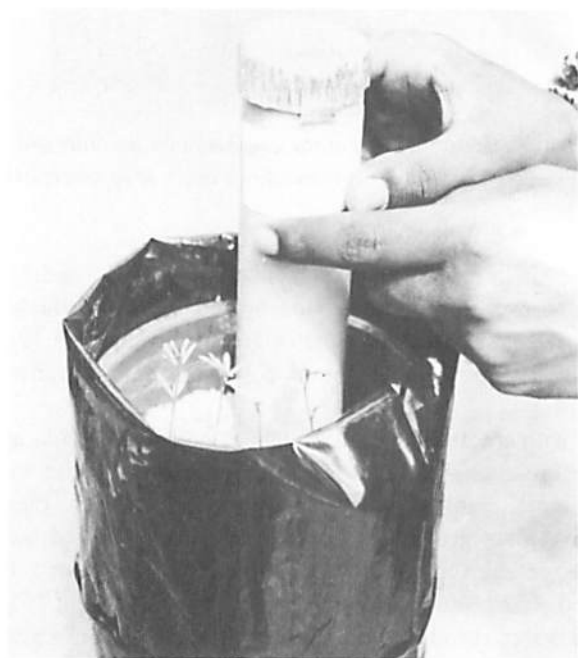


Fig. D3.20. Completed core assembly with watering tube and cover, label, and sterile gravel.



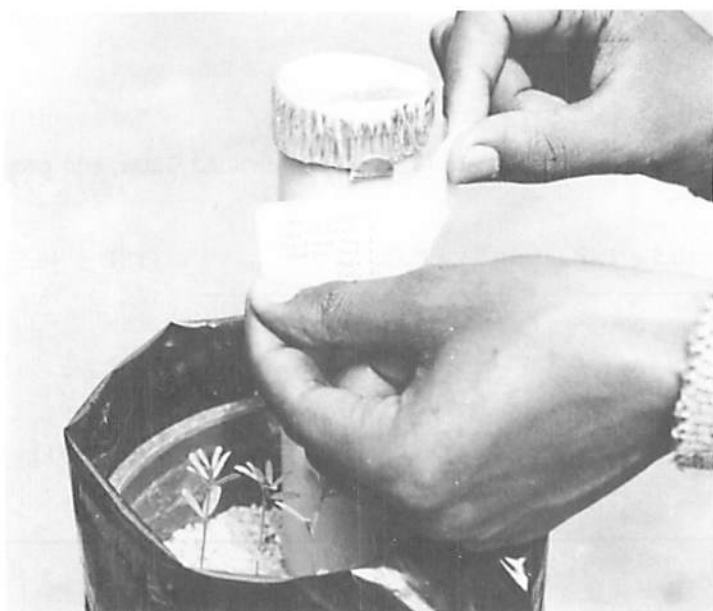


Fig. D.3.21. Labels placed on watering tubes are easy to see.



Fig. D.3.22. Cores are watered periodically to the field capacity weight indicated on the label to provide equal water availability to all treatments.

## Fertilization Calculations

### Phosphorus

Dissolve 10 g triple superphosphate (TSP) in 1 L distilled water, add preplanting 50 ml per core.

$$\frac{10 \text{ g TSP}}{1000 \text{ ml}}; \quad \frac{0.5 \text{ g TSP}}{\text{pot}} = \frac{0.23 \text{ g P}_2\text{O}_5}{\text{pot (approximately 6 kg soil/core)}} \quad (\text{TSP} = 46.5\% \text{ P}_2\text{O}_5)$$

$$\frac{0.23 \text{ g P}_2\text{O}_5}{6 \text{ kg soil}} = \frac{39 \text{ mg P}_2\text{O}_5}{\text{kg soil}} = 39 \text{ ppm P}_2\text{O}_5 = \text{approximately } 100 \text{ kg P}_2\text{O}_5 \text{ per ha}^*$$

\* Given

$$1 \text{ ppm} = \frac{1 \text{ kg fertilizer}}{10^6 \text{ kg soil}}; \text{ then } \frac{? \text{ kg}}{\text{hectare}} = \frac{? \text{ kg}}{\frac{18 \text{ cm} \times 10^4 \text{ cm}^2 \times 1.4 \text{ g/cm}^3}{(\text{depth}) (\text{area}) (\text{soil density})}}$$

$$1 \text{ ppm} = \frac{\text{kg}}{2500000 \text{ kg/ha}} = \frac{2.5}{\text{hectare}}$$

To convert ppm to kg per hectare, multiply ppm by 2.5.

### Nitrogen

Dissolve 3 g urea in 500 ml distilled water, add preplanting 50 ml per core and 50 ml at four to five weeks after planting.

$$\frac{3.0 \text{ g urea}}{500 \text{ ml}} \times \frac{100 \text{ ml}}{\text{pot}} = \frac{0.6 \text{ g urea}}{\text{pot}} = \frac{0.3 \text{ g N}}{\text{pot}} \quad (\text{urea} = 48\% \text{ N})$$

$$\frac{0.3 \text{ g N}}{6 \text{ kg soil}} = \frac{50 \text{ mg N}}{\text{kg soil}} = 50 \text{ ppm} = 125 \text{ kg N/ha (ppm} \times 2.5\text{)}.$$

### **D.3.d. Observations and Evaluation of Experiments**

In all strain-evaluation studies, the objective is to determine the symbiotic effectiveness of the strain(s) concerned, compared with a control which is standard between similar experiments. Nodulation is often correlated with symbiotic effectiveness; the most common observations are nodule number, nodule color, and nodule dry weight. Nodule number and color indicate the infectiveness and, to some extent, the effectiveness (red color = effective, green to white = ineffective). Nodule dry mass is probably the best consistent measurement to score nodulation, with observation of the color recorded to indicate whether the nodules appear to be effective.

Plant dry weights should always be recorded, as this is the simplest measurement to evaluate effectiveness and will generally indicate the strain's performance compared with uninoculated and N-fertilized controls. The best measurement of  $N_2$  fixation effectiveness will be plant N content; dried plant material is ground and analyzed for %N using the Kjeldahl digestion method, with plant dry weight  $\times$  %N giving a quantity of total N in the plant. This parameter will consistently give the best indication of  $N_2$ -fixing effectiveness.

Statistics must be used to determine where significant differences between strains exist; a detailed description of the applicable statistics is given in Section F.8.

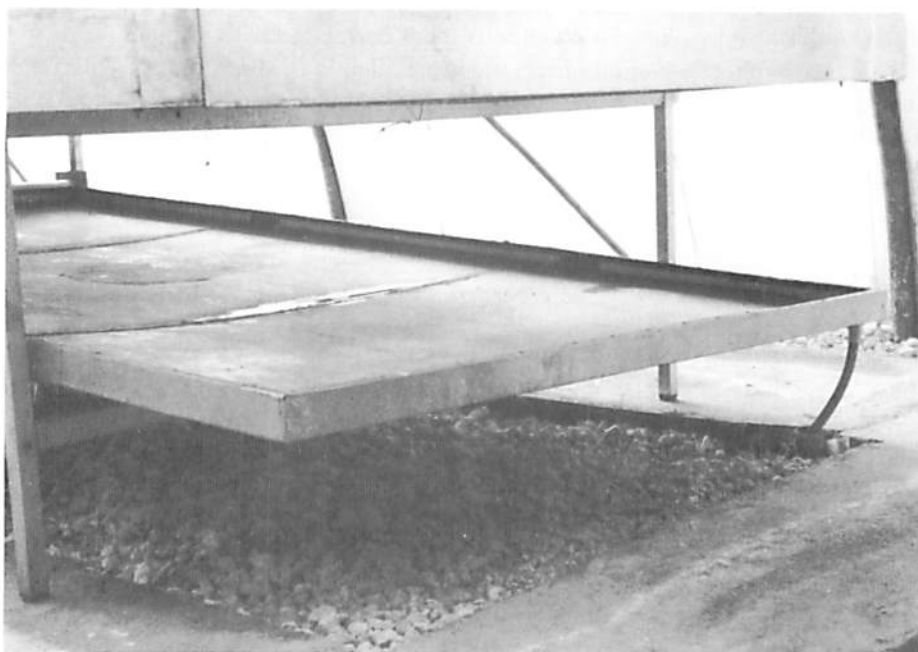
## **D.4. Controlled Environment Systems**

### **Greenhouses**

The most important considerations in operation of any greenhouse are the maintenance of cleanliness (hygiene) and provision of suitable light and temperatures. Cleanliness is achieved through regular maintenance and good operational procedures, while temperature and light control are generally a function of greenhouse design and local conditions.

Hygiene must be practiced at two levels. The amount of airborne contaminating rhizobia must be kept to a minimum. This can be achieved by minimizing drainage from pots, and by having a floor that can be washed on a daily basis (e.g., cement). Drainage saucers under pots should be avoided as they provide a constant source of contamination. Runoff from draining pots should be minimized by using nondraining systems or by watering carefully; if possible, water draining from pots should be caught in trays with drainage outside the greenhouse (Figure D.4.1). All parts of the greenhouse should be kept as clean as possible. No work with soil (other than that

already in pots), filling of pots, or harvesting should take place inside the house, and the number of personnel entering the facility should be limited only to those necessary.



**Fig. D.4.1. Trays under drilled steel table tops in greenhouse to catch drainage from cup system. Drainage is channeled out of greenhouse to reduce overall contamination levels in the experimental area.**

The largest benefit of greenhouses over growth chambers is the quantity and spectrum of light. If coverings and shading are of the proper type, no limitations for plant growth with respect to light will be encountered (the exception being photoperiod requirements of legumes for some experiments). In the Mediterranean region, temperatures and light intensity during the summer months will be high, and some type of shading is generally used. The most inexpensive is whitewash — a mixture of lime ( $\text{CaCO}_3$ ) and vegetable oil — which is brushed on the outer surface of the greenhouse as light intensity and temperatures increase (usually May) and washed off in September when temperatures begin to drop. Of the commercial shading materials available, we have found the 'polyester foil strip' shading (Ludvig Svensson Int'l BV, Marconiweg 2, 3225 LV Hellevoetsluis, Netherlands) with a light interception of 30% to be suitable for local conditions. This material has a three- to five-year life and reflects solar radiation, reducing temperatures in the greenhouse more effectively than

other 'interception'-type materials such as dark-colored netting.

Both nodule formation and the level of nitrogen fixation are influenced strongly by temperature, especially temperature in the root zone. Some temperate legume species are more tolerant of temperature extremes, but in general good experimental results can be obtained with temperatures between 15° and 28°C. Above 30°C, both nodule formation and function rapidly decrease. For greenhouse studies, black or dark pots should be avoided, as the temperatures in these pots may be up to 5°C higher than ambient. Evaporative cooling is effective in many parts of the region, except in coastal regions or where relative humidity is high during summer months. If relative humidity is low (< 40%) but the evaporative cooling system is unable to bring temperatures to the desired level, increasing the pad size and/or fan capacity may improve efficiency.

### Growth Chambers

Controlled environment cabinets, or growth chambers, have both advantages and disadvantages. Light and temperature are easily controlled, and it is possible to repeat the same conditions for different experiments. The most important consideration is that the light source should be adequate in terms of quality and quantity. Unfortunately, light intensity provided by most units is relatively low in relation to field conditions (< 40%);  $8-10 \times 10^3$  lux ( $80-100 \mu\text{E m}^{-2} \text{ sec}^{-1}$ ) is the minimum which should be uniformly available in the experimental area. Spacing between plants may have to be increased where light intensity is on the low side of this figure. Some legumes are particularly sensitive to spectral composition, and both nodule formation and plant growth are affected by far-red wavelengths. The inclusion of incandescent light bulbs or grow-lux fluorescents in the light bank will overcome the unbalanced spectrum of normal fluorescent tubes.

A wide range of commercial growth cabinets is available, and details should be obtained from suppliers. Because of the high cost of commercial cabinets (U.S. \$15,000–\$20,000), many experimenters fabricate their own growth chambers, using an air conditioner and fluorescent tubes in a small, closed room. Ballasts for fluorescent tubes (which produce 65% of the light system's heat) should be located outside of the growth room itself to decrease the heat load. Close spacing of fluorescent tubes, and mixing of regular high-intensity fluorescent tubes, 'grow-lux' tubes, and incandescent lights will provide adequate light intensity and quality. Wise use of reflectors (aluminum foil) will maximize light available to plants.

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## **E. Inoculum Production and Use**

In a strict microbiological sense, an inoculant is anything that contains bacteria and that can be used to infect (or inoculate) an environment previously free of that bacterium. Thus, for example, a loopful of culture used to inoculate a flask of broth can be termed an inoculant. In this section, we define a legume inoculant as a preparation of live rhizobia designed for application to leguminous seeds or the soil to ensure abundant and effective nodulation of the legume seedlings.

The application of inoculum has become an increasingly common practice since the discovery in 1887 that nodules, formed by *Rhizobium* bacteria, were required for nitrogen fixation. The science has evolved from a time in which soil where legumes were grown was used as an inoculant, to the current practice of inoculant production involving selection of superior rhizobia strains, production of dense populations of the strains in broth culture, and incorporation of high numbers of the bacteria into a carrier material designed to maintain the living organisms until they are added to soil.

Use of inoculants is usually at the heart of any program of improvement in biological nitrogen fixation. Commercial production of inoculants is limited in the West Asia-North Africa region, and importation of inoculants from developed countries is restricted because of expense and deterioration (often due to exposure to high temperatures) during shipping and customs clearance. In this section, we present simple but detailed methodologies for production of quality inoculants with scale ranging from production for experimental use in field trials to small-scale commercial production.

Regardless of scale, techniques of preparation are basically the same; the fundamental methodologies have not been modified in the last 20 years. Scale of production will relate directly to the perceived demand for inoculants (i.e., the potential market, related to the necessity for inoculation), and availability of facilities, human resources, funds, and suitable raw materials. The production of high-quality inoculants involves the selection and maintenance of appropriate strains (discussed in Sections D and A, respectively), suitable carrier selection and preparation, broth culture production, inoculant mixing, curing, and packaging. Quality control practices are necessary to ensure consistent production of high-quality inoculants.

### **E.1. Required Facilities**

Trained, qualified personnel are the primary requirement. In the best case, the laboratory will be supervised by a professional microbiologist with at least one technically trained assistant.

Requirements regarding main equipment and laboratory facilities are fairly constant; larger-scale production will, of course, require larger facilities. For the physical plant itself, two separate sections are generally needed. The first contains a laboratory, transfer room, and fermentor room, all kept clean and as sterile as possible. In the second, carrier preparation, mixing, curing and packaging are performed, making this the 'dirty' section.

The laboratory should be designed for microbiological work; it is here that the research and quality control functions are performed. Laboratory bench space, gas outlets, sinks, refrigerator, microscope, shaker, incubator, large autoclave and storage facilities are essential equipment. Especially important is to provide an area in which the transfer of cultures between tubes and flasks, plating, and general manipulation of pure cultures can be performed with a minimum chance of contamination. This is essential not only for production, but in maintenance of a culture collection of *Rhizobium*. A laminar-flow bench, which provides a constant flow of sterile air over the working area, is the best location to perform all transfers. A clean bench (described in Section A.8) is adequate, but a laminar-flow bench is necessary for larger-scale work. A separate transfer room to house the laminar-flow bench is even better, so that the entire area can be kept aseptic. The transfer room should have no windows (unless completely sealed), and there should be only one door from laboratory to the transfer room (Figure E.1.1).

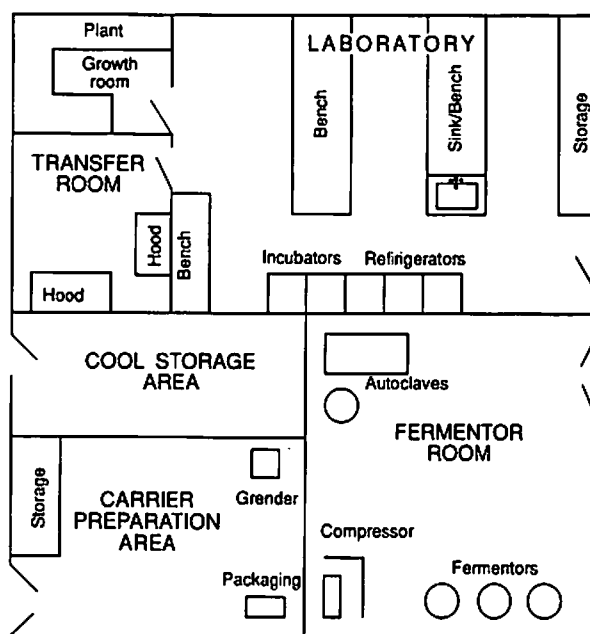


Fig. E.1.1. General layout of inoculant production facility.



The fermentor room can be a part of the laboratory in small-scale production, where the growth vessels (fermentors) are usually glass flasks. With larger stainless steel vessels, particularly if they are self-contained and need to be heated for sterilization, a separate room with good ventilation may be required (Figure E.1.1). The large autoclave and possibly the air compressor (where required) may also be kept in this room.

Carrier preparation, which involves grinding and sieving of the material as well as mixing with the culture, packaging and curing, should be performed in a separate 'dirty' room (Figure E.1.1). This is especially important where nonsterile carriers are utilized; if presterilized carriers are used, this room is used to fill and seal the bags, and broth culture is aseptically transferred to the sterilized bags in a clean area. This carrier preparation room should be well lit and ventilated, with sufficient area for the various operations. This room may also include storage facilities, if moderately low temperatures ( $< 28^{\circ}\text{C}$ ) can be maintained.

Storage of the finished inoculants (following curing) will ideally be under refrigeration, at  $6-8^{\circ}\text{C}$ ; this will allow production of inoculants up to six months before use. If refrigeration is not available, a basement or air-conditioned room with cool temperatures ( $18-25^{\circ}\text{C}$ ) is adequate. As most inoculants are needed during the period October–December in the region, production will take place during the hot months of August–September, so especially designed cool storage should be considered.

## **E.2. Broth Culture Production**

Growth of rhizobia in liquid medium is a standard laboratory practice, and will only require a change of scale to be used for commercial production. Rhizobia are relatively easy to grow, but are not competitive with other microorganisms so will grow poorly if not in pure culture. Therefore, complete sterilization of the growth vessel and the medium must be accomplished before adding the 'starter' rhizobia culture. The process of inoculation of the fermentor with the starter culture is a common source of contamination (introduction of foreign bacteria), and special precautions must be taken to ensure sterile conditions during this procedure. The only requirements for growth are proper temperature, adequate nutrients and an oxygen source. These are discussed below.

### **E.2.a. Broth Media**

The culture medium, rhizobial strain, temperature and aeration are the main factors that affect population density of fermentor-grown rhizobia. Rhizobia are aerobic

bacteria, with a generation time of 2-8 hours. They will grow on the surface of solid media and also in still-liquid media if the surface area is large. Growth in submerged culture in fermentors with aeration is necessary for maximum viable cell production. The aeration requirements of rhizobia are only 5–10 L of air per liter of medium per hour. Optimum growth occurs at 28–30°C. It is not difficult in the laboratory to provide conditions suitable for the production of a reasonably dense population of rhizobia ( $> 1 \times 10^8$  viable bacteria per ml) for most common legumes.

The ingredients used in the formulation of the liquid culture medium must be cheap and available in order to produce inoculants with cost efficiency, but it should be kept in mind that the nutritional requirements of the selected strains may vary. In general the liquid media consist of a suitable carbohydrate, a nitrogen source, micronutrients, and a source of vitamins and minerals. Sucrose is the most commonly used carbon source because it is universally available, satisfactory, and inexpensive. At ICARDA, we prefer to use mannitol, as it has given best results with the strains we use. The slow-growing rhizobia are reported to prefer pentose sugars, such as arabinose. In some laboratories a mixture of mannitol and sucrose is used to provide a greater concentration of cells than that obtained with a single carbohydrate source. Some industrial by-products, such as corn steep liquor, proteolyzed pea husks, malt sprout extract, and unsupplemented whey (by-product of the cheese industry) have been used successfully to grow rhizobia (see suggested reading section).

It is important to utilize the media that will give the maximum number of rhizobia per volume of media. Since rhizobia strains within a species may vary in their abilities to utilize different carbohydrates it is desirable that selected strains for inoculants be evaluated for maximum utilization of the sugar or carbohydrate in the fermentor medium.

Yeast extract is the most commonly used growth factor supplement for rhizobia, but may be unavailable or expensive if imported. Alternatively, yeast water can be made cheaply from locally obtainable materials. Fresh starch-free cakes of baker's yeast are preferred in making yeast water. Suspend 100 g of yeast in 1 L of water and boil slowly or steam for three to four hours, replacing the lost water regularly. Allow the cooked suspension to stand until yeast cells have settled to the bottom, usually 10–12 hours. Pour off the clear, straw-colored liquid; adjust the liquid to pH 6.6–6.8 with 1 N sodium hydroxide; bottle and autoclave for 30–40 minutes at 121°C. Following sterilization, the yeast water can be stored at room temperature until needed. Dried yeast can be substituted in the above preparation for yeast cake; 0.5 kg of dried yeast is equal to about 1 kg yeast cake, so 50 g of dried yeast is sufficient to prepare 1 L of yeast water. Broth medium containing the yeast preparations may foam excessively when it is aerated in fermentor vessels. Foaming can be controlled by adding a small amount (5 ml/L) of sterile clear mineral oil with the yeast.

In commercial production the most inexpensive but effective ingredients are used since the economics of production must be considered. Most often, the chosen formula will depend on locally available materials, but this medium should be tested to be sure that it will support growth of high numbers of the particular rhizobia strains to be used. Some commonly used formulations which have been successfully used in culturing rhizobia are given in Table E.2.1. Two of the media contain mannitol only, one contains sucrose only and another contains both sucrose and mannitol as carbon source. The micronutrient stock solution indicated in the Burton recipe is given in Table E.2.2. Micronutrients are essential for N<sub>2</sub> fixation by the legume, but may not be required for growth of rhizobia.

A concentrate of the medium is prepared by dissolving the ingredients first in warm water and straining before adding to the fermentation vessel. The water used should be potable. Reagent grade chemicals are not necessary; chemicals of the USP grade are satisfactory and much cheaper.

**Table E.2.1. Composition of media for growth of rhizobia.**

| Ingredient                                                    | Waksman<br>1928 | VanSchreven<br>1963 | Date<br>1976 | Fred and<br>Burton<br>1967 |
|---------------------------------------------------------------|-----------------|---------------------|--------------|----------------------------|
| grams per liter                                               |                 |                     |              |                            |
| Mannitol                                                      | 10.0            | --                  | 10.0         | 2.0                        |
| Sucrose                                                       | --              | 15.0                | --           | 10.0                       |
| Dipotassium phosphate<br>(K <sub>2</sub> HPO <sub>4</sub> )   | 0.5             | 0.5                 | 0.5          | --                         |
| Tripotassium phosphate<br>(K <sub>3</sub> PO <sub>4</sub> )   | --              | --                  | --           | 0.2                        |
| Monopotassium phosphate<br>(KH <sub>2</sub> PO <sub>4</sub> ) | --              | --                  | --           | 0.4                        |
| Magnesium sulfate<br>(MgSO <sub>4</sub> ·7H <sub>2</sub> O)   | 0.2             | 0.2                 | 0.2          | 0.2                        |
| Sodium chloride<br>(NaCl)                                     | 0.1             | --                  | 0.2          | 0.06                       |
| Calcium carbonate<br>(CaCO <sub>3</sub> )                     | 3.0             | 2.0                 | --           | 0.2                        |
| Calcium sulphate<br>(CaSO <sub>4</sub> ·2H <sub>2</sub> O)    | --              | --                  | --           | 0.04                       |
| Iron chloride<br>(FeCl <sub>3</sub> ·6H <sub>2</sub> O)       | --              | --                  | 0.1          | --                         |
| Yeast water                                                   | 100.0           | 100.0               | 100.0        | --                         |
| Yeast extract                                                 | --              | --                  | --           | 0.5                        |
| Paraffin oil                                                  | --              | 0.5                 | --           | --                         |
| Ammonium phosphate<br>(NH <sub>4</sub> ) <sub>2</sub> HPO     | --              | --                  | --           | 0.1                        |
| Water                                                         | 900             | 900                 | 900          | 1000                       |
| Micronutrient solution*                                       | --              | --                  | --           | 0.3                        |

\* See special nutrient solution - Burton, Table E.2.2.

**Table E.2.2. Micronutrient stock solution (after Burton).**

| Ingredient                                                   | Quantity for 1 liter |
|--------------------------------------------------------------|----------------------|
| Boric Acid, $\text{H}_3\text{BO}_3$                          | 2.780 g              |
| Manganese sulfate, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ | 1.540 g              |
| Zinc sulfate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$      | 0.210 g              |
| Sodium molybdate, $\text{Na}_2\text{MoO}_4$                  | 4.360 g              |
| Ferric chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$   | 5.000 g              |
| Cobalt sulfate                                               | 0.004 g              |
| Lactic acid (88%)                                            | 580.0 ml             |
| Distilled water                                              | 420.0 ml             |

- Addition of 1.0 ml per liter of final medium gives: boron 0.5 mg; manganese 0.5 mg; zinc 0.05 mg; molybdenum 1.0 mg; iron 100 mg and cobalt 0.0005 mg per liter or parts per million (ppm).

### **E.2.b. Equipment Needed for Broth Culture Production**

In practice, the growth of a pure broth culture of *Rhizobium* requires strict aseptic control in a vigorously aerated culture vessel, termed here a fermentor. Fermentors of any capacity used in the mass culture of rhizobia must be capable of being sterilized, have a supply of sterile air (with the exception of small units on a shaker), aseptic air exhaust unit, separate inoculation and sampling ports, facilities for aeration/agitation, and a culture discharge device.

Two types of fermentors are discussed in this section. The most simple is an autoclavable vessel of glass or stainless steel, usually of 2–15 L capacity, which is filled one-third to two-thirds full of media and sterilized in an autoclave at 121°C. The other type is a stainless steel vessel, capable of withstanding 30 psi internal pressure, which is filled (one-half to three-fourths full) with media then heated from an external source until temperature of the media reaches 121°C. The latter type is generally used in commercial inoculant production, and the former in laboratory production of inoculants, although in Australia the autoclavable vessel is used for commercial production.

One of the first steps in preparing for legume inoculant production is to estimate as accurately as possible the kinds and amounts of various inoculants which will be needed during each planting season. Consideration must be given to the number of rhizobia strains which will be used in each inoculant and the number of legume crops to be inoculated. The equipment and schedule chosen should allow flexibility and production of 130–150% of the anticipated needs because actual production will be less owing to unavoidable contamination of some batches. With well-designed equipment and good techniques, it is not unusual to obtain 90% of theoretical production. In planning an inoculant production facility, it should be remembered that small culture vessels are always needed regardless of the production capacity required. The small fermentors are needed to grow starter cultures for the larger fermentors as well as for inocula required in small amounts. Also, with sufficient small fermentors, a larger fermentor may not be required, or production capacity of the large fermentor can be greatly increased by using larger starter (5–10%) volumes to shorten the final multiplication phase.

Use of compressed air is most common in providing oxygen for growth. Small aquarium pumps, usually available on the local market, work well to aerate 10–15 L of media (Figure E.2.1); an air compressor may be used where requirements are larger (Figure E.2.2). Growth is not increased by violent aeration or agitation. The low oxygen requirement of rhizobia is undoubtedly associated with the organism's ability to grow in the interior of an active nodule where a low oxygen tension exists.



Fig. E.2.1. About 15 L of liquid media can be aerated by a small aquarium pump.

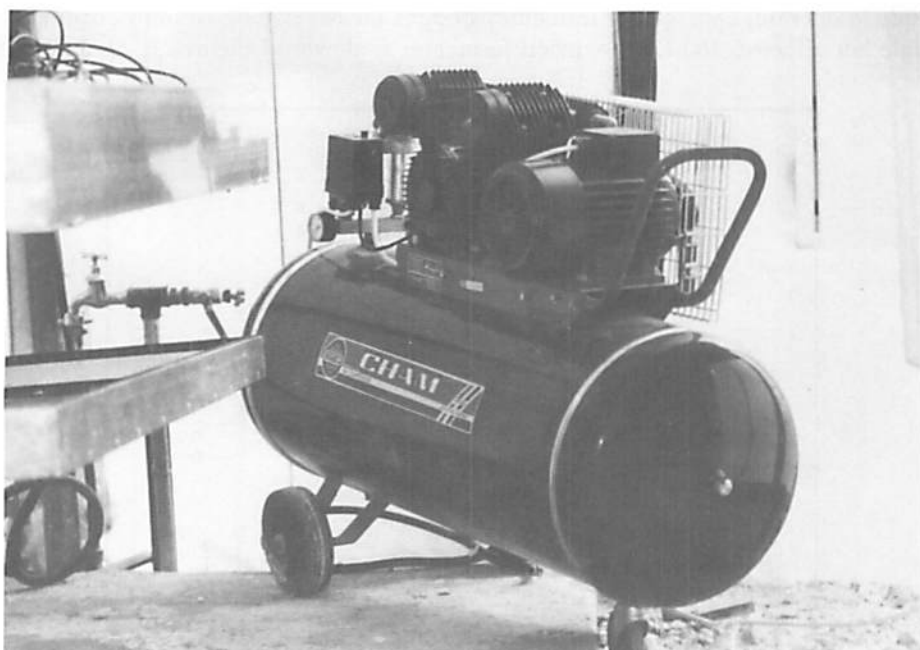


Fig. E.2.2. Air compressor of 50 L capacity for aeration of large fermentors.

**E.2.b.1. Autoclaved Simple Fermentation Vessels.** are most commonly used in laboratory-scale production of inoculants. The simplest form is a glass bottle or flask which is either aerated by air bubbles (compressed or pumped air, Figure E.2.3) or by shaking (Figure E.2.4). After introducing media ingredients into the flask, it is plugged with a cotton wool stopper (Figure E.2.5), the plug covered with aluminum foil, and the flask autoclaved for a given period to kill all bacteria and fungi. If shaken, no air introduction into the vessel is necessary, but some degree of air exchange is necessary; for this reason the cotton wool plugs are used. During the shaking process, these cotton plugs must not be wet by the media, as this will introduce a pathway for contamination of the rhizobia culture with foreign bacteria.

**E.2.b.2. Self-Sterilizing Large Scale Fermentors.** are mostly used in commercial-scale production of inoculants. Steam is the source of sterilization, and is generated either by an external source (steam generator - Figure E.2.6) or within the vessel by applying external heat using a gas burner (Figure E.2.7). In most developing countries, acquisition of suitably sized, affordable fermentors and the level of skilled expertise for the operation can present severe problems for establishing inoculant production capability. The NIFTAL Project has been involved in the development of low-cost,

simple to operate, appropriate fermentor designs for developing country conditions. A simple but effective 100-L pressurized fermentor is shown in Figures E.2.8 and E.2.9.

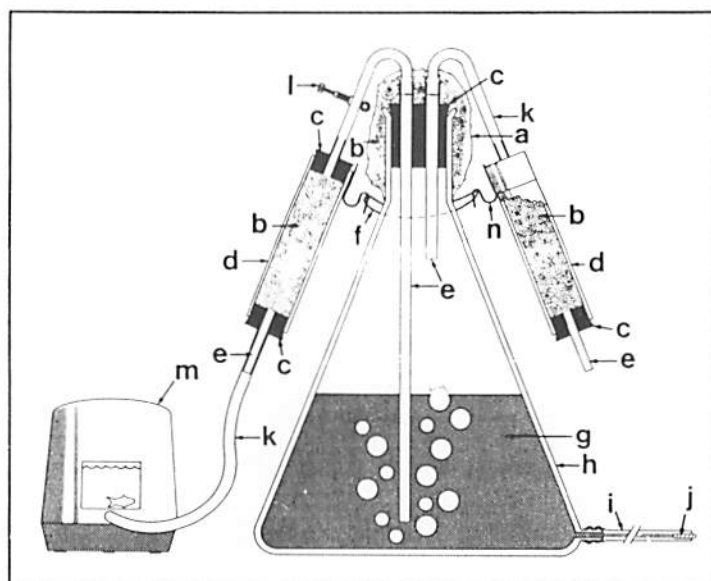


Fig. E.2.3. Scheme of simple fermentor unit: a) aluminum foil outer wrap; b) non-absorbent cotton wool; c) autoclavable stoppers; d) glass or plastic syringe filter housing; e) glass tubing; f) wire ring; g) growth medium; h) Erlenmeyer flask; i) sampling tubing; j) glass plug; k) latex tubing; l) hose clamp; m) aquarium pump; and n) wire hook (after NIFTAL Project Illustrated Concepts series).

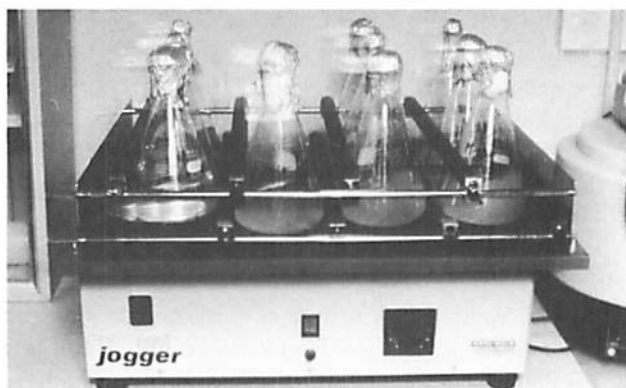


Fig. E.2.4. Flasks containing liquid cultures of rhizobia aerated by shaker action.



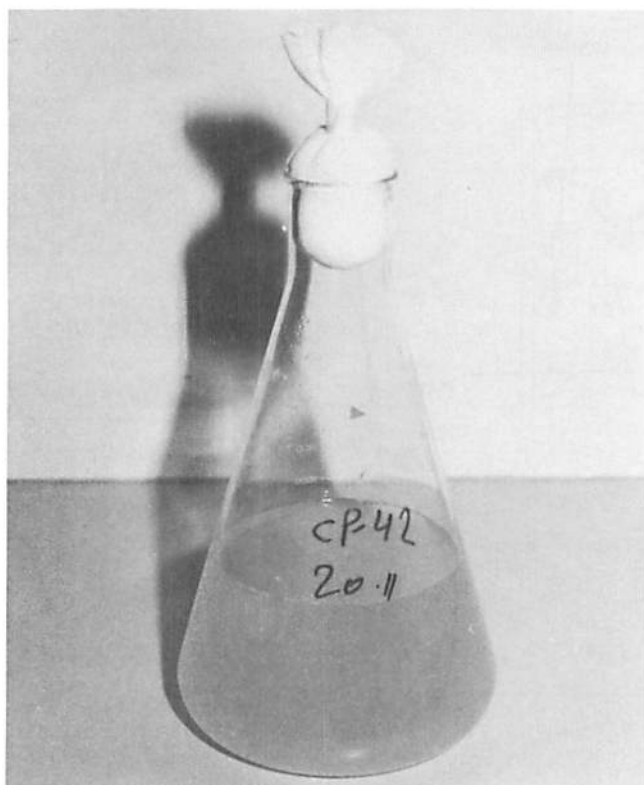
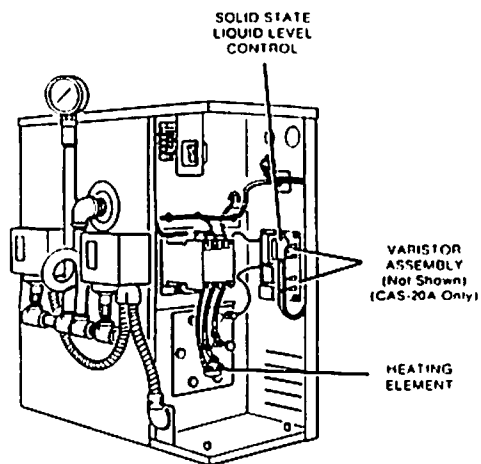


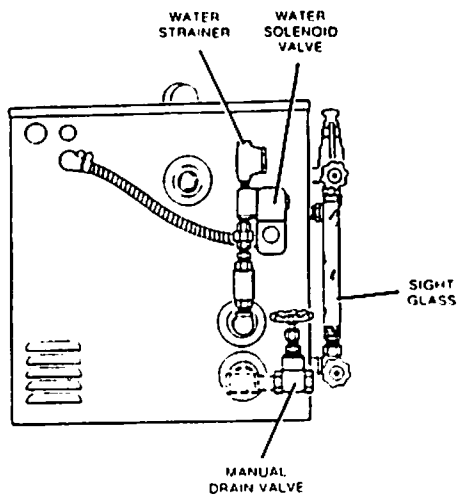
Fig. E.2.5. Flask covered by reusable cotton wool stopper to permit gas exchange but exclude contaminants.

