METHODS IN LEGUME-RHIZOBIUM TECHNOLOGY

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FOREWORD

There is no doubt that in the near future the emerging biotechnology based on genetic engineering and somatic cell fusion will contribute significantly to solving agricultural problems. Presently, however, so much of the available technology, i.e., inoculum technology, is not being fully enough utilized in agriculture. It would be prudent to devote major efforts to their adoption. Serious obstacles to adoption of modern technologies, especially in developing countries, is the shortage of trained personnel. It is, therefore, essential for all development support projects to include a training component.

This book is the culmination of several years of experience in training of scientists and technicians from developing countries. The six-week Training Course, for which this book is intended, was developed at NifTAL and, in the early years, taught there. Subsequently, the course was taken to the field and offered at host institutions in Africa, Asia and Latin America. Somasegaran and Hoben have done a commendable job of drawing from their experience with these courses. They have compiled an “All You Ever Wanted To Know About …” style book that is not only valuable to developing country scientists, but also useful for technicians and graduate students starting work with the legume/Rhizobium symbiosis.

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INTRODUCTION

The symbiosis between the root-nodule bacteria of the genus Rhizobium and legumes results in the fixation of atmospheric nitrogen in root-nodules. This symbiotic relationship is of special significance to legume husbandry as seed inoculation with effective strains of Rhizobium can meet the nitrogen requirements of the legume to achieve increased yields. Obviously, such a phenomenon is of world-wide interest because it implies lesser dependence on expensive petroleum based nitrogen fertilizers for legumes.

In all regions of the world where food consumption exceeds production or where nitrogenous fertilizer has to be imported, leguminous crops have a special relevance. Self-sufficiency for nitrogen supply and the high protein and calorific values of food, forage and feed legumes make them increasingly attractive. Greater use of legumes can have a significant beneficial impact in tropical countries where population increase and food production are most out of balance, and where the purchasing power for imported fertilizers is least adequate.

The University of Hawaii NifTAL Project was funded by the United States Agency for International Development to promote greater use of symbiotic nitrogen fixation through Legume-Rhizobium Technology. An essential component in

NifTAL's overall objective was specialized training in Legume-*Rhizobium* Technology. This strategy would provide the means of transferring the techniques in Legume-*Rhizobium* Technology for the implementation of viable research and development programs in nitrogen fixation in tropical countries.

Training was initiated in 1976 when Professor J.M. Vincent prepared the first course outline for NifTAL. Since then, the authors have conducted similar training courses in Hawaii, Kenya, Malaysia, Mexico, Thailand and India. The valuable experiences gained in these intensive six-week training courses led the authors to identify and develop the key research and development activities essential for Legume-*Rhizobium* Technology.

PREFACE

It was in 1970 that Professor J.M. Vincent published his excellent book entitled “A Manual for Practical Study of Root-Nodule Bacteria.” Unfortunately this book is out of print at the present time.

The motivation for this book of methods grew out of the ever-increasing role of the Legume-*Rhizobium* symbiosis in agricultural production in tropical countries where the benefits of this unique symbiosis can only be realized through correct practices in Legume-*Rhizobium* Technology.

This book is designed for the practicing technologist to provide competent technical support to research and development activities relevant to Legume-*Rhizobium* Technology. Teachers and students will also find this volume useful in addressing the applied aspects of the Legume-*Rhizobium* symbiosis especially when exercises are supported by well prepared lectures.

There are four sections to this book and they related the sequence of activities which should be followed in *Rhizobium* research. Each exercise is structured to include all the steps required to accomplish the particular experiment. Certain activities in the exercise require a knowledge or a source of information and this is given in

the Appendix. At the end of each section are recommended journal articles and textbooks for more background on principles or greater detail on methods. In putting together this book, we have indicated to the user by cross-reference that the various sections support each other. For example, the serological techniques in Section C are not meant only for strain identification in nodules but also for checking strain contamination in inoculant production and quality control.

It is hoped that this volume will serve as an instrument of self-instruction since the skills can be acquired by careful practice of techniques. Satisfactory completion of the exercises should impart to the user a good working knowledge and competence in Legume-Rhizobium Technology. The exercises in this volume were tested successfully in all the NifTAL training courses and intern training programs and we hope that this volume will be useful in organizing similar courses by other institutions.

The authors would appreciate comments and suggestions for effecting further revisions to improve this volume.

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Heinz J. Hoben

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In the final analysis, the production of this volume was the result of teamwork and support given to us by several NifTAL Project staff members. We express our sincere appreciation to all these helpful people. They are Ms. Princess Ferguson who handled all logistics pertaining to publication besides editing; Ms. Judith Dozier for the painstaking checking, proofreading and editing of the typed manuscripts; Mr. Keith Avery who patiently handled much of the graphics and artwork; and Ms. Karean Zukeran and Ms. Mary Rohner for the administrative and secretarial support.

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SECTION A

GENERAL MICROBIOLOGY RHIZOBIUM INTRODUCTION

The bacteria of the genus Rhizobium (family Rhizobiaceae) are a genetically diverse and physiologically heterogeneous group of microorganisms that are nevertheless classified together by virtue of their ability to nodulate groups of plants of the family Leguminosae. This classification scheme is usually referred to as “cross-inoculatin” grouping. A cross-inoculation group is a group of legumes in which one species of Rhizobium nodulates all the legumes within that group. In the old system, species of Rhizobium fall into two groups based on their growth characteristics.

Group I

Rhizobium leguminosarum – nodulates peas (pisum spp.), vetch (Vicia spp.), lentils, (Lens culinaris).

Rhizobium phaseoli - nodulates beans (Phaseolus Vulgaris and the scarlet runner bean) (Phaseolus coccineus).

Rhizobium trifolii - nodulates the clovers, e.g.,

	Trifolium subterraneum, T. Semipilosum, T. repens, and Other Trifolium spp.
Rhizobium meliloti	- nodulates alfalfa (Medicago sativa) and other Medicago spp., Melilotus spp., fenugreek (Trigonella).

Group II

Rhizobium lupini	- nodulates lupins (Lupinus Spp.) and serradella (Ornithopus spp.).
Rhizobium japonicum	- nodulates soybean (Glycine max).
Rhizobium spp.	– nodulates members of the “cowpea miscellany” group of legumes, e.g., Vigna spp., peanut, Desmodium spp., Macroptilium spp., Lablab sp., Lima bean, Stylosanthes spp., etc.

In Group I are the fast-growing acid producers which develop pronounced turbidity in liquid media within 2-3 days and have a mean doubling time of 2-4 hours. The cells are rod-shaped to pleomorphic, 0.5 to 0.9 microns in diameter and 1.2 to 3.0 microns long, and are motile by 2-6 peritrichous flagella. They can grow on a wide range of carbohydrates, but usually grow best on glucose, mannitol, or sucrose. Rhizobia of this group are generally infective on temperate legumes.

In group II are the slow-growing, alkali producing rhizobia. They require 3-5 days to produce moderate turbidity in liquid media and have a mean doubling time of 6-7 hours. Most strains in this group grow best with pentoses as their carbon source. The cells are predominantly rod-shaped, and motile by a single polar or subpolar flagellum. This group nodulates tropical legume species.

Rhizobia are characteristically Gram-negative and do not form endospores. Uneven Gram-staining is frequently encountered with rhizobia depending on the age of the culture. Cells from a young culture and nodule bacteroids usually show even Gram-staining while older cells and longer cells show unstained areas along the cell giving a banded appearance. These unstained areas have been identified to be large granules of polymeric-hydroxybutyric

acid (PHBA). These granules are refractile under phase contrast microscopy.

Though the host-dependent cross-inoculation group system of classifying rhizobia has been subjected to much criticism, because it is not a taxonomic one, it is the best practical system currently available. Classification of rhizobia is becoming increasingly complex because of new findings, e.g. some soybeans are now known to be nodulated by a distinct group of fast-growing acid-producing rhizobia. Thus, a new system has been formulated to classify rhizobia. This new system recognizes three genera for the Rhizobiaceae. In this new system Genus I and Genus II include the rhizobia while Genus III is for the agrobacteria. All fast-growing acid-producing rhizobia now fall under the new genus Rhizobium (Genus I) and all slow-growing alkali-producing rhizobia under the new genus Bradyrhizobium (Genus II). Also, under this system, R. trifolii, R. phaseoli, and R. leguminosarum are combined as one species, designated as Rhizobium leguminosarum, comprising three biovars (trifolii, phaseoli, and viceae). R. meliloti remains as before and R. loti has been assigned to the fast-growing Lotus rhizobia. Genetically related to R. loti are rhizobia from Lotus corniculatus, Lotus tenuis, Cicer arietinum, Leucaena leucocephala, Sophora microphylla etc. The soybean rhizobia are now in two genera, i.e. R. japonicum (fast-growing and acid-producing) and

Bradyrhizobium japonicum (slow-growing and alkali-producing). Rhizobia from Vigna, Arachis, Desmodium, Macroptilium, Stylosanthes etc. are still unclassified, but grouped as Bradyrhizobia spp. The non-legume Parasponia (called Trema previously) is also nodulated by Bradyrhizobium sp. Besides Leucaena, whose rhizobia are now R. loti, there are other legumes (Sesbania, Neptunia, Calliandra, Acacia) which are nodulated by fast-growing acid-producing rhizobia and the taxonomic status of these organisms may be resolved in the future.

As predominantly aerobic chemoorganotrophs, rhizobia are relatively easy to culture. They grow well in the presence of oxygen and utilize relatively simple carbohydrates and amino compounds. With the exception of a few strains, they have not been found to fix nitrogen away from their host legume. Some strains of Rhizobium require vitamins for growth. Optimal growth for most strains occurs in a temperature range of 25-30^{o}C, and at a pH of 6-7. Despite their usual aerobic metabolism, many strains are able to grow well at oxygen tensions less than 0.01 atmosphere (microaerophilic).

Rhizobia are somewhat unique among soil microorganisms I their ability to form nitrogen-fixing symbioses with legumes. To enjoy the benefits of this partnership, however, the rhizobia must not only exhibit saprophytic

competence among other soil microorganisms, but also out-compete other rhizobia for infection sites on legume roots. Potential for physiological versatility is therefore an important trait contributing to their adaptation to the competitive and complex soil environment.

EXERCISE 1

TO COLLECT NODULES AND ISOLATE RHIZOBIUM

The purpose of this exercise is to become familiar with legumes in the field, examine their nodules, isolate rhizobia from nodules and preserve the isolates.

The subfamilies in the *Leguminosae* will be discussed and identifications will be made with the help of a botanical key. Nodules will be sectioned and examined. Simple stains of nodule smears will be examined under the microscope. Rhizobia will be isolated from nodules and grown on presumptive test media. The isolates will be authenticated on their original host plants and then preserved on ceramic beads.

Key steps/objectives

1) Identify legumes in the field, collect nodulated specimens and preserve nodules
2) Examine nodules and bacteroids under the microscope
3) Surface sterilize nodules and isolate rhizobia on differential media
4) Perform Gram stain and reisolate on differential media
5) Store isolates on agar slants
6) Surface sterilize and pregerminate seeds for authentication

7) Plant and inoculate seedlings for authentication
8) Examine plants periodically for nodulation
9) Terminate experiment, examine nodules and reisolate
10) Prepare broth culture of authenticated isolate for desiccation on beads
11) Prepare bead storage vials
12) Impregnate sterilized beads with broth culture of rhizobia
13) Regrow rhizobia stored on beads

(a) Recognizing legumes and identifying them in the field

(Key step 1)

Become familiar with the general taxonomic characters of the *Leguminosae*. Study the different flower types of the three subfamilies: *Caesalpinoideae*, *Mimosoideae* and *Papilionoideae* (Appendix 1).

Note the main similarities among all legumes in their compound leaves and the seed placentation in pods as shown in Appendix 1, Figure A.4 and A.5. However, in many *Acacia* species (e.g., *Acacia auriculaeformis*, *Acacia mangium*, *Acacia koa*) the compound leaves are only formed and seen in seedlings. The compound leaves are replaced by phyllodes as the plants mature (Figure A.5). Compound leaves are also characteristic of numerous non-leguminous families such as: *Bignoniaceae* (e.g.,

Jacaranda, *Spathodea*); *Caprifoliaceae* (e.g., *Sambucas mexicana* var. *bipinnata)*; *Solanaceae* (e.g., *Lycopersicon*, *Solanum tuberosum*), *Passifloraceae* (e.g. *Passiflora* spp.). Familiarize yourself with the basic characteristics of each subfamily as outlined in Appendix 1. Learn to identify legumes in the field and become familiar with the appearance of the most common agricultural legumes in your area.

It is not essential to identify the less common legumes. Many aspects of classification within the *Leguminosae* are in dispute even amongst plant taxonomists. The course followed by many collectors is to recover a good plant specimen (including flowers and fruits), dry and press it, and forward it to a reliable herbarium (Royal Botanical Garden, Kew, Richmond, Surrey TW 3 AE, England) for precise identification.

(b) Recovering nodules in the field

(Key Step 1)

Identify plants of several legume species in the field and select one representative of each for sampling. With a spade, describe a circle with a radius of approximately 15 cm around the plant and cut out this section to a depth of at least 20 cm. Still using the spade, slowly lift out the clump. Carefully remove the soil from the root material with your hands. Avoid detaching secondary roots from the plant as

nodules may be found on the lateral roots as well as the tap root. Carefully place the whole plant into a plastic bag. If the legume has seed, collect the seeds and store them in the refrigerator for the authentication test.

In the laboratory, place a sieve of an appropriate size and mesh under each root sample to catch nodules that may become detached from the root. Carefully wash the roots under a gentle stream of water from a tap or a hose.

The distribution of the nodules on the root system is dependent on the legume species and rhizobial strain as well as soil structure and composition. Examples of nodule types and distribution on some species are illustrated in Appendix 1.

(c) Preserving nodules

(Key step 1)

Fresh nodules may be stored in the refrigerator overnight. Do not freeze nodules as ice crystals may rupture and kill the bacteroids. Frozen nodules may, however, be used for serological typing.

For long term storage, desiccation in glass vials is recommended. A preservation vial is shown in Appendix 2,

Figure A.8.

(d) Examining nodules and bacteroids

(Key step 2)

Note the shape and size of the nodules recovered from the collected plants. Nodule size and shape vary with the rhizobia and host plant species. Large round nodules may be found on cowpea and soybean plants. *Leucaena* and *Acacia* are among legumes which do not have round nodules. See Appendix 1, Figure A.6 for description of nodule shape.

Cut thin sections of nodules with a razor blade and float them on a drop of water on a microscope slide; use a cover glass and examine under low power (10x) and high power (40x) objectives.

An active N-fixing nodule contains a protein called leghaemoglobin. Its presence in the nodule can be noted by the characteristic pink, red, or brown coloration. Active nodules may also be black. Black nodules are not very common. They have been reported on *Lablab purpureus*, *Dolichos biflorus*, and *Vigna unguiculata* when inoculated with some strains of rhizobia.

Senescent nodules are usually grayish green. When nodules on

the soil surface are exposed to sunlight, they may develop a green exterior. This green color is due to chlorophyll development on the cortical region of the nodule. Most ineffective rhizobia cause nodules with white interiors that lack leghaemoglobin.

Gently rub the cut surface of a nodule on a clean microscope slide to make a smear. Allow the smear to air dry and then pass the slide through a flame. Cool the slide and stain the smear with dilute carbol fuchsin for 10-20 seconds. Wash in water, blot off excess moisture, and air dry. Examine under the oil immersion objective. Note the difference in morphology between the "bacteroids" in this smear and bacteria of the same rhizobial species grown in pure culture. Note the size and shape of the bacteroids compared to the rod forms found in pure culture (Figure 3.1).

(e) Isolating rhizobia from a nodule

(Key step 3)

Wash roots thoroughly to remove soil. Collect about 10 nodules from each plant. Sever the nodule from the root by cutting the root about 0.5 cm on each side of the nodule. When moving the nodule, use forceps on the root appendages to reduce the risk of damaging the nodule.

Immerse intact, undamaged nodules for 5-10 seconds in 95% ethanol or isopropanol (to break the surface tension and to remove air bubbles from the tissue); transfer to a 2.5-3% (v/v) solution of sodium hypochlorite, and soak for 2-4 min. Rinse in five changes of sterile water using sterile forceps for transferring. Forceps may be sterilized quickly by dipping in alcohol and flaming. Utilize sterile glass or plastic petri dishes as containers for the alcohol, sodium hypochlorite and water.

Alternatively, nodules may be placed into an Erlenmeyer flask (125 ml). The sterilizing and rinsing fluids may be changed as required, leaving the nodule in the flask each time.

An acidified mercuric chloride solution (0.1% w/v) or a solution of hydrogen peroxide (3% v/v) may be used for sterilizing nodules. However, mercuric chloride is highly toxic and hydrogen peroxide is expensive, making sodium hypochlorite (available as commercial bleach) the preferred choice. When hydrogen peroxide is used, the 5-6 rinses with sterile water may be omitted.

Desiccated nodules must be rehydrated before sterilizing. Place nodules into a small beaker with clean cool water and leave in the refrigerator to imbibe overnight. An one hour soaking at room temperature is sufficient for nodules which have been desiccated for only a short time.

Crush the surface sterilized nodule with a pair of blunt-tipped forceps in a large drop of sterile water in a petri dish. Alternatively, the nodule may be crushed in a sterile test tube with a sterile glass rod. Streak one loopful of the nodule suspension on a yeast-mannitol agar (YMA) plate containing Congo Red (CR). Similarly treat one loopful of the nodule suspension on a yeast-mannitol agar (YMA) plate containing bromthymol blue (BTB) (Appendix 3).

The primary isolate may be streaked in one continuous motion as shown in method 1 of Figure 1.1.

Well isolated colonies may be obtained with method 2 which is most commonly used with isolations from primary plates. It is performed as follows:

Deposit culture on agar with inoculation loop then streak out to 1. Resterilize loop, and cool by touching the agar surface near the side of the Petri dish, then streak from 1 to 2. Repeat the procedure until 4 is reached.

The isolation procedure lends itself well to improvisation and many variations exist. Here are some variations try them and compare your success at isolation by at least two methods.

The needle method of isolation is especially useful with

freshly harvested nodules 2 mm or larger in diameter. Wash the nodule first in water, then alcohol, then hold it with forceps and briefly pass it through a flame. Place this surface sterilized nodule on a small piece of sterile filter paper (2 cm x 2 cm) in a sterile Petri dish. A new piece of filter paper should be used for each nodule. The same Petri dish can be used for several nodules. Dip the blunt tipped forceps into 95% alcohol and flame momentarily. While holding the nodule with the forceps and

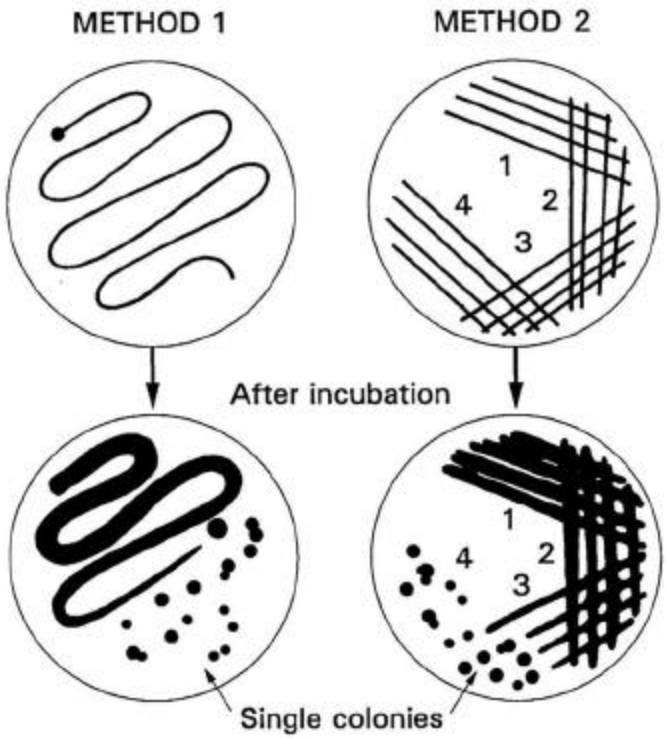

Figure 1.1 Streaking the plate.

resting the nodule on sterile filter paper, quickly slice off a small section with a flamed, hot scalpel. Still holding the

nodule with the forceps on the filter paper, insert the tip of a sterile inoculation needle (with a 1 mm loop) into the cut surface. Load the loop with inoculum. Streak directly onto a YMA plate containing CR and a YMA plate containing BTB.

When using the needle method, the nodule can also be held in the fingers of one hand while inserting the needle with the other hand. Brace the heels of the hands together to steady them.

Another method consists of serially diluting the nodule bacterial suspension and then pour-plating it. This is done as follows: lay out four sterile plastic Petri dishes marked A, B, C, and D. With a sterile Pasteur pipette, place two separated drops of water into each dish. Crush the sterilized nodule in a sterile Petri dish or test tube.

Flame the transfer loop and cool it in drop-1 of dish-A, then transfer the bacteroid suspension from the crushed nodule to drop-2 of dish-A and mix.

Next, flame the loop, cool it in drop-1 of dish-B, and transfer one loopful from drop-2 of dish-A to drop-2 of dish-B and mix. Continue until drop-2 of each dish has been inoculated and mixed with the diluted nodule suspension of the previous one.

Pour 15-20 ml liquid YMA (48◦C) to the inoculum in each dish. Ensure mixing by gently moving the covered dish first clockwise and then counter-clockwise on the table top. Allow three full circles for each movement. Continue mixing by moving the dish from the left to the right and from the right to the left three times. Then, without pausing, move the Petri-dish forward and backward and backward and forward, also three times. Allow the agar to set before incubating. Invert the plates during incubation.

Additional procedures are illustrated in Figure l.2.

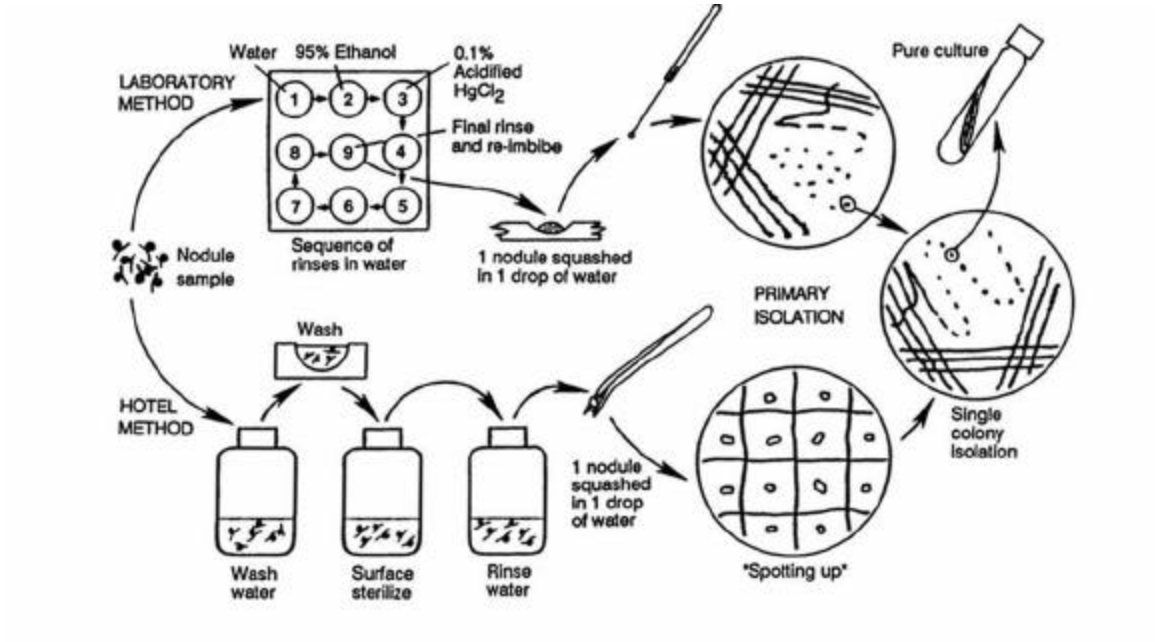

Figure 1.2. Isolation procedures as used by Date and Halliday (1979b)

(f) Performing the presumptive test

(Key steps 4 and 5)

The plates prepared from the three methods described above are referred to as primary isolation plates. Incubate these at 25-30°C in the dark. (Some slow-growing tropical rhizobia absorb Congo Red when incubated in light.)

After 4-10 days, look for well isolated colonies. Pick off a single colony typical of rhizobia (Exercise 3) and perform a Gram stain (Exercise 3), then reisolate by streaking on:

a) YMA containing BTB
b) YMA containing CR
c) Peptone glucose agar

Select isolated typical colonies. It is possible that more than one type colony (e.g. small and large colonies; mucoid and dry, etc.) may appear on a plate streaked from a single nodule. Each of these should be streaked on the three media listed above and considered an individual culture. More than one type of colony in a pure culture of rhizobia may be indicative of variants of the same strain or the occupancy of two different strains in the same nodule.

If no isolated colonies develop, restreak a little of the confluent growth again onto each one of the three media.

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. There are, however, exceptions (eg. some strains of *R. meliloti* absorb Congo red strongly). A blue color indicative of an alkaline reaction on BTB should be obtained with slow-growing *Bradyrhizobium* spp. A yellow color (acid) reaction is usually produced by the fast-growing *Rhizobium* spp. No growth or poor growth should be obtained on peptone glucose agar. Plates should be read for reactions after 3-5 days (fast-growers) and 5-7 (slow-growers). (Unless one is definitely working with fast-growers, an incubation of 7-10 days should be routine.) Check Exercise 3 for details. Check secondary isolates for colony morphology typical of rhizobia, then perform a Gram stain (Exercise 3) to check for purity of culture. Transfer three separate colonies to culture tubes to be added to stock cultures. Stock cultures obtained at this time are considered presumptive rhizobia. The authenticity of these isolates as pure cultures of rhizobia is confirmed later by the nodulation test (authentication) under bacteriologically controlled conditions. Select two representative colonies of the presumptive rhizobia from the isolation. Prepare 20-50 ml broth cultures in duplicates from each of the two colonies. Incubate on a shaker for use in the authentication tests.

(g) Authenticating the isolates as rhizobia

(Key steps 6, 7, 8, and 9)

The importance of determining that the isolate is a pure culture which can form nodules on legume roots cannot be over stressed. It proves the authenticity of a pure culture of rhizobia.

For large seeded legumes like beans (*Phaseolus vulgaris*) and soybean (*Glycine max*), Leonard jars and growth-pouches are recommended as growth units for authentication. Smaller seeded legumes, like clovers (*Trifolium* spp.) and Siratro (*Macroptilium atropurpureum*), may be grown in growth-tubes. Recommended hosts and growth systems to authenticate isolates are given in Appendix 9. Ideally, a rhizobial strain is tested for its ability to produce nodules on the legume species from which it was originally isolated. However, it may be more convenient to substitute another legume from the same cross-inoculation group particularly when a small-seeded legume can be substituted for a large-seeded one. Chickpea, although a large seeded legume, can be successfully grown in tubes by excising the cotyledons. This process produces dwarfed chickpea plants. Siratro is used in authenticating most bradyrhizobia from tropical legumes because it nodulates with more than 90% of all bradyrhizobia. Rhizobia from

specific hosts (e.g.. soybean, *Lotononis*, chickpea, etc.) are not authenticated on Siratro.

Set up two suitable growth units for each of the isolates plus at least two extra units that will serve as uninoculated controls. Consult Appendix 11 for the preparation of Leonard jars. Growth pouches are described in Exercise 5 and Appendix 8.

Surface sterilize and pregerminate seeds as detailed in Appendix 10.

Inoculate 1 ml of broth culture for each isolate onto each of the pregerminated seeds in two growth units. The extra growth units are not inoculated and will serve as controls. Plant and inoculate in a clean area. Take precautions against wind drafts and insects which may cause cross-contamination between treatments.

Examine plants for differences in vigor and color between the inoculated and uninoculated at 15-30 days of growth. Remove the plants from the rooting medium and note the presence or absence of nodules. The presence of nodules in the non-inoculated treatment invalidates the test. Sparse nodulation or nodulation restricted to distal parts of the roots of control plants indicates external contamination and points to a need to improve general hygiene. The

authentication test must be repeated with adequate bacteriological control.

If the presumptive tests are satisfactory, the isolates are regarded as fully authenticated cultures. The cultures of presumptive isolates are now confirmed as rhizobia and may be given collection numbers. When added to a culture collection, other relevant information should be added for each strain e.g. parent host, site of collection, soil pH, etc.

(h) Preserving culture of rhizobia

(Key steps 10, 11, 12, and 13)

There are a number of satisfactory methods for preserving rhizobial cultures including yeast mannitol agar (YMA) slant in screw-cap tubes, desiccated on porcelain beads, lyophilized (freeze-dried), and as frozen liquid suspension under liquid nitrogen. The choice of method will depend on facilities, experience, and finances (Table 1.1). The porcelain bead method is recommended for laboratories with limited resources.

To prepare for storage on beads, inoculate a loopful of culture from a YMA slant into 3 ml of sterile YM-broth and incubate to maximum turbidity on a rotary shaker.