This pressure-type fermentor should have a simple design with the following specifications:

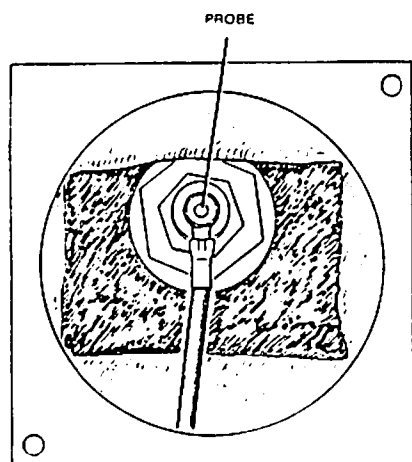
1. Ability to withstand internal pressure of 30 psi steam or greater.
2. Handy access port to facilitate adding medium and washing, and closure which provides a dependable seal during and following sterilization.
3. Composition should be stainless steel which will not corrode, is nontoxic to bacteria and is easy to clean. Types 304 and 316 stainless steel can be used.
4. Ability to withstand direct heating with a gas or oil flame for easy sterilization.
5. Equipped to supply sterile air through a sparger to aerate the broth media and provide oxygen and mixing for the rhizobia.
6. Inoculum port for adding the starter culture aseptically.
7. Sample port which is easy to sterilize to facilitate monitoring of the growth and purity of the culture.
8. Air exhaust tube with valve to regulate aeration.



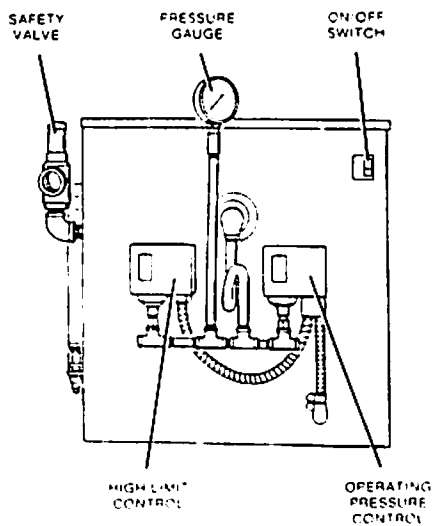
CONTROLS (Cover Removed)



BACK VIEW



TOP VIEW (Cover Removed)



FRONT VIEW

# STEAM GENERATOR

**Fig. E.2.6. Steam generator on large commercial autoclave which can also be used for sterilizing large fermentor vessel.**

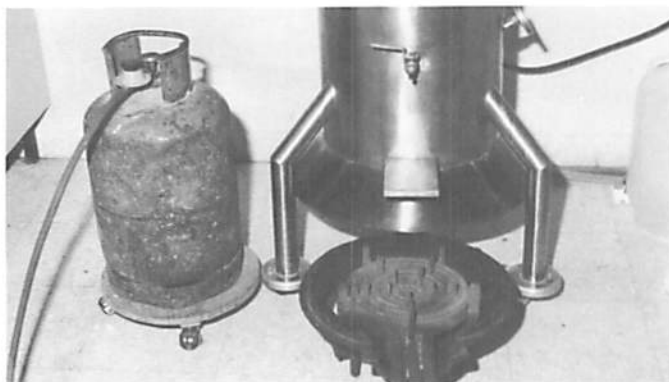


Fig. E.2.7. Gas burner fired by bottled gas, to provide heat for sterilization of pressure-type fermentor.

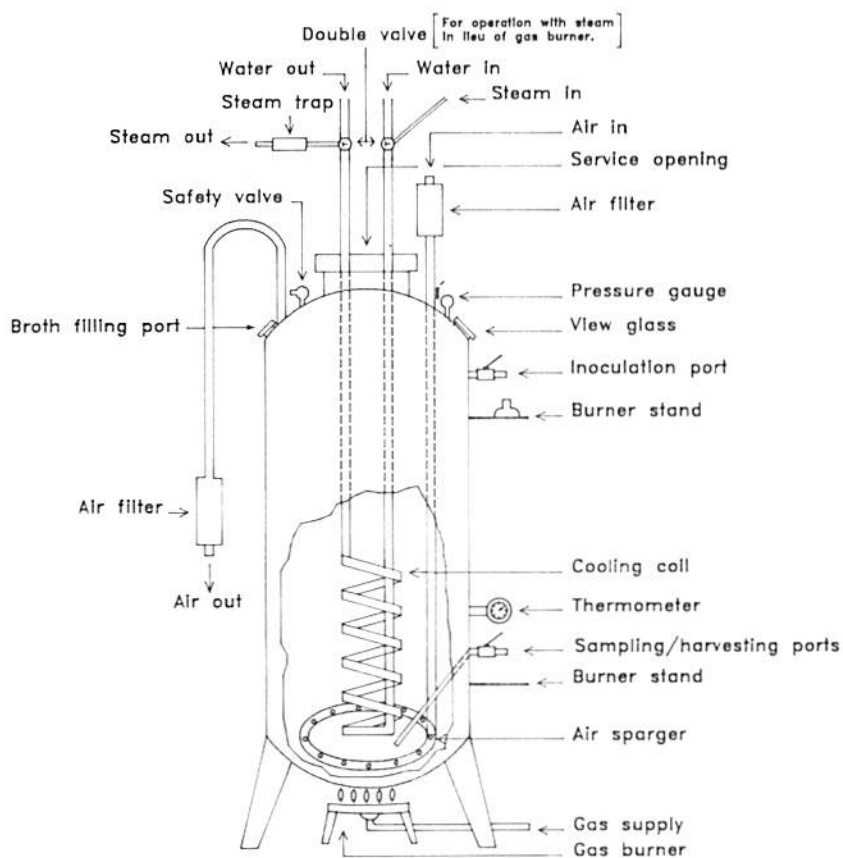


Fig. E.2.8. A 100-liter pressure-type fermentor (NifTAL design).

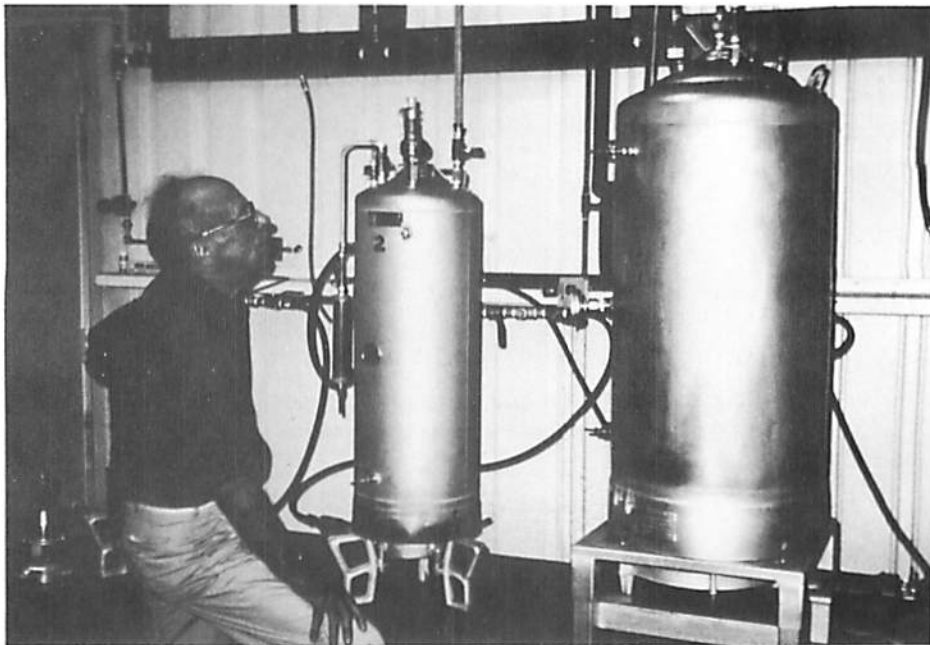


Fig. E.2.9. Two sizes of the NifTAL type fermentor with designer Dr. Joe Burton.

9. Fermentor should be equipped with an accurate, rugged thermometer of the bimetallic type, a pressure gauge, and a pop safety valve.
10. Strength and durability to withstand handling and use over a long period of time.
11. Permit aseptic removal of the broth culture and easy cleaning.

Generally, the fermentor is filled approximately two-thirds full with water; nutrients and sugars are added; and the vessel is sealed and then heated until internal pressure reaches  $121^{\circ}\text{C}$ . The gas flame is adjusted to maintain this temperature for a period of time long enough to kill all bacteria. Sterilization times increase as media volumes increase; care must be taken to autoclave media long enough for full sterilization. If sterilization time is too long, sugars may become caramelized (darker color may indicate caramelization) and therefore unusable by the rhizobia as a food source. If sterilizing time is too short, not all bacteria in the media will be killed, and the result will be a contaminated culture. Preliminary tests should be made to measure precisely the total capacity of the fermentor and just how much time is needed at  $121^{\circ}\text{C}$  to

completely sterilize that medium. The maximum operating capacity of a fermentor is approximately 75% of its total capacity.

Tests also should be made to determine the time needed to cool the medium to 28°C before inoculating with the starter culture. It is a good idea to make a preliminary run with tap water to determine the cooling time required.

Other equipment required will be related to the scale and type of production, but a certain critical mass of equipment items is required before inoculant production above laboratory scale can begin. These basic requirements, and approximate costs, are listed in Table E.2.3. Likewise, supplies and materials must be available in a well-equipped lab. A list of the most commonly used laboratory supplies and materials, and the quantities required for an active microbiology and inoculant production program, is given in Table E.2.4.

**Table E.2.3. Equipment necessary for inoculant production.**

| Equipment |                                                                                                                                                                                                                                                                                                                                                                                                                                 | Approximate<br>Quantity | Cost                   |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|------------------------|
| a.        | <b>Fermentor</b><br>Capacity 50–100 L, stainless steel pressure vessel capable of direct heat (gas burner) sterilization, with copper cooling coils. Includes pressure gauge and thermometer, with ports for sampling, inoculation and air sparging.<br>OR<br><b>Fermentor</b><br>Containers with 20–50 L capacity, modified to serve as fermentors. Must be stainless steel and airtight. Not necessary to withstand pressure. | 1<br><br>3–4            | \$ 3000<br><br>\$ 1000 |
|           | <b>Large capacity vertical autoclave</b><br>Electric, capable of holding above containers                                                                                                                                                                                                                                                                                                                                       | 1                       | \$ 5000                |
| b.        | <b>Autoclave, electric</b><br>Horizontal or vertical chamber, small-medium size.                                                                                                                                                                                                                                                                                                                                                | 1                       | \$ 3000                |
| c.        | <b>Laminar flow hood</b><br>Horizontal laminar flow, 120 cm width minimum, UV and fluorescent light, table support and spare filters                                                                                                                                                                                                                                                                                            | 1                       | \$ 4000                |

|    |                                                                                                                                                        |   |         |
|----|--------------------------------------------------------------------------------------------------------------------------------------------------------|---|---------|
| d. | <b>Incubator</b><br>25–30°C for growing rhizobia, 350 L capacity                                                                                       | 1 | \$ 800  |
| e. | <b>Refrigerator</b><br>350 L capacity                                                                                                                  | 1 | \$ 800  |
| f. | <b>Water distilling apparatus</b><br>Minimum capacity 4 L/hour                                                                                         | 1 | \$ 700  |
| g. | <b>Reciprocal shaker</b><br>Shaking tray with clamping rods to accommodate conical flasks, 100–3000 ml                                                 | 1 | \$ 1000 |
| h. | <b>Automatic syringe</b><br>For injection of peat packages and dispensing media, with extra syringes                                                   | 1 | \$ 1000 |
| i. | <b>Plastic bag sealer</b><br>Can be household type of approximately 35 cm width                                                                        | 2 | \$ 100  |
| j. | <b>Balance</b><br>Weighing range < 1–200 g, readability 0.001 g                                                                                        | 1 | \$ 2000 |
| k. | <b>Balance</b><br>Weighing range 1–5000 g, readability 0.1 g                                                                                           | 1 | \$ 1300 |
| l. | <b>pH meter</b><br>Digital readout, with extra electrode and buffers                                                                                   | 1 | \$ 500  |
| m. | <b>Binocular microscope</b><br>For routine microbiological work, equipped with standard optics and spare bulb                                          | 1 | \$ 3000 |
| n. | <b>Hammer mill</b><br>For grinding peat or soil carrier to pass through a sieve of 0.07–0.25 mm fineness, with spare set of hammers and 30 L container | 1 | \$ 2000 |
| o. | <b>Hot plate</b><br>For agar media preparation, magnetic stirring type                                                                                 | 1 | \$ 300  |
| p. | <b>Air conditioner</b><br>For inoculant storage room                                                                                                   | 1 | \$ 800  |

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**Table E.2.4. Supplies and materials necessary for inoculum production.**

| <b>I. Glassware</b>                                      | <b>Quantity</b> |
|----------------------------------------------------------|-----------------|
| Erlenmeyer flask, 250 ml                                 | 10              |
| Erlenmeyer flask, 500 ml                                 | 15              |
| Erlenmeyer flask, 1000 ml                                | 5               |
| Erlenmeyer flask, 2000 ml                                | 10              |
| Volumetric flask, 500 ml                                 | 4               |
| Volumetric flask, 1000 ml                                | 4               |
| Test tube, 150 × 16 × 1 mm, threaded top with screw caps | 200             |
| Test tube, 140 × 14 mm                                   | 100             |
| Test tube, 160 × 16 mm                                   | 100             |
| Test tube, 200 × 20 mm (plant growth tubes)              | 500             |
| Metallic or autoclavable plastic caps for above tubes    | 400             |
| Graduated glass pipette, TOED, 1 ml capacity             | 100             |
| Graduated glass pipette, TOED, 5 ml capacity             | 50              |
| Graduated glass pipette, TOED, 10 ml capacity            | 50              |
| Petri dish, glass with cover, 80 mm size                 | 200             |
| Graduated measuring cylinder, TAUT plastic, 50 ml        | 5               |
| Graduated measuring cylinder, TAUT plastic, 100 ml       | 5               |
| Graduated measuring cylinder, TAUT plastic, 250 ml       | 2               |
| Beaker, borosilicate glass, 100 ml                       | 6               |
| Beaker, borosilicate glass, 250 ml                       | 6               |
| Beaker, borosilicate glass, 1000 ml                      | 6               |
| Funnel, polypropylene, short stem, fluted, 65 mm         | 6               |
| Funnel, polypropylene, short stem, fluted, 100 mm        | 6               |
| Funnel, polypropylene, short stem, fluted, 160 mm        | 6               |
| Carboy, autoclavable with outlet valve 20 L              | 3               |
| Washing bottle, autoclavable plastic, 250 ml             | 6               |
| Microscope slide, precleaned, 76 × 26 mm                 | 1000            |
| Microscope slide coverglass, 19 × 18 mm                  | 3000            |

## II. General laboratory utensils

|                                                            |        |
|------------------------------------------------------------|--------|
| Sterilizing box for pipettes, 450 × 65 mm                  | 3      |
| Sterilizing box for petri dishes, 100 mm with lifting rack | 3      |
| Brush for test tubes, 18 mm                                | 4      |
| Test tube rack, 24 tube capacity, 22 mm tubes              | 5      |
| Test tube rack, 48 tube capacity, 16 mm tubes              | 5      |
| Test tube basket, stainless steel, about 140 mm            | 6      |
| Automatic syringe, with luer-lock tip, complete, 50 ml     | 3      |
| - spare body, 50 ml                                        | 2      |
| - distributor                                              | 2      |
| - tygon tubing to fit, 5-m long with plunging ball         | 2      |
| - exhaust pipe                                             | 2      |
| - set of gaskets and springs                               | 10     |
| - needles, luer-lock, 20 gauge                             | 1000   |
| Needles, sterile luer-lock, 18 g                           | 1000   |
| Draining rack, wall mounting                               | 1      |
| Bunsen burner with air regulator, 10 mm                    | 3      |
| Tubing for burner, about 10 m                              |        |
| Tripod, stainless steel, diameter 100 mm, height 180 mm    | 1      |
| Wire gauze square, asbestos center 150 × 150 mm            | 3      |
| Inoculating loop handle, mandrel                           | 4      |
| Inoculating loop wire chromo-nickel, 5-6 mm loop           | 20     |
| Inoculating loop wire in chromo-nickel, in meters          | 2      |
| Microscopic forceps, stainless steel, 145 mm               | 6      |
| Spatula, stainless steel, spatula and spoon ends, 220 mm   | 4      |
| Magnetic stirring bar, PTFE coated, 35 × 10 mm             | 10     |
| Magnetic stirring bar, PTFE coated, 65 × 10 mm             | 5      |
| Scaling film, Parafilm, 75 m long, 5-10 cm wide            | 10     |
| Aluminum foil, 300 mm × 10 m roll                          | 10     |
| Cotton wool, nonabsorbent in roll-pack                     | 5 kg   |
| Polyethylene foil, autoclavable, 150 mm, thermowelding     | 1000 m |
| Polypropylene foil, autoclavable, 150 mm, thermowelding    | 1000 m |



|                                                         |    |
|---------------------------------------------------------|----|
| Pencils, glass marking, wax                             | 20 |
| Self-adhesive tape, for autoclave, rolls of 10 m × 5 cm | 20 |
| Filter paper, Whatman no. 4, 20 cm, box of 100          | 10 |
| Gloves, heat protective, pairs                          | 3  |

Autoclavable plastic bags (WINPAK) 12 × 16 cm HDHMW polyethylene

### III. Chemicals

|                                   |       |
|-----------------------------------|-------|
| Agar, standard laboratory (Difco) | 5 kg  |
| Ethanol, pure                     | 5 L   |
| Hydrochloric acid, pure           | 5 L   |
| Magnesium sulfate                 | 1 kg  |
| d-Mannitol, powder                | 20 kg |
| Mercuric chloride                 | 1 kg  |
| Potassium phosphate, $K_2HPO_4$   | 1 kg  |
| Sodium chloride                   | 1 kg  |
| Sodium hydroxide                  | 5 kg  |
| Yeast extract, powder, Difco      | 1 kg  |
| Calcium chloride                  | 1 kg  |
| Clorox (sodium hypochlorite)      | 5 L   |

#### E.2.c. Culture Production and Fermentor Operation

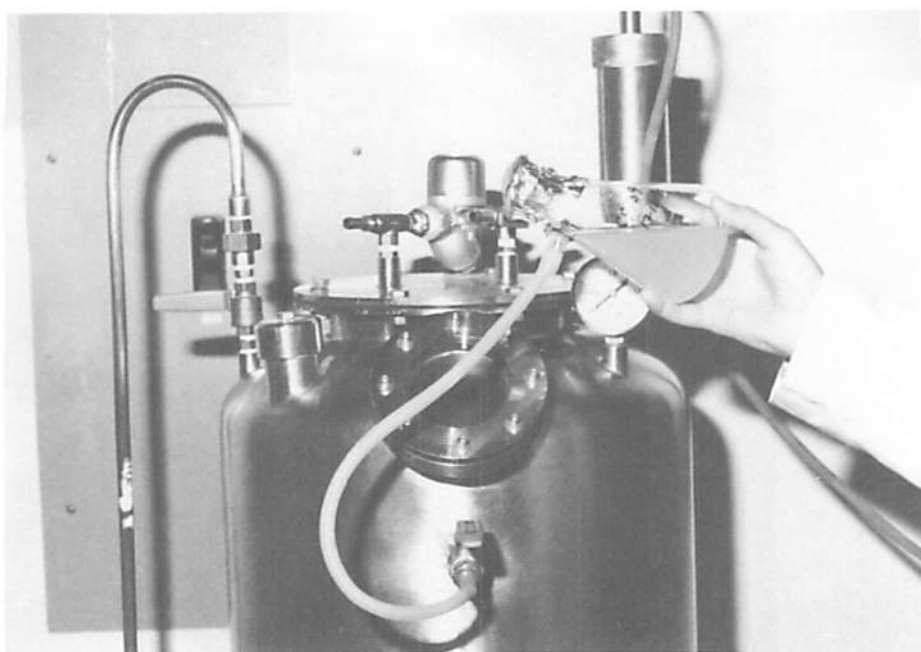
Production of legume inoculants begins with cultures grown in test tubes containing YM agar. The rhizobia grown in tubes are transferred aseptically to larger vessels containing broth medium (YMB), such as Erlenmeyer flasks for shake culture. The rhizobia grown in flasks are then transferred to small or large fermentors based on needs. The rhizobial growth should approach the peak of the logarithmic growth phase in each step before transferring to the next larger fermentor or to the carrier.

Starter cultures for inoculation of the 100-L fermentor are usually prepared in 500 ml sidearm Erlenmeyer flasks (Figure E.2.10). This flask, containing 200 ml YM broth, is sterilized with a portion of rubber tubing attached to the sidearm prior to growth of bacteria. After growth of rhizobia to maximum log phase, the contents are aseptically poured through the sidearm tubing into the inoculation port of the fermentor. This procedure is a common source of fermentor contamination, and should be performed carefully with adequate flaming of the inoculation port (with Bunsen burner or propane torch) and dipping the cut end of the sidearm tubing in alcohol prior to attachment to the inoculation port (Figure E.2.11). In a favorable medium at 28–30°C and a 1% (vol/vol) starter, the population of viable rhizobia should attain a count of 5

$\times 10^8 - 1 \times 10^9$  per ml in 60-72 hours. With a 3-5% starter, the same concentration of viable cells should be reached in less than 48 hours. With an urgent need for inoculants, larger starter inocula can be used to speed up production.



Fig. E.2.10. Starter culture in side-arm Erlenmeyer flask for inoculation of 100 L fermentor.



**Fig. E.2.11. Inoculating the fermentor. The inoculation port is flamed thoroughly before attaching the sterile cut end of tubing from the starter culture flask.**

Growth of rhizobia in the fermentor should be monitored about halfway through the cycle by drawing off a sample from the sampling port, and testing for acidity, purity, and growth (Figure E.2.12). Sampling more often will increase the possibility of contaminating the culture. Again, it is important that the sampling port be thoroughly flamed both before and after sampling to kill contaminating bacteria that may find their way into the fermentor. The pH of the sample can be tested colorimetrically by adding five or six drops of a 0.5% w/v alcohol solution of bromthymol blue indicator to 8 ml of culture in a tube. A yellow color indicates acid conditions and probable contamination. Contaminated cultures also will have an odor distinct from the smell of pure rhizobia cultures; with experience you will be able to tell immediately upon smelling the growing culture if it is contaminated. A Gram stain may be useful if there is a question of purity (Section A.6). Heat-resistant, spore-forming bacilli are the most common contaminants (Gram positive). Contaminated cultures should be discarded, and after cleaning, the fermentor restarted. An estimate of the population (Section B), using either a counting chamber or nephelometry, will help you to decide when to harvest the fermentor. It is useful to have previously produced growth curves for the inoculant strains at varying temperatures so that harvest time for each batch can be forecast (Section B.1.c).



Fig. E.2.12 Collecting sample from fermentor for purity check.  
Sampling port should be flamed thoroughly before and after sample collection  
to prevent contamination of fermentor contents.

#### E.2.d. Single- and Multiple-Strain Inoculants

Legume inoculants may consist of one strain selected for a particular legume host or they may comprise two or more strains effective on that host. The latter is often advantageous, particularly in areas where several varieties or cultivars of the legume are grown or where the soils vary widely in characteristics. In Australia, only single-strain inoculants are produced, to prevent possible dominance and antagonistic effects of a particular strain in the mixture, to be able to diagnose loss of effectiveness, and to facilitate inoculant quality control. It is important to test the strains singly and together in the concerned soils with the concerned cultivars, however, before producing a multiple-strain inoculant for distribution and use by farmers (see Section G). It is necessary also to have compatible strains, and strains must be grown separately in culture before combining them in the carrier to make the inoculant. In recent studies on bean, chickpea and lentil, the amounts of  $N_2$  fixed by multistrain inoculants were significantly lower than that of the most effective strain applied alone.

In general, single-strain inoculants are superior for several reasons. Even where the broth or final inoculant receives equal numbers of cells of more than one strain, subsequent multiplication in the carrier can result in the dominance of one strain. Any loss of infectiveness or effectiveness in a strain is obvious in single-strain inoculants, but may be masked in multi-strain inoculants. Any change in host requirements can be provided for by developing a separate inoculant which can be clearly labelled.

Multiple-host inocula are sometimes desirable (e.g., for several species of medics) and are feasible. For multiple-host inoculants, wide-spectrum strains are preferable. No strain should be included in a multiple-strain inoculant which could produce ineffective nodules on any of the legume hosts for which it is intended. Inoculants with only two or three strains are preferred over inoculants with many strains. Use of multiple-strain inocula also provides some protection against complete loss of effectiveness if one strain for some reason loses its ability to infect the host or fix nitrogen.

When a multi-strain inoculum is desired, it is preferred that each rhizobia strain be grown separately and then mixed in equal volumes at the time of adding broth to the carrier (Figure E.2.13). This method does not ensure that there will be an equal number of each strain in the final product, but it does reduce the risk that one strain (e.g., a faster-growing strain) will dominate over the others at an early stage.

This multi-strain type of production requires a greater number of culture vessels and more precise planning and scheduling, especially if growth rates of the strains differ. This should not be a problem, however, if the number of strains in the mixture is limited to two or three.

## **E.3. Production of Solid Inoculants**

Solid-based inoculants using powdered organic carrier materials are the most common products for inoculation of legumes now on the market. Inoculants based in solid carriers have shown many advantages over other types in terms of preparation, packaging, storage, and distribution. Survival of applied rhizobia is superior and nodulation in the field is generally more effective when solid-based inoculants are applied onto the seed than with other forms of inoculants.

### **E.3.a. Types of Carriers**

The inoculant carrier is the material supporting the living rhizobia from the point of manufacture to the user. The availability of an inexpensive and suitable carrier for

rhizobia is essential for the economic production of legume inoculants. Normally, at least  $5 \times 10^8$  or 500 million living rhizobia should be contained in each gram of carrier; this means that using normal application methods, each seed will have  $10^4$ – $10^5$  rhizobia if there is good attachment. The lower level ( $10^4$  rhizobia/ seed) is a minimum if good nodulation is expected in soils where the necessary rhizobia are not present.

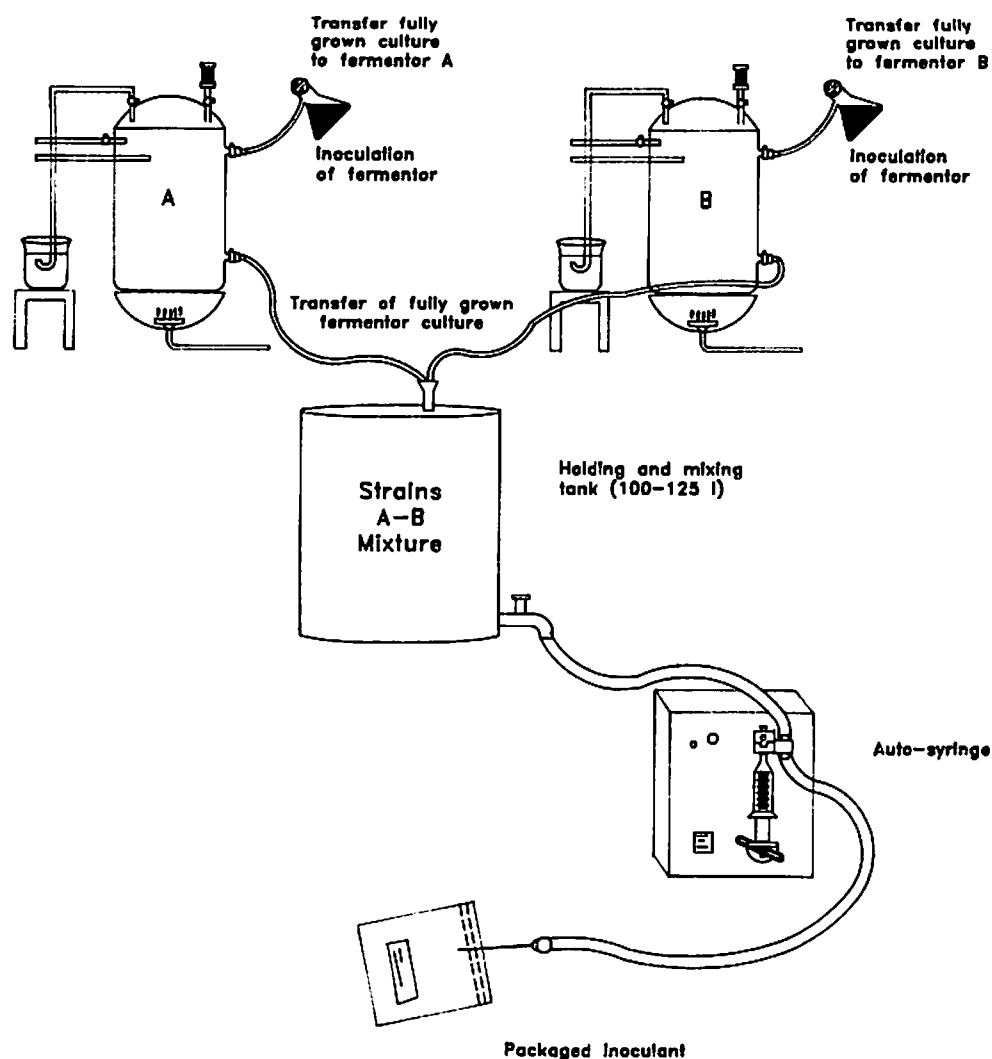


Fig. E.2.13. Schematic of multi-strain production of sterile inoculant.

The suitability of any material used as carrier is determined by its capacity to maintain the viability of high numbers of the added rhizobia over long periods without loss of infectiveness and effectiveness. There are several qualities to look for in acceptable carriers. Most important, the material must be able to maintain high numbers of viable rhizobia, implying that it should provide proper nutrition and moisture for rhizobial growth. Ideally, the carrier will be highly absorptive (high water-holding capacity), easy to process, available in adequate amounts, stick easily to seeds, and provide good pH-buffering capacity (high anion/cation exchange capacity).

The carrier material also must be inexpensive, because farmers are generally not willing to spend more than U.S. \$10 per hectare on inoculation. It must be lightweight for shipping, and must be stable if exposed to adverse conditions during transport and storage. It should be relatively easy to sterilize (autoclaving or gamma-irradiation) so that competing bacteria and fungi can be eliminated. Finally, the carrier must be low in content of soluble salts and phenolic products of organic matter breakdown, and therefore nontoxic both to rhizobia and seed.

The carrier most commonly used in the inoculant industry is peat. Since the composition of peat varies with location of deposits it is necessary that the suitability of specific peat as a carrier be tested for ability to support growth and survival of the rhizobia before it is used in production. In general, high organic matter content soils and peat-based materials have a longer shelf life and give improved survival of rhizobia than other carriers (c.g., lignite or filter mud).

Lack of sources of peat has prompted use of alternate materials. Some other carriers consist of coal dust (coir), bentonite, composted plant materials, bagasse (filter mud) with sugar removed before grinding, pulverized vermiculite, polyacrylamide, lignite, cellulose powder, ground common talc, and others. Chemical and physical analyses of carrier materials are helpful but do not confirm the quality of a carrier. The quality can be determined only by placing viable rhizobia in the material and monitoring the growth and survival over a period of six months. Regardless of the type of carrier chosen for inoculum production, it is important to keep in mind that carrier-rhizobia interactions cannot be avoided. These interactions should be known for all strains of rhizobia used for production, and can only be determined by thorough testing involving growth/survival measurements over time.

Investigations into survival of rhizobia in coal-based inoculants indicate considerable variability, due to the differing properties of various coals. Some finely ground coals also tend to aggregate into lumps and resist wetting at the time of inoculation. Charcoal has proved an adequate carrier for some strains of rhizobia, but is generally inferior to peat, probably because of its low moisture holding capacity. Moisture content greatly affects the ability of a carrier to maintain rhizobial numbers; rhizobial populations decline more rapidly in storage with decreasing moisture content.

Work at ICARDA has shown that Syrian soils high in organic matter (10–15% organic carbon) amended with ground wood charcoal (3:1 wt:wt) proved equally effective as high-quality Australian peat in maintaining high ( $> 10^9$ /g) populations of chickpea rhizobia for periods of 105–126 days. After storage for 280 days, two rhizobial strains differing in growth rate maintained viable numbers in the soil-charcoal mixture above  $10^8$ /g, indicating the suitability of this material as an inoculant carrier. These results imply that quality rhizobial inoculants may be produced with some mineral soils of high organic matter content in areas where peat is not available.

The carrier materials must first be ground in a Wiley mill to pass a 0.25–0.15 mm sieve (the finer, the better). At ICARDA, the mixture of soil and charcoal is made on a dry weight basis, at a ratio of 3:1. Approximate water-holding capacity is determined prior to autoclaving, and materials are moistened with distilled water to approximately 10% moisture (by weight) to increase the effectiveness of sterilization. Moisture percentage is usually expressed in terms of wet weight of peat (carrier), so that when the weight of water equals the weight of dry peat, the moisture content is 50%. All materials should be of approximately neutral pH; where pH of a saturated extract of the material is low, calcium carbonate may be added in sufficient amounts to bring pH to 6.8–7.5.

For production of small quantities of inoculant for field trials and other research purposes, it may be best to purchase prepackaged gamma-irradiated peat, ready for injection. The cost of these packages is relatively low, U.S. \$0.25–\$0.50 per 100-g package depending on quantity (from Root Nodule Pty. Ltd., Australia; telex RTNDLE 25879 A). With this type of sterile, high-quality neutral peat (tested at ICARDA), the probability of having a good research inoculant is greatly increased.

An alternative to solid inoculants is oil-based inoculants, which have the apparent advantage of protecting rhizobia from high temperatures, a common problem during distribution in the region and in some cases at planting. These, however, are difficult to produce as they require mixing of lyophilized (freeze-dried) bacteria with vegetable oil; sophisticated (expensive) freeze-drying equipment is necessary, and the technology of producing large amounts of high-quality freeze-dried rhizobia must be mastered (see Section A.5).

Alginate- and polyacrylamide-entrapped rhizobia as inoculants are complicated to produce, and for mass production, still in the experimental stage. Some literature is available on methods of production, but most information is held under patent protection.



### **E.3.b. Sterilization, Preparation, and Packaging of Carriers**

Inferior-quality inoculants are probably partially responsible for the slow progress in the adoption of BNF technology. Inoculants which contain low numbers of viable rhizobia are inadequate to cause prompt and effective nodulation. Carrier sterilization is a major factor influencing rhizobial growth and survival in the carrier.

Sterilization of the carrier material is important to eliminate competition from fungi and other bacteria, and hence to obtain high numbers of rhizobia. This may be achieved through autoclaving, gamma-irradiation, chemical sterilization or flash drying. The most effective method for sterilization, through exposure of the dry carrier to approximately 5 Mrad gamma irradiation, is limited because of cost and unavailability of a radiation source in many countries. Autoclaving is perhaps the most effective common method of sterilization, but requires a packaging material capable of withstanding the high temperature conditions of repeated sterilization at 121°C. Some high-molecular-weight, high-density polyethylene or polypropylene materials suitable as autoclavable bags are now commercially available. It is essential that these materials conserve moisture but allow passage of gases following sterilization. Sterilization of the carrier through chemical means leaves residues harmful to rhizobia, and excessive heat treatment of carrier materials generally decreases their ability to support rhizobial survival, although successful heat treatment has been reported.

At ICARDA, we successfully sterilize mineral soil in high-density autoclavable polypropylene bags of 0.2 mm thickness (stock No. 402, Kapak Corp., Minneapolis, MN, USA) with a capacity of 470 ml. Each inoculant bag is packed with 50 g of finely ground soil carrier prior to steam sterilization. The open ends of each bag are partially heat-sealed, leaving a 1-cm opening plugged with cotton wool for gas escape during sterilization (Figure E.3.1). The bags are usually arranged in trays in the autoclave with paper towels in between for separation and adequate steam circulation, then sterilized at 121°C for 90 minutes on two consecutive days. Bags are then removed for sealing using a household plastic bag sealer in a laminar flow hood after overnight cooling in the autoclave (Figure E.3.2).

### **E.3.c. Preparation of Inoculants**

Although the types of carriers commonly used vary, there are only two main approaches to production of the inoculant (mixing broth and carrier). The first method maintains sterility in the carrier until it is injected in sealed packages with the pure rhizobia culture; in this way, the rhizobia have no competitors for water or nutrients and can increase further in number if the carrier can support growth. The second method utilizes carriers which have not been sterilized, where the rhizobia

culture is mixed in bulk with the dry carrier prior to curing and packaging of the inoculant. The first method is more labor-intensive and costly, but generally yields a better quality inoculant. The nonsterile method is more adaptable to large-scale inoculant production.

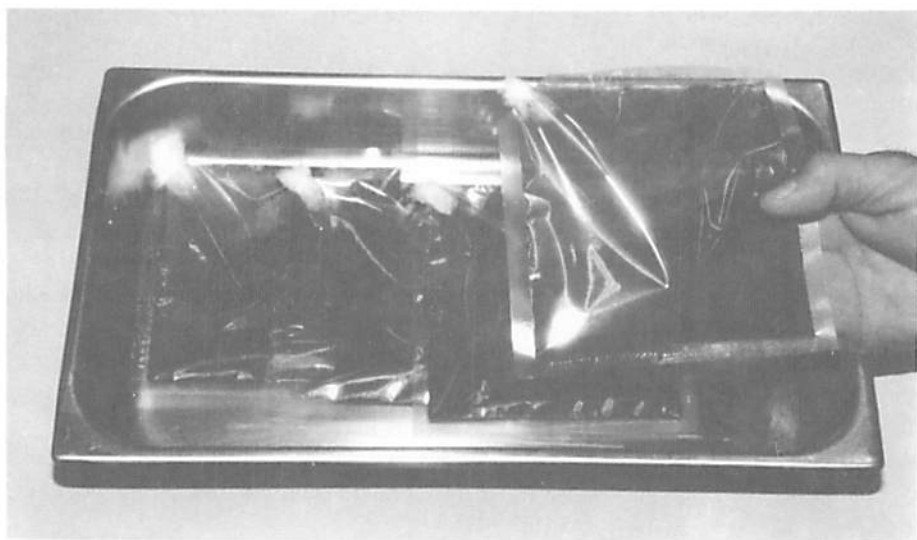


Fig. E.3.1. Sterilizable high density polypropylene bags with ground carrier ready for autoclaving. Note opening in corner, plugged with cotton for gas escape.

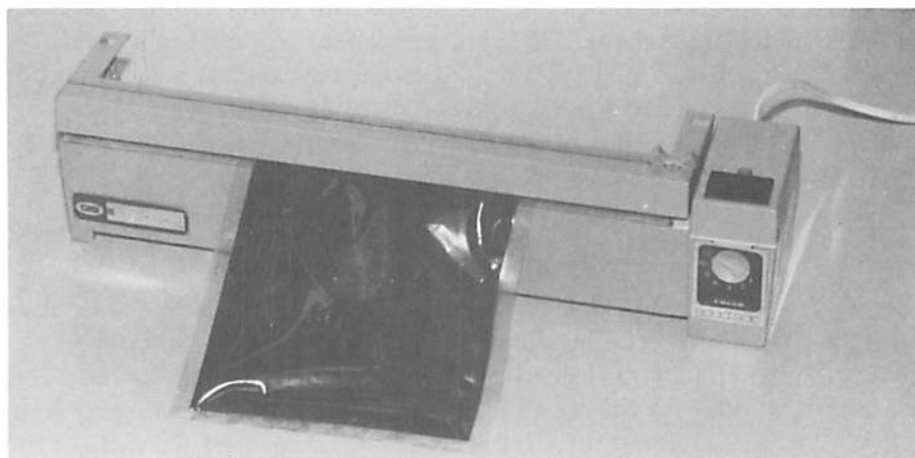


Fig. E.3.2. Sealing bag of carrier using a small commercial plastic bag sealer.

**E.3.c.1. Sterilized Carriers.** The production of solid-based inoculants which contain only rhizobia requires a completely sterile carrier in a sterile package. Packaging films and methods of sterilization have been discussed previously. Generally, each presterilized peat bag containing 50–100 g of peat (Figure E.3.3) is injected aseptically (under a laminar-flow hood if available) by means of a syringe fitted with a sterile needle (18 gauge, 3–4 cm length). An automatic dispensing machine (auto-syringe) may be used to increase efficiency if larger numbers of packages are to be prepared (Figure E.3.4). Prior to injection, the area of the puncture should be disinfected by swabbing the area with a cotton mat soaked with 70% ethanol. A quantity of broth sufficient to adequately wet the carrier (40% by weight for peat) is slowly injected, making sure to insert the needle as horizontal as possible to avoid piercing the opposite site of the bag (Figure E.3.5). The puncture hole is then immediately sealed with a pre-printed (strain identification code and date) self-sticking label (27 × 18 mm) (Figure E.3.6). The bags are then kneaded by hand until the liquid inoculum has been uniformly absorbed into the peat. The final moisture content of a peat inoculant should be between 40 and 50% (weight basis) for best survival of the bacteria.

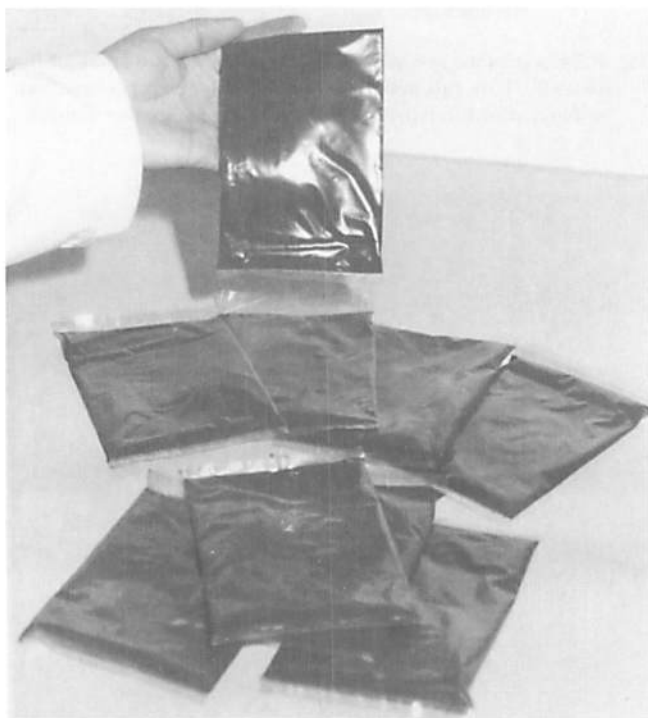


Fig. E.3.3. Gamma-irradiated peat in polyethylene bags is the best rhizobia carrier. These packages each contain 50 g of dry peat.



Fig. E.3.4. Automatic syringe used to inject a specific amount of liquid repeatedly. With this device, large numbers of peat packages can be injected with relative ease and good contamination control.



Fig. E.3.5. Insertion of needle nearly parallel to bag surface for injection of broth into carrier.



**Fig. E.3.6. The puncture hole is wiped immediately after injection with 90% alcohol, then the hole is covered with a self-sticking label to prevent contamination and label the bag contents.**

In a high-quality carrier, such as sterile neutral peat, rhizobia are able to multiply and increase in numbers. This makes it possible to dilute the broth culture injected into the peat as much as 100–1000 times, while continuing to produce inoculants containing  $> 10^9$  rhizobia/g. Smaller quantities of broth, grown in flasks in a laboratory, can therefore be used to inject large numbers of bags of carrier. One liter of  $10^9$  broth could be diluted 100 times (1/100) to inject as many as 2000 50-g packages of peat. When using this dilution method, it is necessary to let the rhizobia grow in the carrier at optimum temperatures (27–30°C) for two or three weeks before placing in cold storage or shipping. Greater care must be taken in all steps to ensure that no contaminants are present; rhizobia are poor competitors and contaminated bags inoculated with the diluted culture method will contain few rhizobia. Quality control is especially important when using this dilution method.

The sterile injection system of production is limited to small packages and is labor intensive even when an automatic syringe is employed in dispensing the broth culture. Even so, this system is being used successfully for commercial production in Australia and other places where pure cultures of rhizobia are considered necessary and sufficiently advantageous to justify the additional cost.

**E3.c.2. Bulk Nonsterile Carriers.** In this method, the broth culture of undiluted rhizobia is mixed with or sprayed onto the carrier in a ribbon or paddle-type mixer under nonsterile conditions (Figure E.3.7). A concrete mixer or bread dough mixer (Figure E.3.8) is adequate for the task. Unsterilized carriers are normally held in the dried form after grinding so that the natural populations of organisms have no opportunity to multiply before the rhizobia broth is added. If the carrier is acid, calcium carbonate is added to neutralize acidity and raise the pH to 6.6–6.8. The final moisture content will vary with different carriers, but with peat the moisture should be approximately 40% in the fresh inoculant. The proportions of broth and peat are controlled by the type and moisture-holding capacity of the carrier, but generally liquid is added until the carrier just begins to stick together in lumps or balls. The ratio of broth to carrier is usually 1:1.5–1:2.

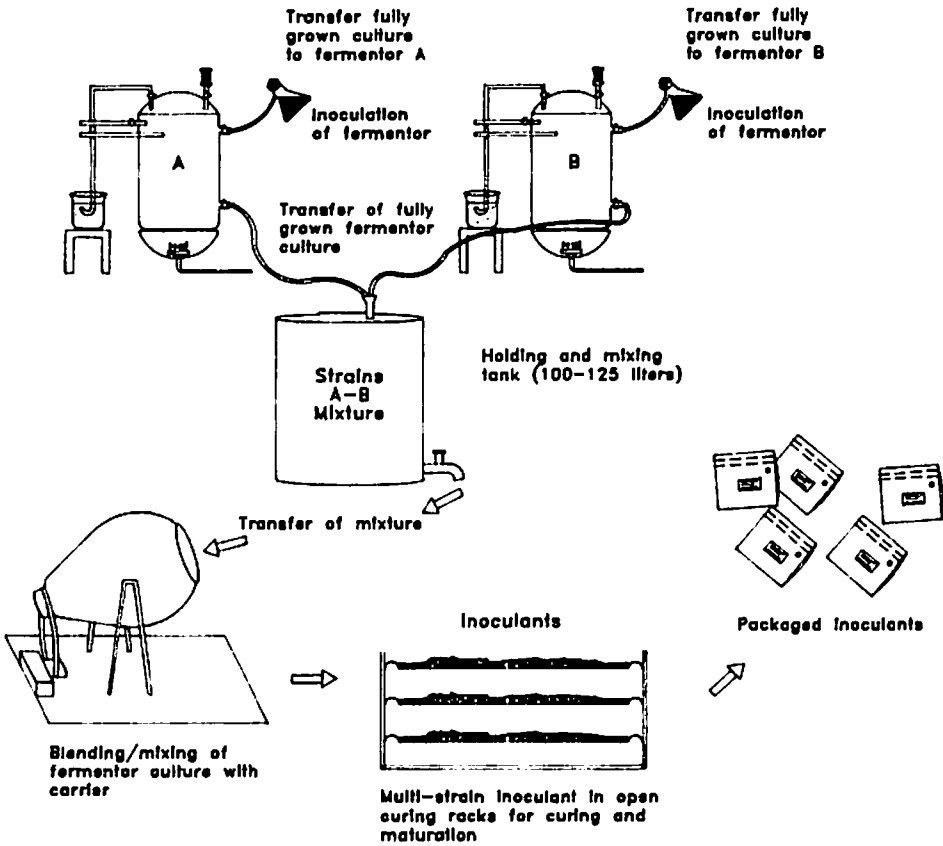


Fig. E.3.7. Non-sterile system of multi-strain inoculant production.



**Fig. E.3.8. Bread dough mixer used to blend peat and rhizobia broth in non-sterile production system in Indonesia.**

If the carrier material is very dry (as will be the case if the carrier is oven or "flash" dried), wetting it will bring about a release of energy and a rise in temperature referred to as the 'heat of wetting'. With a large bulk (e.g., a pile 1 m in depth) of material, this heat of wetting may reach as high as 50–60°C in the center, killing the rhizobia; the freshly prepared inoculant should therefore be placed in relatively thin layers, not over 25–30 cm deep in metal or fiberglass trays. Following mixing, the temperature should be monitored for two to six hours and should not exceed 38°C. After six hours, the heat of wetting will be dissipated.

The broth-carrier mixture (inoculant) is then cured (covered to prevent drying out) for a period of one to six days; during this period moisture levels equilibrate and the rhizobia are established. Following this 'curing' period, during which some multiplication of the rhizobia will take place, the inoculant is again sieved to remove lumps and make a consistent product. This finished inoculant can then be packaged in moisture-retaining containers, usually polyethylene bags (Figure E.3.9). If not bagged immediately after curing, it should be stored in such a way as to retain the moisture, as it will dry out quickly under normal conditions and ability to maintain rhizobial numbers will be lost.



Fig. E.3.9. Commercial bag sealer used for large scale non-sterile inoculant production in Thailand.

#### E.3.d. Inoculant Quality Control

Inoculation of legumes is not a panacea and is still highly underutilized, particularly in the developing world, for several reasons: scope for improvement of legume yields through inoculation is fairly limited for many species, because of large native rhizobial populations; where soil N is not limiting, inoculation may improve the proportion of plant N derived from the atmosphere but not improve yields, leaving little demonstrable benefit from inoculation; and effectiveness is heavily dependent on inoculant quality and how the product is applied.



The decision to make or market inoculants is often made without any evidence that inoculants are beneficial to the crop in the area. Experimentation to successfully define the need for inoculation (see Section G) is usually considered unnecessary by policy makers who do not understand the biological requirements, or too "applied" by developing country researchers, who often feel that they should pursue "basic" research. Ironically it is in these developing countries that applied research is most needed to define problems. This lack of understanding may result in the situation where an unenthusiastic person is loaded with the unwanted duties and responsibility of controlling the quality of inoculants which may not be useful in improving crop yields. It is more often this scenario, rather than poor production and control methodologies, that leads to distribution of "useless" inoculants and subsequent farmer indifference toward inoculant use.

Separation of production and quality control into separate organizations, where the producer(s) accept the authority of the control laboratory, is the ideal situation. There is unfortunately not much incentive for the producer to spend money on quality control. It is a simple fact of life that with self-control, some producers will sell poor inoculants.

During the 1985 workshop on *Rhizobium*/legume inoculants in Puerto Alegre, Brazil, participants concluded that the primary constraints for implementation of quality control programs were: 1) lack of demand for inoculants by farmers; 2) lack of resources to implement testing programs; and 3) the high cost in materials and time of present quality control technology.

**E.3.d.1. Quality Criteria.** The overall aim of legume inoculation is to provide the maximum numbers of suitable rhizobia in the rhizosphere at the time of nodule initiation. Traditionally, quality control means a series of quality checks during and after inoculant production, to ensure that the inoculant package actually contains rhizobia in large enough numbers to provide for a reasonable number per seed at planting. Quality control, however, must really deal with all aspects of quality up to and including the moment of actual inoculation by the farmer. This means that quality control must encompass inoculant storage (before and during shipment and in the retail outlet) and use by the farmer. Clearly, this is logistically beyond the traditional abilities of any quality control unit, and would require close coordination between inoculant producer, tester, distributor, and extension service. The point is that laboratory quality control of inoculants is only as useful as far as the distribution and farmer education system can ensure delivery of the quality product to the soil at planting. The best way for the producer to overcome degradation of inoculant quality during shipment and use is to utilize a carrier able to support the growth of rhizobia (gamma-irradiated sterilized peat is optimum) and to add to the carrier as high a population of viable, effective rhizobia as possible.

Standards for the final product will generally represent a compromise between levels achievable by competent producers and levels theoretically obtainable under the local conditions. Quantitative standards can be simply expressed in terms of a minimum number of infective bacteria available during a given shelf life of the inoculant, and a maximum number of contaminating organisms. Qualitative standards are easily set, and should include use of an agreed-upon strain, demonstrated ability of the strain(s) to form nodules and fix N, and approved packaging with adequate labelling. International standards for minimum numbers of cells at manufacture range from  $10^8$  rhizobia/g (Canada) to  $10^9$ /g in Australia and Holland; minimum levels are  $10^8$  in Uruguay, India, Czechoslovakia and Brazil. Most countries, including the U.S.A. and U.K., have no government standards for quality, but rely on competition and the pressure to market a high-quality product to maintain product quality.

Where regulations exist, minimum standards at the time of inoculant expiry are  $10^8$  to  $10^9$ . The most widely accepted standard for numbers of rhizobia per seed are 1000; Burton (1978) has recommended 1000 for fast-growing rhizobia and 100,000 for the slow-growing rhizobia. France requires a minimum quality of  $10^8$  rhizobia per seed. The numbers of contaminants should never be above 0.001% of the total number of rhizobia, and a maximum of 1000/g is a good standard to adopt for non-sterile carriers. The safest quality standards could be applied to inoculated seed, where  $10^5$  rhizobia per large seed (e.g., soybean, lupin),  $10^4$  rhizobia per medium seed (e.g., mungbean, pigeon pea), and  $10^3$  per small seed (e.g., clover, siratro) are required.

**E.c.d.2. Quality Control Procedures.** The following quality control procedures are derived from those used by the Australian Inoculants Research and Control Service (AIRCS). A lot-numbering system and adequate recordkeeping (in a log book) are absolutely essential to any quality control program. It must be possible to follow a production batch through the production process and to identify a finished product with the specific timing and conditions of manufacture that apply to that batch. An example form for recordkeeping is given in Figure E.3.9.

**Mother cultures** must be known to be pure and true to the stock culture in all respects. Because most inoculant production is seasonal, once-per-year preparation is best, with year-long storage under lyophilized conditions. Ideally, all mother cultures for any one strain should be derived each year from one freeze-dried ampule, streaked and tested for purity (gram stain, glucose peptone medium), identity (serology), and effectiveness and infectiveness (plant test under aseptic N-free conditions). If any variation exists, at least 10 single colony isolates should be subjected individually to all the above tests. Release of the mother culture for production of inoculants should await the results of all tests.

**Broth culture testing** is conducted to eliminate contaminated broths, and may occur at

several stages, including testing of the starter cultures, a short time after inoculation of the fermentor, and fermentor culture at the stage of maximum number of live cells. The most important stage for testing is at maximum fermentor population, at which time a test for purity (gram stain and culture on glucose peptone medium) and a total count (Petroff Hauser counting chamber or turbidity measurements) are necessary. For production of soybean inoculants, contamination checks on glucose peptone agar are particularly useful, as *Bradyrhizobium* will not grow on this medium and contaminants will show up clearly within 3 days. An optional test on pH can be used as an additional check for contaminants. A plate count or Miles and Misra drop count (which will take only 2-5 days for a result) is recommended, but may not be necessary with 'tried and true' strains. New strains should be checked with plate counts.

Use of a plant-infection test (MPN) at this stage may be justified instead of a plant check on the final product, under the assumption that the loss of symbiotic properties during maturation process in the carrier is low. This short-cut will speed the release of an inoculant if MPN is required prior to distribution. With pure broth cultures of rhizobia, the plate count and plant-infection test usually give similar estimates of numbers, but the presence of contamination may interfere with nodulation (Vincent, 1970). It is common in using the MPN count to require positive nodulation at  $10^{-7}$  or  $10^{-8}$  dilutions of an inoculant which reaches  $10^9$  in a plate count.

Solid inoculant should be checked for quality at manufacture. Each batch of inoculant (from a single fermentor or a number of fermentors which have been mixed before or during the process of mixing broth and carrier) should be tested, and inoculants held unreleased in storage until test results confirm acceptability. A sampling protocol, which tests a number of packets from each batch, must be set up. Reserve samples should be available if further testing is needed. All samples should be kept and transported under cool conditions to maintain numbers at the time of collection.

Whether the carrier is sterile or nonsterile, tests should include a plate count on YMA-Congo red (for enumeration and observation of contaminants), plant infection test (may not be necessary with sterile carrier if already conducted on the broth), and a serological test to ensure strain identity (may be conducted on nodules of MPN plants).

Serological techniques, such as ELISA or immunoblot, have been used in quality control and enumeration of rhizobia in inoculants, but have some drawbacks. In most cases in developing countries, newer techniques are not affordable but may be considered for development at the research level.

Inoculants from retail outlets are checked as a monitoring rather than a quality

control activity, in order to develop a sound basis for standards for expiry periods. Randomly selected inoculants should be tested with a viable plate count and plant-infection test, and results compared with those obtained at manufacture. Serological testing may be less important than plant nodulation and  $N_2$  fixation but is a useful check. It also may be useful to check moisture content for research and monitoring purposes.

**E.3.d.3. Packaging.** The inoculant package label must be understandable by users, and should contain the following information:

- manufacturing lot number
- expiry date (date after which the product is no longer usable)
- crop species for which the product is to be used
- instructions for use of inoculant, in simple form
- manufacturer's name and address
- amount of seed that the package will inoculate.

Optional information may include the genus and species of the rhizobia, instructions for proper storage, number of live rhizobia per gram or per seed when used properly, and the weight of the bag. Experience in some developing countries, such as Rwanda, has shown that a more simple package label is most effective.

**Directions for use of inoculants** may be a useful inclusion on the package. A sample from LIPHA indicates "pour the contents of the sachet into a clean container; add about 3/4 liter water on the powder; mix carefully; pour this mixture on the seeds (about 80 kg) into a clean container or on a cemented surface; mix carefully. This operation must be done sheltered from the sun and drying winds. Let the mixture dry and quickly sow the seed; put it deep into the soil. **Important notes:** Do not store at more than 25°C, and do not sow seeds which have been inoculated for more than 4 hours. If they have not been sown in the same day, inoculate the seeds again." It may also be useful to warn that "any bag opened must be used within 24 hours".

It is impossible for a user (farmer) to know the quality of inoculant at the time of planting. The expiry date is the only assurance a user can have from the manufacturer. The period between manufacture and expiry is based on results from experiments and is dependent on the method of inoculant production, carrier material used, and storage conditions. For non-sterile peat carrier stored at 20-30°C, the recommended shelf life is 4 months; when stored at 10°C, 6 months. In sterilized carrier, shelf life is considerably longer, with maintenance of  $10^9$  rhizobia/ g for up to one year.

**E.e.d.4. Quality Control Laboratory.** A technically qualified officer should be in charge with authority to implement the standards. The lab must have adequate operating funds, and if possible, additional funds for associated relevant research. Equipment should include autoclave, drying ovens, incubators, refrigerators, and a safe subculturing area (laminar flow bench). Freeze-drying equipment is optional. Gas and electricity supply should be reliable, and include a backup generator where necessary. One of the most important equipment items is a temperature-controlled cabinet or growth room to allow growing of plants in tubes, plastic pouches, or Leonard jars for plant-infection tests (MPNs) of nodulation and N<sub>2</sub> fixation.

## **E.4. Application of Inoculants**

Inoculants should be used when a legume is introduced into a new area or when a legume is known to have nodulation problems. The prime objective of inoculation is the nodulation of the host legume with the selected inoculant strain. The applied rhizobia should remain alive until the seedling emerges and compete with the indigenous rhizobia for the infection of root-nodulation sites. The rhizobia also should persist in the soil in sufficient numbers to nodulate perennial and annual species of legumes.

Proper inoculants should be used according to specifications on the package, with special care that the plant and the rhizobia are compatible. It is important that outdated, poorly stored, or damaged packages not be used.

Methods of inoculation may be classed as direct, where the inoculant is placed in direct contact with the seed, or indirect, where the inoculant is placed alongside or beneath the seed but separate from it. Either liquid- or solid-based inoculants can be used.

### **E.4.a. Application of Powdered Inoculant to Seed**

As stated previously, legume inoculants based on solid carriers—mainly soil and peat but also other materials—have advantages over other forms of inoculants in terms of preparation, packaging, storage and distribution. Also, the survival of rhizobia applied to seed is superior and nodulation in the field is generally better with the solid inoculants. There are several methods of application.

The finely ground peat inoculant is applied to the seed in the following ways:

- 1) **Dusting.** The most popular method of inoculation is to mix the inoculant with the dry seed directly in the seed hopper. This is generally the least effective

- method because of poor adherence of rhizobia to seed.
- 2) **Sprinkle or slurry method.** Legume seed is sprinkled with water and then mixed with the inoculant powder, or the peat culture is applied as a water suspension. The seed must be dry before sowing (if damp, it will block the seeder), and in the drying process much of the applied inoculant will fall off. In this case the adherence of the inoculant to the seed may be less than desirable. Better attachment of the powder to the seed can be obtained by using an adhesive in the slurry, e.g., a 25% solution of molasses or 1% milk powder in water (Figure E.4.1). Other commercial stickers such as gum arabic or cellulose gum are stronger adhesives. Proportions vary according to the kind of sticker. For example, solutions of 4% gum arabic, 10% sucrose, or 5% methyl ethyl cellulose are frequently used to make a slurry that will easily stick to the seed surface. Some substances have been reported to promote rhizobial survival and have the additional advantages of offering protection against the effect of toxic seed coats and promoting early nodulation. Caution should be exercised in checking that these sticking agents do not contain any preservatives toxic to rhizobia. Amounts of sticker liquid should be minimal to allow rapid drying of seeds (not dried under direct sunlight).



Fig. E.4.1. Good adherence to seed is obtained with various sticking agents.

- 3) **Seed pelleting.** This method is the most effective when rhizobia must be placed in hostile environments. Finely ground (> 300 mesh) lime, gypsum, clay, rock phosphate, superphosphate, peat, charcoal, dolomite, bauxite, calcium silicate, and talc may be used if they have low solubility. To stick the materials plus the rhizobia to seed, solutions or suspensions of gum arabic, methyl cellulose, or other materials may be used. Peat-base inoculant serves well for incorporation into pellets. Major advantages of pelleting are: protection of rhizobia against deleterious effects of sunlight, low soil pH, desiccation, acidic fertilizers, and fungicides and insecticides often used to pretreat seeds. Major disadvantages of pelleting are die-off of rhizobia if seed are not planted soon after inoculation, and the increased seed size and weight.

#### **E.4.b. Granular Implant Inoculants (Indirect Inoculation)**

In the indirect inoculation method, granules of peat, clay (kaolin or sodium bentonite) or vermiculite containing rhizobia are applied to the seedbed beside or beneath the seed (Figure E.4.2). Peat granules are naturally formed during the drying of peat, and can be collected during the screening (sieving) process (Figure E.4.3). Granules also can be produced by coating solid granulated material (e.g., sand) with peat inoculant using an adhesive in a cement mixer. The granules should flow freely through field-implantation equipment. Granular inoculants are used when: (1) conditions for seed inoculation are difficult because of seed morphology and germinating behavior. For example, soybeans and subterranean clover lift the seed coat out of the soil during emergence of the cotyledons, removing seed-applied inoculant from the rooting area; (2) the seed must be treated with fungicides or insecticides that are toxic to the applied rhizobia; (3) small-seeded legumes are used, in which only a small amount of inoculant can adhere to the seed, providing a limited number of rhizobia to effectively nodulate the plant; and (4) inoculant strains require high levels of inoculum to outcompete the indigenous populations.

#### **E.4.c. Liquid or Slurry Application**

The simplest form of inoculant is the liquid inoculant. It is easily produced in the laboratory by inoculating liquid growth medium with a rhizobial culture. This is the most common form used in growth room and greenhouse experiments. Excellent nodulation can be obtained by spraying or pouring inoculum into the row on or beside the seed during planting. A peat culture of rhizobia mixed into a paste is suspended in water just prior to application. Alternately, concentrated broth culture may be diluted in water, but this method is less effective because of poorer survival of rhizobia. Any spray equipment is satisfactory, but must be free from any residues of toxic chemicals. For small field experiments, the suspended peat inoculant can be poured directly over

the seed after placement by hand using watering cans (Figure E.4.4). Liquid inoculants have the advantage of increasing the inoculation rate since there is plenty of room to add more cells of rhizobia into the planting furrow. The major disadvantages for commercial production are encountered in the storage, distribution, and loss in viability of the rhizobia.

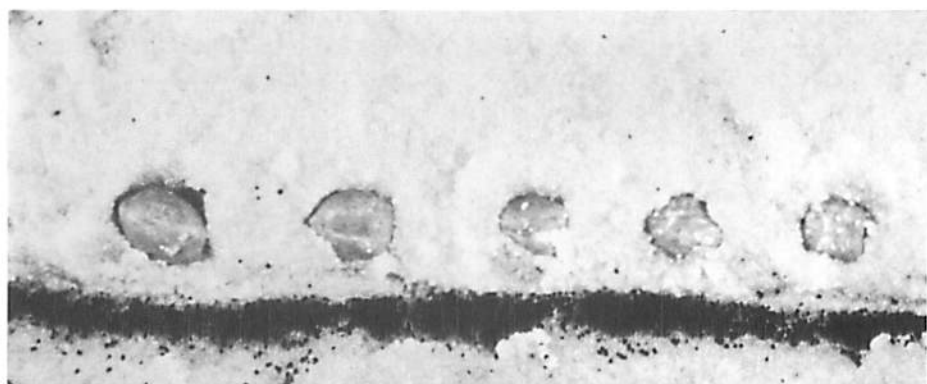


Fig. E.4.2. Implant (granular) inoculant beneath seed.

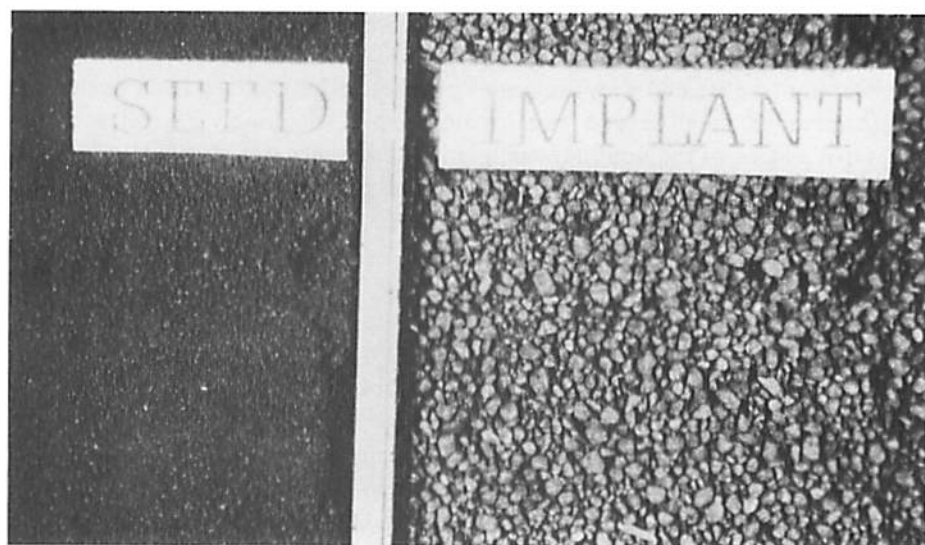


Fig. E.4.3. The two major types of peat inoculants include finely-grained seed inoculant (left) and coarser granular implant inoculant (right).





Fig. E.4.4. Field inoculation of small experiments by direct application of peat suspended in water over seed in the row.

#### E.4.d. Enumeration of Viable Rhizobia on Seed

It may be useful during experimentation to know the numbers of viable rhizobia applied to the seed. Inoculated seed samples should be handled carefully to prevent inoculant from falling off, causing significant reduction of rhizobial numbers prior to the enumeration test. Labelled seed samples should be transported to the laboratory in a cooled container and the enumeration conducted as soon as possible after collection. The determination of the number of rhizobia on seed is difficult by the plate count method since the number of rhizobia may be lower than the number of contaminants. Although the use of a differential medium may aid rhizobial identification and counting, a plant-infection test (MPN) is the preferred method. The MPN technique is described in Section B.3. A sample of 100 seeds can be mixed in 100 ml saline solution (0.85%), shaken vigorously for several minutes, then diluted in a 10-fold series to allow counting. Plate counts and MPN results are expressed as rhizobia per ml or per seed.

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## **II. Field and Greenhouse Assessment of N<sub>2</sub> Fixation**

The methodologies provided in the preceding sections have dealt with manipulations of rhizobia, and are intended to provide the necessary microbiological background to enable the experimenter to conduct complete, well-designed field and greenhouse experiments for improvement of  $N_2$  fixation. With the knowledge and facilities to isolate, count, identify, select, and mass-produce superior strains, it is possible to effectively perform the essential final steps of a program designed to evaluate or improve  $N_2$  fixation. Some knowledge of legume agronomy is assumed in the following sections; where microbiologists lack experience in conducting field experiments, it is hoped that they will combine efforts with agronomists, who are generally familiar with local farming practices and constraints to production. In our experience, the best BNF work will, in fact, come from such teams.

Objectives of field or greenhouse experiments may be to assess  $N_2$ -fixing capacity of host plant or *Rhizobium* strain, determine the necessity for inoculation, determine strain competitiveness or persistence, or to select for superior host plants, rhizobia strains, or host-strain combinations. These questions may be partially answered in greenhouse and laboratory experiments, but ultimately must be resolved under natural conditions in the field.

## F. Planning and Setup of Field Experiments

Field experiments of any kind are nearly always difficult to conduct and usually absorb a large investment in time, effort and money. Field trials are exposed to problems with weather, insects, diseases, and many other difficulties; in many cases, no useful information will be collected because of one or more of these problems. In field work with rhizobia, there is the increased difficulty that living bacteria are part of the experimental system (a fairly important part!), and additional care must be taken to be aware of factors affecting rhizobial behavior and survival and to ensure that rhizobia treatments remain intact. It is therefore critical that field experiments be well planned. It is important to define the characteristics of native rhizobial populations at the chosen site, to inoculate effectively with known numbers of viable rhizobia that can preferably be identified later when they are reisolated from the experiment, to use adequate controls, and to choose an experimental design and a field location that will meet the objectives of the experiment.

Perhaps most important in planning a field experiment is a clear reason for conducting the field study. The purpose of the study will affect selection of the field site, treatments chosen, experimental design, sampling parameters, and data interpretation. Some most common objectives may be to answer the following questions:

1. Is it possible to increase traditional legume crop yields by using inoculation?

2. What is the effect of inoculation, and what savings can be made in terms of N fertilizer application on the following crop in the rotational system?
3. Is one inoculum more efficient than another for a given area or region?

## **F.1. Site Selection and Preparation**

The actual field sites for trials or soil-core collection should be selected on the basis that they are typical of the area in which the legumes are or will be grown. It is strongly recommended that farmers' fields be used, because soils at experiment station sites often tend to be of high fertility (especially N) which may overcome potential treatment differences. In attempting to develop an inoculation policy for a region (such as introducing winter-sown chickpea or a new medic species into an area of low rainfall), the trial should be conducted at (or from, in the case of soil cores or sampling) as many sites in the area as possible.

Sometimes the site is determined from circumstance, being the place that requires evaluation or the place that is available (e.g., experiment station farm). It is suggested, however, that sites be chosen to fit the requirements of the experiment. For example, the ideal site for strain testing is one where soil nitrogen is low and native rhizobia are few in number; if strain selection has been performed effectively, this site will give a yield response to inoculation. If experiments are being conducted to evaluate rhizobial competition, the site soil must have an established native rhizobial population of determined effectiveness and identity. In all cases, the site should be nearly level and present the appearance of being homogeneous in soil type and composition.

In locations where weed growth has been abundant, it is good practice to use a herbicide to control weeds before planting the experiment; type of herbicide will depend on the primary weed species present (see local agronomists for advice). Herbicides do not generally affect soil rhizobial populations.

It is desirable to know any deficiencies or problems with soil fertility in advance of planting the experiment (see Section F.3) so that any amelioration necessary is taken care of. A basal fertilizer application, where necessary (e.g., phosphate, where P Olsen < 8 ppm in Mediterranean soils), can solve problems later in data interpretation. Fertilizer can be spread over the whole of the experimental area and incorporated during cultivation. Banding of the fertilizer will reduce general weed growth and improve fertilizer use efficiency, but it must be remembered that contact of rhizobia with concentrated bands of fertilizer will be lethal to the rhizobia and may reduce nodulation. Since most inoculation experiments measure  $N_2$  fixation, basal application of N fertilizer is not practiced. When it is applied in specific treatments, urea as a source should be avoided, as losses due to soil urease activity may be high. Applied N

is easily lost (see N cycle in Section I.1), so maintenance of soil N for a nitrogen control treatment is best accomplished by side-dressings throughout crop growth. For example, 120 kg N/ha could be applied as 40 kg preplant, 40 kg at early flowering and 40 kg at the beginning of pod fill, or 60-60 preplant and mid-flowering. Nitrogen is fairly mobile in the soil, and side-dressings applied in a shallow furrow next to the row (and covered with soil) will be used easily and effectively by the crop.

As mentioned, the soil fertility and size/effectiveness of native rhizobia population (Section B.3.b) in the soil should be determined by soil testing, to back up trial result interpretation. Since fertility and the size of field populations of rhizobia may vary within short distances in a field, much care should be taken in sampling so that a truly representative sample of the field soil is obtained.

In **soil sampling** the area should be mentally divided into equal squares, approximately 10 for each 0.25 ha, and a subsample taken from each square. A subsample should be roughly cylindrical in shape, 3–5 cm in diameter, and taken from the 5–20 cm depth. A presterilized coring implement or a long-nosed trowel (Figure F.1.1) can be used to extract the subsamples, which are lumped together in a plastic bag. To prevent cross-contamination if another area is to be sampled, a second presterilized sampling tool should be used, or the first one cleaned to remove all soil, washed with 95% alcohol and sterilized by flaming. Because of rapid changes in the bacterial populations of extracted soils, the soil samples should be tested immediately using MPN technique after collection. If this is not possible, the samples should be stored (after air-drying if high in moisture content) at 4°C but for no longer than is necessary.



Fig. F.1.1. Long-nose trowel for the extraction of soil samples.

In the laboratory, the lumped subsamples are thoroughly mixed on a clean, sterilized, solid surface, quartered, mixed again, and so on until a small (100–200 g) composite sample, as homogeneous as possible, is obtained. Stones should be removed, but sieving is not necessary and may remove bits of organic matter in which colonies of rhizobia reside. If desired, soil moisture content can be determined to express population figures on a soil dry-weight basis.

Air-dried samples are sent for analysis to a soils laboratory (to ICARDA if one is not available locally). It is wise to keep a duplicate sample after submission for analysis since samples are sometimes lost in transit to the testing laboratory.