Place 20-30 ceramic beads (washed and oven dried) in a

screw-cap test tube, cover the mouth of the tube with foil, and sterilize in the oven for 1-2 h at 160-170°C. Prepare storage tubes as depicted in Figure 1.4, using 6-7 g silica gel and sufficient cotton or glass-wool to keep the silica gel in place. The rubber lined caps for the tubes must be autoclaved separately in a rubber beaker, then dried in an oven at 80-90°C.

The glass-wool may be oven sterilized in the storage tube with the silica gel. When cotton is used, it should be autoclaved in small balls in a foil covered beaker. These cotton balls should be of a suitable size to facilitate easy aseptic transfer to the storage tube with forceps. Residual moisture is removed in the oven at 70-80°C before transferring it aseptically to the sterile storage tubes. The autoclaved caps are then added to the tubes.

Transfer the sterilized beads aseptically to the broth culture in the tubes and replug. Soak the beads for 1-2 h, then invert the tube and allow the excess broth culture to soak into the cotton plug.

Transfer the beads impregnated with rhizobia into the storage tube aseptically, replace and tighten the screw caps securely.

Examine the tubes after a day or so to ensure that the silica gel is still blue. If it turns pink or colorless, then too

much moisture was transferred with the beads or an improper seal is permitting entry of moisture.

To regenerate a culture, inoculate YM-broth with one or two beads. These are easily speared from the storage tube using a sterile needle with a slight hook. A week or more may be needed to obtain visual signs of growth. Once the broth becomes turbid, loopfuls should be streaked on presumptive test media to check for purity. Subculture from the broth onto YMA slants as desired.

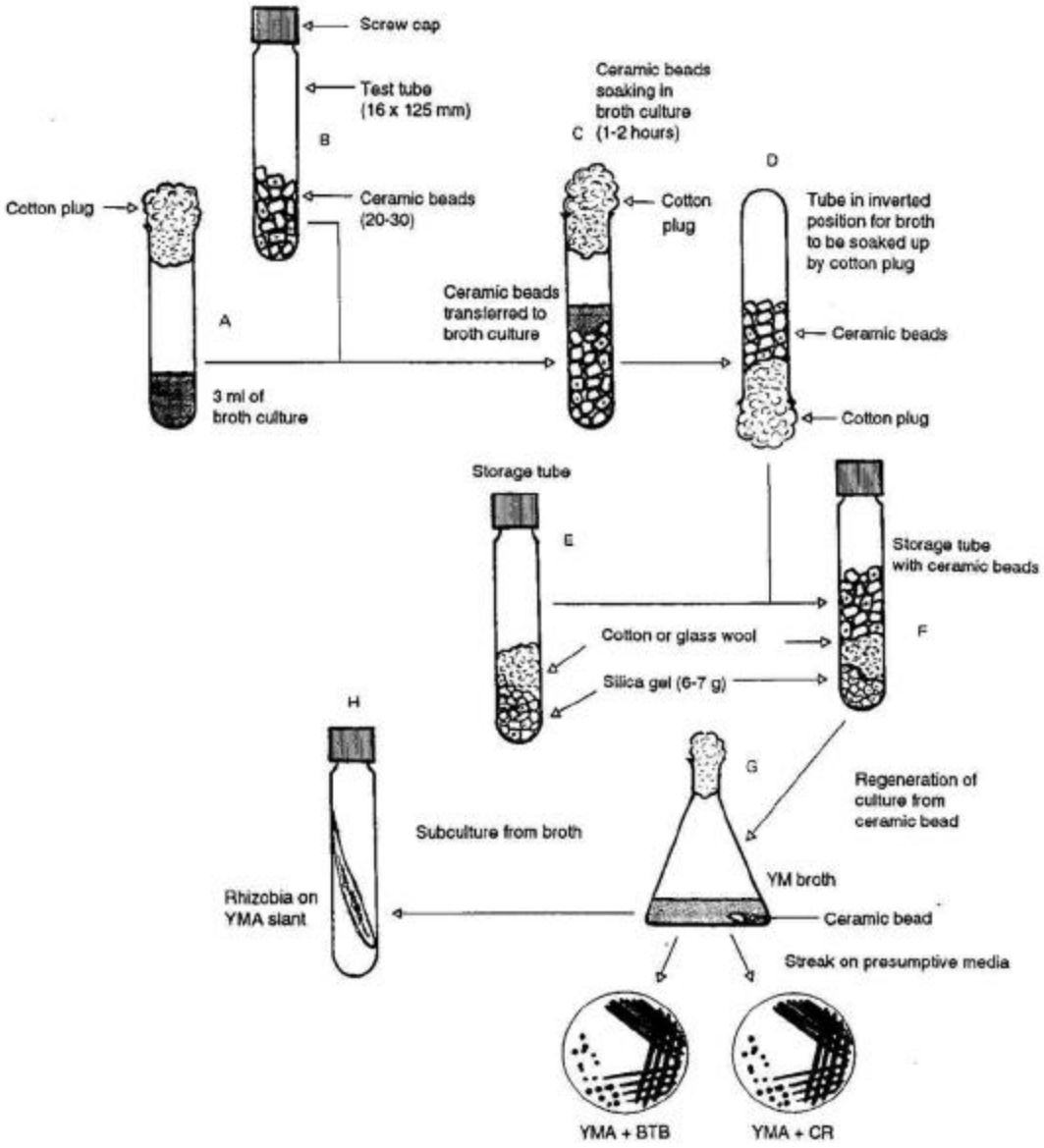

Figure 1.3. Ceramic bead method for storing Rhizobium

Method	Expertise and facilities required	Length of useful storage period	Advantages	Disadvantages	Remarks
Agar slopes in screw cap tubes or agar covered with paraffin oil (Vincent 1970 p. 10)	Basic microbiological knowledge and facilities for pure culture (autoclave, clean transfer area, tubes, media, etc.)	1-2 years without transfer at 25-30° but can be longer, if held at 5°C	Simplicity, low cost minimum facilities and expertise	Short storage time, increased chance of contamination and variants because of more frequent subculturing	Least desirable for long term storage
Porcelain beads	As above, plus availability of beads, suitable airtight containers and dry sterilizing facilities for silica gel desiccant	3-4 years, with some rhizobia significantly shorter with others	Low cost and longer storage time and therefore more time before re-beading. Facility for number of sub cultures (i.e., one bead) from original	Not as long term as lyophilization and risk of contamination and variants when re-beading. Time required for re-beading	Good for 6-12 month storage*
Lyophilized or freeze dried	Basic microbiological facilities lyophilizing equipment (vacuum pump, freezing facility under vacuum) ampoules, glass blowing burner, etc.	Minimum 15-20 years experience suggests much longer	Once ampouled, minimal risk of variants or contamination. virtually permanent storage. Can be at room temperature	Expensive for equipment and materials	Preferred
Liquid N storage	Expertise as above, plus cryostat and liquid N source	Years, but not much information available	Rapid operation	Very expensive; special precautions during freezing and thawing	None

Table 1.1 Methods for preservation of strains of Rhizobium

*Poor survival with some fast-growing rhizobia (e.g. *R. phaseoli* and *Leucaena* and *Sesbania* rhizobia) fused $CaCl_2$ can be used as a substitute for silica gel

Requirements

(a) Recognizing legumes and identifying them in the field.

A suitable botanical key describing the identification of legumes.

(b) Recovering nodules in the field.

Refrigerator
Spade
Sieve
Running water
Plastic bags for plants, smaller bags for seeds

(c) Preserving nodules

Refrigerator
Collection vial (Appendix 2)
Nodules from (b)

(d) Examining nodules and bacteroids

Microscope
Bunsen burner
Microscope slides, cover slips, mounting fluid
Razor blade, inoculation loop

Distilled water

Carbol fuchsin stain (Appendix 3)

Rhizobial cultures

Nodulated plants from (b)

(e) Isolating rhizobia from a nodule

Refrigerator

Scissors, forceps, inoculation loop, isolation needle

Scalpel

Sterile Petri dishes

Sterile test tubes, glass rods

Erlenmeyer flask 125 ml (optional), small beaker

Bunsen burner

Sterile Pasteur pipettes

Sterile filter paper (cut into small pieces)

Running water, sterile water, ethanol or isopropanol

Sodium hypochlorite solution, 3% (may be made from commercial bleach)

Plates of plain YMA, plates of YMA + Congo Red and YMA + BTB

Liquid YMA (50°C)

Nodulated plants from (b)

Desiccated nodules

f) Performing the presumptive test

Transfer chamber

Incubator

Bunsen burner

Inoculation loop

YMA slants

Flasks (125 ml) with 50 ml YM broth

Plates of YMA + BTB, YMA + Congo red, and YMA and peptone glucose agar

Gram stain solutions

(g) Authenticating the isolates as rhizobia

Transfer Chamber

Greenhouse, growth-room or shelf

Drying oven

Kjeldahl N determination equipment (optional)

Racks for growth pouches and growth tubes

10 ml pipettes (sterile)

Alcohol burners

Scissors, paper bags for plant tops

Growth pouches, growth tubes (Exercise 5, Appendix 8)

Leonard jars (Appendix 11)

Materials and glassware for seed sterilization (Appendix 10)

Broth cultures from (f)

(h) Preserving rhizobial cultures

Transfer chamber

Rotary shaker

Sterilizing oven, drying oven

Forceps, inoculation loop, flame, hooked needle

Test tube racks, screw capped test tubes

Ceramic beads (washed and dried)

Silica gel (with indicator), absorbent cotton, aluminum foil

Beaker (400 ml)

Capped tubes with 3 ml YM broth, culture tubes with YMA slants

Cultures of rhizobia

EXERCISE 2

TO OBSERVE THE INFECTION PROCESS

Clover rhizobia enter their host's roots through the root hairs. Infection is preceded by a deformation of root hairs and the forming of an infection thread which can be observed directly under the microscope. Root hair deformations may also be caused by non-nodulating strains of *Rhizobium.* Non-nodulating strains used in this chapter cause no infection threads to form.

Key steps/objectives

l) Culture strains of *Rhizobium* in YM broth
2) Sterilize and germinate clover seeds
3) Mount seedling on microscope slide
4) Incubate the seedlings in inoculated mineral medium
5) Observe root hair deformation and infection threads
6) Compare root hair deformations caused by different kinds of rhizobia strains

(a) Culturing strains of rhizobia in YM broth

(Key step 1)

Inoculate 50 ml flasks or test tubes containing 20 ml of YM

broth in duplicate with the strains listed below:

1) *Rhizobium leguminosarum* bv. trifolii (TAL 382) isolated from nodules of *Trifolium semipilosum*
2) *R.l.* bv. trifolii non-infective isolated from nodules of *Trifolium* sp.
3) *R.l.* bv. trifolii (TAL 1185) isolated from nodules of *Trifolium repens*
4) *R.l.* bv. phaseoli (TAL 182) isolated from nodules of *Phaseolus vulgaris*
5) *R. meliloti* (TAL 380) isolated from nodules of *Medicago sativa*
6) *Bradyrhizobium* sp. (TAL 764) isolated from nodules of *Lupinus angustifolius*

Other strains of the same species of rhizobia may be substituted.
Incubate at 25-30°C for 5-7 days on a rotary shaker.

(b) Germinating seeds

(Key step 2)

Choose a small-seeded legume. Clover, especially *Trifolium repens* or *T. glomeratum,* is most suitable for this exercise. Surface sterilize seeds according to the procedure outlined in Appendix 10. Some clover species may need scarification with

sulfuric acid. Others, like Nolan's white clover and strawberry clover (*Trifolium fragiferum*) germinate easily without scarification. Wash seeds with at least eight changes of sterile distilled water. Aseptically place the seeds onto water-agar plates for germination. Incubate the plates inverted for 48 h or more until roots are 6-8 mm long.

(c) Preparing a Fahraeus slide

(Key step 3)

Prepare 10 ml of Fahraeus carbon and nitrogen free medium (Appendix 3) containing 0.6% agar in a 15 ml tube. Cool the liquid agar medium to 48°C in a water bath.

For each strain of *Rhizobium* used, prepare two sterile 50 ml boiling-tubes containing 25 ml Fahraeus carbon and nitrogen-free medium without agar. Set up two additional tubes for uninoculated controls. Cover with 50 ml beakers.

Transfer approximately 0.2 ml of agar medium to a sterile microscope slide using a Pasteur pipette fitted with a rubber bulb. Leave one-half of the slide empty. This is best done by lining up the slide and a long coverslip side by side in a sterile Petri dish (Figure 2.1). Place the agar in five or six drops onto the bottom half of the slide. Immediately, transfer a well formed seedling to the slide with a sterile

inoculation loop. Place the seedling onto the slide in such a way that the root tip is immersed in the agar and the cotyledons are in the empty half of the slide. With sterile forceps, carefully place the long coverglass over the agar and the root tips. If the seed coat adheres to the cotyledons on the seedling, carefully remove it with sterile fine tipped forceps.

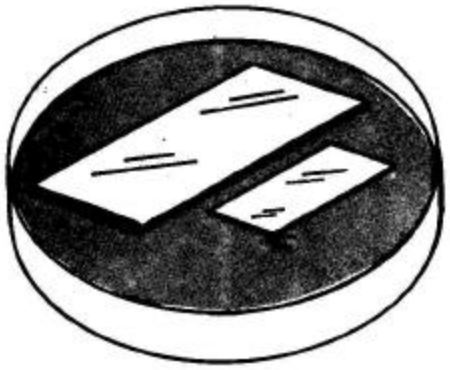

Figure 2.1. Petri dish with Components of Fahraeus slide

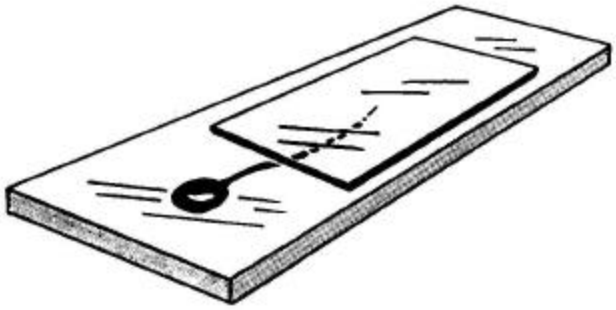

Figure 2.2. Placement of seedlings on Fahraeus slide

Transfer the slide mounted seedlings to the tubes containing the Fahraeus mineral medium.

(d) Inoculating the seedlings

(Key step 4)

Using the broth cultures which have been set up for this

experiment in (a), inoculate two seedlings with each of the six strains of *Rhizobium* by adding five drops of the cell suspensions to individual tubes containing the mineral medium and the Fahraeus slides.