## **F.2. Naturalized Rhizobial Populations in Soils**

Because of the long-term cultivation of currently popular legume species in the region, most soils contain large, stable populations of rhizobia capable of nodulating these species. The exceptions are where land is being newly cultivated (e.g., newly irrigated desert) or exposed to the crop for the first time (e.g., introduction of new medic species, or winter-planted chickpea in areas of low rainfall not previously planted to chickpea).

The characteristics of this native rhizobial population that are most likely to affect field experiments are the size of the population and its symbiotic effectiveness with the legume host. Both of these factors will affect competition with introduced strains and therefore will influence the success of the inoculum in forming nodules on the legume. A preliminary estimate of the competition from soil rhizobia likely to be encountered by field inocula and an idea of its effectiveness can be obtained by enumeration of the native population using a plant infection (MPN) test with the concerned legume host. The procedure for the most probable number (MPN) test is fully explained in Section B.3.b.

The numbers of native rhizobia likely to overwhelm any introduced strains have been estimated by various investigators to be in the range of 50–1000/g soil, but in our work we have found that the infectiveness, competitiveness, and effectiveness of native strains with the particular cultivar or species also have a great influence on whether a response to inoculation can be achieved. Certainly, in soils where high native populations exist (i.e.,  $> 10^4$  rhizobia/g soil), the likelihood of an inoculation response is greatly decreased, but if these native strains are not effective with introduced cultivars, a successful selection program for effective and competitive strains may lead to an inoculation response.



### F.3. Assessment of Available Soil Nitrogen

Soil pH, phosphorus, and nitrogen often have large effects on rhizobial growth, invasiveness, and survival; but soil pH in the Mediterranean region (except in limited areas of low pH) is not extreme enough to adversely affect plant or rhizobial growth. The levels of available phosphate and nitrogen, however, may affect plant growth and the symbiosis to a large extent. Lack of phosphorus may limit legume growth, and may further affect the symbiosis itself (see Section G.1 for trial to determine P effect on symbiosis). The effect of nitrogen, either mineralized from the soil organic matter or applied as fertilizer, depends on the amount available. Higher levels of available soil N will inhibit nodulation and therefore depress fixation. On the other hand, it may be beneficial to apply small 'starter' doses of N fertilizer (e.g., 20 kg N/ha) where soil N levels are extremely low. The interference with  $N_2$  fixation where soil N levels are high will increase the proportion of plant N derived from the soil while decreasing the amount from fixation, and will tend to mask effects in inoculation response studies. However, unless soil N is measured before planting or  $^{15}N$  is used to differentiate N-source in the plant, you cannot know that high soil N has interfered with fixation.

Collection of soil samples for analysis is explained thoroughly in Section F.1. Depending on locally available facilities, however, it may be difficult to have these samples accurately analyzed. As mentioned, it is possible to have samples sent to the ICARDA headquarters for analysis, but problems may be incurred in getting samples to the Center intact, and in waiting for the results to arrive. We therefore suggest that in all field experiments, duplicate plots of a nonfixing crop that generally matches legume growth (see Section H.3.d for reference crops) should be planted with and without 120 kg N/ha added. Growth of this 'reference' crop without applied nitrogen will clearly indicate the approximate availability of soil N; if plant growth of -N reference is similar to +N reference, the soil N level is high enough to strongly interfere with  $N_2$  fixation. If the -N reference plant produces little growth (with older leaves turning yellow before podfill stage), and +N reference responds strongly to N, soil N level is low and soil N level is suitable for BNF studies (Figure F.3.1).

Where it is possible to measure soil N in a suitable laboratory, the best test is generally one that which measures  $NO_3^-$  in the soil KCl extract. Where nitrate levels in the soil surface 60 cm are above 10 ppm,  $N_2$  fixation will be adversely affected, at least during early crop growth. If levels are very high (> 20 ppm), it may be best to utilize a different field or to modify the soil.

If soil N levels are high (as is often the case in experiment station soils), the level of available N can be decreased by: (1) growing a cereal crop (with no N addition) on the land before its use for nodulation experiments to decrease available N; and/or (2) incorporation of heavy dressings (0.3–0.5%) of low C:N ratio organic matter (e.g., cereal straw or chaff) several weeks before planting. In the first case, soil N will be

depleted. In the second case, the available N will be immobilized until the added carbon is broken down; this period is usually long enough for growth of the legume crop, given that enough carbon has been applied.



Fig. F.3.1. Soil cores planted with barley to indicate N status of the soil. The core on the right has received no N fertilizer; the left equivalent of 80 kg N ha<sup>-1</sup>. This Tel Hadya soil is clearly N-deficient.

## F.4. Selection of Treatments and Experimental Design

Field experiments should be designed and performed carefully so that conclusions may be reached from collected data. To make the conclusions as accurate as possible, replication and randomization of the experiment are necessary. The heterogeneity of the soil is generally the greatest source of experimental error, so treatments must be replicated. Replication reduces the uncertainty of the data and provides an estimate of experimental error. Multiple observations from a plot are not enough to resolve experimental error; these only allow estimation of sampling error. Replication is in itself also not sufficient; treatments must be assigned to plots at random to account accurately for field heterogeneity.

Each field experiment contains variables that must be considered in the choice of an appropriate experimental design. The simplest design, which can be used in most N<sub>2</sub> fixation experiments, is the randomized complete block (RCB); this is usually effective for experiments having up to 12 treatments. The RCB design has the advantages of being easy to set out and maximizing the number of degrees of freedom for error. Where more than one set of treatments (or factors) is to be examined, a factorial experiment may be set up. The factorial is not an experimental design, but contains all combinations of several levels of several factors. The simplest example is a 2 × 2 factorial, with two cultivars inoculated and uninoculated; this can be arranged in a RCB design. A split-plot design may be used when it is possible to arrange the plots of a multiple-factor experiment (e.g., strain × cultivar) so that all factors are not distributed over the replication and factors are not of equal importance. The split-plot design provides a more precise comparison of treatments assigned to subplots and a less precise comparison of the whole-plot treatments.

Work at ICARDA on nodulation specificity and strain effectiveness in all ICARDA-mandated legumes has revealed considerable host-strain interaction as well as favorable responses to inoculation. Inoculation response trials can be factorial and contain different cultivars as well as different strains to find strains most effective across cultivars or a potential strain mixture for all cultivars. In dealing with numbers of cultivars and strains, we have found it useful to use the split-plot design, with rhizobia treatments as main plots to minimize cross-contamination between these treatments.

Some suggested trial layouts for specific experiments are given in detail in Section G.

#### **F.4.a. Control Treatments**

Any trial which aims to assess response to inoculation must contain an uninoculated control treatment against which inoculation treatments can be compared. Minus-nitrogen, uninoculated controls are necessary to judge the nodulating and nitrogen fixing abilities of the native rhizobial population with the test cultivar(s) at the trial site. Therefore, a replicated uninoculated control treatment should always be included in a field experiment.

A nitrogen-fertilized treatment, receiving enough N to provide optimum growth of the test plant and thereby removing the 'symbiosis' variable, ideally can be used to measure the growth or yield potential of the crop under the given environmental conditions. In practice, however, the difficulties in maintaining a constant and sufficient supply of nitrogen mean that the legume will not rely solely on fertilizer N, but will form some nodules and fix N. We still consider it worthwhile to include a N-fertilized control in our experiments, and modify N fertilization so that N is provided

over the growing season. For grain and forage legumes, 120–150 kg N/ha will be sufficient to provide maximum growth, and if applied as split application, with 50 kg preplant, 50 kg side-dress at early flowering, and 20–50 kg at beginning of podfill, it will be available as required by the plant.

For pasture legumes, 90–100 kg N/ha is applied to uninoculated N control treatments. The nitrogen fertilizer (ammonium nitrate or ammonium sulfate recommended in high pH soils) is evenly distributed in the plot by broadcast application and the total dose split into three. The first application is done when most of the medics or clover plants have germinated. The second is applied three weeks after the first application and corresponds to the second leaf stage. The last portion is applied approximately three weeks after the second application when plants have three to four leaves.

#### F.4.b. Plot Size and Layout

The size and spacing of the plots will be based on the growth habits of the legumes. For forage and food legumes, recommended densities are: faba bean, 15–22 plants/m<sup>2</sup> (lower density for large-seeded type); chickpea, lathyrus, and vetch, 33–45 plants/m<sup>2</sup>; and lentil, 180–200 plants/m<sup>2</sup>. These legumes are commonly planted with 30-cm spacing between rows, and plant spacing is adjusted accordingly; this allows for uniformity in setup of cultivation and planting tools (Figure F.4.1). With isolated



Fig. F.4.1. A small plot planter like the one pictured enables the experimenter to plant quickly and uniformly. Leaving furrows uncovered will allow inoculation using the liquid slurry application method after planting.

individual plots, edge effects (increased availability of N and/or water to outside rows) can be considerable, so we advise a minimum of three rows per plot (or adequate use of guard rows), using only the middle portion of the middle row for measurement of treatment effects. Examples are given in NTI and IR experiments in Sections G.1 and G.2.

For pasture legumes, we generally use experimental plots of approximately 2 m<sup>2</sup>. Seed can be broadcast or planted by hand in rows. When seed is broadcast we use plots of 4 m<sup>2</sup> (2 × 2 m). Seed can be planted in rows separated by a distance of 17–24 cm depending on the growth habit of the pasture legume. At ICARDA we use 17.5 cm for all medic species. Each plot consist of four 2-m- long rows. Alleyways for both types of planting systems are 1 m wide to allow mechanical cultivation with the available rotovator (Figure F.4.2).



Fig. F.4.2. Plots should be clearly labelled at planting time.

## F.5. Precautions in Field Experiments

Throughout the earlier parts of this manual, we have repeated the importance of sterile technique, or hygiene, so that the particular strain under study is not confused with another strain or type of bacteria. Nowhere is this more important than in field experiments. Contamination of uninoculated controls or treatment plots has ruined results in many well-planned, otherwise well-conducted field experiments.

*Rhizobia* are mobile organisms, and are easily transferred from plot to plot by soil on hands, shoes, or animals, or by surface movement of wind or water. The easiest of precautions is to always handle uninoculated treatments before inoculated treatments, in all applications, from planting to weeding to sampling. It is therefore necessary to wash hands, clean shoes, and sterilize equipment and implements between operations with one treatment and the next. Use different or sterilized implements for inoculating batches of seed with different treatments. If hand-planting inoculated seed, the operators should wash their hands with 70% alcohol between treatments. Seed drills for sowing preinoculated seed in large experiments are extremely difficult to sterilize between treatments; if possible, clean the seed box and seed lines with compressed air, then sterilize with spray of 95% alcohol for rapid drying. Any shovels or cultivation tools used in sampling or plot maintenance should be sterilized by washing with water and dipping in 20% alcohol between use in different treatments. Take care not to walk across plots, but between them. Finally, drainage ditches between plots of different treatments will ensure that overirrigation or heavy rainfall will not cause movement of waterborne surface soil, and with it *rhizobia*, to contaminate other treatments; this is especially important if the field is not level.

Applying biologically active materials such as fungicides, insecticides, herbicides, etc., to the soil or seed should be avoided unless their use is essential and the experimenter has positive evidence that they have no effect on *rhizobia*, the host legume, or nodulation.

Although it is not often considered, conducting and evaluating a trial has a great deal to do with the person performing the activity. It is generally considered that any basic operation is performed the same by all persons, but this is not the case; personal viewpoints cause different persons to perform tasks differently. For example, in hand sowing, one person may plant more deeply than another, with the result that seeds will emerge later. If more than one person is planting, therefore, they should not each plant different treatments but should plant different replications of the same treatment before moving on to a different treatment. The same is true with making observations, where one person may judge plant disease, vigor or nodulation in a different way than another. For example, in scoring an experiment for nodulation, one technician may score on a slightly different basis than another technician. It is better, therefore, to have one person score the whole experiment than to share the task with

another. Where the experiment is too large for one person, then the work load should be shared on the basis of replications, with the two technicians finishing replications of one treatment before moving to another treatment.

Data analysis and interpretation will be made considerably easier if a few simple rules are followed in planning the experiment.

1. Obtain statistical advice early, for it will always be easier to change the design than to repeat the experiment (with a changed design).
2. Decide which controls and comparisons may be needed to determine your objectives, and include them.
3. Make sure you have the resources to cope with the sampling and analysis which will be necessary for the planned experiment (try to imagine how much time everything will take). If you feel that resources will be stressed, cut back treatments or experiments.

## **F.6. Methods of Inoculation**

One of the primary considerations to be made before applying inoculants in the field is inoculant quality. If inoculants are prepared freshly in the laboratory prior to planting, the operator is relatively certain that the inoculant contains high numbers of the desired strain(s). If, however, inoculants are purchased locally or from suppliers abroad, how can the experimenter be certain that the inoculant is of high quality, or if it actually contains any living rhizobia? Shipments, whether by air, ship or truck, are often exposed to high temperatures ( $> 40^{\circ}\text{C}$ ), particularly in late summer; even brief exposure to these high temperatures will kill rhizobia. It is a fact that processed peat containing no rhizobia looks exactly like a high-quality peat inoculant, so visual observation is no assurance (Figure F.6.1). The only way to assure quality of an unknown inoculant is to perform a plant-infection test (MPN) prior to inoculant use; this requires a lead time of six to eight weeks before planting (Section B.3), and more time if the inoculant is of poor quality and must be replaced. This quality check, however, is of utmost importance when conducting inoculation trials; failure to achieve inoculant response in the field because of poor inoculant may otherwise be attributed to other causes, misleading the investigator in future experiments.

The methods that can be used for inoculating legume seed are numerous, and sometimes are determined by the objectives of the experiment itself. Some general principles, however, apply to all methods of application.

1. Uninoculated controls always should be handled before inoculated treatments to reduce the risk of contamination of controls.
2. The numbers of rhizobia in the inoculant should be as high as possible

(depending somewhat on objective) since the presence of large numbers of inoculant rhizobia will improve the probability that inoculant strains will infect the legume host.



Fig. F.6.1. Visual appearance of the inoculant is never an indication of quality.

3. Field experiments should be designed and conducted with the awareness that rhizobia are easily transferred from plot to plot by water movement, human operations, or animal movement; or by moving soil or water from plot to plot.
4. Rhizobia may be killed by many pesticides or fungicides used as seed treatments, so treated seed should not be used where rhizobia come into contact with the seed.

Methods of inoculation have here been classed as direct, where inoculant is applied directly to the seed, or indirect, where the inoculant is applied as a liquid or solid into the sown row with the seed. More detailed information on methods of inoculant application is given in Section E.

#### **F.6.a. Direct Application of Rhizobia to Seed**

Legume inoculants based on solid carriers (see Section E.3.a) are the most common because of their advantages in preparation, packaging, storage, and distribution. Also,



high-quality solid inoculants have been shown to be superior to other types in terms of rhizobial survival and nodule formation in the field. There are several methods of application, which are presented here in order of least effective to most effective.

**Dusting** is the simplest method of application, but also the least effective, because most of the applied inoculant does not adhere to the seed and therefore is not in the place to produce maximum effect at the time of seed germination. With this method (the method used most commonly by farmers in developing countries), the powder inoculant is mixed with the dry seed immediately before sowing. Very little of the inoculant sticks to the seed; most is lost.

**Slurry inoculation** involves utilizing something to increase the amount of powder inoculant that sticks to the seed through the process of sowing. In the simplest case, the peat is mixed with some water and poured over the seeds; a slightly better method is to first wet the seed with water then mix the powder with the moistened seed. The seed must be dried before sowing (but never in direct sunlight), and as it dries and during the sowing operation, most of the inoculant will fall off. Better attachment of the powder to the seed can be obtained by adding a 'sticker' to the liquid or slurry. This sticker can be household sugar (10% w/v in water), molasses (10% v/v in water), powdered milk (3 g/L water), or commercial adhesives like gum arabic or cellulose compounds. The most effective method of application is to first wet the seed with the sticker, then thoroughly mix in the powder inoculant. We have found large plastic bags (of up to 50 L capacity) to be ideal for mixing without damaging seed (Figure F.6.2). Alternately, the powder may be mixed into seed in a seed box or on a ground-cloth, using whatever method comes to mind for distributing the powder evenly over the seed. Seed must be dried before sowing, but seed inoculation should not be performed more than a few hours before sowing. An example of an inoculation procedure is given in Section G.2, using cellulose gum as a sticker.

**Seed pelleting** is a procedure commonly used with small-seeded pasture legumes, and has been utilized widely to establish pasture species in acid soils. Its advantages include protection of the inoculant rhizobia against unfavorable physical and chemical conditions in soil and against competition from other soil bacteria. Pelleting also ensures better survival of rhizobia when delays between inoculation and sowing are unavoidable.

The process of pelleting involves first sticking the finely powdered inoculant to the seed, then covering the inoculated seed with a pellet coating of calcium carbonate, gypsum, bentonite, rock phosphate, charcoal, or any other of a wide choice of materials. Adhesives used to attach the coating material include synthetic glues, vegetable or animal glues, gelatin, and sugars. Adhesives must be free from preservatives.



Fig. F.6.2. Mixing sticker, seed, and inoculant in a large plastic bag is easy and effective. Up to 50 kg of seed can be inoculated in large plastic bags with this method.

Further details on all methods are given in Section E, on production and use of inoculants.

### F.6.b. Indirect Application of Rhizobia in the Field

In some cases, it may be preferable not to apply the inoculant to seed, but to add the inoculant to the seed row before or after placing the seed in the furrow (but always before covering seed with soil). The most common reasons for indirect application are: (1) where use of fungicides or pesticides as seed dressings are necessary because pre-emergence diseases or insect attack are problems; and (2) it may be advantageous to apply inoculant at a higher rate than is possible with seed inoculation with very small-seeded legumes or where native rhizobia offer strong competition.

**Solid inoculant**, or soil-implant inoculant, is composed of large granules of peat (see Figures E.4.2 and E.4.3), or an inert material covered with peat to make granules that can be placed in the row with the seed. Solid inoculant can be applied through the fertilizer box or insecticide attachment of a seed drill, and for smaller experiments can be placed by hand in the row beneath or alongside seed. The major drawback to commercial use of this method is the higher quantity of inoculant required (10–20 kg/ha vs 0.5 kg/ha for powder), and hence the higher cost involved.

**Liquid inoculation** involves mixing a peat culture of rhizobia with water, and spraying or pouring the liquid onto the seed at planting. Freshly grown liquid or agar cultures are not generally suitable for dilution because of poor survival when applied directly to the field soil. Any spray equipment is usable, given that it has not been used previously for toxic chemicals. Nozzles should be checked frequently for plugging. A small-scale hand application method (Figure F.6.3) is described in the inoculum response trials (Section G.2).

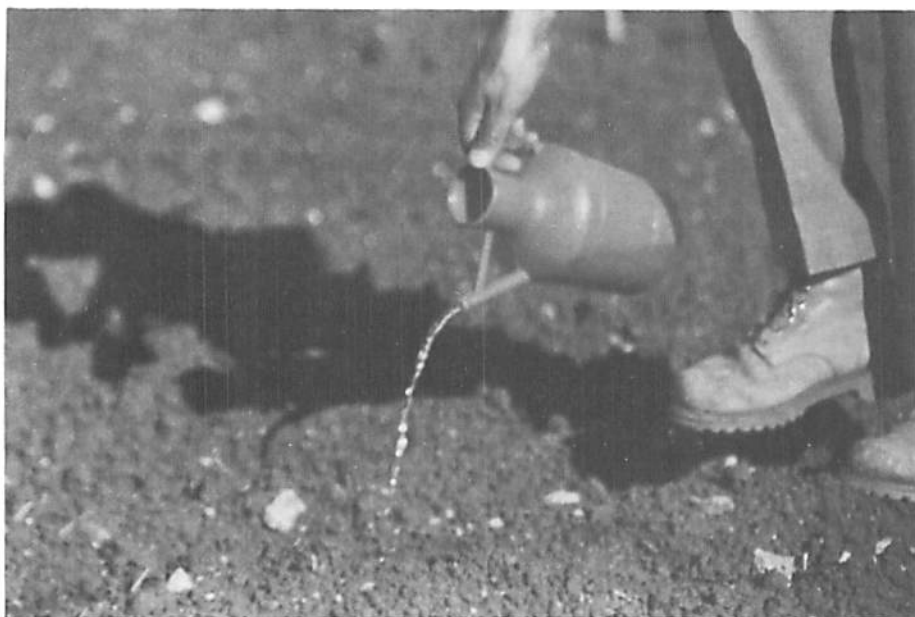


Fig. F.6.3. A small watering can is used to effectively inoculate small plots with a liquid slurry of peat inoculant.

## F.7. Trial Evaluation and Interpretation of Data

Response to inoculation is generally measured by plant dry-matter production, total plant-N accumulation, and seed yield (grain legumes). In addition, nodulation can be evaluated at different stages to indicate the activity of inoculant strains.

It is fairly common for legumes to show an early, visible response to inoculation that does not translate to a seed yield increase. For this reason, it is desirable to measure early response of treatments to distinguish between no response and a potential for response.

### F.7.a. Nodulation

Nodulation may be assessed several times during early growth of the crop, when nodule number, size, and distribution can be indications of a response to inoculation. For food/forage legumes, we have found that a single observation made at mid-flowering usually indicates whether inoculation has been successful (Figure F.7.1). For medics, sampling for nodulation assessment is also usually performed once. We usually take five plants per plot, 10–14 weeks after germination. The time depends on the season; dry, cold weather will delay the sampling date. Usually plants have four to five leaves at nodule sampling.



Fig. F.7.1. The period from mid-flowering to early podfill is the best time to evaluate nodulation in food and forage legumes.

Nodule number and nodule dry mass are the parameters most often measured, but data collection can be extremely tedious and time consuming, especially where nodules are small and numerous (as in lentil or medics). In many laboratories, a nodule scoring system (Table 1) is used which takes into account nodule number, distribution, and effectiveness; this system has been used at ICARDA on grain legumes with success, as indicated by a high correlation between nodule scores and dry matter production/N accumulation at mid-flowering. The scoring system may be modified to fit nodulation characteristics of the legume. Nodule effectiveness is determined by nodule color (internal pigmentation) and nodule size; ineffective nodules are not considered. Effective nodules generally have pink-red internal tissue,

while tissue of ineffective nodules is green or white. It is suggested that 6–10 plants from each treatment and replication be carefully dug out of the soil with a minimum of disturbance (Section A.1). Root systems are placed in a plastic bag, and transported to the laboratory where the soil is carefully washed away from the roots under running water in a screen to catch detached nodules (Figure F.7.2). Roots are scored (one person scoring all samples), and root systems with intact nodules kept in a refrigerator for nodule typing (if desired).

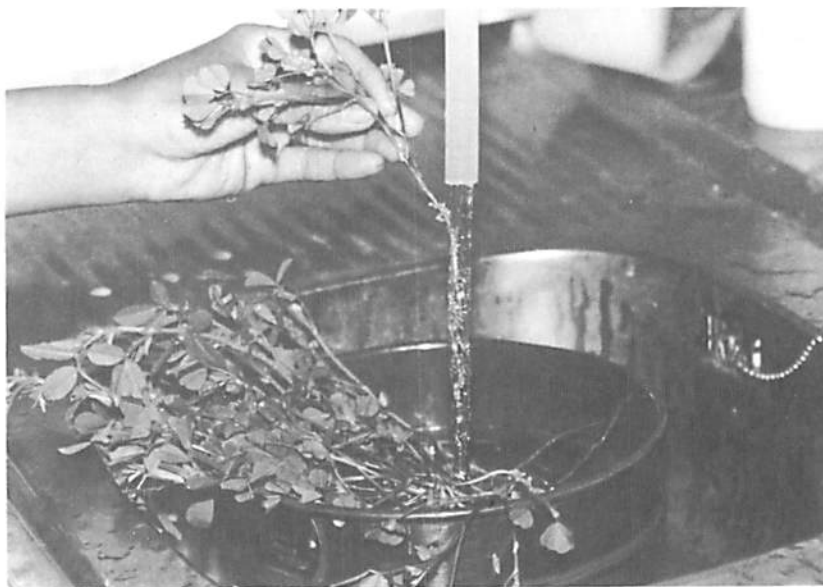


Fig. F.7.2. Roots being washed in running water using a screen to trap detached nodules.

The scale used at ICARDA for scoring medic nodulation is based on a qualitative criterion and ranges from 1 to 4: 1 corresponds to no nodulation; 2 to ineffective nodulation (nodules abundantly distributed, with white internal tissue; 3 to nodules laterally distributed, with red-pink coloration inside; and 4 to highly effective nitrogen fixing nodules usually located at the upper region of the primary root or along the primary root. Nodule number per plant is also recorded. Nodule weight, although a desirable characteristic, is not currently measured for medics in ICARDA trials. Morphology of the nodules varies from coralloid to single ovaloid types. Coralloid nodules are generally effective and characteristic of certain medic species (e.g., *Medicago polymorpha*). Some species produce a greater number of effective nodules than others; this may depend on the availability of water during the growing season. In general, nodule shape in medics is ovaloid.

The identification of rhizobia forming the nodules can be useful in inoculation experiments. If inoculant strain(s) can be distinguished from the native bacteria, invasiveness of an inoculant can be determined easily by testing whether the inoculant strain has been successful in occupying the majority of nodules. Procedures and a full explanation of the uses of nodule identification are given in Section C.

**Table 1. Nodule scoring system (after Corbin *et al.*, 1977).**

| Distribution/number of effective nodules |        |           |
|------------------------------------------|--------|-----------|
| Nodule score                             | Crown* | Elsewhere |
| 0                                        | 0      | 0         |
| 1                                        | 0      | few       |
| 2                                        | few    | 0         |
| 3                                        | many   | 0         |
| 4                                        | many   | few       |
| 5                                        | many   | many      |

\* crown regarded as top 5 cm of root

### F.7.b. Yield Parameters

Yield may be measured at any stage during plant growth, but objectives of the experiment should be kept in mind. After seasons of multi-point sampling during early plant growth, we have found that a single early plant sampling in food legumes for plant dry-matter production and total N accumulation at mid-flowering is sufficient. Samples for dry matter measurement should be placed in loosely woven cloth bags or paper sacks and dried in a forced-air oven at 100°C where dry matter determination is the only objective, or at 60°C if N content is to be analyzed. If a drying oven is not available, samples can be dried in bags hung in the shade until they achieve a constant weight.

With pasture legume, yield is based upon the amount of dry herbage the legume has produced at harvest. Seed is sometimes an important criterion. Harvest is normally done at the stage of late seed pod filling. When harvesting experimental units planted by the broadcast method usually an inner 2.25 m<sup>2</sup> is collected by using a rod template square having a side of 1.5 m. All plants inside this square are harvested.

Alternatively, when experimental units contain four rows, the two inner rows are harvested by hand except the top and lower portions. Rows are usually 2 m long, of which the inner 1.5 m is harvested. Samples are collected in cloth bags (one bag per plot) and dried in an oven at 80°C for two days. After weighing, subsamples are taken for fine grinding and analyzed by the Kjeldahl method for N determination. Results

are expressed as mass of herbage yield per unit plot. These can be extrapolated to tons of dry herbage per hectare.

With food legumes, seed yield is the parameter most researchers consider of greatest importance. Response to inoculation, however also may be indicated by straw yield or total N increases. When harvesting, as much area as possible should be collected from each plot and placed into carefully labelled cloth bags (one per plot) for further drying (if necessary) before threshing. Care should be taken to avoid harvesting plants at plot borders, which often display greater growth due to increased water and/or N availability. With small experiments, we suggest that the material be hand threshed; the time involved is worth the accuracy. If many or very large experiments are harvested, a mechanical thresher may be used if all plant material is removed from drum and rollers (using a stream of compressed air where possible) between plot samples (Figure F.7.3). We normally express crop yield as total seed yield, total aboveground plant dry matter, and total straw yield, usually on a kg/ha basis.



**Fig. F.7.3.** A small mechanical thresher can be used if a large quantity of plant material is harvested.

Total crop nitrogen, whether measured during early plant growth and/or at harvest, is popularly used to evaluate  $N_2$  fixation. It is important to realize, however, that increased  $N_2$  fixation (as measured by  $^{15}N$ ) is not always accompanied by increased total N (see Section H). The dried sample (or subsample) is ground to a fine powder

with a standard laboratory mill (Figure F.7.4). Care must be taken to thoroughly clean working parts of the grinder between samples. Ground material is kept in moisture-proof containers or bags and stored in the dark until analyzed. Sample analysis involves measurement of  $\text{NH}_3$  following Kjeldahl digestion; the procedure is given in reference section documents. Crop nitrogen is often expressed on the basis of total N (kg/ha), and may be broken into seed N and straw N (if separate samples are analyzed) to view effects on N distribution.

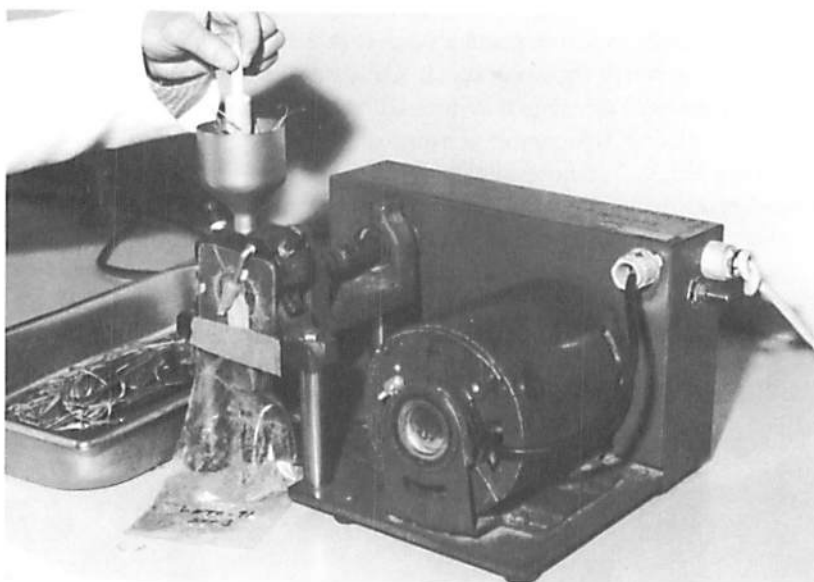


Fig. F.7.4. Wiley mill for grinding plant and soil samples.

Methods of measuring  $\text{N}_2$  fixation are fully discussed in Section H.

## F.8. Statistics and Analysis of Experiments

Experimental design is the planning of field experiments so that with proper manipulation of the data general conclusions can be drawn about the effect of the treatments applied upon the yield or other characteristics of the crop. It is essential that one have an understanding of the concepts of experimental design before attempting to conduct field experiments.

There is great uncertainty in any field experiment. Results often can be due to chance occurrence rather than any natural truth. Field-plot size can vary greatly, but we do



not generally use very large plots. To get meaningful information we must use small plots that can be managed by the resources at hand. To draw general conclusions we use inductive reasoning; that is, we observe small plots and then attempt to draw general conclusions concerning the effect of the treatment applied.

Conclusions about the effect of a given treatment applied to plots in a field experiment are made with the help of statistical analysis. This consists of using various mathematical procedures to manipulate the data so that we can better evaluate the effects of our treatments. Statistics will not tell the research worker what the data indicate, but they are useful in helping draw the right conclusions. The need for statistical analysis in field research is obvious to the research scientist. In fact, it is so obvious that the researcher tends to forget that it is not so evident to everyone. Biological material is variable, and soil is seldom uniform over an experimental area. Differences in crop yield due to these variables are referred to as 'experimental error'; research workers must design experiments that separate the effects of these factors from the effects due to applied treatments. The steps to be taken in conducting a field trial are listed in sequence below. Most important is experimental design, which will determine what information can be obtained from the trial.

There are several ways by which it is possible to overcome the effect of these extraneous factors, which are contained in our experimental material and so cannot be eliminated. **Randomization** and **replication** are the two most important procedures used. Soil differences generally will not be apparent so we assign the treatment to each plot by chance. That is, we 'randomize' the treatments. There are several ways of doing this. A simple and effective method is to put numbers on slips of paper and pull them out of a bag. If the experiment has many treatments and replications we can use tables of random numbers found in books of statistics. Error can be reduced further by splitting the land into smaller plots with more replications, although obviously there is an effective lower limit to plot size. Often in practice, plot size and the number of replications needed are decided first, and then the amount of land needed is determined.

The replications are generally referred to statistically as blocks, and the most common simple form of trial as a 'randomized block design'. With proper statistical procedures all variation due to differential behavior of the experimental materials (in this case the legume plants) because of the soil differences can be identified and separated from the effect due to treatments.

To determine whether the yield differences observed were indeed true yield differences we proceed to our statistical analysis. These mathematical manipulations allow us to evaluate the effect of the land ('blocks') separately from other variation due to the crop itself, or the research worker's technique. Therefore, we are able to correctly determine the effect due to the treatments added, and also to judge how well

the experiment was done. The statistical procedure most commonly applied to field-plot data to determine whether the differences observed represent real differences is called 'analysis of variance' (ANOVA). It is always the first step in data analysis from field experiments. Other procedures are sometimes used but these generally build upon the values obtained in the analysis of variance.

Some essential statistical terms are necessary:

1. The '**coefficient of variation**', generally noted by 'C.V.' or 'CV', and expressed as a percentage, is a measure of the variability among the individual plots. Therefore, it is a measure of the care with which the experiment was performed. It measures the variation not accounted for by treatments or by the basic variability of the experimental material. Extremely low CVs will not normally be encountered in field plots. Although it is desirable to keep the CV below 20, ranges of 20-30 are common in field experiments and can give useful information if the differences between treatments are great enough. With CVs above 30 the size of the differences between treatments needed before they can be detected becomes larger than the differences generally encountered. Lack of treatment response along with a high CV may indicate a difference masked by poor management of the experiment rather than a lack of treatment response. Therefore, experiments with high CVs should be repeated using more care. If good management does not reduce the CV it will be necessary to increase the number of replications.
2. The '**level of significance**' tells us how likely certain events are to occur. It is the probability of an event being due to chance rather than to added treatments. In this case the event is the yield increase due to the treatments. A significance level of 5% indicates that there are fewer than 5 chances out of 100 that the results obtained were due to chance alone, which indicates that there is a greater than 95% chance that the results were due to the applied treatments. Likewise with a significance level of 1%. Often results that are significant at the 5% level are referred to as 'significant' and those at the 1% level as 'highly significant'. The former is often indicated by the mark '\*\*' after the data means and the latter by \*\*\*.
3. '**F tests**' and '**t tests**' are used to compare treatment means. With all of these various statistical values you should not be concerned about what they represent mathematically, but rather what a given value tells you about your data. Both 'F tests' and 't tests' measure the same thing so we will not go into detail of when to use which one. This can be dictated both by the experimental design and by the whim of the researcher. Both 'F' and 't' tests indicate the likelihood of the yield differences obtained being due to chance rather than to the effects of the applied treatments. They may be given as a probability of occurrence or as a percentage.
4. The '**arithmetic mean**' or just the 'mean' is the value we use to compare one

treatment with another. The various mathematical manipulations we use in statistics merely tell us the likelihood of the means we obtain being the correct ones. The mean is often indicated in statistics as 'x bar'. Most people refer to it as the 'average'. Mathematically it is the sum of all of the numbers in a group divided by the number of individual items.

5. The '**standard error of the mean**' (SEM), or just 'standard error', is a measure of how much the treatment means we calculate will vary from the actual mean. Therefore, if an experiment is repeated you expect to get a mean for a given treatment that is within the range of the mean of the first experiment plus or minus the SEM.
6. '**Interaction**'. This refers to whether one applied experimental treatment behaves in the same way when in combination with all of the other treatments. Two factors are said to interact if the effect of one factor changes as the level of the other factor changes. An interaction between two factors can be measured only if the two factors are tested together in the same experiment (i.e., in a factorial experiment).

Determining if comparisons are important is the most important step and the one which requires that the research worker stop and think about what his data mean. These procedures are designed to measure the differences between means in a series, such as crop varietal trials, or to array such means in order from highest to lowest or to form them into groups of like performance. There are many such tests, but the ones most likely to be encountered are the 'least significant difference' (LSD) and Duncan's multiple range procedures. Results of the latter are usually expressed by placing the same letter after all means not significantly different or by connecting them with spanning lines. These would be useful in conducting an evaluation of a series of strains of *Rhizobium*, but not in an experiment to compare the effects of inoculation with other treatments.

For example, suppose that an experiment had a significance level of 0.2. That is, the yield increase obtained could be expected to be due to inoculation only 80 times out of 100. On this basis would you recommend to farmers that they inoculate? If the yield increase was worth enough over the cost of the inoculation then you might want to make such a recommendation. But would you tell the farmer that the recommendation was based on data that had only an 80% chance of producing a real increase in yield? No, rather you would say that there was a good probability of the farmer getting a yield increase of whatever amount was indicated by the experiment.

All treatments should directly address the question you wish to answer with the experiment. You also must be careful not to leave out any treatment that is crucial to answering your main question. As has already been stressed, a field experiment represents a big investment in time and resources. Experimental design and treatment

selection should be discussed with knowledgeable colleagues. It is always possible for even an experienced investigator to miss a point that a disinterested but knowledgeable person would note.

Especially important is checking with a statistician. All research workers have a statistician available to them somewhere in the system who should always be consulted early in the planning. This will eliminate the problem of collecting data that cannot be manipulated to give answers to your questions. This does not mean that you should let the statistician dictate your research. You know what you want to find out and you know what is practical to do. The statistician may make suggestions that are not practical and it is your responsibility to point this out so that an alternative might be found. The basic role of a statistician, in this case, is to advise whether there would be a problem with the statistical interpretation of your data if you conduct the experiment according to your plan.

**Data interpretation** involves determination of whether the yield differences obtained, or differences in any other observation made, are important. This is not the same as determining whether they are statistically 'significant'. Data may be highly significant statistically but not be important to the researcher. However, the converse is not true. If data are not significantly different at a level acceptable to the researcher then they cannot be considered. The researcher must determine how much uncertainty can be tolerated in the results. Although 5% and 1% levels are most often used, the researcher must consider what value is important in a given study. It must be kept in mind, however, that not obtaining a yield difference in a carefully controlled experiment tells the researcher just as much as finding one does. People like to get positive results, but negative ones can be just as important; it is as useful to know that inoculation does not increase yield of one crop or in one location as it is to know that it does increase yield of another or in a different location.

Steps in the establishment of an experimental field trial:

1. Define the problem.
2. Determine the treatments.
3. Determine the experimental design.
4. Consult with the biometrician/statistician.
5. Select suitable land.
6. Acquire treatment materials.
7. Prepare the land properly.
8. Prepare treatment materials.
9. Plant the experiment.
10. Manage the experiment well:
  - a. Make detailed notes on any unusual occurrence.
  - b. Control weeds.

- c. Control pests.
- d. Avoid plant disease.
- e. Make all experimental observations in a timely manner.
- f. Organize field notes promptly.
- g. Irrigate when, and if, required.
11. Harvest properly, and at the proper time.
12. Organize and file data carefully.
13. Analyze the data.
14. Draw conclusions.
15. Plan for continuing or follow-up trials if needed.

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## **G. Assessing the Need for Inoculation and Inoculation Effects**

Two major factors affect symbiotic nitrogen fixation of legumes in field soils: (1) the presence and effectiveness of indigenous rhizobia populations, and (2) soil fertility. Improvement of the nitrogen-fixing capacity of a legume crop may be possible where either of these factors is a constraint to maximum fixation; inoculation is performed where native rhizobia are present in low numbers or are ineffective, and fertilization (e.g., with phosphate) where nutrition is the limitation on fixation.

### **G.1. Necessity for Inoculation Trials**

A need for inoculation may be indicated in several ways: low yield in a crop that is not well nodulated, the yield response of a crop to proper inoculation, or a response of the legume crop to fertilizer N. The presence of compatible rhizobia in the field can be detected rather easily; the appearance of nodules on the roots of legumes growing at the site means that infective rhizobia are present. However, it is important to look closely at the interior of the nodules. The tissue should be sound, and the color pink or reddish; nodules are generally ineffective if they do not show this color. White or green nodules usually indicate infection but not effective  $N_2$  fixation. Nodules with a green base and red tissue at the tips (distal nodulation) often indicate that the nodule has entered a senescent stage, following active fixation.

The position and size of the nodules also can be an indicator of the presence, number, and effectiveness of the bacteria. The appearance of many large nodules on the main primary root—in the region of the root crown (the juncture of the root and the stem)—indicates abundant, competitive bacteria. Conversely, sparse nodulation, usually indicated by small nodules scattered widely on the secondary or finer roots, often indicates delayed nodulation due to low bacterial populations.

The physical appearance of the host plant also is a good indicator of the presence of effective bacteria. Most field soils are not high in plant-available N; therefore, a healthy, green legume is considered to be fixing adequate nitrogen if normally nodulated. Chlorotic (yellow), stunted plants can result from many causes, including moisture stress, diseases, insect damage, nematodes, nutritional factors, etc. Often, farmers seek complicated or subtle explanations for poor legume crop yields, without considering the possibility that inadequate nodulation caused by the lack of the proper rhizobial strain or low numbers of rhizobia may be the main limiting factor. If the legumes are pale or light green and lack vigor, but the roots are well nodulated, the possibilities are that (1) the rhizobia that infected the root are ineffective or (2) some other environmental or nutritional factor is limiting growth. If the legumes lack vigor

and the roots are not nodulated, the conclusion is that no infective native rhizobia are present in numbers adequate to cause nodulation. Inoculation in this case should increase yields, unless factors such as P availability or pH are limiting the crop, the bacteria, or both.

The need-to-inoculate trials described here have two purposes: (1) to determine if there exists a need for inoculation, accomplished through evaluation of the nitrogen fixing effectiveness of the indigenous rhizobial population, and (2) to evaluate how the basic fertility of the soil affects the capability of the legume to produce good yields.

Prior to field tests at a chosen site, the cropping history of the field should always be considered. An N-fertilized cereal grown during the previous season may not have depleted the soil of nitrogen. Depending on previous fertilization practices and environmental conditions, some residual N may be available for the legume and may even be high enough to interfere with successful nodulation and N<sub>2</sub> fixation (see Section F.3). If, on the other hand, unfertilized cereal or legume crops have been grown at the site for several seasons, the level of mineral N available in the soil will be low. In this case, a poor legume crop might be due to inadequate or ineffective rhizobial populations, but also could be caused by low P fertility or accumulated pathogens, nematodes, or other pests. A vigorous legume crop would indicate adequate N<sub>2</sub> fixation.

If a natural stand of medics is being evaluated, the appearance of associated species, particularly grasses, often will provide an indication of the general environmental impact on that season's growth. The grasses serve as indicators of the N-supplying power of the soil. If patches of grass not close to the legumes are a healthy green, the soil N level is probable adequate. In this case, the condition of the legumes, nodulated or not, must be attributed in part to this soil N supply.

Another aspect to be considered when evaluating nodulation of a legume crop is the weather pattern preceding observation. Drought may cause premature senescence of the nodules; legumes which have been excavated after a dry period may have few or no active nodules on the roots. However, if infective rhizobia are present in the soil in adequate numbers, a secondary infection of the host legume may occur upon restoration of favorable conditions, often resulting in many small nodules on secondary roots. Depending on the stage of crop growth, this kind of reinfection may be effective in supplying the nitrogen needs of the host later in the season.

### **G.1.a. Nonmicrobiological Trial to Determine Need to Inoculate**

The described trial requires no microbiological input (e.g., enumeration of native rhizobia or inoculation), but utilizes various fertility treatments to determine potential



yield under prevailing conditions, effects of fertility on plant growth, and the symbiotic effectiveness of the native rhizobia with the selected host.

*The trial described has been used successfully for field experiments with the food legumes as an international cooperative trial. In the case where the trial shows a positive response to nitrogen fertilization, the experimenter is encouraged to follow up by conducting an inoculation response trial. Where comparison of treatments indicates a requirement for P and/or K additions, it may be necessary for further definitive fertility experiments to indicate optimum economic levels of each amendment. This can be accomplished through soil pot trials followed by field trials for yield data.*

The actual field sites should be selected on the basis that they are typical of the area under consideration. It is strongly recommended that farmers' fields be used because of the artificially high fertility levels present in most agricultural experiment station soils. In attempting to develop an inoculation policy for a region (such as introduction of a new crop into an area), the trial should be conducted at as many sites in the area as possible.

Conducting trials in soil cores allows greater control of environmental variables, while greatly decreasing the costs and labor involved in multi-site trials; it does, however, not give yield results, but utilizes total dry matter and nitrogen yield data at mid-flowering to extrapolate treatment effect (see Section D.3.b for intact soil core methodology).

### **Treatments**

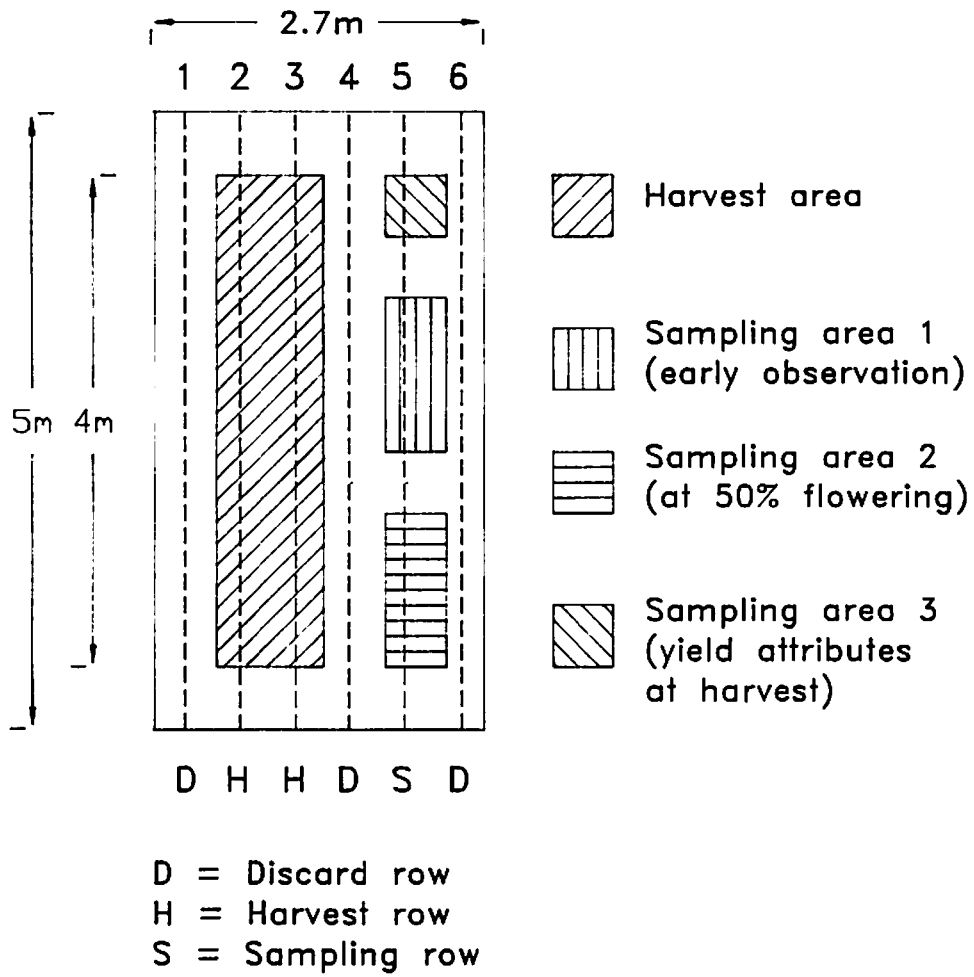
**F-levels** are fertility levels actually used by local farmers, in most cases involving no fertilizer applications of P or K.

- F-N** - No nitrogen application
- No applications of P, K, etc.
- F+N** - 120 kg N/ha, split dose as: 60 kg N/ha at planting; 60 kg N/ha as side-dress at beginning of flowering.
- No application of P, K, etc.

**O-levels** are optimum fertility levels designed to maximize yield, given local environmental conditions.

- O-N** - No nitrogen application
- Phosphate at 80 kg  $P_2O_5$ /ha (or as required) preplant.
- Potash at 60 kg/ha (or as required) preplant.
- O+N** - 120 kg N/ha split dose as: 60 kg N/ha at planting, 60 kg/ha at beginning of flowering.
- Phosphate application as in O-N.
- Potash application as in O-N.

**Plot layout.** Experience with this trial indicates that four replications are required. The 16 plots are arranged in a complete randomized design. All of the sampling areas are fully bordered (see Figure G.1.1).



**Fig. G.1.1. Illustration for a faba bean plot showing the sampling and harvesting areas in a need-to-inoculate experiment.**

**Fertilizing the plots.** All the fertilizers are to be applied at the time of planting except the second portion of 60 kg N/ha in the nitrogen-fertilized plots, which is to be applied as a side-dressing at mid-flowering. In plots receiving more than one nutrient, the different fertilizers should be mixed together in one bag or bucket uniformly. The whole fertilizer amount per plot may be subdivided into as many lots as there are rows in each plot. Each lot should be uniformly placed in a furrow (8–11 cm depth), opened approximately at the place where the seed row will lie. After placing the fertilizer, the bottom of the furrow should be covered with soil before seeding is done by opening a more shallow furrow immediately to the side of the fertilizer furrow. Care has to be taken that seeds and emerging roots do not come into direct contact with the fertilizer.

If placement of the fertilizer in furrows is not possible, the fertilizer for the whole plot should be subdivided into two lots and each lot should be uniformly broadcast over the whole plot (2.7 m × 5.0 m). After broadcasting, the fertilizer should be incorporated well in the top 15 cm of the soil utilizing a hand hoe or spade.

Side-dressing of the 60 kg N/ha in the nitrogen treatments should be done at the mid-flowering stage of the crop. A shallow furrow may be opened on one side of each row and the required quantity of fertilizer nitrogen uniformly spread there. The furrow should then be covered with soil. If the crop is being grown with irrigation, a light irrigation may be given after the side-dressing.

**Weed and pest control.** Weed competition and pest damage should be minimized to avoid obscuring the treatment effects. Control treatments should be applied when needed and a record should be made of when and how they are used. Damage caused by nodule-grazing insects, such as *Sitona* (Figure G.1.2) and nematodes, should be periodically checked as these underground pests are more likely to escape early detection than aboveground pests. Nodules damaged by *Sitona* are often left intact but hollow, with a small hole where the larvae entered and exited (Figure G.1.3).

For lentil and faba bean trials in locations where *Sitona* is commonly a problem, it is recommended that the granular insecticide carbofuran (Furadan) be applied in the optimal fertility plots. This insecticide is to be applied at the time of planting, when it is broadcast over the plot, then incorporated with a hand hoe or spade before seeding. During the course of the experiment, and especially when nodules are observed at 50% flowering, nodule damage from *Sitona* in the farm and optimal fertility plots should be carefully recorded. It may be desirable, under conditions of high probability of *Sitona* attack, to employ an additional fifth plot per block at farm fertility levels, where pest control is utilized.

**Planting.** Cultivar, row spacing, and seeding rate should be according to local recommendations. Numbers of seed needed per plot are given for some common

plant populations in Table G.1.1. Seed should be selected for uniformity and should be free from off-types.



Fig. G.1.2. *Sitona* larvae happily feeding on a lentil nodule.

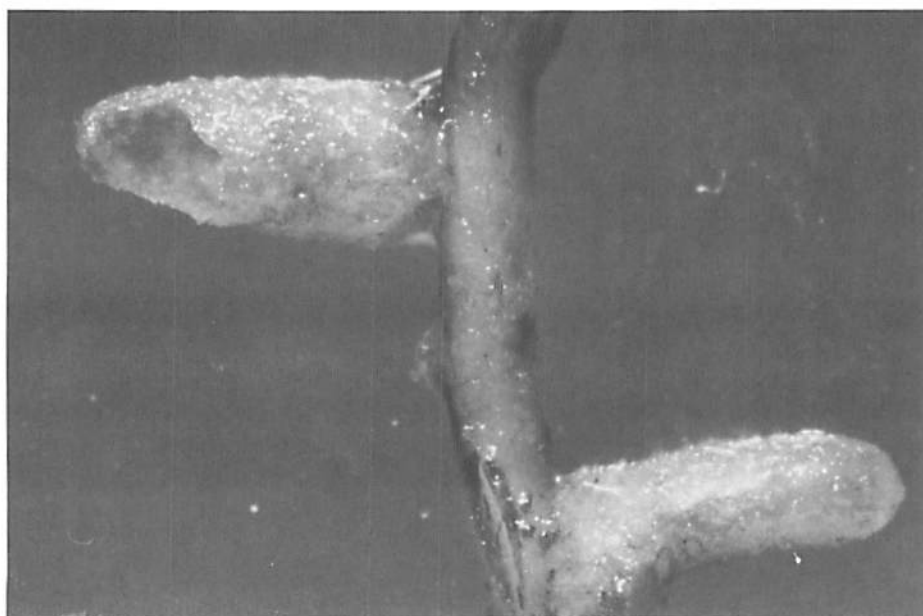


Fig. G.1.3. Hollowed-out nodules damaged by feeding *Sitona* larva.

**Table G.1.1. Standard plant populations and seeds per plot for need-to-inoculate trial.**

| Crop      | Row spacing (cm) |        | Seeds/m<br>of row | Plants/<br>hectare | Seeds<br>needed/<br>plot | Grams<br>seed/<br>plot* |
|-----------|------------------|--------|-------------------|--------------------|--------------------------|-------------------------|
|           | Between          | Within |                   |                    |                          |                         |
| Chickpea  | 30               | 10     | 10                | 333,333            | 450                      | 95                      |
| Lentil    | 22.5             | 1.5    | 67                | 2,977,778          | 4020                     | 170                     |
| Faba bean | 45               | 10     | 10                | 222,222            | 300                      | 40                      |

\* The seed size of some cultivars may vary by a factor of 2 or 3. The values given here are for the larger-seeded varieties. If seed supply is a critical factor, then the weight of seed needed should be calculated for the specific batch of seed to be used.

**Nodule collection.** Where a need-to-inoculate experiment shows a positive response to nitrogen fertilizer at the mid-flowering stage, a deficiency in the symbiotic efficiency of the native rhizobia is indicated. Here the experimenter is encouraged to select the most promising strains for use in the following season's inoculation trial. Strains for screening should be obtained from nodules in other experiments where the native soil rhizobia show high capacity for nitrogen fixation with the same local cultivar. Superior strains also may be requested from ICARDA, where sets of serologically distinct, highly effective strains have been selected for use in cooperative inoculation response trials. Instructions for nodule collection are given in Section A.1. Methodologies and details of *Rhizobium* strain-screening experiments are given in Section D.

**Data collection and records.** Grain yield and total dry matter (plant tops) are the main parameters by which the soil fertility and effectiveness of the native rhizobia are measured. However, nitrogen-fixing activity that is clearly apparent early in the growth cycle may not be translated into a yield response at final harvest because of limiting factors such as disease or drought. Therefore, an early sampling is made at mid-flowering. At this time, nodulation (nodule dry mass) in the various treatments is measured along with total plant dry matter to quantify the potential nitrogen fixing activity of the native rhizobia (if any) at that stage. The sampling plan is given in Table G.1.2 and Figure G.1.1. Sample formats for collecting and recording data, and suggestions for observations are given below.

**Table G.1.2. Details of areas for sampling and harvest.**

|                                           | Chickpea         | Crop<br>Faba bean | Lentil          |
|-------------------------------------------|------------------|-------------------|-----------------|
| Number of rows/plot                       | 9                | 6                 | 12              |
| Row nos. of sampling                      | 8                | 5                 | 11              |
| Row nos. for discard                      | 1,7,9            | 1,4,6             | 1,10,12         |
| Row nos. for net plot harvest             | 2,3,4,5,6        | 2,3               | 2,3,4,5,6,7,8,9 |
| Row length harvested per plot row         | Central 4 meters |                   |                 |
| Total row length harvested per plot       | 20               | 8                 | 32              |
| Area harvested per plot (m <sup>2</sup> ) | 6                | 3.6               | 7.2             |

### **Suggested Data Collection**

#### **During Establishment (sampling area 1)**

- Days to germination: days taken from planting to complete emergence of about 90% of the seedlings
- Stand observations: observations on germination and stand, with reference to insect, disease, or other problems
- Early nodulation: age of plants when first nodules appear, usually 10–20 days after emergence (observations of plants from border rows)

#### **Early Sampling (sampling area 2)**

- Days to 50% flowering: when 50% of plants have flowers
- Total plant dry matter per sampling area
- Number of plants per sampling area on which total plant dry matter is based
- Foliage color: scored 1–5; 1 = very yellow, 5 = deep green
- Nodulation per plant: average dry weight (mg) of nodules per plant, from representative five plants per plot
- Nodule color: overall internal color of fresh cut nodules, e.g., white, green, red

Date of sampling: .....

| Plot no. | Treat-ment no. | No. of plants in sampling area | Days to 50% flowering | Total dry matter g/plant | Nodule dry wt. mg/pl | Foliage color (1 to 5) |
|----------|----------------|--------------------------------|-----------------------|--------------------------|----------------------|------------------------|
|----------|----------------|--------------------------------|-----------------------|--------------------------|----------------------|------------------------|

#### Replication 1

|     |    |       |       |       |       |       |
|-----|----|-------|-------|-------|-------|-------|
| 101 | T1 | ..... | ..... | ..... | ..... | ..... |
| 102 | T4 | ..... | ..... | ..... | ..... | ..... |
| 103 | T2 | ..... | ..... | ..... | ..... | ..... |
| 104 | T3 | ..... | ..... | ..... | ..... | ..... |

#### Replication 2

|     |    |       |       |       |       |       |
|-----|----|-------|-------|-------|-------|-------|
| 201 | T3 | ..... | ..... | ..... | ..... | ..... |
| 202 | T2 | ..... | ..... | ..... | ..... | ..... |
| 203 | T1 | ..... | ..... | ..... | ..... | ..... |
| 204 | T4 | ..... | ..... | ..... | ..... | ..... |

#### Replication 3

|     |    |       |       |       |       |       |
|-----|----|-------|-------|-------|-------|-------|
| 301 | T4 | ..... | ..... | ..... | ..... | ..... |
| 302 | T3 | ..... | ..... | ..... | ..... | ..... |
| 303 | T2 | ..... | ..... | ..... | ..... | ..... |
| 304 | T1 | ..... | ..... | ..... | ..... | ..... |

#### Replication 4

|     |    |       |       |       |       |       |
|-----|----|-------|-------|-------|-------|-------|
| 401 | T1 | ..... | ..... | ..... | ..... | ..... |
| 402 | T4 | ..... | ..... | ..... | ..... | ..... |
| 403 | T3 | ..... | ..... | ..... | ..... | ..... |
| 404 | T2 | ..... | ..... | ..... | ..... | ..... |

#### Harvest (sampling area 3 and harvest area)

- Days to maturity: number of days after planting when 90% of the whole plot is ready for harvesting
- Area of net plot: area of the plot (m<sup>2</sup>) actually harvested for yield estimates
- Air-dry weight of aboveground dry matter: total produce from the net plot harvested so that all of the aboveground parts are obtained, without roots (air-dry weight recorded in g per net plot)

- Air-dry weight of seeds per net plot: after harvest the total produce from the net plot to be threshed, and cleaned seeds to be weighed after air-drying (weight recorded in g per net plot)
- Total nitrogen content of plant tops (optional): N content of plant tops including grain, taken on subsample from five representative plants in sampling area 3

Date of harvest .....

| Plot no.      | Treat ment no. | Area harvested (m <sup>2</sup> ) | Dry matter harvested |         | Seed yield |         |
|---------------|----------------|----------------------------------|----------------------|---------|------------|---------|
|               |                |                                  | (g/plot)             | (kg/ha) | (g/plot)   | (kg/ha) |
| Replication 1 |                |                                  |                      |         |            |         |
| 101           | T1             | .....                            | .....                | .....   | .....      | .....   |
| 102           | T4             | .....                            | .....                | .....   | .....      | .....   |
| 103           | T2             | .....                            | .....                | .....   | .....      | .....   |
| 104           | T3             | .....                            | .....                | .....   | .....      | .....   |
| Replication 2 |                |                                  |                      |         |            |         |
| 201           | T3             | .....                            | .....                | .....   | .....      | .....   |
| 202           | T2             | .....                            | .....                | .....   | .....      | .....   |
| 203           | T1             | .....                            | .....                | .....   | .....      | .....   |
| 204           | T4             | .....                            | .....                | .....   | .....      | .....   |
| Replication 3 |                |                                  |                      |         |            |         |
| 301           | T4             | .....                            | .....                | .....   | .....      | .....   |
| 302           | T3             | .....                            | .....                | .....   | .....      | .....   |
| 303           | T2             | .....                            | .....                | .....   | .....      | .....   |
| 304           | T1             | .....                            | .....                | .....   | .....      | .....   |
| Replication 4 |                |                                  |                      |         |            |         |
| 401           | T1             | .....                            | .....                | .....   | .....      | .....   |
| 402           | T4             | .....                            | .....                | .....   | .....      | .....   |
| 403           | T3             | .....                            | .....                | .....   | .....      | .....   |
| 404           | T2             | .....                            | .....                | .....   | .....      | .....   |



Weather records, and weed and pest management records should also be kept. A sample data sheet is given below.

- 1. Location address: .....  
.....
  - 2. Latitude: ..... Longitude: .....  
Altitude: ..... m above mean sea level
  - 3. Soil type (name): .....  
Soil texture: Sandy Loam/Loam/Clay Loam.....  
Soil color: Red/Brown/Grey/Black.....  
Soil depth: Shallow/Intermediate/Deep.....  
General status of soil fertility: Poor/Intermediate/Rich.....
  - 4. Past cropping history of the experimental plot:  
previous season: .....
  - 5. Fertilizer if any applied to the previous seasons crop:  
Nitrogen ..... kg/ha  
 $P_2O_5$  ..... kg/ha  
 $K_2O$  ..... kg/ha
  - 6. Date of planting: .....
  - 7. Spacing details at planting: .....  
(a) Number of rows per plot : .....  
(b) Row length : .....  
(c) Row-to-row distance : .....  
(d) Seeds per row : .....
  - 8. Dates of Irrigation, if any : .....
  - 9. Major weeds and herbicides applied, if any, for controlling the weeds:  
Weeds : .....  
Herbicides : .....  
Rate : .....  
Date of application : .....
  - 10. Pesticides applied, if any (specify type, rates and dates of application):  
.....
  - 11. Name of the cultivar used: .....  
.....
  - 12. Weather details for the growing period:  
.....  
.....
  - 13. General comments on the weather conditions during the season:
-

## G.2. Inoculation Response Trials

The final decision on whether inoculation is required at any given site must be based on an economic criterion, usually evaluated as yield response to inoculation. Yield response to inoculation can only be determined definitively in field trials brought to harvest; greenhouse trials are only indicative of potential yield response. In the semiarid areas of WANA many native legumes respond to inoculation. Pasture legumes such as annual medics and clovers require specific rhizobial strains in order to fix nitrogen and be produced economically. The food and forage legumes are generally more promiscuous in terms of nodulation, but introduced cultivars also may require appropriate strains of rhizobia to obtain maximum  $N_2$  fixation. In particular, chickpea exhibits a large degree of strain-cultivar interaction, and introduction of chickpea into new areas of cultivation often requires inoculation for efficient fixation and good yields.

Where inoculum is inexpensive relative to the expected gain, inoculation is often practiced as a form of 'insurance'. Farmers may prefer to make the necessary investment as a means of avoiding losses if the legume crop yield, given prevailing conditions, is less than potential yield. It must be kept in mind that inoculation of legumes *per se* is not a panacea. It represents only one aspect of a total management program, and must be viewed in context with other management factors. Weed control, fertility, moisture stress, and insect and disease control, and crop, cultivar, or ecotype adaptation are other facets that should be considered in any specific environment.

Under any circumstances, three questions must be asked if one is to evaluate a crop's response to inoculation:

1. Does the added inoculum measurably improve the agronomic (herbage or grain) yield of the crop?
2. Is lack of N the factor limiting herbage or grain yield or is it something else?
3. Have the majority of nodules been formed by the inoculant strain of *Rhizobium*?

These questions may be answered by the use of three basic treatments in a field experiment:

1. Plant the legume without inoculation, under traditional management practices. If it is a newly introduced species, plant it according to the best available information but without inoculation. These plants will be nodulated by the native rhizobia inhabiting the soil.
2. Plant the legume as above but inoculate with the rhizobial strain(s) to be tested (viability of the inoculum and adequate number of rhizobia per seed must be

assured). Levels between  $10^7$  to  $10^9$  rhizobia/g carrier in the inoculant and  $10^3$  to  $10^4$  rhizobia/seed for small-seeded legumes and  $10^6$ /seed for larger seeds are assumed adequate. The inoculant rhizobia will compete for nodulation sites with native rhizobia.

3. Plant as in (1) but fertilize with an appropriate quantity of mineral nitrogen. The amount used for uninoculated nitrogen controls for pasture legumes is 90 kg N/ha, and for the food and forage legumes 120-150 kg N/ha, preferably in split application to assure a continuous supply of N. These experiments assume that the level of P in the soil is adequate; if experience indicates that P is deficient, a preplant broadcast application of 60-80 kg  $P_2O_5$ /ha is normally sufficient.

Possible responses to these three treatments are shown in Table G.2.1.

**Table G.2.1. Possible plant growth and nodulation results from a three-treatment inoculation trial to determine inoculation response.**

| Case | <u>Noninoculated</u> |        | <u>Inoculated</u> |        | <u>Nitrogen</u> |        |
|------|----------------------|--------|-------------------|--------|-----------------|--------|
|      | Nodulation           | Growth | Nodulation        | Growth | Nodulation      | Growth |
| 1    | -                    | Poor   | -                 | Poor   | -               | Poor   |
| 2    | -                    | Poor   | -                 | Poor   | -               | Good   |
| 3    | -                    | Poor   | + E               | Good   | + or -          | Good   |
| 4    | -                    | Poor   | + I               | Poor   | + or -          | Good   |
| 5    | + I                  | Poor   | + E               | Good   | + or -          | Good   |
| 6    | + I                  | Poor   | + I               | Poor   | + or -          | Good   |
| 7    | + E                  | Good   | + E               | Good   | + or -          | Good   |

- = plants not nodulated    E = effective nitrogen fixation

+ = plants nodulated    I = ineffective nitrogen fixation

The interpretation for the above cases is:

1. No nodulation and plants looking small, pale-green, and stunted. This indicates that native rhizobia compatible with the test legume are not present in the soil. Similarly, no nodules formed and no growth response from either the inoculated or nitrogen treatments indicates that some factor other than N (e.g., phosphorus supply) is limiting plant growth.
2. No nodulation and poor growth of uninoculated and inoculated plants as above, but vigorous green plants with added N. This indicates that the inoculum strain or the quality of the inoculant supplied was unsatisfactory, or soil conditions were adverse for growth of the rhizobia and infection of the root. In this case soil mineral nitrogen is limiting for plant growth. Native rhizobia are absent.

3. No nodulation and poor growth of the uninoculated plants as above, but effective nodulation and vigorous growth of inoculated plants, indicating success of the inoculum strain in forming nodules and fixing nitrogen. Native rhizobia absent.
4. No nodulation and poor growth of uninoculated plants, and inoculated treatment plants ineffectively nodulated with poor growth compared with a vigorous green plus-N treatment. This indicates that the inoculum strain was inappropriate for the test legume.
5. Uninoculated plant growth poor and with ineffective nodules indicating nodulation by incompatible native strain(s); inoculated plants effectively nodulated and vigorous demonstrating that the inoculum strain was both competitive with the native rhizobia for nodule sites and effective in  $N_2$  fixation with the test host.
6. Both uninoculated and inoculated treatment plants nodulated effectively but with poor growth compared with the plus-N treatment. This situation suggests that native rhizobia are ineffective and competitive for nodule sites, thus keeping out the inoculum strain, and/or that the inoculum strain was ineffective in  $N_2$  fixation. It is necessary to use a strain identification technique such as antibiotic-resistant markers or serology to determine the proportion of nodules in the inoculated treatment occupied by the applied inoculum strain.
7. Uninoculated plants are vigorous and effectively nodulated, indicating compatibility of native rhizobia with host legume. If there has been no response to inoculation, nodules on the plants in the inoculated treatment may be from native or inoculant strains. Again, this could be checked by strain identification techniques.

### **G.2.a. Field Trials for Small- and Large-Seeded Legumes**

Where a 'need to inoculate' exists, the researcher is encouraged to conduct a strain-screening experiment to select the most promising strains for use in the inoculation response trial (see Section D). Strains for screening should be obtained both from nodules in other experiments where the native soil rhizobia show high capacity for nitrogen fixation with the same cultivar, and from the ICARDA *Rhizobium* collection, following recommendations of ICARDA microbiologists. Nodules from superior nodulated local plants can be sent to ICARDA for isolation of rhizobia and incorporation into inoculants for use by the experimenter. By previously testing strains for their capacity to fix N under local conditions with the relevant cultivars, the inoculation-response field experiment will be conducted using the optimal strains of *Rhizobium*. The opportunity for a positive yield response to inoculation is thereby maximized.

## Medics and other pasture legumes

One of the attractions of the ley farming system is the independence of the system from applications of nitrogen fertilizer. When associated with the concept of medic self-regeneration, the ability of pasture legumes (e.g., annual medics) to fix nitrogen and transfer it to cereals makes the ley farming system a truly low-input system. However, owing to the marked specificity of annual medics to strains of *Rhizobium meliloti* it is important to test the need to inoculate and response to inoculation if the system is to be successful.

A field trial consisting of a split-plot design in which a given number of species of annual medics are inoculated with superior and local rhizobial strains is currently recommended to cooperators. The size of the experiment depends on several factors: (1) number of species or ecotypes that are to be exploited; (2) natural occurrence of the species; (3) environmental conditions; and (4) land and labor force availability.

Assume that two annual *Medicago* species need to be tested for adaptation as pasture legumes in a region with an annual average precipitation below 300 mm; one species being an ecotype native to the region of interest and the other a commercial cultivar. In general, we use two strains which are known to effectively nodulate the two species and are tolerant to the soil moisture conditions implied by the given level of precipitation. If available, we also use two strains native to the general location of study. The 'strain' treatments are set up as the main plots and the species as subplots in a split-plot design. A minimum of three replications are used. Treatments include uninoculated controls with and without mineral nitrogen (90 kg N/ha). Plots are usually 2 to 4 m<sup>2</sup>. This trial will require (2 species × 6 treatments × 3 replications) 36 plots. Fertilization with superphosphate at a rate of 60 kg P<sub>2</sub>O<sub>5</sub>/ha and application of carbofuran (25 kg commercial product/ha) to control any infestation of nematodes or *Sitona* weevil are recommended. Herbage harvests are conducted at flowering and podding stages. Observations on nodulation characteristics are recorded.

An analysis of variance with separation of means is performed on the data, the sources of variation being blocks, strains, block by strain interaction (error A), species, species by strain interaction, and block by species interaction pooled over strain (error B: the error for species and species by strain interaction). The various combinations of interactions are then compared by either the least significant difference (LSD) or the Duncan's method at 5% probability level.

Differences between the inoculated and uninoculated treatments are easily observed when the local populations of *R. meliloti* are absent or produce an ineffective symbiosis with the medic host. Selected strains can then be used as seed inoculants, providing the inoculant strains are competitive with the native rhizobia. In cases where native rhizobia are moderately effective, we recommend no inoculation.

## Food and Forage Legumes

There are five treatments recommended for this inoculation response trial: three inoculated with different single strains of *Rhizobium*; nitrogen fertilized; and control without N or inoculation. The N-fertilized treatment receives a split application of 120 kg N/ha to provide an indication of yield potential of the crop under given environmental conditions. Yield of the uninoculated control will indicate effectiveness of the native soil rhizobia, if present. Three treatments, each having a selected single strain of rhizobia, are necessary because of possible strain-cultivar specificity (particularly with chickpea). All treatments should receive basal dressings of phosphate (and potash where required) at 60–80 kg P<sub>2</sub>O<sub>5</sub>/ha and 60 kg/ha potash (if required) broadcast presowing, so that fertility constraints (except N) on yield will be alleviated.

### Treatments

- T1 - 120 kg N/ha as urea, with application split as 60 kg N/ha presowing, 60 kg N/ha as side-dress at 50% flowering, no inoculation
- T2 - Uninoculated control
- T3 - Inoculation of seed with selected *Rhizobium* strain No. 1
- T4 - Inoculation of seed with selected *Rhizobium* strain No. 2
- T5 - Inoculation of seed with selected *Rhizobium* strain No. 3

Details of site selection, fertilization, plot sizes, planting, nodule collection, pest control, and design and sampling are given in Section G.1. Methods of inoculation are given in Sections E and F. Details for application of inoculants to seed are given in Table G.2.2. A system of nodule scoring is given in Table G.2.3.

Table G.2.2. Inoculation of large legumes seeds using two inoculation methods.

|                                         | Lentil | Chickpea | Faba bean |
|-----------------------------------------|--------|----------|-----------|
| <b>Seed coating method</b>              |        |          |           |
| Volume sticker <sup>+</sup> /100 g seed | 2.0 ml | 6.0 ml   | 1.5 ml    |
| Grams peat/100 g seed                   | 15 g   | 20 g     | 20 g      |
| <b>Liquid application method</b>        |        |          |           |
| Number of rows/plot                     | 12     | 9        | 6         |
| Volume of water/row                     | 500 ml | 500 ml   | 500 ml    |
| Volume of water/plot                    | 6 L    | 4.5 L    | 3 L       |
| Grams peat/L water                      | 4.5 g  | 10 g     | 15 g      |
| Packages of inoculant/plot              | 3/4    | 1        | 1         |

+ prepared cellulose gum adhesive solution

**Table G.2.3. Method of nodule scoring for food legumes (after Corbin *et al.*, 1977).**

| Nodule score | Distribution and number of effective nodules* |           |
|--------------|-----------------------------------------------|-----------|
|              | Crown**                                       | Elsewhere |
| 0            | 0                                             | 0         |
| 1/2          | 0                                             | 1-4       |
| 1            | 0                                             | 5-9       |
| 1-1/2        | 0                                             | > 10      |
| 2            | Few                                           | 0         |
| 2-1/2        | Few                                           | Few       |
| 3            | Many                                          | 0         |
| 4            | Many                                          | Many      |
| 5            | Many                                          | Many      |

\* Effectiveness judged on basis of nodule size and internal coloring; ineffective (green or white) nodules not considered.