Alternatively, the seedlings may be inoculated by incorporating a cell suspension into the Fahraeus agar medium before the seedling is placed onto the slide. This speeds up the infection process. Add five drops of sterile broth medium to the controls.

Incubate at 25-30°C in a well-lighted environment.

(e) Observing the root hairs under the microscope

(Key step 5)

After 24 h remove Fahraeus slide from the nutrient tube and examine it under the microscope. Remove the excess solution with absorbent filter paper. Observe with phase contrastor ordinary bright field microscope under low and high power magnifications. Search for root hair deformations and/or curling and infection threads. Mark the position of your slide on the microscope stage so that the same spot may be found in later observations of the same root hair infections. Make observations in intervals of 12-24 h. Periodic observation may be made at shorter intervals if inoculation

was done by including the cell suspension into the agar medium. Return the slide to its tube between observations.

Take precautions against undue contamination when returning the slide to the mineral medium. Aseptic conditions cannot be maintained beyond the first observation. However, contamination usually does not interfere provided the root hairs chosen for observations are not located at the edges of the microscope slide.

(f) Comparing root hair deformations

(Key step 6)

Photograph or draw the root hair deformations or curling caused by each strain. Distinguish full curling from slight curling and root hair branching. Note the effects of noninvasive strains on the root hairs. Compare the deformations caused by the various strains used.

Typical root hair deformations, like the shepherd's crook, are shown in Figure 2.3.

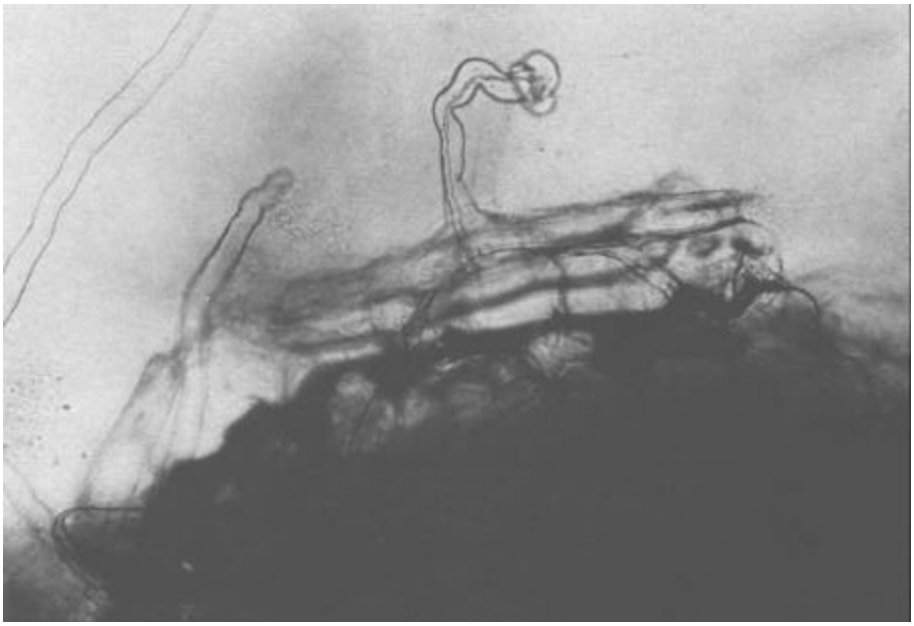

Figure 2.3. Deformed white clover root hair infected with R. trifolii 0403. Note the sheperd's crook and the infection thread. (Photo courtesy of F. Dazzo)

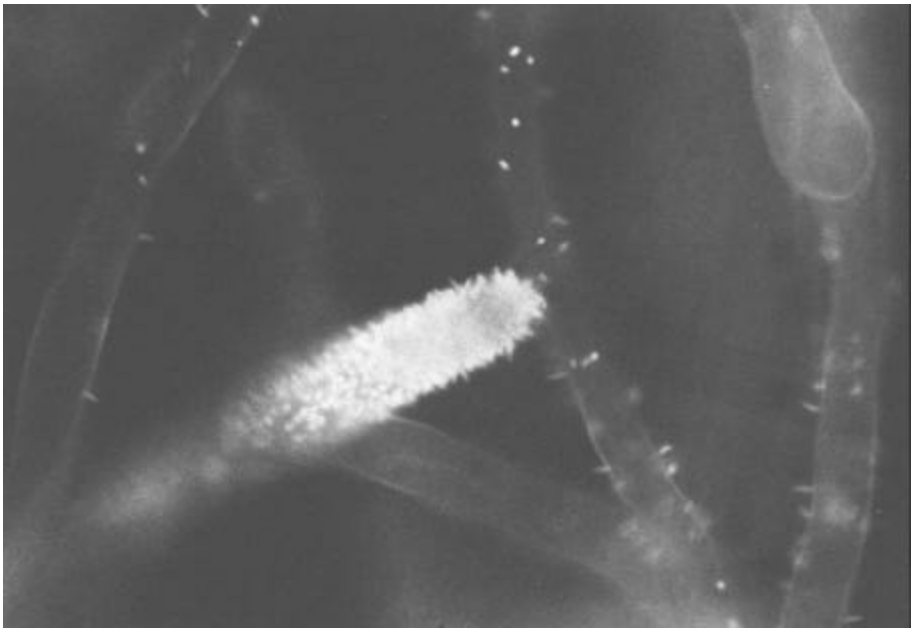

Figure 2.4. Selective proliferation and colonization of Rhizobium trifolii on a root hair of its host legume clover in a Fahraeus slide system. (Photo contributed by B.B. Bohlool)

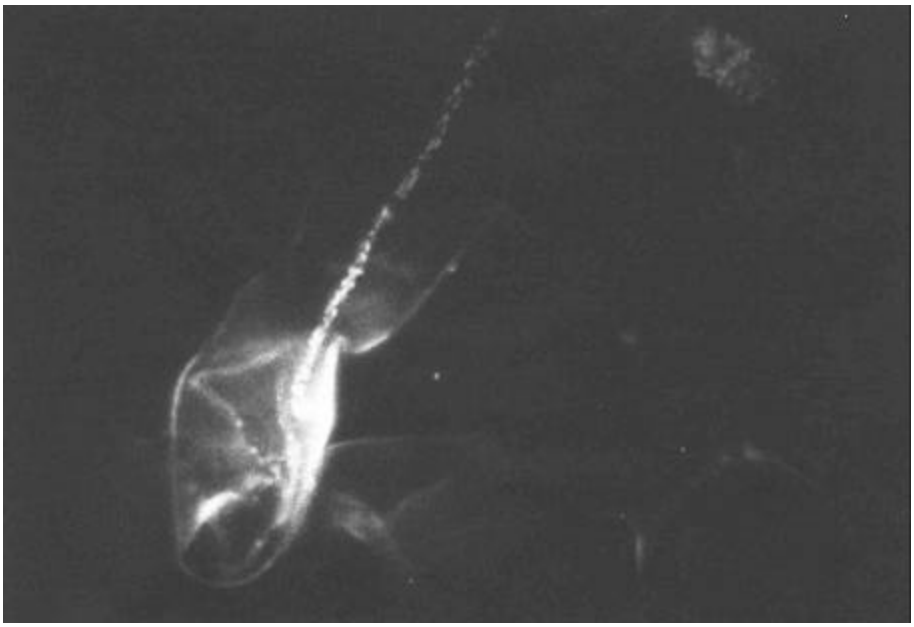

Figure 2.5. Rhizobium trifolii inside infection thread in the root hair of its host clover (Trifolium repens).

(Photo
contributed by B.B. Bohlool)

Requirements

(a) Culturing *R. leguminosarum* bv. trifolii strains in YM broth

Rotary shaker
Twelve 50 ml flasks (or tubes) containing 20 ml culture broth each
Inoculation loop, flame
Slant cultures of clover rhizobia strains TAL 382, TAL 1185, TAL 182, TAL 380, TAL 386, noninfective strain of clover rhizobia

(b) Germinating seeds

Incubator
Materials and tools for sterilizing seeds (Appendix 10)
Plates of water agar (7.5 g agar per liter distilled water)
Seeds of clover (*Trifolium repens, T. glomoratum* or other)

(c) Preparing a Fahraeus slide

Water bath

Sterile microscope slides (1 mm x 24 mm x 40 mm)

Coverslips (kept in sterile Petri dishes)

Pasteur pipettes (sterile); rubber bulbs

Inoculation loop, forceps, flame

Fahraeus C and N free medium

Fahraeus medium plus 0.6% agar in 15 ml tube

Seedlings of clover

Fahraeus medium (25 ml) in tubes (39 mm x 150 mm) with covering 50 mm beakers

(d) Inoculating the seedlings

Growth chamber (or well lighted environment) at 25-30°C

Pasteur pipettes (sterile); rubber nipples

Tubes with seedlings from (c)

(e) Observing the root-hairs under the microscope

Microscope with phase or bright field condenser

Forceps

Filter paper (sterile and absorbent)

Seedlings in inoculated Fahraeus solution from (d)

(f) Comparing root hair deformations

Microscope as in (e) with camera attachment

EXERCISE 3

TO STUDY CULTURAL PROPERTIES, CELL MORPHOLOGICAL CHARACTERISTICS AND SOME NUTRITIONAL REQUIREMENTS OF RHIZOBIA

The aims of this exercise are to learn to distinguish rhizobia from other microorganisms by cell morphology, staining reactions, growth responses on various media, and to show how media for rhizobia can be modified.

Key steps/objectives

1) Subculture rhizobia and other bacteria
2) Observe cell morphology of rhizobia and other bacteria under phase contrast microscopy
3) Examine rhizobia and other bacteria for cell morphology using a simple stain (carbol fuchsin) and the Gram stain
4) Culture rhizobia and other bacteria on indicator media
5) Observe colony morphology and growth reaction on the indicator media
6) Prepare agar media with different carbon and nitrogen sources
7) Inoculate media with rhizobia
8) Observe growth reactions on each medium

(a) Preliminary subculturing of different bacterial cultures

(Key step 1)

Make subcultures on agar slants from the stock cultures of the following microorganisms using YMA slants for the rhizobia, and nutrient agar slants for the other bacteria: *Rhizobium meliloti*; *R. leguminosarum* bv. phaseoli; *Rhizobium* sp. (chickpea); *Bradyrhizobium* sp. *(Lotononis)*; *B. japonicum*; *Bacillus subtilis; Escherichia coli*; *Staphylococcus aureus*; and *Pseudomonas* sp.

Also needed are:

1) surface sterilized nodules (saved from Exercise 1)
2) a homogenate of non-surface-sterilized nodules of slow-growing and fast-growing rhizobia, and
3) a broth culture of rhizobia mixed with other species of bacteria.

(b) Comparing cell morphology and Gram stain reactions of rhizobia with those of other microorganisms

(Key steps 2 and 3)

Make wet mounts of the cultures provided and examine under the phase contrast microscope. Note the motility, size and shape of the rhizobia compared to other bacteria.

Place a loopful of sterile distilled water onto a clean, pre-flamed and cooled microscope slide. Flame the loop and transfer a small sample of the bacterial growth from the slant culture to the water on the slide. Mix thoroughly and make a thin smear approximately 1 cm^2 in diameter.

For broth cultures, transfer a loopful and make smear directly on the dry slide. Air dry, heat fix, and allow to cool.

Flood the smear with diluted carbol fuchsin for 60 seconds. Rinse carefully in a gentle stream of water and blot dry.

Locate smears under low power (10x, 25x, or 40x) objective. Apply a drop of oil to the smear and observe with the 100x oil immersion objective using bright field illumination.

The carbol fuchsin stain makes the bacteria easily visible (cells appear pink). Note the characteristic rod shape of the cultured cells of rhizobia and compare the size and shape of these to that of bacteroids seen in the nodule preparation. Also compare rhizobia with the other bacteria and note the difference in size and form. Refer to Figure 3.1 for the morphology of the microorganisms.

(c) Determining Gram stain reactions of various bacteria

(Key step 3)

1) Make thin smears of the various bacteria provided and heat fix.
2) Stain the smears with solution 1 (crystal violet) for 1 min.
3) Wash lightly with water and flood with solution II (iodine).
4) Drain immediately and flood again with solution II for 1 min.
5) Drain solution II and decolorize with solution III (95% alcohol) for 15-30 seconds in the case of a thin smear and 60 seconds if the smear is thick.
6) Wash with water and blot dry carefully.
7) Counter stain with solution IV (safranin) for 1 min.
8) Wash with water and air dry.

Observe the preparation under oil immersion.

The Gram stain procedure separates bacteria into two groups: **Gram-positive** and **Gram-negative** organisms.

Gram-positive organisms retain the crystal violet stain after treating with iodine and washing with alcohol, and appear dark violet after staining (e.g., ***Bacillus subtilis*** and ***Staphylococcus aureus)***.

Gram-negative organisms lose the violet stain after treating

with iodine and washing with alcohol but retain the red coloration of the counter-stain, safranin (e.g., *Rhizobium*, *Pseudomonas*, *Escherichia coli*).

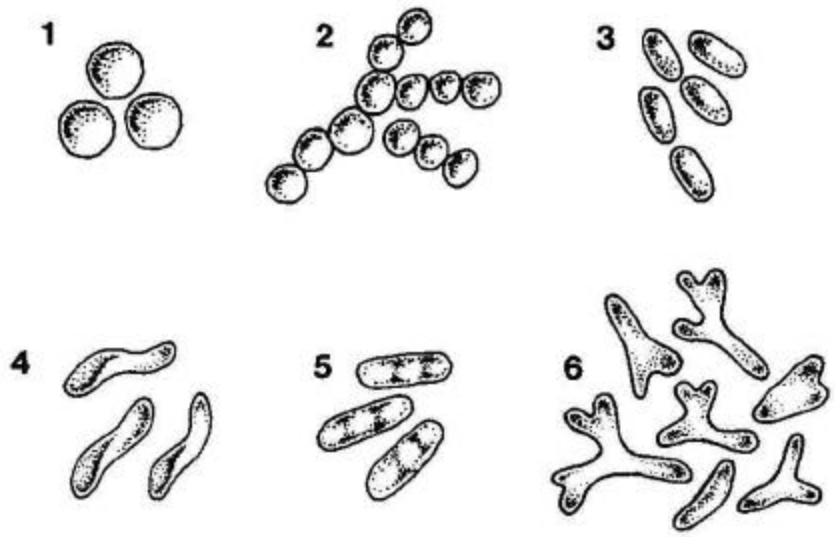

Figure 3.1. Shapes of bacteria

l) coccus
2) Staphylococcus
3) Rod (e.g., *Escherichia coli)*
4) *Spirillum*
5) Cultured cells of rhizobia
6) bacteroids of rhizobia (e.g., *Lens* sp.)