\*\* Crown regarded as top 7 cm of root system.

### **Suggested Data Collection (see Section G.1)**

#### **During Establishment (sampling area 1)**

- Days to germination: days taken from planting to complete emergence of about 90% of the seedlings
- Stand observations: observations on germination and stand, with reference to insect, disease, or other problems
- Early nodulation: age of plants when first nodules appear, usually 10-20 days after emergence. Weekly observations for nodule score (Table G.2.3)

#### **Early Sampling: (sampling area 2, at mid-flowering stage)**

- Days to 50% flowering: when 50% of plants have flowers
- Total plant dry matter: 10 plants per plot sample
- Foliage color: score 1-5: 1 = very yellow, 5 = dark green
- Plant nodulation: average dry weight (mg) of nodules per plant or nodule score (Table G.2.3), from a representative group of five plants per plot
- Nodule color: overall internal color of fresh cut nodules, e.g., white, green, pink, red

### **Harvest (sampling area 3 and harvest area)**

- Days to maturity: number of days after planting when 90% of the whole plot is ready for harvesting
- Area of net plot: area of the plot (m<sup>2</sup>) actually harvested for yield estimates
- Air-dry weight of total plant tops: total produce from the net plot harvested so that all of the aboveground parts are obtained, without roots (air-dry weight recorded in grams per net plot)
- Air-dry weight of seeds per net plot: after harvest the total produce from the net plot to be threshed, and cleaned seeds to be weighed after air-drying (weight to be recorded in grams per net plot)
- Total nitrogen content of plant tops (optional but recommended): N content of plant tops including grain, to be taken on subsample from five representative plants in sampling area 3.

Weather records, and weed and post management records also should be kept as per data sheets (Section G.1).

### **G.2.b. Soil Core and Pot Studies**

The potential response to inoculation also may be determined under controlled conditions in the greenhouse, but field trials are necessary to determine the effect on yield. There are two greenhouse methods used at ICARDA to determine inoculation response. One utilizes disturbed soils collected from the field; the other involves collection of intact soil cores. Undisturbed soil cores are more suitable for assessing the effect of inoculation than taking bulk soil samples for pot experimentation. Disturbing the soil destroys the spatial relationship of the rhizobial population in the original soil, and favors mineralization of soil nitrogen, thus reducing the differences between effectively nodulated and non-nodulated or ineffectively nodulated plants. Procedures for soil core experiments are detailed in Section D.3. Treatments may duplicate those presented in the preceding field experiment sections.

For the disturbed soil (pot) method for use with medics, soil samples are collected in the region where the crop is to be grown. The samples are taken from the first 20 cm of the soil profile and kept in sealed containers. The soil samples are brought to uniform consistency by passing them through a 2 mm-mesh sieve. Ten grams of this soil are diluted into 90 ml of water. The dilution is agitated vigorously for 10 minutes in a reciprocating shaker and aliquots of 1 ml used to inoculate seedlings growing in test tubes as described in Section B.3.b. Six seedlings per species or ecotype are recommended for each soil dilution. This system will allow determination of the symbiotic effectiveness of potential inoculants as well as the indigenous population



present in the area of study. The biomass produced is compared with those of the uninoculated treatments with and without mineral nitrogen (see Section B.3.b).

An analysis of variance is performed on the data collected corresponding to shoot dry masses of all treatments of the completely randomized design (RBD) experiment, sources of variation being the two factors (soil inoculation and medic species) and their interaction.

In cases where need to inoculate or inoculation response is established we recommend two alternatives: (1) inoculate the seed of the tested medic species with the best strain and fertilize the field with superphosphate; (2) if the native population of rhizobia produces herbage yields not significantly different from the mineral nitrogen control and/or a standard strain, then we recommend no inoculation. In some cases, indigenous ineffective populations of medic rhizobia are more competitive than the effective inoculant strains. Other medic hosts are tested against these bacteria and, if compatible, their introduction is then recommended. This alternative is possible as there are many species of medics native to the Mediterranean area and because of the marked specificity to native populations of *R. meliloti*.

At harvest, plants are graded according to their response to inoculation and on their vigor and color. The shoot dry mass is used to measure symbiotic response through comparison with the shoot mass of the uninoculated controls, with evaluation according to that outlined previously in Section D.1.

### G.3. Host $\times$ *Rhizobium* Associations (Cultivar $\times$ Strain Studies)

The legumes are an immense group of plants estimated at approximately 750 genera containing some 19,700 species. Most nodulate naturally with native rhizobia present in the soil, but some species are more specific in their rhizobial requirements than others. Success in the  $N_2$ -fixing symbiosis is largely a matter of genetic compatibility between the plant and rhizobia. If one takes into account the  $N_2$ -fixing effectiveness of the *Rhizobium*-host association, a complex pattern of specificity grading from a whole ineffective condition to levels of maximum efficiency is found. Philogenetically diverse genera such as *Trifolium* and *Medicago* show marked specificity for their rhizobial partners. Many other legume genera also show diversity in response to strains of *Rhizobium* extending from species to the level of cultivars.

The importance of matching host-genotypes and rhizobial strains has long been recognized. Studies on the specificity of medics at ICARDA are conducted using the tube system described in Section D. Different species, ecotypes and cultivars are tested against a number of strains of rhizobia. Each plant entry is tested against a

rhizobial inoculum. Usually an aliquot of 1 ml of a  $10^9$  rhizobia/ml suspension is added to the tube containing a seedling of a legume plant. Four replicate tubes are recommended per treatment combination. Plant response parameters such as herbage yield, nodule number, nodule mass and plant vigor are evaluated after four weeks of growth. Strains that are compatible in one species may not be compatible in another. This is evident by observing the type of nodulation and plant growth response.

Strain-cultivar interactions have been observed in faba bean and chickpea, but appear minimal in lentil, lathyrus and vetch. Interactions are most critical in chickpea, where newly introduced cultivars may display rhizobia requirements quite different from landrace cultivars grown for centuries, and two cultivars may behave differently with a single rhizobia strain. Optimum strains for each cultivar are selected at ICARDA using the hydroponic system described in Section D (Figure G.3.1), with the objective of developing a set of rhizobia strains having broadly based effectiveness with a range of diverse plant germplasm. After strain selection under aseptic conditions, screening is continued in soils to select strains effective on many cultivars under varying conditions.



Fig. G.3.1. Aseptic hydroponic system used at ICARDA to evaluate symbiotic effectiveness.

## G.4. Ecological Studies

### G.4.a. Rhizobial Competition

Competition between infective strains of rhizobia during the nodulation of a host plant is a complex phenomenon which occurs between the strains composing the natural soil population, between indigenous strains and inoculum strains, and also between individual inoculum strains if these are applied in a mixed inoculum. The relative success of different strains of rhizobia is probably the result of many factors interacting to affect rhizobial survival and root colonization as well as competition for nodule sites (Figure G.4.1). Some of these factors are controlled by the environment, some by the host plant genome, and some by the genome of the strain of *Rhizobium*. These factors interact and influence the outcome of field inoculations. The successful nodulation of a legume largely depends on the ecological components, which affect competition both directly through their effect on rhizobia and indirectly through their effect on plant growth.

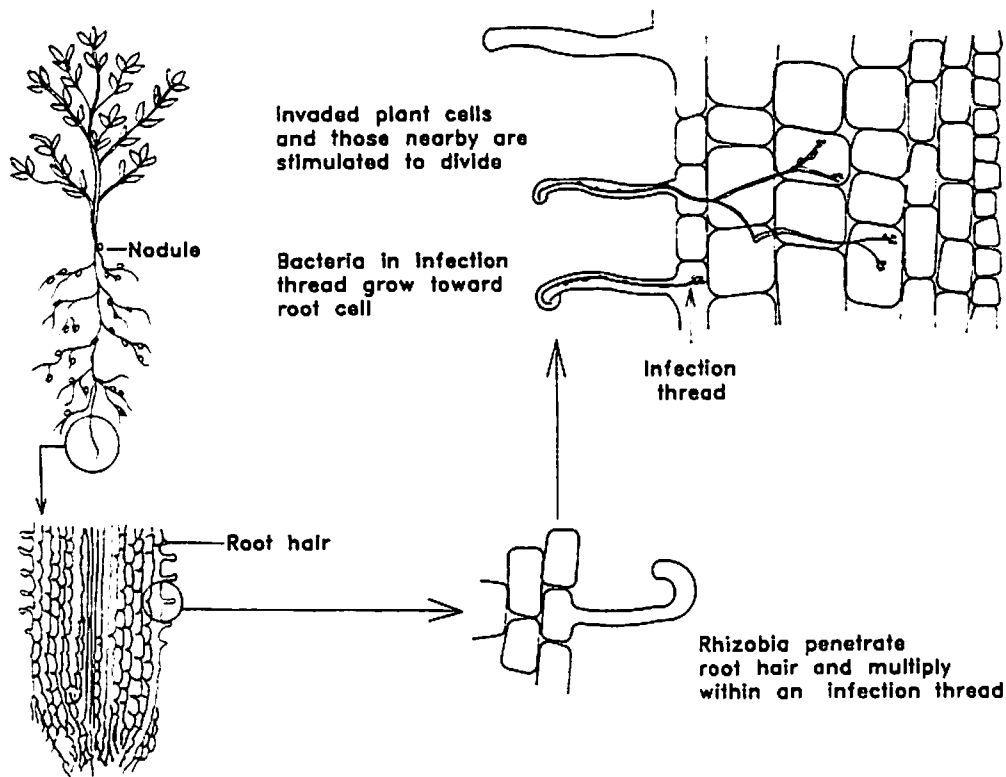


Fig. G.4.1. Nodulation process in a legume. Strains present will compete for nodulation sites on root hairs.

It is difficult to establish an inoculant strain in a soil containing the same rhizobial species, especially if the existing indigenous population is already large at the time of planting. Unless it is possible to select for increased competitiveness of applied strains at the expense of those already in the soil, more effective strain-genotype combinations are academic and the adoption of legume cultivars with a greater ability to fix  $N_2$  could be hampered because their performance may be adversely affected by less effective soil rhizobia. In most cases the effectiveness of resident naturalized rhizobia populations at plant breeding sites is not known. There are cases in which plants raised under certain conditions and selected for their symbiotic properties at a particular location have failed in other areas where the rhizobial populations are different.

Competition studies make it possible to evaluate the 'success' of inoculants in cases where inoculum strains must enter into competition with infective but less effective strains of rhizobia occupying the soil. Some strains have a much greater likelihood of success—defined as the ability to fully colonize the host and form the majority of nodules—as inoculants. *Rhizobium* added as inoculum at planting must compete with the microflora of the soil and other rhizobia for available substrate necessary for multiplication and survival in the soil. Most important, the more desirable inoculant strain is the more competitive for nodule sites on the plant root.

Various means of identifying strains occupying nodules are described in Section C; these methods should be attempted with their strengths and weaknesses in mind. The proportion of nodules occupied by the inoculant strain(s) is expressed as a percentage of the total number of nodules on the plant. Higher proportions indicate that the inoculant strain was more competitive than the indigenous population of rhizobia.

Competition for successful nodulation of the host is difficult to regulate because it involves the plant, the bacteria, and the environment as described above. However, present science suggests that: (1) there appears to be a genetic basis for selection of certain rhizobia by the host legume; (2) it seems to be possible to select rhizobia that are resistant to various unusual environmental factors, and thus to tailor *Rhizobium* to most agricultural conditions; (3) some genes involved in competition among strains for nodulation have been cloned using recombinant DNA techniques; and (4) presumably in the future it will be possible to manipulate both the host and the rhizobial genes in such a way that nodulation will only be possible with specific *Rhizobium* combinations. This will reduce the likelihood of allowing ineffective rhizobia to take valuable root nodule sites. A multidisciplinary approach involving breeding programs based on an understanding of molecular biology, nitrogen fixation, and microbial ecology is necessary to tackle these challenges.

### **G.4.b. Rhizobial Persistence**

The concept of persistence involves all those traits that permit rhizobia to live as a continuing member of the soil microflora, even in the absence of its host legume. Persistence of strains from season to season is generally a desirable trait in annual legumes as it makes yearly inoculation unnecessary. The regularly reseeded legume will provide an opportunity for fresh establishment of its rhizobia by inoculation at planting time. In annual self-regenerating legumes, such as medics, their rhizobia must persist in the soil over the year corresponding to the cereal rotation phase. During the summer period, soils are exposed to high temperatures and dry conditions which may drastically affect the survival of rhizobia. Some strains are known to be better able to survive and colonize the soil under these conditions. This characteristic, which we term here persistence, also has been called 'saprophytic competence'.

The persistence of rhizobia in the soil after inoculation, nodulation, and plant seasonal growth is a subject that requires attention in ecological studies of rhizobia. Factors most commonly affecting persistence include: (1) continued or periodic presence of a host; (2) suitability of soil for rhizobial survival (pH and soil type in particular); (3) soil temperature; (4) availability of soil moisture; and (5) presence or absence of competitors or antagonists, including other rhizobia.

To determine rhizobial persistence, it is necessary to enumerate and identify strains simultaneously; the most common methods are the plant infection (MPN) test and serological techniques (ELISA or FA) (Sections B and C). In attempting persistence studies, the introduced strain must be stably identifiable; MPNs alone will not indicate the numbers of introduced bacteria that have survived, but will measure the whole population of infective rhizobia for the particular host. Antibiotic resistance is generally less stable than are antigenic properties (see Section C). Persistence is generally measured over a period of three to five years without cultivation of the host legume. In the most straightforward evaluation, an MPN is conducted and nodules formed are tested for occupancy by the strain in concern. This method does not directly indicate survival; the result also is affected by the competitiveness of the strain. Optimally, the whole rhizobial population would be isolated from the soil and tested for population size and identity without mediation of the legume host. In practice, however, this is extremely difficult as selective media for rhizobia are not available (see Section B.3).

## **G.5. Statistical Analysis and Data Interpretation**

Statistical analysis is essential for all experiments because variation due to soil, water, nutrients, or other factors must be separated from variation due to experimental

treatments. Only when the variation due to treatments has been determined can you make conclusions about trial results.

Statistics need not be complicated, but we urge you to consult a statistician both at planning stage and during analysis. A simple analysis of variance uses the means of blocks and treatments to estimate the amount of variation and source. The remaining variation is due to differences between treatments within blocks, i.e., error from differences in soil or other factors. The F value (variance ratio) will indicate whether the mean square from one of the sources of variation is significantly greater than that for error. The significance of the F value is determined by the use of tables, given for example in Snedecor and Cochran's *Statistical methods*, Sixth ed., Iowa State University Press (1967). Significance at the 5% level is the accepted minimum for biological experiments. The coefficient of variation (CV) will indicate the overall efficiency in conducting the experiment, i.e., whether the level of variation is acceptable. A CV below 20% usually indicates that the experiment was properly conducted. If significant treatment differences exist (i.e., F-test is significant), the least significant difference (LSD) will indicate where treatments vary. Again, the tables of a statistical reference are necessary.

Calculations and application of various statistical methods are beyond the goals of this manual, although we have treated the subject briefly as applicable to field experiments briefly in Section F.8. If statistical help is not available locally, results can be referred to ICARDA for assistance.

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## H. Measurement of Dinitrogen Fixed

Accurate measurement of  $N_2$  fixation is necessary to understand the N economy of the legume, and in a broader context, to improve the contribution of legume  $N_2$  fixation in the cropping system N cycle (Figure H.1.1). Using the information gained in trials measuring  $N_2$  fixed, strategies can be developed to overcome deficiencies in agricultural and natural systems, e.g., improved legume genotypes, more efficient rhizobia, and cropping/tillage strategies to increase soil N available to a crop following the legume. With use of methods to quantify  $N_2$  fixation comes an understanding of the factors affecting fixation, and increased opportunities to maximize legume N input into systems. It is important not only institutionally but also regionally to collect accurate and relevant data on  $N_2$  fixation by legumes in a variety of systems in the region and to publish these findings. Given a sufficient number of cooperators working with similar methods in several locations, it may be possible to model  $N_2$  fixation and predict rates given relevant information.

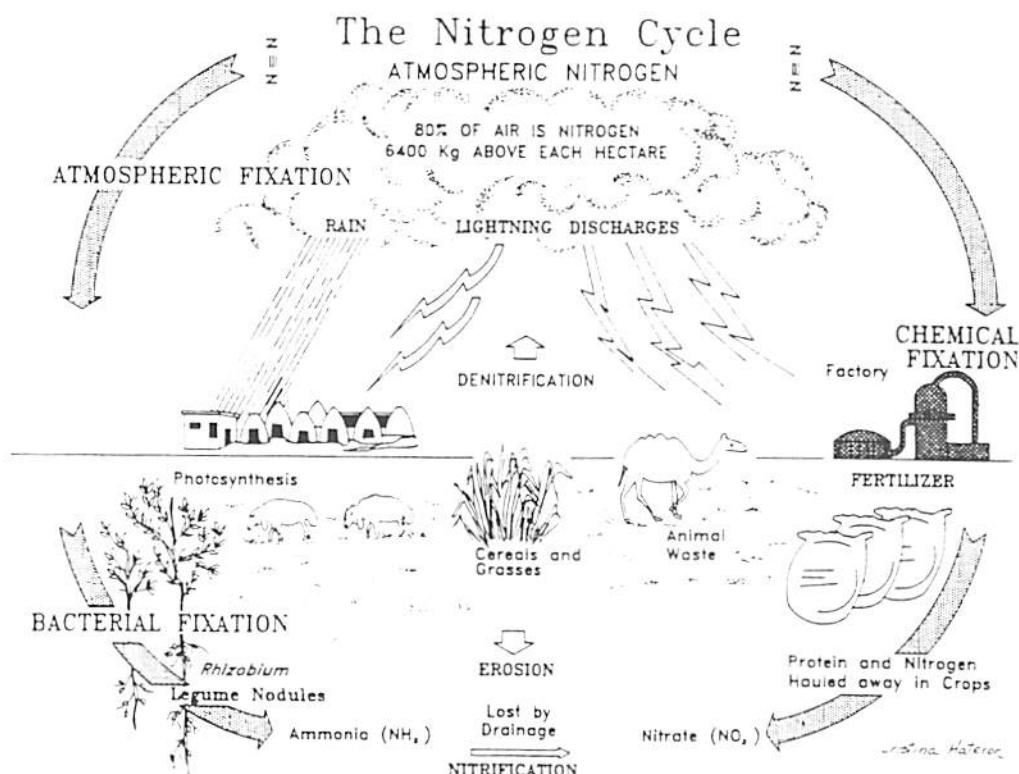


Fig. H.1.1. Schematic of some components of the nitrogen cycle.

Most of the variation in legume  $N_2$  fixation can be accounted for by: (1) the amount of available soil N (nitrate); (2) plant growth, as affected by available water and nutrition; and (3) the number and effectiveness of rhizobia present in the soil. Given the generally good agreement between the N-difference and  $^{15}N$  methods and the poor accuracy of the acetylene reduction method, it is suggested that greater effort be dedicated to developing expertise and using the former techniques in research programs. In all studies of legume  $N_2$  fixation, measurement of plant N (Kjeldahl), nodulation, and levels of soil-available N provide a good basis for interpretation of results.

In the following sections the assumptions, advantages, and limitations of some of the more popular and applicable methods are discussed.

## H.1. Acetylene ( $C_2H_2$ ) Reduction Assay (ARA)

### H.1.a. Theory, Advantages, Disadvantages

The acetylene reduction assay was developed following the observation that the nitrogen-fixing enzyme, nitrogenase, reduced acetylene to ethylene. The reliability of acetylene reduction as an indicator of nitrogenase ( $N_2$  fixing) activity in legume nodules has been well established and the technique has played an important role in  $N_2$  fixation studies. Briefly, the nodulated legume roots are enclosed in gas-tight containers and exposed to an atmosphere containing acetylene. The atmosphere is sampled after a suitable incubation period (0.5–24 hours, depending on the system) and analyzed for ethylene using gas-liquid chromatography with a flame ionization detector. The ARA was appealing initially and gained rapid acceptance after its initial development because it was simple, inexpensive, and sensitive compared with the  $^{15}N$  methods. The methodology also was rapidly adapted for field studies of  $N_2$  fixation. Techniques for field assays include excavating the nodulated root minus soil and incubating in a gas-tight vessel of the appropriate size; excavation of the nodulated root in a soil core; and incubating, or assaying *in situ*. A good review of these three methods is given in Masterson and Murphy (1980).

There are two major problems with use of this method as a field assay of  $N_2$  fixation in legumes: (1) it is difficult to obtain accurate time-integrated measures of total plant activity because of incomplete recovery or incubation of nodules, short-term fluctuations, and diurnal effects. The problem of incomplete recovery of nodules is the same whether the nodulated roots are excavated or contained in soil cores. This problem is greater with legumes where nodules are poorly attached (as in medics) or with deep root systems (such as faba bean and chickpea). Late nodulation (and hence fixation) on the lateral roots cannot be accounted for using this method as nodules cannot be retrieved; and (2) interpretation of rates of  $C_2H_2$  reduced in terms of  $N_2$

fixation varies widely. The acetylene reduction technique is an indirect measurement of  $N_2$  fixation gained through evaluation of the activity of nitrogenase, and rates of  $C_2H_2$  reduced must be converted to amounts of  $N_2$  fixed using a conversion factor. The theoretical relationship between  $C_2H_2$  reduction and  $N_2$  fixation states that 3 moles of  $C_2H_2$  are reduced for each mole of  $N_2$  fixed, and originally this was the ratio proposed for use. With later investigations, however, the ratio was calculated (by correlating with  $^{15}N$  methods) to be between 0.5:1 and 10:1. It is now considered almost impossible to determine an accurate ratio because of environmental, diurnal, and plant effects (such as age or water status) acting independently on  $C_2H_2$  and  $N_2$  assimilation and reduction. Other factors likely to affect the  $C_2H_2:N_2$  ratio are nonlinearity of  $C_2H_2$  reduction over the period of assay, impurities in the  $C_2H_2$  resulting in reduced rates of  $C_2H_2$  reduction, and effects of nodule removal from roots or soil, such as changes in nodule membrane function.

Early estimates of seasonal fixation were not calibrated against estimates using other methods. When comparisons were made, the  $C_2H_2$  reduction assay usually underestimated fixation significantly, although there were many exceptions.

It is clear that the major problems with this technique relate to sampling the belowground nodules and interpretation of rates in terms of  $N_2$  fixed. In addition, there appears to be an acetylene-induced decline of nitrogenase activity during assay. It has been used effectively in biochemical and physiological studies in artificial growth systems. Most of the time, however, data on rates of acetylene reduction as a measurement of  $N_2$  fixation in the field are of little use. We recommend that, unless it is planned to do biochemical studies of the fixation process or the necessary equipment already exists in your lab, you do not plan to include this technique in your work. If it is used, the article by Witty and Minchin (1988) is recommended reading.

Following, we briefly outline the methodology for field assay, using medics as the host legume.

### H.1.b. Field Assay Methodology

The main objectives of conducting the acetylene reduction assay of pasture legumes at ICARDA involve the evaluation of the symbiotic properties of different species and genotypes of annual medics. The method has been similarly used in the evaluation of introduced strains of *R. meliloti* and in assessing treatments on inoculation techniques.

It must be kept in mind that the acetylene reduction assay is a sensitive method for estimating the activity of nitrogenase at a given time. Results cannot be extrapolated into nitrogen fixed because of variations in the energy use of the two reduction pathways by nitrogenase as previously described in this section. We have found that

the method is somewhat difficult to apply to annual medics in our region because of the highly variable error encountered in removing root systems from the soil. The major problems are the medics' extensive root system and the fact that nodules of annual medics may be easily lost owing to their very light attachment to the root system.

The methodology adopted at ICARDA for taking root samples for the acetylene reduction assay from annual medic plants growing in the field is as follows:

1. Open a trench in the field by the plots where the medic stands are to be sampled. The trench should have a depth of approximately 30 cm.
2. Dig out four nodulated plants and exercise extra care not to detach the nodules. Carefully remove extra soil debris and cut the root at the crown area.
3. Place the four roots inside a plastic container having a lid that seals tightly (Figure H.1.2). The volume of the container used at ICARDA is 500 cc.



Fig. H.1.2. Airtight plastic container with subseal port in the removable lid for injection and removal of gas.

4. Ten ml of acetylene taken from a basketball previously filled with this gas are injected through the 'subseal' port on the lid of the container (Figure H.1.3). The nitrogenase enzyme in the nodule will reduce the acetylene to ethylene instead of reducing nitrogen molecules to  $\text{NH}_3$ .
5. The nodules are exposed to or 'incubated' with the gas for 30 minutes. The containers are buried in the trench to allow the incubation process to have the same temperature as that of the soil (Figure H.1.4).
6. After the incubation time remove 10 cc from the container and transfer them to a vacutainer tube (Figure H.1.5). These tubes are then stored and their contents analyzed in a gas chromatograph (GC).
7. Calibrate the gas chromatograph (Figure H.1.6) using pure ethylene as the standard gas (see Section H.1.c on gas chromatography).
8. Take a 0.1-ml sample and inject it through the port of a gas chromatograph.
9. From the peaks obtained, calculate the amount of ethylene found in the sample.
10. Nitrogenase activity then can be expressed in terms of moles of ethylene produced per plant per hour.
11. Coefficients of correlations can be obtained by plotting the mass of dry or fresh nodules against nitrogenase activity, the dependent variable.

### H.1.c. Gas Chromatography

The procedure for operation of gas chromatograph (GC) and measurement of ethylene produced is as follows:

1. Use 3 mm diameter  $\times$  150 cm length preppacked stainless steel column with Porapak N, 80–100 mesh.
2. Adjust the flow of gases to the following rates:
 

|                |            |
|----------------|------------|
| a) hydrogen    | 20 ml/min  |
| b) air (clean) | 200 ml/min |
| c) carrier gas | 15 ml/min  |
3. Set the temperatures as follows:
 

|                   |       |
|-------------------|-------|
| a) injection port | 80°C  |
| b) oven           | 100°C |
| c) detector       | 100°C |
4. Switch on power, then ignite flame.
5. Check the flame by putting a glass slide on the top of the flame ionization detector (FID); if moisture is formed on the glass the flame is on. If not, repeat ignition sequence.
6. Continue running GC unit for at least 15 minutes or until baseline of the recorder becomes stable.
7. Calibrate the GC unit by injecting 0.1 ml of standard pure ethylene gas of a

- known concentration.
8. Estimate the area under the ethylene peak (known quantity).
  9. Inject 1.0 ml of the unknown sample and estimate the area under the peak (unknown sample).
  10. Calculate the concentration of  $C_2H_4$  in  $\mu\text{mole}$  for unknown sample as follows:

$$\mu\text{mol } C_2H_4(\text{unknown}) = \frac{C_2H_4 \text{ peak area (unknown)}}{C_2H_4 \text{ peak area (known)}} \times \mu\text{mol } C_2H_4 (\text{unknown})$$

## H.2. N-Difference Method

The simplest field estimates of  $N_2$  fixation are obtained by measuring total amount of N in the legume crop. Kjeldahl analyses for N content of plant dry matter can be used to estimate total N yield of a field of legumes. This determination is, however, based on the assumption that all crop N comes from fixation; N derived from  $N_2$  fixation and from soil as mineral or fertilizer N are not distinguished. To estimate fixation, it is therefore necessary to determine the quantity of plant N which is obtained from soil. This is achieved by measuring the N content of a nonfixing reference plant (non-nodulated legume or a nonlegume). This quantity is subtracted from total legume N to determine  $N_2$  fixed.

This technique is based on the assumption that the test legume and reference plant remove identical amounts of N from the soil. This, however, is largely dependent upon the success of the researcher in matching growth rates of fixing and nonfixing crops. Other factors known to affect plant growth, such as water availability, insects and diseases, should be similar in both crops. Reference plants commonly used include cereals (usually wheat, barley, or ryegrass), non-nodulating isolines of legumes (available for chickpea, peanut, and soybean only), and uninoculated or ineffectively nodulated legumes (Figure H.2.1). Of these, the non-nodulating isolines probably give the best results, although differences in root morphology may cause considerable error. The potential for N assimilation by most nonlegumes is not as high as for legumes, generally resulting in an overestimation of  $N_2$  fixation. If levels of soil N are low and reference plants accumulate much less N than the fixing legume, error due to plant type will be minimal.

Advantages of this assay are simplicity and the fact that it is a direct measure of plant growth and N fixation/uptake. It is also a time-integrated measurement, giving a total fixation estimate for the whole season. Matching growth rates and N uptake in reference and fixing crops remains the major disadvantage of this technique. There are three possibilities: (1) approximately equal amounts of soil N will be assimilated by test and reference plants, resulting in an accurate estimate of  $N_2$  fixation; (2) in a

low N soil, the more vigorous nodulated plant will produce greater root mass and therefore explore a greater volume of soil.  $N_2$  fixation will be overestimated because the  $N_2$  fixing legume will take up more soil available N than the reference plant; and (3) the fixing legume with an active efficient symbiosis will require and take up less soil N than the reference plant, and fixation will be underestimated.

Several variations of the N-difference method exist. Generally the quantity of legume N derived from  $N_2$  fixation (Q) is calculated as:

$$Q = N \text{ yield (legume)} - N \text{ yield (reference)}$$

In the case where legume and reference are not well matched, measurement of postharvest soil mineral N can be determined in the fixing and nonfixing plots and added to the differences in total N yields of the two crops:

$$Q = [N \text{ yield(leg)} - N \text{ yield(ref)}] + [N \text{ soil(leg)} - N \text{ soil(ref)}]$$

The use of this second equation assumes that mineralization, leaching, and denitrification are identical under each crop, which may not be generally true. More importantly, data may be confusing owing to the lack of precision in soil-N measurement techniques.

### H.3. $^{15}\text{N}$ Methodologies

#### H.3.a. Isotope Dilution

The methods based upon the measurement of  $^{15}\text{N}$  in test and reference plants are basically an extension of the N-difference method. Fertilizer enriched with  $^{15}\text{N}$  is added to the soil in small amounts, either as labelled organic or inorganic N, and the concentrations of  $^{15}\text{N}$  in the fixing plant relative to the reference plant are used to calculate the proportion of soil-derived N in the fixing plant and, by difference, the proportion of  $N_2$  fixed.

$$\text{In a simple form: } \%N \text{ fixed} = \frac{\%^{15}\text{N excess (legume)}}{\%^{15}\text{N excess (reference)}} \times 100$$

The major assumption made is that the fixing and reference plants take up N from a similar volume of soil having the same isotopic composition, although the total amounts taken up may be different. This is potentially a more accurate technique than the N-difference method which assumed equal total uptake of soil N by the test and reference plants.

The choice of an appropriate non-N<sub>2</sub>-fixing reference plant is a major factor affecting whether the test and reference plants take up N from the soil at the same isotopic (<sup>15</sup>N:<sup>14</sup>N) composition. The ratio of <sup>15</sup>N:<sup>14</sup>N is unlikely to be uniform with depth and time unless a long period of equilibration (or thorough mixing) has taken place. This problem will be increased if the test and reference plants have different rates of growth and therefore N uptake (see Witty, 1983, for a complete explanation). As with the N-difference method, three possible situations may occur when assessing nitrogen fixation: (1) nitrogen fixation will be overestimated if the fixing plant explores both enriched and nonenriched volumes of soil, while the roots of the reference plant are contained within the enriched area; (2) underestimation results if the reference crop root system is more vigorous and explores labelled and unlabelled soil N; and (3) N<sub>2</sub> fixation is estimated accurately if the test and reference plants explore the enriched volume of soil or the labelled and unlabelled volumes equally.

The three different possibilities are illustrated in Figure H.3.1. With normal surface application of <sup>15</sup>N-labelled material to soil, a highly <sup>15</sup>N-enriched layer is created at the surface, with the <sup>15</sup>N enrichment of plant-available soil N decreasing rapidly with depth. In Figure H.3.1(a) the roots of the legume have explored deeply into the region which has not been artificially enriched (labelled) with <sup>15</sup>N, while the roots of the non-N<sub>2</sub>-fixing reference plant have been restricted to the uppermost enriched zone. In this case fixation will be overestimated. In the reverse situation [Figure H.3.1(b)], where the roots of the reference plant have extended below the labelled zone, the dilution of the <sup>15</sup>N content of the reference plant with N taken up from unenriched soil will result in an underestimation of N<sub>2</sub> fixation by the legume. Only in the ideal situation, where roots of the legume and reference plant explore a similar volume of soil over a similar time span and utilize soil N of similar <sup>15</sup>N enrichment [Figure H.3.1(c)], can accurate determinations of N<sub>2</sub> fixation be expected.

Cost is a major limitation to the adoption of <sup>15</sup>N techniques for routine and general use. The major problem relates to analyses of samples rather than to the cost of the isotope itself. Much of the literature relating to the use of <sup>15</sup>N in field studies of N<sub>2</sub> fixation originates from a few laboratories, and in developing countries work is often supported by the International Atomic Energy Agency of the FAO (Vienna). Cost of <sup>15</sup>N per microplot will range from U.S. \$5 to \$20, while each sample analyzed will cost from U.S. \$10 to \$80, depending on the laboratory and the number of samples. This translates to a cost of about U.S. \$50–\$300 per treatment (three replications)—with just a few treatments or sites, this figure rapidly goes beyond the annual budget of many national N<sub>2</sub> fixation programs.

The overall advantage of the isotope dilution method is that it is potentially the most accurate method for measuring N<sub>2</sub> fixation in the field.



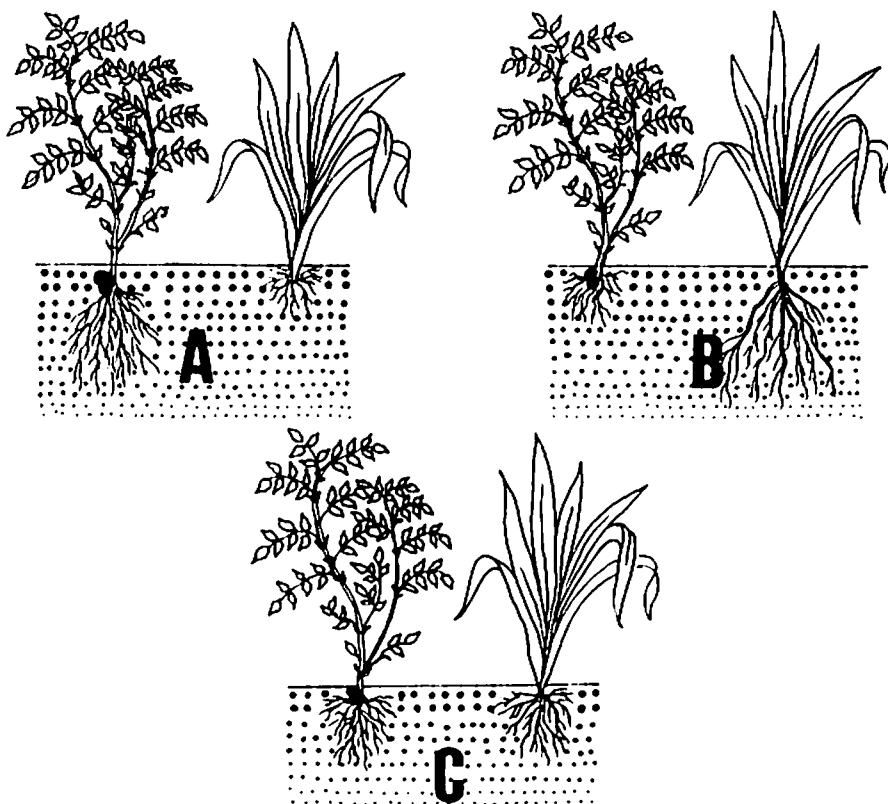


Fig. H.3.1. Differential growth in fixing and reference plants and their effect on the estimation of nitrogen fixation using the  $^{15}\text{N}$  dilution method

### H.3.b. "A"-value Method

It is essential that addition of  $^{15}\text{N}$ -labelled material does not affect fixation. The effect of mineral N on  $\text{N}_2$  fixation is well established and therefore the use of low rates of N application (e.g., 5–20 kg N/ha) are preferable. Use of the "A"-value method, as a variation of the isotope dilution technique, allows application of low amounts of N to the fixing crop, and high amounts of N (adequate to produce a root system equivalent to the fixing plant) to the reference crop.

The soil nutrient supply measured in terms of available units of applied fertilizer N has been referred to as the 'A-value'. The A-value is based on the concept that when a plant is confronted with two or more different sources of a given nutrient, it will absorb from each in direct proportion to the respective quantities available to that crop.