(d) Characterizing growth of rhizobia using a range of media

(Key steps 4 and 5)

Rhizobia can be described according to their growth in solid and in liquid media. The size, shape, color, and texture of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in defining strains or isolates. Typical colony characteristics, when grown on standard yeast-mannitol medium, are described below.

Shape: Usually discrete, round colonies varying from flat () to domed () and even conical () shape on agar surface. Colonies usually have a smooth margin. When growing subsurface in the agar, colonies are typically lens-shaped.

Color and texture: Colonies may be white-opaque or they may be milky- to watery-translucent. The opaque colony growth is usually firm with little gum, whereas the less dense colonies are often gummy and soft. Colonies may be glistening or dull, evenly opaque or translucent, but many colonies develop darker centers of rib-like markings with age. Red or pink (e.g., from *Lotononis)* and yellowish (e.g., from *Stylosanthes)* occur, but are not common.

Growth rate: Generally 3-5 days for fast-growers (e.g., from *Leucaena*, *Psoralea*, *Sesbania*), 5-7 days for slow-growers (e.g., from *Macroptilium, Desmodium, Galactia*), to 7-12 days (e.g., some *Stylosanthes*, *Lupinus*) to achieve maximum colony

size on agar or growth in liquid medium. Growth rate varies according to the temperature of incubation (optima 25-30°C), origin (culture or nodule), aeration (in liquid cultures), and composition of medium.

Size: When well separated on agar plates, colony size may vary from 1 mm for many slow-growing strains (e.g., from *Stylosanthes*, *Zornia, Aeschynomene*, *Lupinus*) to 4-5 mm for faster-growing strains (e.g., from *Leucaena*, *Psoralea, Sesbania*). In crowded plates colonies remain smaller and discrete but coalesce to confluent growth when colonies join.

Select two preparations from the pure cultures, the nodule homogenates or the mixed cultures of rhizobia and other bacteria. Streak out on plates containing each of the following media: YMA, YMA + bromthymol blue, YMA + Congo Red, and peptone glucose agar + bromcresol purple.

These indicators and selective media are used as presumptive tests for purity of cultures. Their interpretation is as follows:

Rhizobia generally do not absorb Congo Red when plates are incubated in the dark. Colonies remain white, opaque or occasionally pink. Contaminating organisms usually absorb the red dye. However, reactions depend on the concentration of Congo Red and age of the culture. Rhizobia will absorb the

red dye if plates are exposed to light during the incubation or exposed to light for an hour or more after growth has occurred.

Freshly prepared YMA plates containing bromthymol blue have a pH of 6.8 and are green. Slow-growing rhizobia show an alkaline reaction in this medium, turning the dye blue. Fast-growing rhizobia show an acid reaction, turning the medium yellow.

Rhizobia grow poorly, if at all, on peptone glucose agar and cause little change in pH, when incubated at 25-30°C. Heavy growth is indicative of contamination.

Rhizobia will change the pH and appearance of litmus milk only slowly, if at all, and may produce a clear serum zone at the surface. Common contaminants of Rhizobium cultures metabolize the medium; they may turn it brown or they may acidify and curdle it. R. meliloti produce a pink acidic reaction to litmus milk. Curdling, digestion, or reduction (white color) of litmus milk indicates contamination.

(e) Observing growth reactions on modified media

(Key steps 6, 7 and 8)

If mannitol and yeast extract are not available, the basic

growth medium may be modified, since rhizobia can utilize carbon and nitrogen from various sources.

Make up media as shown:

1) Prepare 1500 ml of a mineral salts solution containing the inorganic constituents of YMA (Appendix 3). Add 28 grams of agar and heat in the autoclave or water bath. Dispense the melted mineral salts agar solution in 12 ml portions into test tubes. Sterilize in the autoclave and keep melted in the water bath at 48°C.
2) Prepare carbon source stock solutions (10 g/100 ml) of mannitol (M), sucrose (Su), arabinose (A), and glycerol (G). Sterilize by autoclaving, except for A which should be filter sterilized.
3) Prepare stock solutions of the nitrogen sources (Appendix 3), yeast-water from baker's yeast (B), yeast extract (Y) 0.5 g/100 ml, soybean extract (S), and ammonium chloride NH_4Cl (N) 0.4 g/100 ml. Sterilize by autoclaving.

Pipette into separate, sterile Petri-dishes 1.5 ml of (2) and 1.5 ml of (3). Pour one test tube (12 ml) of the melted (48°C) mineral salts agar preparation (1) to each plate, so as to provide the combinations in duplicate.

Nitrogen Source

Carbon Source	B	Y	S	N
	----------Media----------			
M (mannitol)	MB	MY	MS	MN
Su (sucrose)	SuB	SuY	SuS	SuN
A (arabinose)	AB	AY	AS	AN
G (glycerol)	GB	GY	GS	GN

Mix immediately after adding the agar by rotating each dish gently three times clockwise and counterclockwise, and three times to the right and to the left, as well as forward and backwards.

Allow the plates to cool overnight for the agar to solidify. Remove any contaminated plates.

After the agar media have solidified, streak one of each of the four provided strains onto one separate plate as you would streak for isolation (Exercise 1) Alternatively, broth cultures of the strains may be surface spread using 0.1 ml portions on separate plates.

The streaking of two or more cultures onto one plate may be necessary if there is a shortage of plates. However, this practice should be avoided if possible because of an aerosol effect during the process of streaking.

Incubate and compare growth on the various media at 3, 7, and 10 days after plating.

Requirements

(a) Preliminary subculturing

Transfer chamber
Incubator
Inoculation loop; flame
Microorganisms on slants, surface sterilized nodules, homogenate of non-surface sterilized nodules, and a mixed broth culture as indicated in step (a) of the chapter.

(b) Comparing cell morphology and Gram stain reactions of rhizobia with those of other microorganisms

Microscope
Inoculation loop; flame
Microscope slides
Immersion oil, lens paper
Running water or wash bottle with water
Carbol fuchsin solution (Appendix 3)
Tissue paper or paper towels
Microorganisms from (a)

(c) Determining Gram stain reaction of various bacteria

All requirements of (b) not including phase contrast equipment

Gram stain solutions (Appendix 3)

(d) Characterizing growth of rhizobia using a range of media

Transfer chamber

Incubator

Inoculation loop, flame

Plates of yeast mannitol agar (YMA)

Plates of YMA + bromthymol blue

Plates of YMA + Congo Red

Plates of peptone glucose agar and bromcresol purple

Tubes of litmus milk

Consult Appendix 3 for growth media preparation

(e) Observing growth reactions on modified media

Incubator, autoclave, water bath, pH meter, suction pump, balance

Bacteriological filter unit (0.2 micron pore size)

Volumetric flasks (8 of 100 ml)

Pipettes (sterile, 5 and 10 ml)

Spatula, weighing paper

Large flask or beaker (2-3 liter)

Test-tubes, test tube rack

Petri dishes (sterile)

Inoculation loop, flame

Mineral salts: K_2HPO_4, $MgSO_4 \cdot 7H_2O$

Distilled water

Agar

Mannitol, sucrose, arabinose, glycerol

Brewers yeast, yeast extract (Difco), soybean extract,

ammonium chloride

Rhizobia from (a)

EXERCISE 4

TO QUANTIFY THE GROWTH OF RHIZOBIA

This exercise deals with routine enumeration techniques for pure cultures of rhizobia. The total or direct count is performed using the microscope. Optical density measurements are used to estimate the number of cells in broth culture. The viable count is accomplished through plating methods. The mean generation times of a *Rhizobium* sp. and a *Bradyrhizobium* sp. in broth culture are computed.

Key steps/objectives

l) Inoculate yeast-mannitol broth with rhizobia
2) Calibrate Pasteur pipettes
3) Determine the total count
4) Measure the optical density of the broth-cultures
5) Make a serial dilution and plate by the pour-plate, spread-plate, and drop-plate methods
6) Read and calculate the viable counts obtained by the three methods
7) Compare results of the counting methods
8) Inoculate flasks with diluted culture(s) for the generation time experiment
9) Determine viable counts periodically
10) Plot growth curve and determine the mean generation time.

(a) Preliminary culturing of fast- and slow-growing rhizobia

(Key step 1)

Inoculate two flasks each containing 50 ml of YM-broth with fast-growing *Rhizobium leguminosarum* bv. phaseoli strain TAL 182, and two other flasks with a slow-growing *Bradyrhizobium japonicum* strain TAL 379. Other strains of *Rhizobium* and *Bradyrhizobium* may be used instead. Incubate the flasks at 25-30°C on a rotary shaker at 20 rpm. TAL 182 should be started 4-5 days in advance of the exercise; TAL 379, 7-9 days in advance. Take the culture flask of TAL 182 from the shaker after 4-5 days and remove a 20 ml sub-sample for procedures (b) and (c).

(b) Becoming familiar with the Petroff-Hausser and Helber counting chambers

(Key step 3)

The Petroff-Hausser counting chamber (Figure 4.1) is a precision-machined glass plate that has a sunken platform at its center. The depth of this sunken platform is exactly 0.002 cm. Two sides of the platform are bordered by a channel. The surface of the platform is etched with a grid system which consists of 25 large squares, each of which is divided into 16 smaller squares. Because of the precisely machined

gap between the grid surface and the overlying glass coverslip, it is possible to relate the number of cells observed in a field to the volume of fluid in which they are suspended. Knowing the volume above each square, the concentration of rhizobia (total cells ml^{-1}) can be calculated.

The data in Table 4.1 apply to the Petroff-Hausser counting chamber and also to the Helber counter which has a grid system of an identical design.

Table 4.1. Brief details of the Petroff-Hausser counting chamber

Area (cm^2)		Corresponding Volume (ml)	Factor
Total grid	$1x10^{-2}$	$2x10^{-5}$	$5x10^{4}$
Large square	$4x10^{-4}$	$8x10^{-7}$	$1.25x10^{6}$
Small square	$2.5x10^{-5}$	$5x10^{-8}$	$2x10^{7}$

The large squares are most suitable for counting rhizobia. The countable range is 8-80 cells per large square.

(c) Using the Petroff-Hausser and Helber counting chambers.

(Key step 3)

Chamber and coverslip should be soaked in a mild liquid

detergent and thoroughly rinsed with distilled water and then air dried.

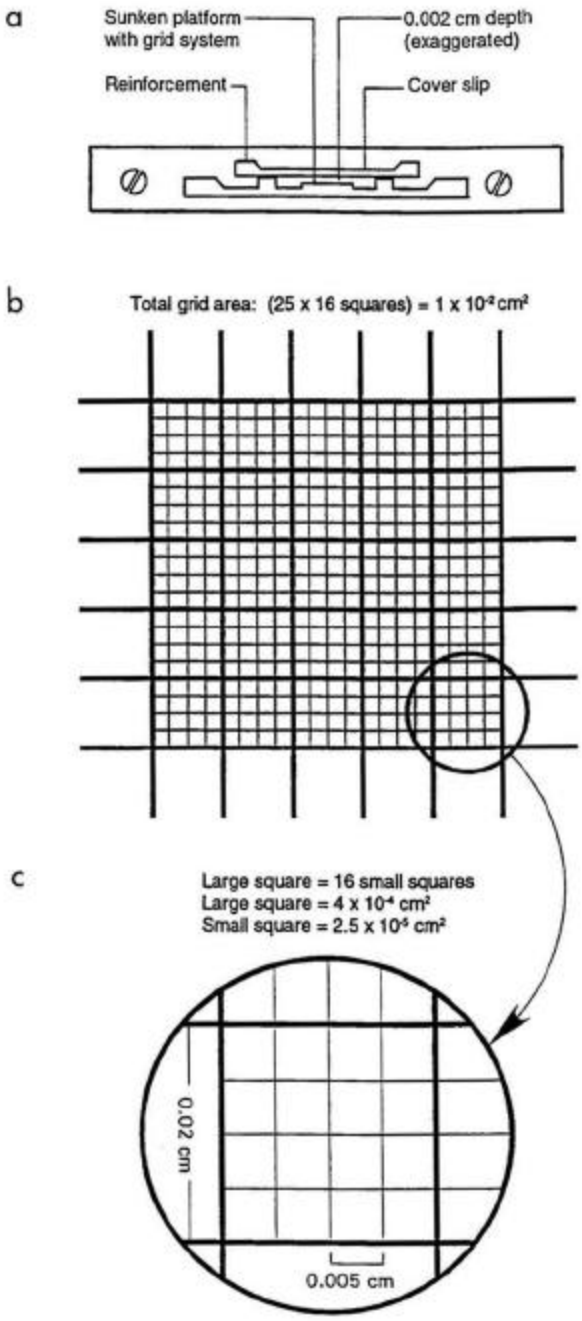

Figure 4.1. Petroff-Hausser counting chamber

(a) Cross section

(b) Top view of entire grid

(c) Magnified view of grid

This will assure an even flow of the liquid into the chamber and prevent the formation of air bubbles.

A fully grown broth-culture contains approximately 10^9 cells per ml. Make a 1:10 dilution with sterile water to bring the suspension within a countable range. From this dilution, make another dilution series in sterile water of 10, 20, 40, and 80%. Choose the dilution which you consider is within the best counting range. Trial runs with the various dilutions may be needed to select the best dilution for the count. This may require washing and drying the counting chamber several times, until the best dilution has been determined.

Slide the clean Petroff-Hausser chamber into its frame and place the coverslip into position and press it down lightly to assure a firm seating on the supporting surface of the chamber.

The frame of the Petroff-Hausser chamber has a small indentation on the inside of one of its long edges. To this area, deliver a small drop of the diluted culture suspension using a fine tipped Pasteur pipette.

The Rhizobium suspension will quickly spread over the grid. Excess culture (if a large drop was added) will overflow into

the two channels at the edges of the etched platform. If these channels flood completely, the coverslip may not rest flush on the surface of the glass plate. If this happens, clean the counting chamber and start again.

Place the chamber under the 40x objective of a phase-contrast microscope. Count cells in individual large squares. To avoid counting the same cells twice, omit bacteria on the upper and left borderlines of each square. Count at least ten fields (eight-80 cells per large square) to obtain coefficients of variability of 10%.

Use the following formula to calculate the number of cells: Bacteria in 1 ml of original cell suspension = dilution x cells per square x factor for square.