In a simple case of a nonfixing plant growing on a soil with an applied labelled fertilizer, the roots can absorb the nutrient in question from only two sources, i.e., the native soil nutrient pool and the known quantity of fertilizer supplied. In this case, it is only necessary to determine the respective quantities absorbed from each source to determine the A-value of soil. The A-value is therefore a yield-independent measure, and can be used to estimate the amount of native soil nutrient available to the plant. This is expressed in fertilizer equivalents.

$$\frac{\text{fertilizer nutrient in plant}}{\text{fertilizer nutrient applied}} = \frac{\text{soil nutrient in plant}}{\text{soil nutrient supply (A-value of soil)}}$$

and:

$$\text{A-value of soil} = \frac{\text{soil nutrient in plant}}{\text{fertilizer nutrient in plant}} \times \text{fertilizer nutrient applied}$$

Using nitrogen (N) as an example,

$$A_N = \frac{fNd_{fs}}{fNd_{ff}} \times \text{rate of fertilizer application}$$

$$\text{but } fNd_{fs} = 1 - fNd_{ff}$$

thus,

$$A_N = \frac{1 - fNd_{ff}}{fNd_{fs}} \times \text{rate of fertilizer application}$$

where  $fNd_{fs}$  = nitrogen in plant derived from soil

and  $fNd_{ff}$  = nitrogen in plant derived from fertilizer

In using the A-value concept, it is important to realize the following:

1. Any changes in the placement methodology of the labelled fertilizer, or in the conditions of plant growth, may affect the magnitude of the A-value for soil.
2. The A-value for a particular soil remains constant even at different rates of application of the same labelled fertilizer. In soil fertility studies, it is therefore sufficient to use only one rate of application to assess the nutrient-supplying potential of a soil.

3. The A-value of a given soil often changes with time.

Calculations and examples using this method are given in Section H.3.f.

### H.3.c. Natural Abundance Method

In this isotope dilution technique, variation in natural abundance of  $^{15}\text{N}$ , caused by discrimination during  $\text{N}_2$  fixation in favor of the lighter ( $^{14}\text{N}$ ) isotope, is measured. In a wide variety of (but not all!) soils, abundance of  $^{15}\text{N}$  is on average 9.22% higher than in the atmosphere. Therefore, plants dependent upon soil N (nonfixing plants) rather than atmospheric N (fixing plants) should contain higher abundance of  $^{15}\text{N}$ . Successful application of this technique is only possible if natural enrichment of the soil is high, soil variability is low, isotopic discrimination within the plant is identical in test and reference plants and is known, and precise isotope-ratio measurements are possible.

This technique has become increasingly popular with availability of the more sensitive mass spectrometer needed for analysis. Advantages and disadvantages are similar to those detailed above for the isotope dilution method. Although it is not necessary to purchase  $^{15}\text{N}$ -enriched fertilizer for application, testing of soils for enrichment and homogeneity prior to experimentation and the higher cost of sample analyses using a more expensive mass spectrometer make the technique generally more expensive than isotope dilution.

### H.3.d. Use of Reference Crops

As mentioned earlier, the accurate application of isotope dilution methodologies depends on proper choice of reference crop. The following criteria should be followed when deciding on a reference crop to use in  $^{15}\text{N}$  experiments:

1. Reference must not fix  $\text{N}_2$ . Within the ICARDA region, associative  $\text{N}_2$  fixation (nonsymbiotic) is negligible, so most nonlegumes with a similar growth and rooting pattern may be used. Non-nodulating legumes (isolines) are generally reliable, but the non-nodulating characteristic may be strain-specific, and reversion of genotypes to a nodulating type is not uncommon. Therefore, root-systems of 'non-nodulating' legume lines should be excavated and examined for nodules during late-flowering or early pod-fill stages.
2. Rooting patterns and depth of reference and legume crops must be similar. This can be evaluated by excavation of roots from plants at varying stages of growth, i.e., mid-flowering, pod-fill, and harvest. If using different species for reference, enrichment in the different potential reference crops can be

- compared. Differences in rooting patterns are often indicated by different  $^{15}\text{N}$  recoveries.
3. Relative N-uptake patterns of legume and reference should be similar. In simplest terms, the legume and reference should start to accumulate N and reach their maximum N contents at the same time (see Witty, 1983, for full discussion).
  4. Because the  $^{15}\text{N}$ -enrichment of the available soil N pool will change with time (change is faster during early crop growth), growth rate and duration of growth should be similar in reference and legume. It is therefore best if the reference plant does not mature or stop accumulating N before final legume harvest.
  5. The choice and management of reference crop is quite different in legume pastures, or in forage legumes which are grazed or cut more than once. Essentially, the reference crop must be treated exactly as the legume, with clippings made to correspond with grazing or cutting. Obviously, accuracy in determination of  $\text{N}_2$  fixed in a grazing situation is greatly diminished.

At ICARDA, research into choice of reference crop under varying agro-environments has determined the following reference crops to be appropriate for legumes grown in the region:

|                       |                                          |
|-----------------------|------------------------------------------|
| lentil/vetch/lathyrus | locally adapted barley, non-nod chickpea |
| chickpea              | non-nod chickpea ('PM 233')              |
| faba bean             | locally adapted barley or wheat          |
| medics                | locally adapted ryegrass                 |

It should be realized that errors derived from poor matching of reference and  $\text{N}_2$ -fixing plants become most important when only small proportions of legume N are derived from the atmosphere (as with spring-sown chickpea). Errors become smaller and mismatching less important as the amount of  $\text{N}_2$  fixed by the legume increases.

### H.3.e. Use of $^{15}\text{N}$ -Enriched Fertilizers and Analysis

The most commonly used form of enriched fertilizer is ammonium sulfate, usually purchased with an enrichment of about 10%  $^{15}\text{N}$ . Other forms of enriched material may be purchased, such as ammonium nitrate or urea. The nitrate ion is highly mobile in soils, and may be more prone to loss than ammonium (although, depending on soil conditions, ammonium may be quickly nitrified to nitrate); urea-N may be quickly volatilized, and therefore lost under some conditions. It must be kept in mind that it is important to keep the N added as  $^{15}\text{N}$  available to the growing plant for as long as possible. Slow release  $^{15}\text{N}$  fertilizer formulations to measure  $\text{N}_2$  fixation by isotope dilution are highly recommended. Even with a "well-matched" reference crop (e.g., a non-nodulated isoline) estimates of fixation vary with fertilizer formulation.

Even application of the enriched fertilizer over the area of the microplot is essential. Uniform labelling of the soil in this area forms the basis of the methodology; if applied unevenly, misleading results and poor replication will be obtained. This is true for both fixing and reference crop microplots. In sampling the microplot, plants from the center of the microplot only are taken; plants near the borders of the microplot will take soil N from outside the microplot, and will therefore be exposed to a different  $^{15}\text{N}$ : $^{14}\text{N}$  ratio.

Enriched fertilizer is usually applied just after planting but before emergence of the seedlings. Low doses of fertilizer, applied as a liquid, may be applied after emergence without affecting the plants or N uptake. However, application of high rates of ammonium fertilizer after emergence may result in damage to seedlings. A small amount of the  $^{15}\text{N}$  fertilizer solution used should be kept for analysis of actual  $^{15}\text{N}$  enrichment; if different from theoretical calculations, this figure will be used in final calculations of  $\text{N}_2$  fixation.

With the modern equipment now available in most labs that analyze  $^{15}\text{N}$  enrichment using mass spectrometry, ground plant material can be measured directly without digestion. The sample is combusted to gas and the N in the gas is analyzed. It is important that the plant material be ground as finely as possible ( $< 0.2$  mm particle size) to obtain best results. Plant samples from microplots should be ground, mixed, and a subsample from the total material ground again to obtain very fine material. During the grinding process, care should be taken not to heat the plant material.

Alternately, the plant material may be digested using the Kjeldahl method, and the dried crystals from the digestion sent to the analyzing laboratory for determination of  $^{15}\text{N}$ -enrichment levels. Check with the laboratory when enquiring as to costs whether they will accept ground plant samples.

### H.3.f. Sample Calculations for Isotope Dilution

Legumes, in symbiosis with the appropriate strains of *Rhizobium* bacteria, are capable of supplementing N derived from soil by converting atmospheric  $\text{N}_2$  into forms available to the plant. Such a legume crop growing in the presence of nitrogen fertilizer will thus derive its N from soil, fertilizer, and the atmosphere. It is, however, impossible to label all the N in an atmosphere over a field crop with  $^{15}\text{N}$ . Using the indirect labelling technique, it is possible to assess how much of the plant's total N supply was derived from atmospheric  $\text{N}_2$ . The method, as previously discussed, involves the use of a reference crop (which does not fix  $\text{N}_2$ ) to assess the A-value of the soil, or to determine the relative amounts of available soil and applied  $^{15}\text{N}$  labelled fertilizer N available to a crop. The basic assumption is that if the reference is an appropriate standard, it will absorb the soil N and fertilizer N in the same ratio as the

legume, thus reflecting the same amount of available N (in fertilizer units) present in the soil to both crops. The A-value obtained for the legume crop would therefore comprise the available amounts from both soil and  $N_2$  fixed. Thus, by applying the A-value concept, we can determine:

from the reference (nonfixing) crop  $A_{\text{soil}}$  (1)

from the legume (fixing) crop  $A_{\text{soil}} + \text{fixation}$  (2)

and  $A_{\text{fixation}}$  will equal  $A_{\text{soil} + \text{fixation}} - A_{\text{soil}}$  (3)

The fractional utilization equation for the legume crop is as follows:

$$\frac{\% \text{ Ndff}}{A_{\text{fert}}} = \frac{\% \text{ Ndfa}}{A_{\text{fix}}} = \frac{\% \text{ Ndfs}}{A_{\text{soil}}} = \frac{\% \text{ Ndfa} + \% \text{ Ndfs}}{A_{\text{fix}} + A_{\text{soil}}} \quad (4)$$

where Ndff = nitrogen derived from fertilizer

Ndfa = nitrogen derived from fixation

Ndfs = nitrogen derived from soil

Thus, if they have the same amounts of soil plus fertilizer N available to them, they will reflect the same amounts of available soil and fertilizer N by absorbing them in the same ratio. By means of this relationship it is possible to calculate % Ndfa, since % Ndff can be determined experimentally by the use of  $^{15}\text{N}$ -labelled fertilizer.  $A_{\text{Fert}}$  also is known, since it is equivalent to the amount of fertilizer N applied, and  $A_{\text{Fix}}$  is the difference in the A-values obtained for the fixing and nonfixing treatments.

From an experimental point of view there can be two situations:

1. The simplest case is where the  $N_2$  fixing and nonfixing crops have available to them the same amounts of soil and fertilizer nitrogen; the same amounts of a  $^{15}\text{N}$ -labelled material are applied to both crops.
2. In the case where the  $N_2$  fixing and nonfixing crops have available to them the same amount of soil N but receive different amounts of fertilizer N, a "starter" amount or a low rate of N fertilizer is applied to the fixing crop to avoid interference with N fixation, but a higher rate may have to be applied to the reference crop to ensure normal growth.

- (1) **Method of calculating N fixed when fixing and nonfixing crops receive the same rate of  $^{15}\text{N}$  fertilizer**

To illustrate the methodology, consider the following examples:

| <sup>15</sup> N at. excess |                |               |
|----------------------------|----------------|---------------|
| Fixing crop                | Nonfixing crop | Remarks       |
| 0.5                        | 0.5            | No fixation   |
| 0.0                        | 0.5            | 100% fixation |
| 0.25                       | 0.5            | 50% fixation  |
| 0.1                        | 0.5            | 80% fixation  |

This relationship is described in the following equations:

$$\% \text{ Ndfa} = \left( 1 - \frac{\% ^{15}\text{N at. excess legume crop}}{\% ^{15}\text{N at. excess reference crop}} \right) \times 100 \quad (5)$$

$$\text{or} = \left( 1 - \frac{\% \text{ Ndff legume}}{\text{Ndff reference crop}} \right) \times 100 \quad (6)$$

$$\text{N Fixed (kg/ha)} = \frac{\% \text{ Ndfa}}{100} \times \text{Total N yield} \quad (7)$$

### Exercise

Estimate the percentage of symbiotic nitrogen fixation by faba bean and chickpea, given the following data (the crops were grown in soil labelled by incorporating <sup>15</sup>N-labelled plant material into the soil):

|    | Treatment               | <sup>15</sup> N at. excess | N yield in kg/ha |
|----|-------------------------|----------------------------|------------------|
| 1. | Barley (reference crop) | 0.335                      | 90               |
| 2. | Nodulated chickpea      | 0.198                      | 160              |
| 3. | Faba bean               | 0.112                      | 200              |

The above experiment is a case where both the legume and nonlegume received the same rate of <sup>15</sup>N fertilizer, and can tap the same amount of available soil N, since they are grown on the same soil. In such a situation, it is not necessary to calculate the A-values for soil and the following equation can be used directly:

$$\% \text{ Ndfa} = \left( 1 - \frac{\% ^{15}\text{N at. excess in legume}}{\% ^{15}\text{N at. excess in reference}} \right) \times 100$$

For chickpea therefore,

$$\begin{aligned}\% \text{ Ndfa} &= (1 - 0.198/0.335) \times 100 = 41 \\ \text{N Fixed} &= (41/100) \times 160 \text{ kg N/ha} = 66 \text{ kg N/ha}\end{aligned}$$

For faba bean,

$$\begin{aligned}\% \text{ Ndfa} &= (1 - 0.112/0.335) \times 100 = 67 \\ \text{N Fixed} &= (67/100) \times 200 = 134 \text{ kg N/ha}\end{aligned}$$

**(2) Method of calculating N fixed when fixing and nonfixing crops receive different rates of  $^{15}\text{N}$  fertilizer**

In this case, it is necessary to calculate A-value for the non-nodulating crop and that for the nodulating crop. The non-nodulating crop estimates the A-value for soil, while the A-value obtained for the nodulating crop is the sum of the A-values of the soil plus the N fixed. The difference between the two A-values then represents the A-value of fixation in fertilizer equivalent units, which can be converted into the actual amount of N fixed by multiplying this figure by the N utilization efficiency of the applied fertilizer by the legume.

**Example:**

- I. **Nodulating crop**
  - $^{15}\text{N}$  at. excess = 0.2% in plant
  - 20 kg N/ha  $(\text{NH}_4)_2\text{SO}_4$  with 5%  $^{15}\text{N}$  atom excess was applied at planting
  - Total N yield = 200 kg N/ha
- II. **Non-nodulating crop**
  - $^{15}\text{N}$  at excess = 0.4% in plant
  - 100 kg N/ha  $(\text{NH}_4)_2\text{SO}_4$  containing 1%  $^{15}\text{N}$  atom excess was applied at planting

**Calculations:**

$$\% \text{ NdfF} = \frac{\% \text{ }^{15}\text{N at. excess in legume plant}}{\% \text{ }^{15}\text{N at. excess in fertilizer}} \times 100 \quad (8)$$

$$\text{therefore, } \% \text{ NdfF (nodulating crop)} = \frac{0.2}{5} \times 100 = 4$$



$$\% \text{ Ndff (non-nodulating crop)} = \frac{0.4}{1} \times 100 = 40$$

$$\text{A-value} = \frac{100 - \% \text{ Ndff}}{\% \text{ Ndff}} \times \text{rate of fertilizer N application} \quad (9)$$

therefore,

$$\text{A-value (nod. crop)} = \frac{100 - 4}{4} \times 20 = 480$$

$$\text{and A-value (non-nod. crop)} = \frac{100 - 40}{40} \times 100 = 150$$

thus,  $A_{\text{soil} + \text{fix}}$  = 480 kg N/ha as  $(\text{NH}_4)_2\text{SO}_4$  equivalents

and  $A_{\text{soil}}$  = 150 kg N/ha as  $(\text{NH}_4)_2\text{SO}_4$  equivalents

therefore  $A_{\text{fix}}$  = 330 kg N/ha as  $(\text{NH}_4)_2\text{SO}_4$  equivalents

To determine the actual amount of  $\text{N}_2$  fixed, we must multiply  $A_{\text{fix}}$  by the % fertilizer utilization efficiency of the nodulated crop.

$$\begin{aligned} \text{Fertilizer N yield} &= \frac{\% \text{ Ndff} \times \text{Total N yield}}{100} \\ &= \frac{4 \times 200}{100} = 8 \text{ kg N/ha} \end{aligned} \quad (10)$$

% Fertilizer utilization efficiency (% F.U.E.)

$$= \frac{\text{N fert. yield in plant}}{\text{fertilizer applied}} \times 100 \quad (11)$$

$$\text{therefore, \% F.U.E.} = \frac{8}{20} \times 100 = 40\%$$

$$\text{Total amount of N fixed (kg/ha)} = \frac{A_{\text{fix}} \times \% \text{ F.U.E. (nod.crop)}}{100} \quad (12)$$

$$= \frac{330 \times 40}{100} = 132 \text{ kg N/ha}$$

$$\text{and \% Ndfa} = \frac{132}{200} \times 100 = 66\%$$

Since for a nodulating crop, total N yield = N yield from fertilizer + N yield from soil + N yield from fixation, the amount of N derived by the nodulated crop from soil (Ndfs) can be calculated as follows:

$$\begin{aligned} \text{Ndfs} &= \text{Total N yield} - (\text{NdfF} + \text{Ndfa}) \\ &= 200 - (8 + 132) \text{ kg N/ha} \\ &= 60 \text{ kg N/ha.} \end{aligned} \quad (13)$$

Thus, the amounts of N derived by the nodulating crop from fertilizer, soil and fixation are 8, 60, and 132 kg N/ha respectively.

There are other ways of calculating N fixed, and below is another method for calculating the above data:

#### For the Nodulating Crop

$$\begin{aligned} \% \text{ NdfF} &= \frac{\% \text{ }^{15}\text{N at. excess in sample (plant)}}{\% \text{ }^{15}\text{N at. excess in fertilizer}} \times 100 \quad (14) \\ &= \frac{0.2}{5} \times 100 = 4 \end{aligned}$$

The fractional utilization relationship for the nodulating crop is the following:

$$\frac{\% \text{ NdfF}}{\text{Fert. applied}} = \frac{\% \text{ Ndfs} + \% \text{ Ndfa.}}{A_{\text{soil}} + A_{\text{fix}}} \quad (15)$$

$$\begin{aligned} \text{But } \% \text{ NdfF} + \% \text{ Ndfs} + \% \text{ Ndfa} &= 100 \\ \% \text{ Ndfs} + \% \text{ Ndfa} &= 100 - \% \text{ NdfF} \end{aligned} \quad (16)$$

$$\text{Therefore, } \frac{4}{20} = \frac{96}{A_{\text{soil} + \text{fix}}}$$

$$A_{\text{soil} + \text{fix}} = \frac{96}{4} \times 20 = 480 \text{ kg N/ha as } (\text{NH}_4)_2\text{SO}_4 \text{ equivalents}$$

#### For the Non-Nodulating Crop

$$\% \text{ NdfF} = \frac{0.4}{1} \times 100 = 40$$

In this case, since there is no fixation, only soil and fertilizer N are available for this crop, and therefore:

$$\frac{\% \text{ NdfF}}{\text{Fert. applied}} = \frac{\% \text{ Ndfs}}{A_{\text{soil}}} = \frac{100 - \% \text{ NdfF}}{A_{\text{soil}}}$$

$$\text{Therefore, } \frac{40}{100} = \frac{100 - 40}{A_{\text{soil}}}$$

$$\text{and } A_{\text{soil}} = \frac{60}{40} \times 100 = 150 \text{ kg N/ha as } (\text{NH}_4)_2\text{SO}_4 \text{ equivalent}$$

$$\begin{aligned} \text{Thus, } A_{\text{fix}} &= A_{\text{soil} + \text{fix}} - A_{\text{soil}} = 480 - 150 \\ &= 330 \text{ kg N/ha as } (\text{NH}_4)_2\text{SO}_4 \text{ equivalents} \end{aligned}$$

Furthermore, it was shown that for the nodulating crop

$$\frac{4}{20} = \frac{\% \text{ NdfFix}}{330} = \frac{\% \text{ Ndfs}}{150}$$

$$\text{Therefore \% Ndfa} = 330 \times \frac{4}{20} = 66$$

$$\% \text{ Ndfs} = 150 \times \frac{4}{20} = 30$$

$$\begin{aligned} \text{and } \% \text{ Ndff} &= 4 \\ \text{Total} &= 100\% \end{aligned}$$

$$\text{Since the total N yield/ha} = 200 \text{ kg N/ha}$$

$$\begin{aligned} \text{Therefore the amount of N fixed} &= \frac{\% \text{ Ndfa}}{100} \times \text{N yield} \\ &= \frac{66}{100} \times 200 = 132 \text{ kg N/ha} \end{aligned}$$

$$\text{The amount of N derived from soil} = \frac{30}{100} \times 200 = 60 \text{ kg N/ha}$$

$$\text{The amount of N derived from fert.} = \frac{4}{100} \times 200 = 8 \text{ kg N/ha}$$

### H.3.g. Trial Setup

Although the  $^{15}\text{N}$  isotope dilution technique is not without limitations, if the basic assumptions of the procedure are considered in experimental setup, it provides the most reproducible and precise estimates of  $\text{N}_2$  fixation in field-grown annual legumes. Following is a sample trial detailing the various steps in setting up and conducting a trial.

**H.3.g.1. FIXT: Field Trial Using "A" Value Method.** In this trial, the amount of plant nitrogen derived from biological nitrogen fixation will be determined using  $^{15}\text{N}$  and isotope dilution methodology. This experiment has been designed primarily to determine the effect of rhizobial inoculation on nitrogen nutrition of the legume crop in general, and to quantify  $\text{N}_2$  fixed and N taken from soil for crop growth in particular. For this reason, this FIX trial has been imposed on the ICARDA food

legume 'inoculum response' trial, or IRT, with minor differences. The IRT methodologies should be followed for treatments, inoculation, layout, etc., with differences occurring only in the addition of microplots in three replications and addition of three plots for the reference crop.

The details of IRT are given in Sections G.1 and G.2. Modifications will occur in trial layout, fertilization, details for sampling and harvest areas, and data collection. These modifications will be dealt with in the pages that follow.

## **Experimental Treatments**

### **Fertilization**

High levels of inorganic N can depress  $N_2$  fixation. This therefore necessitates the application of low amounts of labelled N fertilizer to the fixing crop to estimate  $N_2$  fixed. However, such amounts may be too low to support the proper growth of the nonfixing reference plants, especially in soils of low nitrogen content. For these reasons, it is practical to give a reasonable dose of  $^{15}N$ -labelled fertilizer to the reference crop, while the fixing crop receives only a low quantity. Using the A-value concept of Fried and Broeshart (Section H.3.b), it is still possible to determine  $N_2$  fixed under these conditions. In studies of nitrogen fixation by various grain legumes in the field, 20 kg N/ha of 5%  $^{15}N$  atom excess fertilizer and 100 kg N/ha of 1%  $^{15}N$  atom excess fertilizer have generally been applied to fixing and nonfixing crops, respectively, with good results.

In order to impose minimum N fertilization on the legume, 10 kg N/ha, applied at planting, will be provided for all *Rhizobium* treatments, including the uninoculated treatment. The high dose nitrogen treatment will receive 120 kg N/ha, as a split application, as indicated in the IR trial methodology. Reference crop plots will receive 100 kg N/ha. Microplots will receive the same amount of total N as whole plots, but it will be in the form of diluted  $^{15}N$  fertilizer.

Calculations for application of nitrogen fertilizers in plots with and without microplots will be given.

### **Microplots**

Replications I, II, and IV have  $^{15}N$  microplots in all the treatment plots (4), not including the high dose nitrogen fertilized (120 kg N/ha) treatment. Location of microplots is shown in Figure H.3.2. Microplots are 1.2 × 1.2 m, large enough for sampling one time only, at physiological maturity of the crop.

Microplots of  $1.2 \times 1.2$  m are also located in the three reference crop plots. Fertilization of microplots is as follows; using  $^{15}\text{N}$  enriched and unenriched ammonium sulfate (21% N) (see calculations):

### **Fixing Crops**

#### **Microplot Fertilization**

For each microplot of 0.000144 ha ( $1.44 \text{ m}^2$ ), application of 10 kg N/ha will need 7.6 g ammonium sulfate with 10%  $^{15}\text{N}$  atom excess. Therefore, for three microplots a total of 22.8 g ammonium sulfate with 10%  $^{15}\text{N}$  atom excess will be needed. The  $^{15}\text{N}$  fertilizers should be mixed with enough water to evenly cover the  $1.2 \times 1.2$  m microplot by either spraying or by evenly distributing with a plastic wash bottle (Figure H.3.3), usually at  $200 \text{ ml/m}^2$ . Fertilizer for each microplot should be dissolved in water and applied separately.

When applying  $^{15}\text{N}$  to microplots be sure to distribute  $^{15}\text{N}$ -water mixture evenly over the entire microplot (Figure H.3.4). No  $^{15}\text{N}$  should be placed out of microplot. The  $^{15}\text{N}$  should be applied after planting, but before emergence of seedlings.

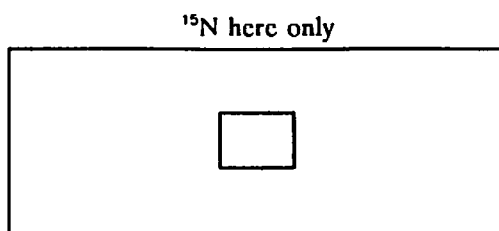
#### **Whole Plot Fertilization**

All treatment plots must then receive 10 kg N/ha or 120 kg N/ha depending on the treatment, so that growth in microplots is equivalent to that in the rest of the plot. For the remainder of the plots containing microplots (non-microplot areas to receive normal unenriched fertilizer), use ammonium sulfate, at the same rate of 10 or 120 kg N/ha, depending on the treatment. If ammonium sulfate is not available, another N fertilizer such as urea may be used (urea is usually 46% N).

When applying normal unenriched ammonium sulfate to the remainder of plots in replications I, II, and IV prior to planting, cover the microplots with plastic or cloth to assure that no fertilizer is placed in the microplot. Normal fertilization should be done preplant, with fertilizer incorporated into the top layer of the soil.

#### **N Fertilization: Fixing Crop for Remainder of Plot (Outside the Microplot)**

The area outside the microplot is 0.001206 ha per plot. At the rate of 10 kg N/ha, this area will need 12.1 g N which can be provided through 57 g ammonium sulfate to be spread evenly over plot area except for microplot.



### **N Fertilization: Fixing Crop for Plots Without Microplots**

The area of whole plot is 0.00135 ha. At the rate of 10 kg N/ha this whole plot will need 13.5 g N which can be provided through application of 64.3 g ammonium sulfate spread evenly over plot, preplant.

### **Reference Crop**

The accuracy of the isotope dilution method depends on selecting a suitable reference crop which does not fix N but is similar in volume of soil explored by the root system and in the length of the growing period. See Section H.3.d. for full description and suggested reference crops for the various legume species.

### **Microplot Fertilization**

For each microplot of 0.000144 ha, application of 100 kg N/ha will require 66.6 g ammonium sulfate with 1% <sup>15</sup>N atom excess. As with fixing crop microplot fertilization, this amount is to be spread evenly over entire microplot area in solution after planting but before emergence of seedlings.

### **N Fertilization for Remainder of Plot**

The area outside the microplot in the 1.5 × 5 m reference crop plot is 0.000606 ha. At a rate of 100 kg N/ha, 60.6 g of N will be needed which is provided as 289 g ammonium sulfate. This amount should be spread evenly over the area in the reference crop plot outside the microplot, before planting.

### **Layout**

Treatments, given in the inoculum response trial, Section G.2, include three treatments with single-strain inoculants, and one treatment each of uninoculated and nitrogen-fertilized controls. All layouts shown include the five suggested treatments, but treatments can be adapted to the needs of the experimenter. Randomization and

plot numbers can be allocated according to this layout. The modified layout for the FIX experiment, including reference plots, is shown in Figure H.3.2.

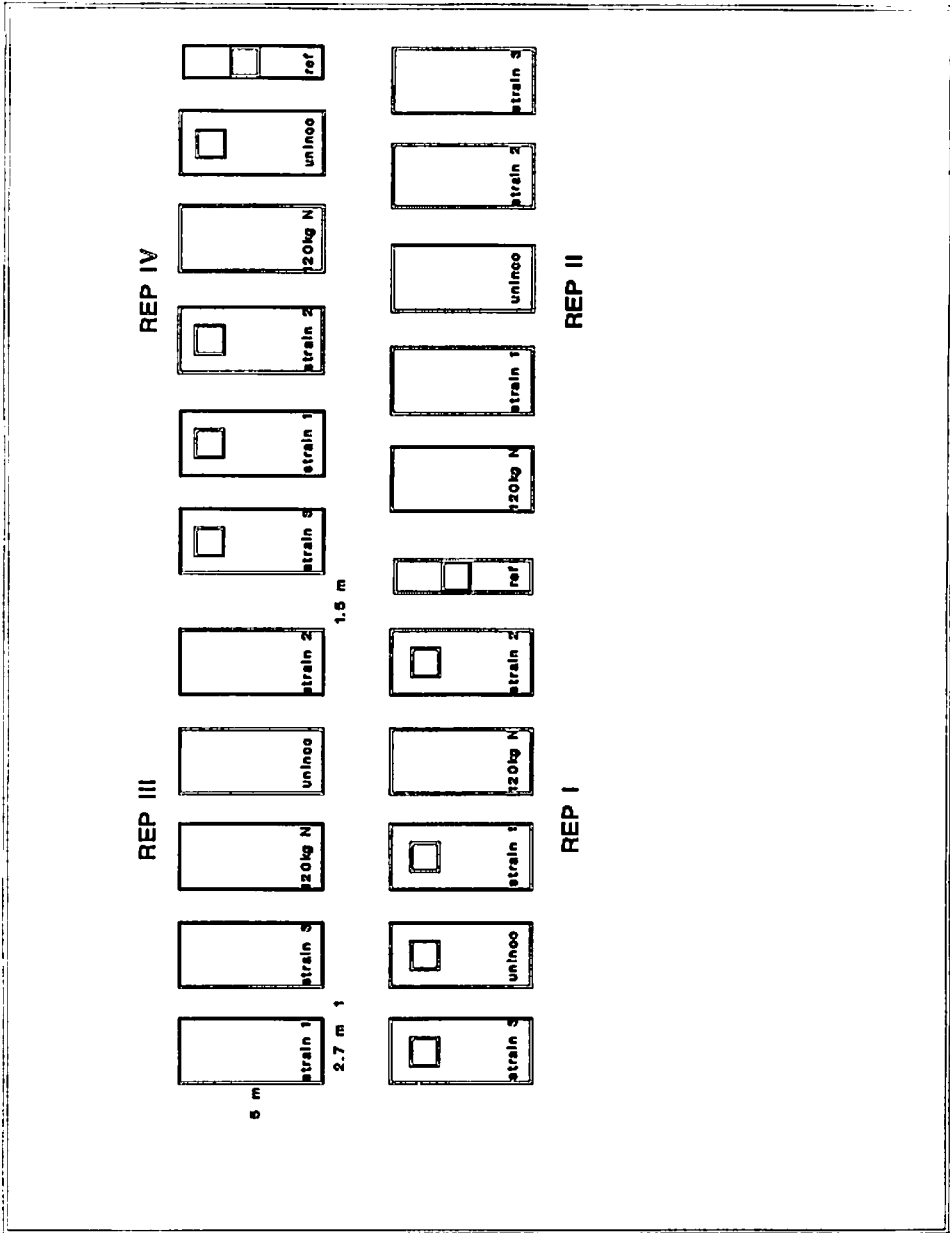


Fig. H.3.2. FIX trial layout



## Data Collection

Suggested data collection for IR experiment is given in Section G.2. The same data set should be collected, if possible, for the FIX experiment. In addition, it is necessary that the microplots be harvested at **physiological maturity**. Ten plants from the center of the microplot should be harvested, before loss of leaves but after seeds have matured, and oven-dried at 60°C. The dried plant material should then be ground finely in a Wiley mill. From these samples from each microplot, a 10-g subsample can be used for analysis of total N. Five grams from each microplot will be needed for  $^{15}\text{N}$  analysis.

On receipt of  $^{15}\text{N}$  analysis data, calculations for amounts of  $\text{N}_2$  fixed can be calculated from equations shown on the following pages.

**H.3.g.2. Preparation of  $^{15}\text{N}$  Solutions.** The isotope dilution equation can be used to obtain the desired  $^{15}\text{N}$  enrichment when N fertilizers labelled with different enrichments are mixed:

$$x(a_1) + y(a_2) = (x + y) a \quad (1)$$

where:

$x$  = quantity of material with  $^{15}\text{N}$  abundance  $a_1$  (of higher  $^{15}\text{N}$  enrichment)

$y$  = quantity of material with  $^{15}\text{N}$  abundance  $a_2$  (of lower enrichment)

$a$  = average or desired %  $^{15}\text{N}$  abundance in the final mixture of  $(x + y)$

If the added diluting material is an unlabelled (ordinary) fertilizer, then

$$a_2 = a_o = 0.37\% \text{ } ^{15}\text{N} \text{ abundance} = 0\% \text{ } ^{15}\text{N} \text{ at. excess}$$

$$\text{Equation (1) then becomes} \quad \frac{x(a'_1)}{x + y} = a' \quad (2)$$

where  $a'_1$  = %  $^{15}\text{N}$  at. excess of material of higher  $^{15}\text{N}$  enrichment

$a'$  = %  $^{15}\text{N}$  at. excess desired in the final mixture.

The amounts of  $x$  and  $y$  can be expressed as  $m_1$  and  $m_2$  in mass units (g).

Thus equation (2) can be rewritten as follows:

$$m_1 = \frac{(m_1 + m_2) a'}{a'_1} \quad (3)$$

**EXAMPLES: Making solutions of required  $^{15}\text{N}$  content.**

**Example 1** From a stock of 10.0%  $^{15}\text{N}$  abundance  $(\text{NH}_4)_2\text{SO}_4$  and ordinary  $(\text{NH}_4)_2\text{SO}_4$ , make up a 10-L solution containing 140 g  $(\text{NH}_4)_2\text{SO}_4$  at 1.0%  $^{15}\text{N}$  abundance.

**Calculations:**

$$m_1 + m_2 = 140 \text{ g } (\text{NH}_4)_2\text{SO}_4$$

$$10\% \text{ } ^{15}\text{N} \text{ abundance} = 10.0 - 0.37 = 9.63\% \text{ } ^{15}\text{N} \text{ atom excess} = a',$$

$$1.0\% \text{ } ^{15}\text{N} \text{ abundance desired} = 1.0 - 0.37 = 0.63\% \text{ } ^{15}\text{N} \text{ at. excess} = a'.$$

$$\text{Then, } m_1 = 140 \times \frac{0.63}{9.63} = 9.2 \text{ g } (\text{NH}_4)_2\text{SO}_4, 10\% \text{ } ^{15}\text{N} \text{ abundance.}$$

$$\text{and } m_2 = 140 - 9.2 = 130.8 \text{ g ordinary } (\text{NH}_4)_2\text{SO}_4$$

**Example 2** There are eight plots, each covering an area of  $1 \text{ m}^2$ , to be fertilized at a rate of 100 kg N/ha in the form of urea with 1%  $^{15}\text{N}$  at. excess. The fertilizers available are urea with 10%  $^{15}\text{N}$  abundance and ordinary urea. The fertilizer is to be applied as a solution, each plot receiving 200 ml/ $\text{m}^2$ . To make up the appropriate solution:

**Calculations:**

**Total N requirement:**

$$\text{Total area to receive N fertilizer} = 8 \times 1 \text{ m}^2 = 8 \text{ m}^2$$

$$100 \text{ kg N/ha} = \frac{100 \times 10^3 \text{ g N}}{10^4 \text{ m}^2} = 10 \text{ g N/m}^2$$

$$\text{N content of urea} = 46.7\%$$

$$\text{Amount of urea needed per m}^2 = \frac{100 \times 10}{46.7} = 21.41 \text{ g}$$

Amount of urea needed for total area =  $21.4 \times 8 = 171.2$  g

Total volume of solution needed:

$200 \text{ ml/m}^2 \times 8 \text{ m}^2 = 1,600 \text{ ml}$  or 1.6 L. (However, to allow for spillage, taking solutions for standards, etc., prepare a total of 1.8 L. In this case, the amount of urea required is higher by the following ratio 1.8/1.6)

$$\begin{aligned}\text{the required amount for 1.4 L} &= 1.8/1.6 \times 171.2 \\ &= 192.6 \text{ g}\end{aligned}$$

Dilution calculations:

$$m_1 + m_2 = 193 \text{ g}$$

$$a'_1 = 10 - 0.37 = 9.63\% \text{ }^{15}\text{N at. excess of urea to be diluted}$$

$$a' = 1\% \text{ }^{15}\text{N at. excess desired in final solution}$$

$$m_1 = 193 \times \frac{1.00}{9.63} = 20 \text{ g}$$

$$m_2 = 200 - 20 = 180 \text{ g}$$

The required solution is thus obtained by mixing 20 g of 10%  $^{15}\text{N}$  abundance labelled urea and 180 g of normal urea in 1.8 L of water.