If 20 cells (mean of ten squares) were counted in a large square and the original broth culture was diluted by a factor of ten, and if a 20% suspension of the dilution was counted, the total number of rhizobia per ml of undiluted broth would be:

$$20 \times 10 \times (100/20) \times (1.25 \times 10^{6}) = 1.25 \times 10^{9} \text{ cells ml}^{-1}$$

Note that this direct count included dead as well as viable rhizobia and also the cells of contaminants, if present. Most direct total counts are of variable reliability in that they

may overestimate the viable-count by a factor of more than two, as 50% or more of the cells counted may not be viable. This method is suitable only for counting log phase broth cultures in liquid media, and not in peat, soil, or other particulate materials.

d) Estimating cell concentration by optical density

(Key step 4)

The optical density of a bacterial suspension is generally correlated with the number of cells it contains. Optical density measurements are a simple and convenient estimate of cell numbers as they require but little manipulation, and aseptic conditions need not be observed.

Dilute 5-10 ml of the TAL 182 broth culture to 10, 20, 40, and 80% of its original concentration. Measure the light absorbed by each concentration with a spectrophotometer at a wavelength of 540 nm. Use yeast mannitol broth to calibrate the instrument at zero. Relate the different concentrations to the actual cell count obtained with the Petroff-Hausser chamber by plotting the Optical Density (OD) against the total cell number.

This method also has its limitations. It is best suited for initially clear media. Dead cells and contaminants contribute to the O.D. of the culture, as well as gum produced by the

rhizobia, undissolved salt or precipitate in the medium.

e) Determining the number of viable cells in a culture by plating methods

(Key steps 2, 5, 6, and 7)

Make serial dilutions of the TAL 182 broth culture. Based on the total count, the number of viable cells will be approximately 1.0×10^{9} ml^{-1}. A countable range for plate counts is 30-300 cells ml^{-1}. To achieve this concentration, set out eight tubes, each containing 9 ml of sterile diluent (1/4 strength YM broth, pH 6.8). One ml of the broth culture is diluted in steps, tenfold each time (10^{-1} through 10^{-8}). Refer to Figure 4.2 for the serial dilution procedures.

Use a fresh pipette for each strain and for each dilution in the series. Begin with the highest dilution in the series. With the aid of the suction bulb, fill and empty the pipette by sucking in and out 5 times with the diluted culture, then transfer 1 ml aseptically to a sterile Petri dish. Open the Petri dish only sufficiently to allow the pipette to enter and deliver the sample. Flame the pipette briefly (but do not overheat) by passing it through the Bunsen burner flame each time prior to successive removal of aliquots for replication (2 per dilution) from the same tube. Similarly with the same

pipette remove 1 ml aliquots in duplicated from the 10^{-7} and 10^{-6} dilutions into more Petri dishes.

Pour 15-20 ml YMA (kept melted at 50°C in a water-bath) aseptically onto each of the cell suspensions in the Petri dishes. To disperse the cells evenly, gently move each Petri dish clockwise and counterclockwise allowing an equal number of swirls in each direction. To further ensure uniform dispersion of the cells, move the Petri dish three times forward and backward, then to the left and right. Allow the agar to set, invert the dishes and incubate at 26-28°C. Read the plates after 3-5 days. You have now completed the pour-plate method.

Prepare serial dilutions of TAL 379. Make pour-plates with dilutions 10^{-8}, 10^{-7}, and 10^{-6} in duplicates. Incubate the plates for 7-9 days, checking daily during the incubation. Lens shaped colonies develop in the YMA and normal colonies develop on the surface.

Multiply the average number of colonies by the dilution-factor. If the average number of colonies at 10^{-7} dilution is 50, then the original broth culture had a concentration of:

(number of colonies) X (dilution factor) X (vol. of inoculum)

(50 colonies) X (10^{7}) X (1.0ml)

$= 50 \times 10^7$ cells per ml $= 5.0 \times 10^8$ cells per ml

A similar technique called the spread-plate method is also commonly used. Use the same serially diluted samples of TAL 379 prepared for the pour-plate method above. Begin with the 10^{-7} dilution and deliver 0.1 ml of the sample into each of four plates of YMA previously dried at 37°C for about 2 h. Using the same pipette, dispense 0.1 ml samples from the 10^{-6} and 10^{-5} dilutions, in that order. Prepare a glass spreader by bending a

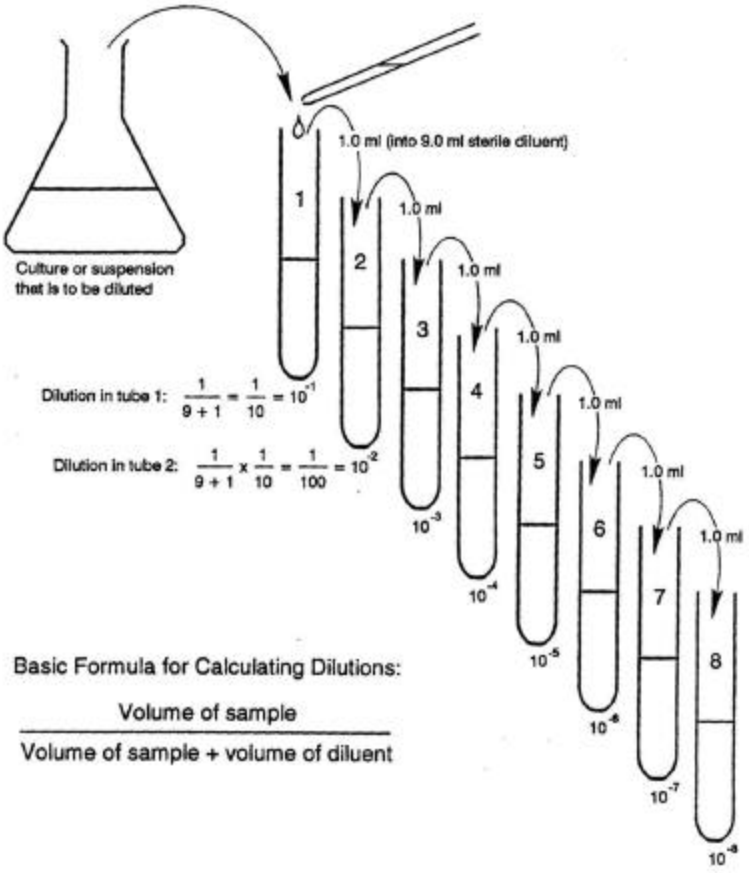

Figure 4.2. Procedure for serial dilution

20 cm glass rod of 4 mm diameter to the shape of a hockey stick, dip it in alcohol, and flame; then cool the spreader by touching it to the surface of a separate YMA-plate. Lift the cover of each Petri-dish just enough to introduce the spreader and place it in position on the agar surface. Spread the

sample evenly over the agar surface, sterilizing and cooling the spreader between samples. Incubate as before. Calculate the number of viable cells as outlined for the pour-plate method, adjusting for the smaller volume that was plated (0.1 ml instead of 1.0 ml).

Both of the above methods are lengthy and require a large number of Petri dishes. A variation known as the Miles and Misra drop-plate method is more rapid and consumes less materials. Use agar plates which are at least 3 days old or have been dried at 37°C for 2 hours. Radially mark off eight equal sectors on the outside bottom of the Petri dish. Label four sectors for replications of one dilution and four for another, allowing two dilutions per plate.

For this technique calibrated pipettes are required. Calibrate at least 10 pipettes by the following method:

Determine the weight of 100 drops of water on a sensitive balance or the volume of 100 drops of water in a small measuring cylinder.

Calculate the weight or the volume of a single drop by dividing the total weight or volume by 100.

Pipettes with the same tip diameter (e.g., external diameter of 1 mm) deliver drops of virtually the same volume. After

the drop size of a calibrated pipette has been established, more pipettes of the same tip diameter may be selected using a wire-gauge. Alternatively, any Pasteur pipette may be cut to the same tip diameter with a fine file after matching its tip with a wire gauge.

Use the dilution series of TAL 379 which had been prepared earlier. Plate dilutions of 10^{-7}, 10^{-6}, and 10^{-5}. Using a calibrated Pasteur pipette fitted with a rubber bulb, begin with the highest dilution and deliver 1 drop to each of the appropriate four sectors of the plate. Two dilutions can be shared by one plate.

To do this, hold the pipette vertically, about 2 cm above the agar surface, exert just enough pressure on the bulb to deliver one drop. Use the remaining four sectors of the plate for the next dilution. Allow the drops to dry by absorption into the agar; then invert and incubate at 26-28° C.

The drop-plate method requires more practice than the other methods. Results may not match those of the pour-plate and spread-plate methods at the first attempts. It is advisable to practice drop-plating with water before using this method for the first time. Fewer colonies per drop require more drops to be counted to provide the same statistical precision.

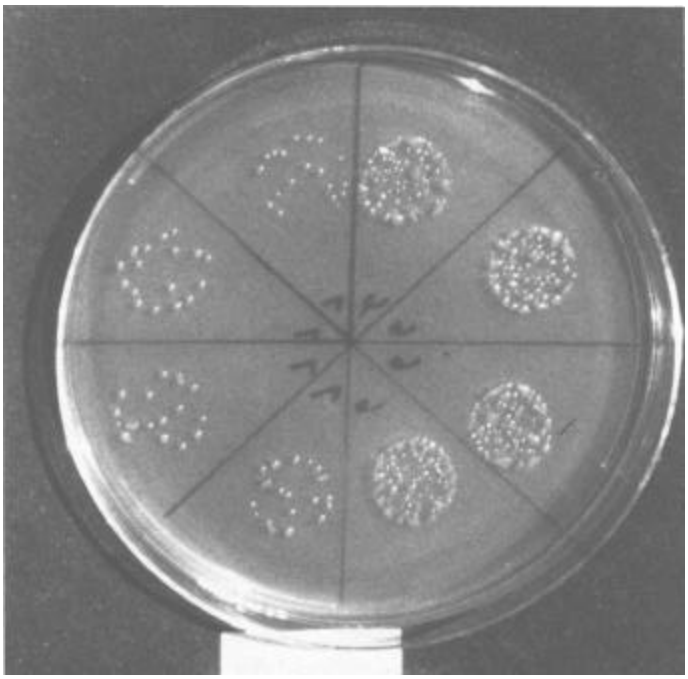

Figure 4.3. Growth of colonies of Rhizobium sp. from drops plated by the drop-plate method.

After 3-5 days of incubation, with daily observations, count the colonies formed by TAL 182. Open the Petri dish, invert it, and place on the illuminator of a colony counter. With a fine tipped felt pen, mark each colony counted while simultaneously operating a tally counter. Record your counts. The preferred counting range should be 10-30 colonies per drop.

If a pipette with a 14 gauge tip is used, one drop will be 0.03 ml. Divide 1 ml by 0.03 and multiply by the dilution factor and the average number of colonies per drop. Example, if the average number of colonies per drop is 30 at 10^{-5} dilution, the number of viable cells are:

$$(1/0.03) \times 30 \times 10^5 = 1000 \times 10^5 = 1 \times 10^8/ml$$

Compare the viable count of TAL 182 with its total count and calculate the percentage viability in the original culture.

At the end of a 7-10 day incubation period, count the colonies of TAL 379 on plates prepared by the three methods. Calculate the number of viable cells per ml and compare the results obtained by the different methods. Discuss the advantages and disadvantages of the three plating methods.

Bear in mind that plate counts, of whatever variety, are of value only for counting the viable rhizobia in pure culture. There is no selective medium that permits growth of Rhizobium alone. Therefore, quantifying Rhizobium in soil is difficult. Also, the plating methods do not distinguish between strains or species of Rhizobium having similar visual colony characteristics on YMA. When it is necessary to quantify the occurrence of viable cells of a particular Rhizobium in non-sterile materials, a plant infection method must be employed (Exercise 5).

f) Determining the mean-generation (doubling) time of rhizobia

(Key steps 8, 9, and 10)

The time required for a doubling of a given cell population is referred to as the generation time.

The growth of Rhizobium in broth culture is followed for a period of 7 days. Viable counts are made each day throughout the duration of the experiment. A growth curve is obtained by plotting the viable count versus time. From the curve, the mean-generation (doubling) time is computed.

Two strains of Rhizobium, the fast growing TAL 182 (R. phaseoli) and the slow-growing TAL 379 (R. japonicum) are used in this experiment.

A total of sixteen 250 ml Erlenmeyer flasks, each containing 100 ml of full strength YM broth will be needed for each strain.

Prepare 32 flasks for the two strains. Measure accurately 100 ml of YM-broth into each flask and sterilize.

Obtain 1 ml each of the fully grown cultures of TAL 182 and TAL 379 from broth cultures prepared previously in this exercise. By the serial dilution procedure, dilute each culture to give 1×10^6 cell ml^{-1}. (It is approximated that when fully grown, each strain will have at least 1×10^9 cells ml^{-1}).

Inoculate each flask with 1 drop (0.03 ml $drop^{-1}$) of the diluted broth culture. Use a calibrated Pasteur pipette for the inoculation. Inoculate 16 flasks with TAL 182 and another 16 with TAL 379. Two flasks will be sampled each day for each strain. Incubate flasks on a rotary shaker (100 rpm) at room temperature (26-28◦C).

Based on the presence of at least 1 x 10^6 cells ml^{-1} in the diluted broth, and by inoculating 0.03 ml or 3.0 x 10^4 cells of this sample into 100 ml of the broth, the starting number of cells at zero-time should be 3.0 x 10^2 cells ml^{-1}.

Perform a zero-time viable count for both strains. Remove 1 ml and dilute in 9 ml of quarter strength YM broth to give a 10^{-1} dilution and plate this dilution in duplicate by the spread-plate method.

Perform viable counts for each culture every day for 7 days, taking care to allow the full 24 hours between counts.

The extent of dilution of a culture, the choice of dilutions to be plated, and the volume (0.1 ml by spread-plate or 0.03 by drop-plate) to be plated will depend on the rate at which turbidity develops during growth.

Obtain the mean viable count for each day and transform the

values to $\log_{10}$. Plot viable count (Y-axis) versus time (X-axis). Draw a smooth curve through the points.

The mean generation time is computed using values from the exponential phase. From the exponential phase, choose a straight line portion of the curve and note the values for viable count and time. Obtain the number of generations by transforming the value for viable count from $\log_{10}$ to $\log_2$ using the relationship:

$$\log_a x = \log_b x / \log_b a$$

when a = 2 and b = 10

then $\log_2 x = \log_{10} x / \log_{10} 2$

since $\log_{10} 2 = 0.3010$

Therefore, $\log_2 x = \log_{10} x / 0.3010$

Divide the time (hours) by the number of generations to obtain the mean generation time.

Compare the mean generation time of TAL 182 with that of TAL 379.