**Example 3** In a field experiment on nitrogen fixation, there is a total of 24 plots. A legume crop is to be grown on 18 of these plots, while the remaining six plots are to be planted to the reference or nonfixing crop. The legume will receive 20 kg N/ha applied as 5%  $^{15}\text{N}$  at. excess  $(\text{NH}_4)_2\text{SO}_4$ , while the reference crop receives 100 kg N/ha of 1%  $^{15}\text{N}$  at. excess ammonium sulfate. The available fertilizer stock consists of  $(\text{NH}_4)_2\text{SO}_4$  with 10%  $^{15}\text{N}$  abundance and unlabelled  $(\text{NH}_4)_2\text{SO}_4$ .

The plot sizes are  $2.0 \text{ m}^2$  for the legume, and  $1.8 \text{ m}^2$  for the reference crop. To prepare these solutions:

**Calculations:**

- (i) **Legume crop:**  
Fertilizer requirement:

$$\text{Area to be fertilized} = 18 \times 2.0 \text{ m}^2 = 36 \text{ m}^2$$

$$\text{Rate of N application} = 20 \text{ kg N/ha} = 2 \text{ g N/m}^2$$

$$\text{Total N required} = 36 \text{ m}^2 \times 2 \text{ g/m}^2 = 72 \text{ g N}$$

N fertilizer to be used is  $(\text{NH}_4)_2\text{SO}_4$ , 21.2% N.

$$\text{Amount of } (\text{NH}_4)_2\text{SO}_4 \text{ required} = \frac{100}{21.2} \times 72 = 340 \text{ g}$$

Preparation of solution:

$$\text{Volume required per plot} = 200 \text{ ml/m}^2 \times 2 \text{ m}^2 = 400 \text{ ml}$$

$$\text{Total solution needed for 18 plots} = 400 \times 18 = 7,200 \text{ ml}$$

To allow for spillage, etc., prepare 7.5 L of solution.

$$\text{Amount of } (\text{NH}_4)_2\text{SO}_4 \text{ required for 7.5 L} = \frac{7.5}{7.2} \times 340 \text{ g}$$

$$= 354 \text{ g}$$

$$m_1 + m_2 = 354 \text{ g}$$

$$a' = 5\% \text{ }^{15}\text{N atom excess}$$

$$a'_1 = 10 - 0.37 = 9.63\% \text{ }^{15}\text{N atom excess.}$$

Then:

$$m_1 = 354 \times \frac{5}{9.63} = 183.8$$

Therefore,

$$m_1 = 183.8 \text{ or approximately } 184 \text{ g } 10\% \text{ }^{15}\text{N abundance } (\text{NH}_4)_2\text{SO}_4 \text{ and,}$$

$$m_2 = 354 - 184 = 170 \text{ g ordinary } (\text{NH}_4)_2\text{SO}_4$$

For this experiment, therefore, 184 g of 10%  $^{15}\text{N}$  abundance  $(\text{NH}_4)_2\text{SO}_4$  and 170 g of ordinary  $(\text{NH}_4)_2\text{SO}_4$  fertilizer should be dissolved in 7.5 L of water, and applied to the legume plots at a rate of 200 ml/m<sup>2</sup>, or 400 ml per plot of 2.0 m<sup>2</sup>.

**(ii) Standard nonfixing crop:**

**Fertilizer requirements:**

$$\text{Area to be fertilized: } 6 \times 1.8 \text{ m}^2 = 10.8 \text{ m}^2$$

$$\text{Rate of N application} = 100 \text{ kg N/ha} = 10 \text{ g N/m}^2$$

$$\text{Total N required} = 10.8 \text{ m}^2 \times 10 \text{ g/m}^2 = 108 \text{ g N}$$

N fertilizer to be used is  $(\text{NH}_4)_2\text{SO}_4$ , 21.2% N

$$\text{Amount of fertilizer needed} = (100/21.2) \times 108 = 509.4 \text{ g } (\text{NH}_4)_2\text{SO}_4$$

**Preparation of Solution:**

$$\text{Volume per plot} = 200 \text{ ml/m}^2 \times 1.8 \text{ m}^2 = 360 \text{ ml}$$

$$\text{Therefore, } 6 \text{ plots} \times 360 \text{ ml/plot} = 2160 \text{ ml}$$

Prepare 2.5 L, to allow for spillage, etc., and also for aliquots of standard to be taken.

$$\text{This makes the required quantity of fertilizer} = (2.5/2.16) \times 509.4 = 590 \text{ g}$$

$$m_1 + m_2 = 590 \text{ g,}$$

$$a' = 1\% \text{ } ^{15}\text{N atom excess, and,}$$

$$a'_1 = 10 - 0.37 = 9.63\% \text{ } ^{15}\text{N atom excess.}$$

Then:

$$m_1 = 590 \times (1/9.63)$$

$$m_1 = (590/9.63) = 61.3 \text{ g } 10\% \text{ } ^{15}\text{N abundance } (\text{NH}_4)_2\text{SO}_4$$

$$m_2 = 590 - 61.3 = 528.7 \text{ g of ordinary } (\text{NH}_4)_2\text{SO}_4$$

Therefore, the 1%  $^{15}\text{N}$  at. excess labelled  $(\text{NH}_4)_2\text{SO}_4$  solution would be prepared by dissolving 61.3 g of 10%  $^{15}\text{N}$  abundance  $(\text{NH}_4)_2\text{SO}_4$  and 528.7 g of ordinary  $(\text{NH}_4)_2\text{SO}_4$  in 2.5 L of water.

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# I. Nitrogen Cycling in Farming Systems

## I.1. The Nitrogen Cycle

The atmosphere above the soil contains 79% nitrogen, but this nitrogen can only be used by plants indirectly through chemical fixation and application of fertilizers or through the less expensive process of biological nitrogen fixation. Nitrogen as a nutrient is a major limiting factor for agricultural production in many parts of the world. The majority of soil nitrogen is present in surface soil where most of the organic matter is located. Organic matter contains an average of 5% nitrogen (w/w); the plow layer of cultivated soils usually contains from 0.02 to 0.4% nitrogen by weight.

Nitrogen is subject to a large number of microbiological, chemical, and physical processes in soil, collectively referred to as the 'nitrogen cycle' (Figure I.1.1). This cycle is dynamic, changing constantly as conditions change to favor one transformation or another. Gains of soil nitrogen occur through biological nitrogen fixation, fertilization, and from the return of ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) in rainwater; losses occur through crop removal, leaching, immobilization, and volatilization. We will briefly discuss the main components of this cycle and its importance to agriculture and the ecosystem.

### I.1.a. Gains or Inputs to the N Cycle

The major pathway by which soil gains nitrogen is dinitrogen fixation by microorganisms, either free-living in the soil, in symbiotic relationships with legumes and certain nonlegumes, or in associative relationships with certain grasses. The magnitude of such inputs varies from less than one kilo to hundreds of kilos of N per hectare per year. Estimation of  $\text{N}_2$  fixation by changes in soil N content and development of a N balance sheet is difficult because of the many facets of the nitrogen cycle.

**Biological nitrogen fixation (BNF)**, the reduction of atmospheric  $\text{N}_2$  to ammonia, is a process carried out not only by rhizobia but by several species of bacteria, algae, and fungi that exist as free-living organisms or in mutually beneficial associations with several species of herbaceous and woody crops. The reduction of  $\text{N}_2$ , catalyzed by the nitrogenase enzyme complex contained in bacteroids within root nodules, proceeds as follows:

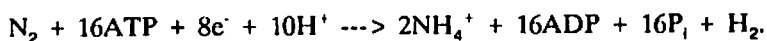
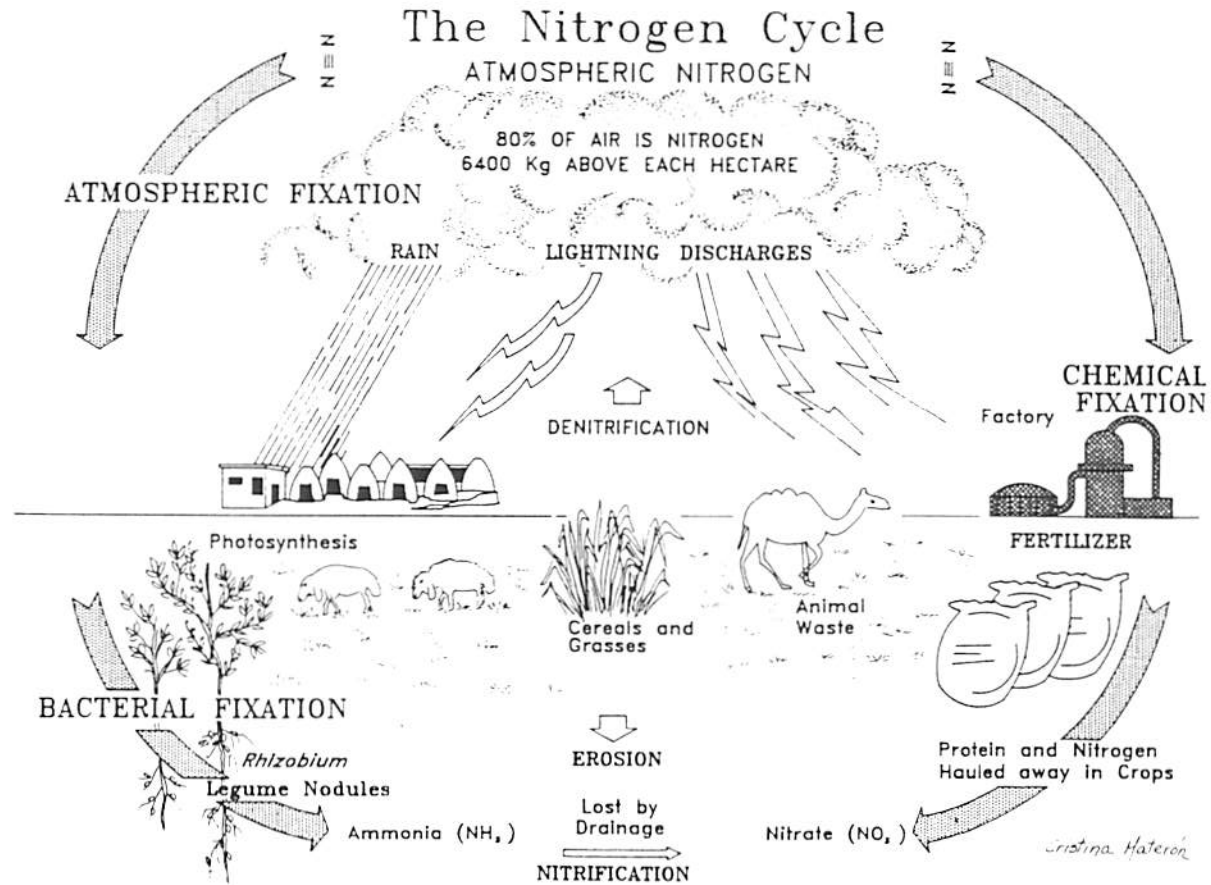


Fig. 1.1. The nitrogen cycle.

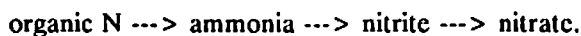


The ammonium ions resulting from fixation are incorporated directly into the amino acids and proteins necessary for plant growth, ultimately for the benefit of humans and animals that consume the high-protein legumes. When residues of legume crops are incorporated into the soil, legume protein N is mineralized to the plant-available form,  $\text{NO}_3^-$ , by the action of soil microorganisms.

Biological nitrogen fixation annually contributes about 140 million metric tons of N on a global basis, of which about 112 million metric tons result from symbiotic fixation and the remainder from associative and free-living nitrogen fixation. Agriculturally important legumes are estimated to fix more than 80 million metric tons of N annually. In comparison, fertilizer N synthesized industrially (using petroleum feedstocks) by the Haber-Bosch process totalled about 50 million metric tons in 1980. The process of  $\text{N}_2$  fixation by agriculturally important legumes provides N equal to that which would require  $21 \times 10^{10} \text{ m}^3$  of natural gas to produce, while about  $6 \times 10^{10} \text{ m}^3$  of natural gas were used for feedstock and process heat to synthesize chemical fertilizer N in 1980.

Precipitation also contributes to systems with an amount of N oxidized from atmospheric dinitrogen by the action of thunderstorms. The amount added is much less than the contributions from nitrogen fixation or fertilizer inputs. Input from precipitation may be measured directly.

**Mineralization of soil nitrogen** is the process by which nitrogen in organic compounds is converted into the inorganic form, i.e., ammonium and nitrate ions. The transformation takes place in the stages:



These transformations are mediated by soilborne microorganisms. The process is affected by those factors influencing microbial activity (temperature, moisture, pH, etc.), as well as by the C/N ratio of decomposing plant residues. Mineralization is nearly always accompanied by conversion of mineral forms of N to organic forms, or immobilization. These two processes are inseparable, one closely depending on the other, and together they result in the formation of organic matter, microbial cells, and plant tissue. The difference between the two processes will be a net effect, net mineralization or net immobilization. The final effect of these processes results in the availability of nitrogen forms for plant production.

Mineralization involves the transformation of organic N to ammonia, nitrite and nitrate by microbial action. Thus when a protein, such as dried blood, is added to a soil, about 80% of the added N is liberated as ammonia and the remainder of the nitrogen is retained in the microbial tissues. As increasing amounts of carbohydrate (e.g., cellulose) in plant material are mixed with the protein, the amount of microbial tissue is increased and the proportion of nitrogen released as ammonia decreases.

When the ratio of carbohydrate to protein reaches 5:1, all the nitrogen is needed by the microorganisms. The C/N ratio is the determining factor for whether net mineralization or net immobilization will take place. If the C/N ratio in soil is high, above 30, immobilization will completely dominate and very small amounts of nitrogen will be available to the plants. If the C/N ratio is below 15, mineralization will dominate and a release of plant available forms of nitrogen will proceed. Incorporating high carbon residues, such as straw, into soil will result in a temporary deficiency of nitrogen for plant growth.

The cyclic nature of these two processes must be kept in mind. When wheat straw, with a C/N ratio of about 25, is added to soil, net immobilization takes place and plant-available nitrogen is 'tied-up'. As the carbon is broken down by microbes and becomes part of the microbial biomass, there is less C available to sustain the increased population of microbes. Part of this microbial population then begins to die, and in turn becomes a source of energy for other microorganisms. As this organic material is broken down, nitrogen is released again as ammonia which rapidly changes to plant-usable  $\text{NH}_4$  and  $\text{NO}_3$ . The small amount of nitrogen in crop residues (usually less than 0.5%) is then eventually released as plant-available N, although the process can take place slowly depending on the total amounts of carbon and nitrogen available. The majority of evidence indicates that 15-25% of the N in legume residues may be available to the first subsequent crop.

**Nitrification** is a component of the mineralization process brought about by the action of soil microorganisms (*Nitrosomonas* and *Nitrobacter* spp.). In this oxidative process, the ammonium ion (in this case from organic decomposition) is transformed first to nitrite and then rapidly to nitrate, through the action of enzymes contained in specific bacteria. Nitrate is the most widely available form of N for plants, both because it moves easily in soil with water, and because its negative charge balances the many positive ions taken up by plant roots.

As a biological process, nitrification is affected by several factors: (1) the amount of ammonium ion available; (2) the amount of oxygen available; (3) moisture availability; (4) temperature; and (5) soil pH. In general, the best conditions for plant growth also are the best conditions for nitrification. Very little  $\text{NH}_4$  will be transformed to  $\text{NO}_3$  in cold or waterlogged soils, or where the pH is below 5 or above 9. Addition of urea, which is rapidly transformed to  $\text{NH}_4$  in soil, will under good conditions of nitrification result in complete transformation to nitrate in six to eight weeks (Figure I.1.2). Nitrification does not take place in very acid soils. Nitrification only proceeds rapidly in warm soils, and proceeds slowly when the soil temperature is below 4–5°C.

Mineral nitrogen in the soil is present either as ammonium or nitrate ions. The nitrates are all dissolved in the soil solution, unless the soil dries out, while much of the ammonium is held on the exchange complex. The total quantity of mineral

nitrogen in the soil is the difference between the rate at which it is being produced from the soils' store of organic matter by the soil microbial population and the rate or its removal by leaching, by growing crops, and by other members of the soil microbial population. The proportion of nitrate to ammonium depends on the rate of nitrification, the uptake of nitrate by the plant and the loss of nitrates by leaching. In general, all field soils contain microorganisms that will oxidize ammonium to nitrate.

### The Nitrogen Cycle

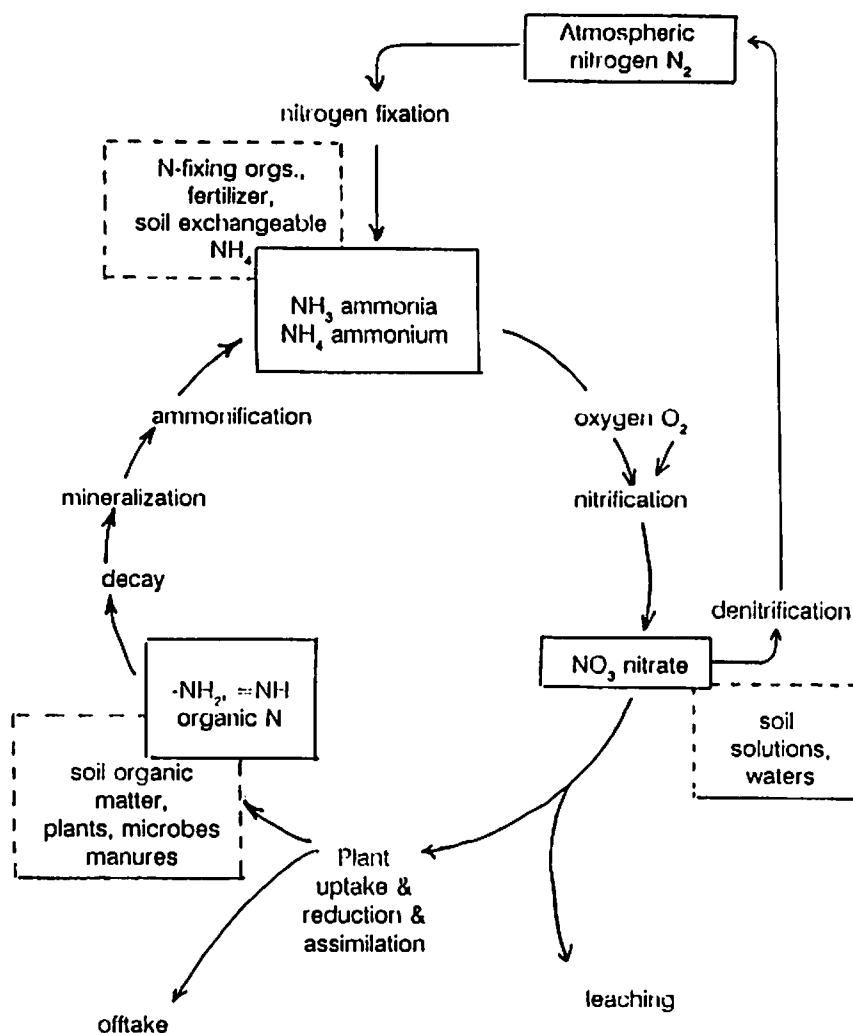


Fig. I.1.2. Schematic diagram of the N cycle.

### **I.1.b. Losses from Soil-Plant Systems**

Loss of N from soil occurs mainly through crop removal, leaching, volatilization, and denitrification. Accurate measurements of losses due to leaching and volatilization are difficult, and to describe the methodologies is beyond the scope of this manual. Often, these losses are not included when estimating rates of N turnover in soils and it is recognized that the estimate for total N losses will be underestimated.

**Denitrification** is important in agriculture as a process in which inorganic N is lost from soil, thus diminishing the amount available to crop plants. Denitrification involves reduction of  $\text{NO}_3^-$  to  $\text{N}_2$  or  $\text{N}_2\text{O}$  gas which requires anaerobic or waterlogged conditions. The process is mediated by the action of anaerobic bacteria (*Nitrosomonas* spp.) that are able to use nitrate instead of oxygen as a hydrogen acceptor. Some of these bacteria may only be able to carry out one stage of the reduction process, from nitrate to nitrogen gas. Denitrification occurs in soils above pH 5.0, under conditions of poor aeration in the presence of an active microbial population, and can therefore be important during wet periods in warm soils well supplied with decomposable organic matter.

**Leaching** is dependent primarily on rainfall and soil permeability. In semiarid areas, nitrate leaches only into the subsoil where it may accumulate. However, in more humid areas where precipitation exceeds evapotranspiration, significant leaching may occur. Lysimeter experiments with growing crops can give more detailed information about this loss, because the amount of nitrogen lost in the drainage water can be measured.

**Volatilization** may cause nitrogen loss to the atmosphere as  $\text{NH}_3$  gas, whenever  $\text{NH}_4^+$  is present at the soil surface. This process is accelerated at high pH (above 7.0) and warm temperature. Ammonia or ammonium-forming fertilizers (such as urea, which is broken down into ammonium by bacteria utilizing urease) may be lost to the atmosphere by volatilization when applied to the soil surface under these conditions. Common situations for loss through volatilization are when ammonium sulfate is added to the surface of calcareous soils, or when large dressings of urea are added to the surface of soils that are not strongly acid, particularly during periods of light showers of rain interspersed with strong drying conditions. These losses can be avoided if the fertilizer is incorporated into the surface soil or placed sufficiently far below the soil surface for any ammonia that is released by volatilization to be absorbed by the soil before it can reach the atmosphere.

## **I.2. Role of Legumes in Systems**

One of the most important tasks for soil scientists at present is to quantify the movement of nitrogen through the various pathways and so optimize use of legumes in systems and N fertilizers, while lessening the impact on groundwater and other environments. An understanding of the processes involved will lead to management practices which minimize losses and improve the utilization of nitrogen in farming systems.

The residual value of leguminous crops is often used as a justification for inclusion of legumes in farming systems. A leguminous crop, particularly forage and pasture legumes (e.g., vetches, medics, and clovers) may increase the level of available nitrogen in the soil, and the following crop may respond to this nitrogen. The residual effect depends on efficiency of  $N_2$  fixation, the legume species, water supply and management practices.

### **I.2.a. Factors Introduced by Legumes in Farming Systems**

In legume-cereal rotations, the amount of fertilizer N that can be replaced by legume N depends on: (1) the quantity of legume residues returned to the soil; (2) the content of symbiotically fixed N in the residues; and (3) the availability of the legume residue N to the succeeding cereal. The application of  $^{15}N$  methodologies (Section H) allows development of N budgets showing how the  $N_2$  fixation capability of the crop, N partitioning among plant organs, and crop management influence the return of N to the soil and its subsequent recovery.

Only part of the symbiotically fixed N in a hay or pasture legume is typically returned to the soil for use by a succeeding crop. This is because a portion of the fixed N is removed from the land when the legume is harvested or grazed, with the balance remaining in unharvested roots and crowns. Likewise, the harvest of leguminous grain crops usually involves removal of all above ground plant matter (seed and straw) from the field, leaving only the root N for return to the system.

Legumes in general, however, appear to confer benefits greater than that possible from N contribution or conservation. Even where fertilizer N is nonlimiting the maximum yield obtainable by a cereal following a legume almost always exceeds that following a cereal. No general explanation for this effect can be given, but some possible (and demonstrated) reasons include: (1) the legume's effectiveness in breaking cereal disease and pest cycles; (2) the reduction of phytotoxic and allelopathic problems; (3) improvement of soil structure; (4) differential water use patterns; and (5) favorable positioning of N and other necessary nutrients in the soil profile.

The usual agronomic approach to assessing the N-supplying capability of a legume preceding a cereal is to compare the yield of the nonlegume following the legume with that obtained from different rates of fertilizer N applied to the cereal, versus that in continuous cereal or in a fallow-cereal rotation. This methodology will likely overestimate the fixed N contribution to the system, however, due to the following factors: (a) cereal response to legume N cannot be separated into response to fixed N in the legume and turnover of soil-derived N; (b) legume N is not the only factor affecting cereal yield response in a legume-cereal sequence; (c) the methodology assumes that applied fertilizer N and the residual legume N are equally available to the cereal; and (d) the N-supplying capacity of the soil often is altered by growth of a legume and incorporation of legume residues. Experiments intended to determine the quantities of fixed N available to a following cereal must take these factors into account.

### **I.2.b. Field Trial for Effect on Following Crop**

The role of food and forage legumes in rotation with wheat through contribution of BNF may be investigated using a two-course rotation trial. Effect on following cereal of legume treatments in comparison with continuous wheat and fallow-wheat will allow evaluation of legume contribution in a farming systems approach. Both phases of the rotation are grown each year. Phase I includes legume treatments, in which quantities of  $N_2$  fixed are measured using  $^{15}N$  methodology. Phase II is planted with wheat or barley, in which varying levels of N fertilization allow calibration of N contribution from phase I treatments against yield and N-uptake from added N fertilizer. Design is a randomized complete block with three replications in phase I, and a split-plot with phase I treatments as main plots in phase II.

From phase I treatments, estimations of N contribution from soil and fixation in the legume crop can be obtained, from which potential N contribution to soil is calculated. Total aboveground biomass is harvested at maturity and removed from the field, with measurements of seed and straw N contents and yield. Whole plants are harvested from the center of microplots at physiological maturity, oven dried and finely ground prior to analysis of  $^{15}N$ -atom excess.  $N_2$  fixation is calculated using the A-value methodology (Section H).

With phase II treatments, fertilizer use efficiency (FUE), as well as amount of fixed N from the previous crop treatment (soil  $A_N$  value), can be measured using  $^{15}N$  enrichment data. In addition, contributions from phase I treatments can be calibrated against yield and N uptake from N fertilizer added to following cereal crop (fertilizer replacement value).



## Methods

Soil mineral N (extractable  $\text{NH}_4^+$  and  $\text{NO}_3^-$ ) should be measured in the surface 40 cm in the three replicates of each treatment plot in both phases before planting the trial each year (84 samples); five or six samples from each plot should be bulked and N analysis conducted on a subsample of the bulked samples. This analysis will indicate soil N differences due to the various treatments. (Note: Year 1 will require sampling only of experimental area as a whole; year 2 will require sampling in only phase II plots; year 3 will require sampling in all treatment main plots.)

## Suggested Phase I Treatments

1. winter-planted chickpea, inoculated
2. winter-planted chickpea, uninoculated
3. spring-planted chickpea, inoculated
4. spring-planted chickpea, uninoculated
5. lentil
6. lentil with Promet seed treatment for *Sitona* control
7. dry peas
8. dry peas with promet seed treatment
9. faba bean
10. *Vicia sativa* ssp. *dasycarpa*
11. *Vicia narbonensis*
12. *Lathyrus sativus*
13. wheat, 40 kg N/ha basal application
14. fallow, weed-free

Plot size is  $20 \times 2.1$  m, with 30-cm row spacing in all legume treatments. Microplots of  $1.0 \text{ m}^2$  (containing 10 kg N/ha using 10%  $^{15}\text{N}$ -enriched ammonium sulfate) are placed in the legume treatments of phase I (location changed each season within the area of the 20 N treatment of phase II). Each season,  $5 \times 1.05$  m strips of reference crop (non-nodulating chickpea and barley) each containing a  $1 \text{ m}^2$  microplot fertilized with 100 kg N/ha (using 1%  $^{15}\text{N}$ -enriched ammonium sulfate) will be placed between phases in each replication both for winter and spring crops (four reference crop plots in each replication). Microplot location within the reference crop plots will be changed each year to avoid previously enriched areas and maintain consistent enrichment levels. Three replications are used for yield and  $^{15}\text{N}$  measurements.

## Microplot Fertilization

Twelve of the 14 treatments will contain microplots for measurement of  $\text{N}_2$  fixation, for a total of 36  $1\text{-m}^2$  microplots in the legume treatments of phase I (30 winter-sown, 6 spring-sown). Each of these microplots will receive 4.75 g of 10%  $^{15}\text{N}$ -enriched

ammonium sulfate dissolved in 300 ml distilled water, applied evenly over the microplot area after planting but before emergence. The easiest method of application is to dissolve the total amount of  $^{15}\text{N}$ -enriched fertilizer in water, and then dilute a fixed amount of this stock solution in the distribution bottle for each microplot (e.g., for the 30 winter-planted microplots, dissolve 190 g 10%-enriched ammonium sulfate in 800 ml distilled water. For each fixing crop microplot, pipette 20 ml of this enriched stock into the distribution bottle, and add 280 ml distilled water). No fertilizer will be applied to the areas outside of microplots in the legume treatments of phase I.

Two microplots of each of the two reference crops (one set sown in winter and one in spring) will be planted each year in the 1.05-m strip between phases in each replication, for a total of 12 reference microplots. These reference microplots will receive 100 kg N/ha of 1%  $^{15}\text{N}$ -enriched ammonium sulfate after planting but before emergence, usually at the same time as fixing crop microplots. Prepare a stock of unenriched ammonium sulfate by dissolving 257 g ammonium sulfate in 300 ml water (enough for six microplots). Add 20 ml of the  $^{15}\text{N}$ -enriched stock (from above) and 50 ml of the unenriched stock solution to the distribution bottle; add 230 ml distilled water and mix thoroughly before even distribution over microplot area.

Areas outside of the reference crop microplots should be fertilized at the rate of 100 kg N/ha at planting time, with care taken to prevent any unenriched fertilizer from entering microplot areas. This fertilization is not critical, as no reference crop will be harvested outside of the microplot area. It is, however, essential to maintain even fertility in the reference crop area with rotation of microplot location.

### **Suggested Phase II Treatments**

1. main treatment plots from phase I are divided into four subplots of  $5 \times 2.8$  m, on which wheat is grown with four levels of N application:
  - 20 kg N/ha added
  - 40 kg N
  - 60 kg N
  - 80 kg N
2. subplots contain  $1.0 \text{ m}^2$  microplots fertilized with  $^{15}\text{N}$ -enriched ammonium sulfate (enrichment at 5% for 20 kg/ha, 2.5% for 40 kg/ha, 1.67% for 60 kg/ha and 1.25% for 80 kg/ha). Placement of microplots must be carefully planned (especially in the 20 kg N subplots, where microplots from both phases are to be placed) to avoid the area which was under  $^{15}\text{N}$  microplots in previous seasons.

Wheat is planted using local practices. Care should be taken in land preparation not to move soil from one treatment plot to another. Fertilization of plots outside of microplot areas is done before or at planting using granular N fertilizer. Microplots

are fertilized after planting but before emergence using  $^{15}\text{N}$ -enriched N fertilizer dissolved in water. Placement of microplots in phase II plots should be arranged so that microplots used in phase I are avoided. New microplots must not overlap the areas of old microplots if consistent soil enrichment is to be maintained.

### Fertilization Outside Microplots

After broadcast P fertilization and land preparation, subplots and locations of microplots should be staked out. Depending on planting methodology, N fertilizer can be broadcast preplant (hand planted) or after planting (tractor planted). Preferably, some incorporation of broadcast N fertilizer will occur to prevent volatilization. It is important that, before broadcast of fertilizer, a plastic sheet  $1\text{ m}^2$  is used to cover the microplot so that no granular fertilizer is applied to the microplot area. It is suggested that the granular fertilizer be broadcast by hand evenly over the subplot area (minus microplot). The best method is to broadcast at planting time while furrows are open; in covering furrows the fertilizer is mixed with surface soil.

Commonly, ammonium sulfate (21% N) is used to fertilize plots outside microplots, but other forms of inorganic N (e.g., ammonium nitrate, urea) may be used. The quantities of ammonium sulfate needed for N treatments in the area outside microplots (subplots  $5 \times 2.8\text{ m}$  each containing a  $1\text{ m}^2$  microplot =  $13\text{ m}^2$ ) are as follows:

| Treatment  | g $(\text{NH}_4)_2\text{SO}_4$ per $13\text{-m}^2$ plot |             |
|------------|---------------------------------------------------------|-------------|
| 20 kg N/ha | 124                                                     |             |
| 40 kg N/ha | 248                                                     |             |
| 60 kg N/ha | 371                                                     |             |
| 80 kg N/ha | 495                                                     | TOTAL 52 kg |

There are 42 subplots for each subplot treatment (total of 168 subplots). The total quantity of unenriched ammonium sulfate needed, including that used to dilute the  $^{15}\text{N}$  enriched fertilizer in microplots, is about 56 kg.

### Microplot Fertilization

Microplots containing  $^{15}\text{N}$ -enriched fertilizer are located in each subplot. As each plot is divided into four fertilization level subplots, there will be a total of 168 microplots of  $1\text{ m}^2$  each.

A total of 808 g of 10%  $^{15}\text{N}$ -enriched ammonium sulfate  $[(\text{NH}_4)_2\text{SO}_4]$  is needed for the phase II portion of the trial (798 g plus 10 g for enrichment measurement and measurement error). A total of 3.8 kg of unenriched  $(\text{NH}_4)_2\text{SO}_4$  will be required to

dilute the enriched  $(\text{NH}_4)_2\text{SO}_4$  to the desired levels for the four fertilizer treatments. Quantities required per microplot are given in Table I.2.1:

**Table I.2.1. Fertilizer composition of treatment microplots.**

| Fertilizer treatment | In microplot | $(\text{NH}_4)_2\text{SO}_4$ , g<br>10% enriched | Unenriched |
|----------------------|--------------|--------------------------------------------------|------------|
| 20 kg/ha             | 9.5          | 4.75                                             | 4.75       |
| 40 kg/ha             | 19.0         | 4.75                                             | 14.25      |
| 60 kg/ha             | 28.6         | 4.75                                             | 23.85      |
| 80 kg/ha             | 38.1         | 4.75                                             | 33.35      |

The amount of 10%  $^{15}\text{N}$ -enriched fertilizer in each treatment microplot is the same, although the total amount of fertilizer is different. The reason for this is that we are attempting to achieve an economical enrichment in the crop, i.e., one which is measurable using a small amount of  $^{15}\text{N}$ . The "A-value" methodology will be used to calculate the fertilizer use efficiency (FUE) and N-supplying capability of the soil with the  $^{15}\text{N}$  enrichment analyses of plant samples at harvest.

Microplots should be fertilized 5–15 days after planting, during a dry period but before plant emergence (see Section H). Five stock solutions of  $(\text{NH}_4)_2\text{SO}_4$  in distilled or deionized water should be prepared prior at the time of fertilization of microplots (Table I.2.2). Note that slightly more stock solution is made than will be used; this is to ensure that you have enough for all treatments and to account for spillage or loss. Make sure that all added fertilizer in stock solutions is dissolved before use. Precise quantities of solution from these stock solutions will be added (Table I.2.3) to each microplot to give replicated levels of enriched  $^{15}\text{N}$  fertilizer.

**Table I.2.2. Composition of stock solutions for fertilization of microplots.**

| Stock solution | $(\text{NH}_4)_2\text{SO}_4$ , g |            | Quantity of distilled water to be dissolved in |
|----------------|----------------------------------|------------|------------------------------------------------|
|                | 10% enriched                     | Unenriched |                                                |
| 1              | 808                              | 0          | 1700 ml                                        |
| 2              | 0                                | 238        | 2000 ml                                        |
| 3              | 0                                | 712        | 2000 ml                                        |
| 4              | 0                                | 1193       | 2000 ml                                        |
| 5              | 0                                | 1668       | 2500 ml                                        |

**Table I.2.3. Quantities of stock solutions necessary for application to one microplot in the different fertilizer treatments.**

| Fertilizer treatment | Stock solution (ml) |    |    |    |    |
|----------------------|---------------------|----|----|----|----|
|                      | 1                   | 2  | 3  | 4  | 5  |
| 20 kg N/ha           | 10                  | 40 | 0  | 0  | 0  |
| 40 kg N/ha           | 10                  | 0  | 40 | 0  | 0  |
| 60 kg N/ha           | 10                  | 0  | 0  | 40 | 0  |
| 80 kg N/ha           | 10                  | 0  | 0  | 0  | 50 |

An applicator bottle with holes in the lid for even solution distribution is suggested for proper application of  $^{15}\text{N}$  solutions. The minimum volume necessary for a  $1\text{ m}^2$  area is about 300 ml. After addition of the exact amounts of stock solutions necessary for each microplot treatment (Table I.2.3), the bottle should be filled with water to a constant volume (300 ml). The contents should then be mixed thoroughly by swirling, and then applied evenly over the microplot area using back-and-forth and crossing motions. Care must be taken that all surfaces of the microplot receive equal amounts of solution.

A 10-ml pipette can be used for precise addition of stock solution 1, which contains the  $^{15}\text{N}$ . A 50-ml graduated cylinder or volumetric flask should be used to accurately measure proper amounts of the other stocks. Care should be taken to mix stock solutions thoroughly during use, and to mix the contents of the distribution bottle well before spreading on the microplot. After completing half the microplots in the experiment, 5 ml of stock solution 1 should be transferred to a clean glass vial, to be analyzed later for  $^{15}\text{N}$  concentration.

Calculations for A-value and  $\text{N}_2$  fixation from enrichment of crops grown in microplots can be made as given in Section H.

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