Requirements

(a) Preliminary culturing of fast- and slow-growing rhizobia

Transfer Chamber
Rotary shaker
Flasks (four) containing 50 ml YM broth each
Pipettes (10 ml sterile)
Slant cultures of TAL 182 and TAL 379

(b) Becoming familiar with the Petroff-Hausser and Helber counting chambers

No requirements

(c) Using the Petroff-Hausser and Helber counting chambers

Phase contrast microscope
Petroff-Hausser counting chamber and covers
Pasteur pipettes, rubber bulb
Pipettes, 10 ml
Wash bottle with distilled water
Small beaker with diluted liquid soap
Test tubes and rack
Tally counter
Broth cultures of TAL 182 and TAL 379

(d) Estimating cell concentration by optical density

Spectrophotometer, cuvettes
Pipettes, 10 ml
Test tubes, rack
Broth-cultures of TAL 182 and TAL 379 from (b)

(e) Determining the number of viable cells in a culture by plating methods

Incubator, balance, water bath, colony counter, tally counter
Wire gauge (obtainable through Scientific Products, USA)
Dilution tubes with 9 ml sterile ¼ strength YM broth
Test tube rack
Pipettes 1 ml, sterile
Suction bulb
Liquid YMA in flask
Pasteur pipettes
Glass rod or spreader; beaker with alcohol, flame
Small beaker with water; small beaker (empty)
Sterile Petri dishes
YMA-plates
Broth-cultures of TAL 182 and TAL 379 from (b)

(f) Determining the mean generation (doubling) time of rhizobia

Rotary shaker, colony counter, autoclave

Spreader, small beaker of alcohol, flame

Pipettes (1 ml, sterile)

Erlenmeyer flasks (32) with 100 ml YM broth each

Dilution tubes with 9 ml sterile ¼ strength broth each

Plates of YMA

EXERCISE 5

TO COUNT RHIZOBIA BY A PLANT INFECTION METHOD

The plant infection count (also called the most-probable-number (MPN) count) is used to determine the number of viable rhizobia in the presence of other microorganisms. This indirect method is commonly used to determine the quality of inoculants produced from non-sterile carrier materials. In this exercise, the quality of inoculants prepared separately from presterilized and nonsterilized peat is determined by the plate count and MPN count methods. The results are compared for agreement between the two methods.

Key steps/objectives

1) Prepare peat inoculants
2) Prepare growth pouches
3) Surface sterilize and pregerminate seeds
4) Transfer pregerminated seeds from seedling agar to growth pouches
5) Prepare serial dilutions of peat sample(s); initiate MPN and plate counts
6) Make periodic observations of plants and water if needed
7) Count colonies on plates
8) Harvest and record nodulation
9) Determine the MPN

10) Compare results of plant infection and plate counts

(a) Preparing inoculants

(Key step 1)

Start in duplicate, 100 ml cultures of a strain of slow-growing Rhizobium e.g., Rhizobium japonicum (TAL 102) in 250 ml flasks. Aerate on a rotary shaker for 7 days.

Test the purity of the fully grown broth culture by Gram-stain (Exercise 3), pH measurement and agglutination with its specific antiserum as described in Exercise 20.

Prepare or obtain two sealed polyethylene bags of 50 g neutralized peat sterilized by gamma irradiation or neutralized peat packaged and sealed in autoclavable bags sterilized by autoclaving (Exercise 21).

Also needed are two sealed polyethylene bags, each of which contain 50 grams of non-sterile peat.

Following the methods described in Exercise 21, inject 40 ml per bag of the fully grown broth cultures (1×10^9 cells ml^{-1}). Prepare two bags of TAL 102 in sterile peat and two bags of TAL 102 in non-sterile peat to produce the peat based inoculants. (Remember to inject the bags of sterile peat

first). Allow the inoculants to mature at 25-30°C for at least two weeks.

(b) Setting up the plant dilution count in plastic growth pouches

(Key step 2)

The pouches used in this exercise are made of polypropylene (16 x 18 cm) with paper wick liners obtainable from Scientific Products, Evanston, Illinois, USA. Growth pouches serve well as inexpensive space saving substitutes for Leonard jars. They are susceptible to contamination introduced by air and insects. Also, they are not shielded against radiated heat. Their use is, therefore, restricted to growth chambers or growth rooms. As in growth tubes, plants cannot be grown to maturity in such a system.

Leonard jars and growth tubes are also frequently used for MPN counts. Leonard jars (Appendix 11) are convenient growth units for large seeded legumes and are primarily used in the greenhouse. Growth tubes are used on growth shelves or in growth chambers where space is limited. As in authentication (Exercise 1), a large-seeded legume of the same cross-inoculation group may be substituted by a small-seeded one for the MPN count. Growth tubes (seedling-agar slants) may then be used to save space and labor (Appendix 7).

Place 30 ml of plant nutrient solution (Appendix 3, Table A.1) into each growth pouches. (The growth pouches purchased are sterile. However, if contamination is suspected, the pouches may be sterilized by autoclaving after inclusion of the plant nutrient solution.) Arrange the pouches in a rack (Figure 5.1). Set up one rack of 60 pouches for each bag of inoculant to be tested. Suggestions for building a growth pouch rack are given in Appendix 8.

(c) Planting seeds in growth pouches
(Key steps 3 and 4)

Surface sterilize and pregerminate 100 soybean seeds as explained in Appendix 10. Select seeds of uniform size and high viability (95-100%). Use more seeds if the viability rate is lower.

Select 60 well germinated seeds of similar size and radical length (1-1.5 cm). Transfer one seed to each pouch aseptically. Place each seed in the trough of the paperwick.

To prevent the growing radical from pushing the seed out of the pouch, a hole is made in the trough of the wick and the radical is inserted into the hole during planting. 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 the other for making the hole.

Figure 5.1. Soybean plants growing in growth pouches.

When the plants are 5-7 days old, reorganize the growth pouches on the rack. Discard plants of poor growth and select 50 healthy plants. You will need forty pouches to count dilutions for 10^{-1} - 10^{-10} in quadruplicate plus one control pouch following each group of four. This brings the number of pouches needed to 50. Repeat this set-up in separate racks for each inoculant to be tested.

(d) Inoculating for the MPN count

(Key steps 5, 6, 7, and 8)

Make a tenfold dilution of each inoculant bags by transferring the content of each bag (100 g) into separate 2.0 liter flasks

containing 900 ml of sterile water. Remove the peat through a cut at one corner of each bag. Close each flask with a sterile rubber stopper and shake vigorously for 5 minutes by hand. Make a dilution series for each of the 4 samples from 10^{-1} to 10^{-10}.

Plate the 10^{-5}, 10^{-6}, and 10^{-7} dilutions of each inoculant.

The drop-plate method (Exercise 4) may be used for inoculants prepared from sterile peat. Plate in quadruplicate on YMA-agar containing Congo Red.

Inoculants prepared from non-sterile carriers should be plated by the spread-plate method on YM-agar containing a fungicide such as Brilliant Green (1.25 p.p.m.) or Pentachloronitrobenzene (PCNB) (0.5 g in 100 ml acetone plus 1 drop of Tween 80 added to 400 ml of medium). Plate in duplicates and, if possible, include an additional YMA plate containing Congo Red for each dilution. Incubate at 25-30◦C for 5-8 days.

Similarly inoculate the plants which have been set up for the MPN count. Pipette 1 ml of each dilution (from 10^{-1} - 10^{-10}) to each one of the four replicates in each set. Begin by taking aliquots from the highest dilution and proceed down the series with the same pipette.

Observe the plants periodically and replenish the nutrient solution if necessary. Nodulation may be evident after 2 weeks. Make the final observation after 3 weeks and record presence (+) or absence (-) of nodules.

Count rhizobia on plates (Ex. 4)

(e) Determining the MPN

(Key steps 9 and 10)

For each set, write down the dilutions used and record the nodulation.

The actual number of nodules on each plant and the number of plants in each replication have no bearing on the MPN count. If replications are in quadruplicate, the reading may be 4, 3, 2, 1, or 0 nodulated units. The highest dilution used should show no nodulation in each replication, indicating the absence of rhizobia.

Refer to tables, (Appendix 14) indicating tenfold dilutions (Table A.10) for the estimation of the number of rhizobia by the plant infection method.

If twofold or fourfold dilutions are used, refer to Tables A.8 and A.9, respectively.

The number of replications is indicated by "n", and "s" signifies the number of dilution steps.
Dilutions may be made in duplicate or quadruplicate. Each series should end with a dilution at which no nodules are formed.

The MPN is calculated from the most likely number (m) found in the MPN tables. To find this number, use the procedure shown in the example below:

l) Record nodulation (+ or -) as shown in Table 5.1
2) Take note of the number of replications used (n=4)
3) Count the number of dilution steps used (s=10)
4) Add up the total number of (+) units (+=18)
5) Find this number 22 in Table A.10 (calculated for tenfold dilutions)
6) Locate the most likely number (m) in column s=10, on the same line as 18, which is 5.8×10^3

The MPN may now be calculated from "m" by using the following formula:

m = likely number from the MPN table for the lowest dilution of the series
d = lowest dilution (first unit or any unit in which all replicates are nodulated)
v = volume of aliquot applied to plant

The MPN per gram of inoculant is:

$$X = \frac{m \times d}{v} = \frac{(5.8 \times 10^3) \times 10^2}{1} = 5.8 \times 10^5 \text{ rhizobia } g^{-1} \text{ inoculant}$$

Table 5.1. Example for recording nodulation for the MPN count

	NODULATION				
	----------Replications----------				NUMBER OF NODULATED UNITS
DILUTION	I	II	III	IV	
10^{-2}	+	+	+	+	4
10^{-3}	+	+	+	+	4
10^{-4}	+	+	+	+	4
10^{-5}	+	+	+	+	4
10^{-6}	+	-	+	-	2
10^{-7}	-	-	-	-	0
10^{-8}	-	-	-	-	0
10^{-9}	-	-	-	-	0
				Total	18

For additional information refer to Appendix 14, essential to this exercise for the evaluation and understanding of the plant infection count.

Compare results obtained by the plant infection (MPN) and plate count methods.

Requirements

(a) Preparing inoculants

Platform shaker, incubator, waterbath

Agglutination tubes and rack; test tubes; rack

Pipettes 1 ml; pipettes 10 ml

Saline; flame; alcohol in spray bottle; adhesive tape

Sterile 50 ml syringe; 18 gauge needles

Requirements for Gram stain (Appendix 3)

Solution of BTB (0.5% in alcohol)

Erlenmeyer flasks (four) of 250 ml containing 100 ml broth

Sterile peat, 50 g polyethylene bag^{-1} (two)

Nonsterile peat, 50 g polyethylene bag^{-1} (two)

Culture of TAL 102; antiserum to TAL 102

(b) Setting up plastic growth pouches

Growth chamber; autoclave

Forceps, flame

Measuring cylinder (50 ml) or adjustable filling unit

Growth pouches 16 x 18 cm with paper wick liners (obtainable from Scientific Products, Evanston, IL, USA.)

Plant nutrient solution (Appendix 3)

(c) Planting seeds in growth pouches

Requirements for seed sterilization (Appendix 10)

Water agar plates

Soybean seeds

(d) Inoculating for the MPN count

Incubator

Pipettes, 10 ml sterile; pipettes, 1 ml sterile

Pasteur pipettes, calibrated, sterile; rubber bulbs for Pasteur pipettes

Flame; spray bottle with alcohol

Erlenmeyer flasks of 2 liter capacity (four) containing 900 ml sterile water each

Rubber stoppers, sterile, to fit 2 liter flasks

Dilution tubes with 9 ml sterile water; racks

Plant nutrient solution

YMA containing Congo Red (YMA-CR)

YMA containing Brilliant Green (YMA-BG)

Plants in growth pouches from (b)

Peat inoculant from (a)

(e) Determining the most probable number in peat

Records of observations

MPN tables (Appendix 14)

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SECTION B

STRAIN IDENTIFICATION INTRODUCTION

Rhizobia that have dramatic differences in such important traits as host specificity, infectiveness (invasiveness) and effectiveness are indistinguishable from each other under the microscope. However, there are many circumstances in which recognition of a particular rhizobial strain and monitoring its occurrence following introduction to a soil environment is important in ecological studies. Indirect procedures are available for this purpose.

Serological markers

Any substance which provokes an immune response when introduced into the tissue of an animal or human is referred to as an antigen. In work with rhizobia, rabbits are commonly used for immunization and the antigens are rhizobial cell preparations. As a result of antigen injections, complex immunological reactions result in the rabbit producing special proteins called globular antibodies (immunoglobulins). These antibodies are found in the serum portion of the blood, and the study of the reactions of the immune serum with the antigens outside the animal is known as serology. Antigen-antibody reactions are highly specific in that the antibody reacts only with the antigen that elicited its

formation.

As in other bacteria, antigens of rhizobia can be categorized into somatic, flagellar, and capsular, depending on their derivation. Somatic antigens are closely related to the rhizobial cell-wall and usually designated by the letter "O". Some somatic antigens may be tightly bound to the cell wall, in which case they are not removed by washing of the cells; therefore, these antigens are only detected when whole cells of rhizobia react with the antibody as in agglutination or immunofluorescence. The somatic antigens that are soluble and easily removed by washing are detected by precipitation in gel, as in the Ouchterlony double-diffusion process. Somatic antigens are also heat stable. They are the most specific of the three groups of antigens.

The tiny whip-like appendages (flagella) of the rhizobia are also antigenic and appropriately called flagellar or H-antigens. They are heat labile and are commonly detected by agglutination or immunofluorescence.

The capsular (extracellular) antigens are surface antigens and are found outside the cell itself. They are usually designated by the letter "K."

In rhizobial serology, both cultured cells and nodule antigens (bacteroids) are used for strain identification. Basic concepts on some serological methods for identification of

rhizobia are described below.

Agglutination. The process in which the antigens are linked together by their corresponding antibodies is called agglutination. The linked antigens may be microscopically or macroscopically visible as clumps, agglutinates or aggregates. The agglutination reaction depends on a firm structural relationship between an exposed bacterial antigen and the antibody. Linus Pauling's lattice hypothesis (Figure B.1) is the widely accepted concept for explaining the agglutination reaction. Pauling postulated that the antibody is bivalent and the antigen is multivalent, and that the antigen-antibody complexes are molded into a lattice or framework of alternating antigen-antibody particles.

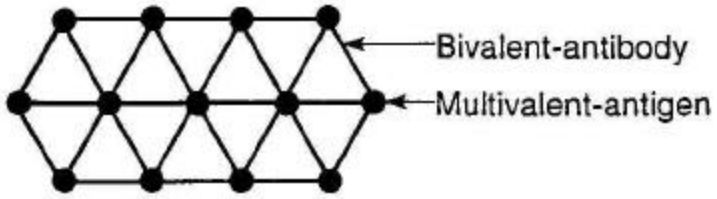


Figure B.1. Lattice formation in an antigen-antibody reaction

Precipitin reaction. In recent years the precipitation reactions of somatic antigens have been used extensively for work with rhizobia. The precipitation reaction occurs when

certain soluble antigens are brought into contact with the corresponding antibody. Precipitation differs from agglutination in that the precipitating antigens are not whole bacterial cells (cellular) but are proteins or polysaccharide molecules in solution. In the double-diffusion technique, gels, usually clarified agar, are used as matrices for combining diffusion with precipitation. The reactants simply diffuse through the gel towards each other and precipitation results when the equivalence points have been reached. Antigen preparation of a rhizobial strain will give rise to one or more lines of precipitation in the presence of the homologous antibody. When two antigens are present in a system, they behave independently of one another. The different types of precipitation reactions are illustrated in Figure B.2.

Rhizobial strains that share some of or all their antigens will cross-react with respective antisera. These cross-reactions may be encountered in both agglutinations and precipitations.

Immunofluorescence. Certain chemical dyes (fluorescein isothiocynate and lissamine rhodamine) have the property of fluorescing when excited by near ultraviolet light. Rhizobial antibodies developed in rabbits can be conjugated to these

fluorescing chemical dyes or fluorochromes. In work with rhizobia, the chemical dye commonly used for labeling the specific antibody is fluorescein isothiocyanate (FITC) which has an apple-green fluorescence upon irradiation with blue light. In practice, a smear of rhizobial cells (cultured, or from a nodule) is made on a microscope slide, and this smear is allowed to react or "stain" with the specific antibody labeled with FITC. After appropriate washing to remove uncombined and excess labeled antibody, the smear may be viewed through a UV-microscope fitted with appropriately complementary filters, and an apple-green fluorescence of the bacterial (rhizobial) cells would mean that the antigen smear has reacted with the FITC-labeled antibody.

There are two types of fluorescent antibody techniques, namely the direct- and indirect-immunofluorescence. In the direct method the specific antiserum is conjugated and is used as a "stain" in the procedure. This is different from the indirect method where the unconjugated (unlabeled) specific or primary antibody is first reacted with the antigen smear, and after sufficient time is allowed for antigen-antibody reaction, the smear is then washed free of excess antiserum. This step is followed by "staining" with the FITC-labeled secondary antibody.

In serological work with rhizobia, the specific or primary antibody against the rhizobial strain is most often developed

in rabbits. The secondary antibody is developed by immunization of goats or sheep with purified rabbit immunoglobulins from a previously unimmunized rabbit. Thus, the rabbit immunoglobulin serves as an antigen for immunization of the goat or sheep. Therefore, the antibody produced in the goat or sheep will not only react with the rabbit antiserum, but also with rhizobial

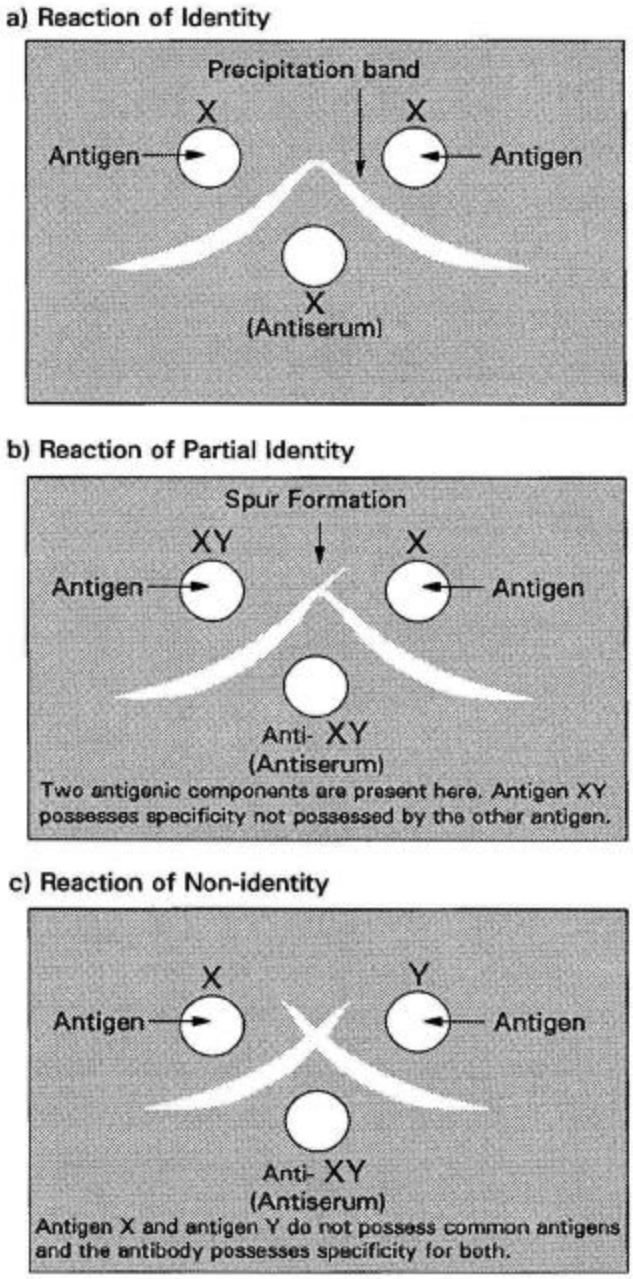


Figure B.2. Precipitation reactions

antigen with specific unlabeled rabbit antibody attached when

the indirect procedure is employed. Though the results are the same, the indirect method is considered more sensitive. The indirect method requires the labeling of only the immune serum from the goat or sheep, and involves two reaction steps; but the indirect method is also known to give more nonspecific staining reactions. In the direct method, each rabbit antiserum developed against each rhizobial strain must be conjugated. The two methods are illustrated diagrammatically in Figures B.3 and B.4.

Antibiotic resistance markers

When high density inocula of a rhizobial strain are inoculated into media containing an antibiotic, a few cells may exhibit resistance as a result of spontaneous genetic changes or mutations. The resistance of a rhizobial strain to a particular antibiotic is a useful marker. If the mutant strain is used to inoculate a legume then nodules occupied by that strain may be identified by plating nodule isolates on media containing the respective antibiotic. The mutant rhizobial strain will grow on the antibiotic media and other bacteria will be suppressed. It is important that antibiotic-resistant mutants that are selected for inoculation experiments have not lost their infectiveness (ability to form nodules) nor their effectiveness (ability to fix nitrogen) in the symbiosis with the host plant. The symbiotic capacity of the mutant should be compared with its parent culture from

time to time. The mutant should be stable throughout the steps of infection, nodulation, nitrogen-fixation and subsequent re-isolation.

Streptomycin resistance is frequently used as a marker for rhizobia. Mutants resistant to this aminoglycoside are stable, have a low incidence of cross-resistance, and infrequently lose their symbiotic capacity. Besides streptomycin, spectinomycin and rifampicin have also been used. Highly resistant mutants with single or double markers (streptomycin-spectinomycin or streptomycin-rifampicin) can be obtained with one exposure of the rhizobia to low concentrations of these antibiotics or by successive selection for resistance.

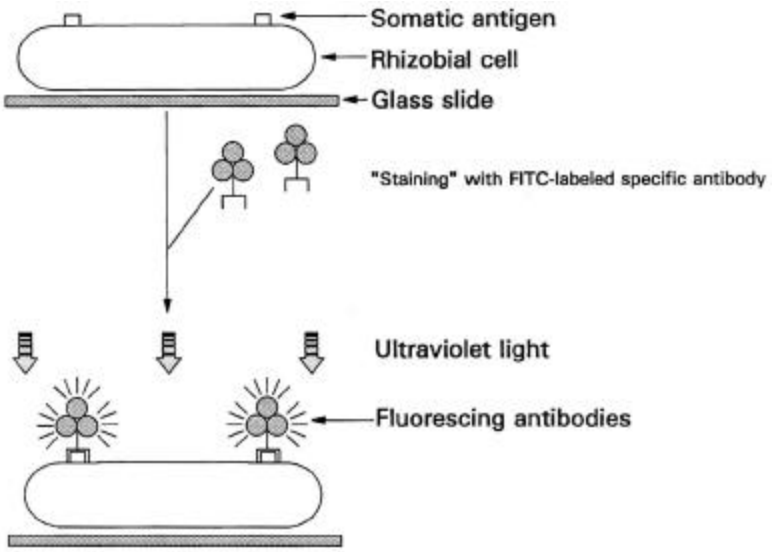


Figure B.3. Direct immunofluorescence

Cross-resistance is a phenomenon whereby a bacterium develops resistance to a second antibiotic as a result of resistance to the first. This may happen if the antibiotics are closely related.

The parallel use of antibiotic and serological markers, both relatively stable in themselves, provides a means of confirming the stability of each marker independently in ecological research with rhizobia. Compared to the serological marker techniques (fluorescent antibody, enzyme immunoassays, gel-diffusion and agglutination) the development and use of antibiotic resistant

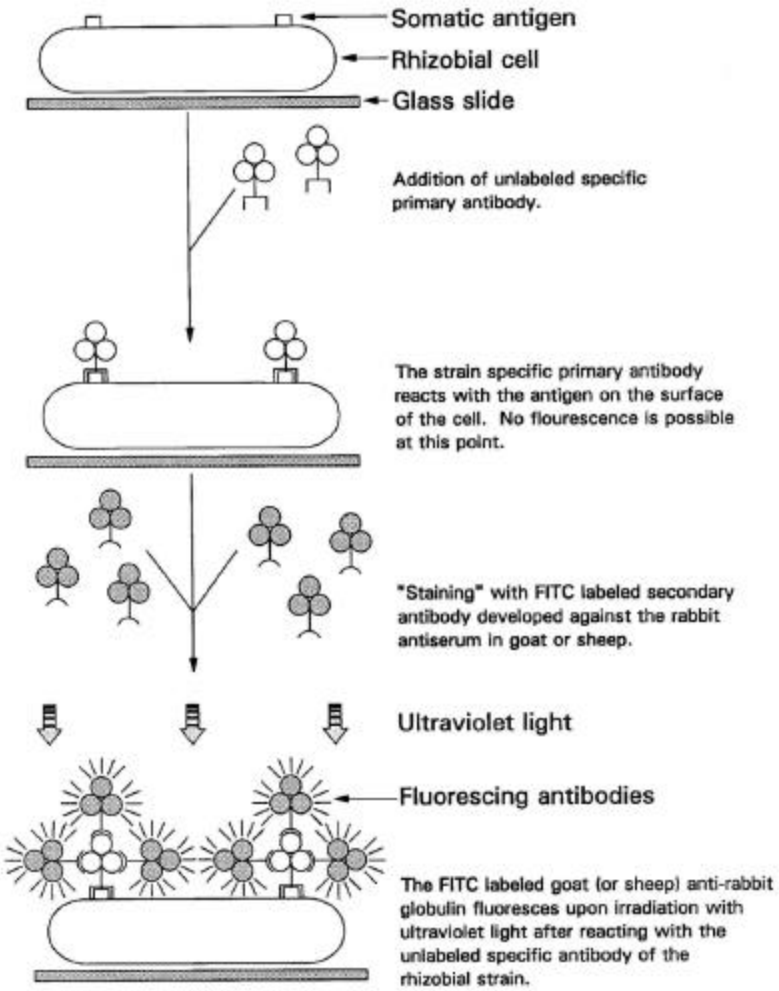


Figure B.4. Indirect immunofluorescence

markers is relatively inexpensive and does not require sophisticated equipment.

Bacteriophage markers: phage typing

Viruses that infect bacteria (bacteriophage) were independently discovered by Twort and by d'Herelle in 1917 and 1919, respectively. Since then, the processes of infection and multiplication have been well defined. The first step

involves the adsorption of the virus to specific receptors on the bacterial cell (somatic), flagella or pilli. This is followed by the injection of the viral nucleic acid into the bacteria. The nucleic acids utilize the machinery of the host cell to replicate, leading to the accumulation of several copies of the viral nucleic acid. These nucleic acids are packaged newly-synthesized viral coat protein and are then released by lysis of the host cell, liberating many infective viruses.

Susceptibility of a certain bacterial strain to a particular bacteriophage forms the basis for phage-typing. One approach in a phage-typing scheme is the use of a group of phages with different host-specificities. The bacteria can then be placed into groups (lysotypes) if they are susceptible to some of the phages and not others. Through this means, bacteriophage-marked rhizobia can be indirectly traced in soil, in isolates from nodules and in laboratory experimentation.

EXERCISE 6

TO DEVELOP ANTISERA

Rabbit antiserum developed against Rhizobium is the basic reagent for strain identification utilizing serological techniques. In this exercise, Rhizobium antigens are prepared and then used for the development of antiserum in rabbits.

Key steps/objectives

l) Culture rhizobia on YMA bottle flats
2) Check for culture purity by Gram stain
3) Harvest and prepare antigens for immunization
4) Begin immunization; inject antigen intramuscularly
5) Inject antigen intravenously
6) Give intraperitoneal injections
7) Trial bleed
8) Determine the antiserum titers
9) Harvest blood through cardiac puncture
10) Give subcutaneous booster injections

(a) Culturing rhizobia for antigen
(Key steps 1 and 2)

Inoculate selected strains of rhizobia on two 500 ml YMA flats and incubate at 26^{o}C. Broth culture in 50 ml Erlenmeyer

flasks may also be used but the medium must be fully defined to avoid complications with antigenic components from the yeast in YM broth (see Appendix 3).

Check for purity (by Gram stain) at the end of the specified time for growth, e.g., 3-5 days for fast-growers and 7-10 days for slow-growers. Strains that produce a lot of "gum" should be harvested earlier.

(b) Preparing antigens for immunodiffusion

(Key step 3)

When the cultures are ready for harvest, aseptically add about 10 ml of sterile, filtered saline and 20 sterile glass beads to the YMA-slants. Close the culture vessel and hold it level so that the saline irrigates the entire surface. Tilt back and forth so that the glass beads dislodge the rhizobial cells into suspension. Transfer the suspension (but not the glass beads) to sterile centrifuge tubes and spin down the cells at approximately 5,000 X g for 15-20 min. Discard the supernatant and resuspend the precipitate again in sterile saline. The gummy substance in the supernatant consists of polysaccharides and is found especially in older cultures. It should be discarded at this point. Do not repeat the centrifugation as excessive washing would remove the soluble antigens essential to the immunodiffusion reaction. Resuspend

the precipitate by dropwise addition of sterile saline and with frequent agitation to obtain a thick suspension of 1 x 10^{10} cells ml^{-1}.

Store about one-half of the thick suspension in the refrigerator, for reference. Dilute the remainder to 1 x 10^{9} cells ml^{-1} using the McFarland standards (Appendix 6). Dispense the diluted suspension into small (5 ml) sterile serum vials in 2 ml portions to be used for injections. Add a preservative (1% merthiolate) to each 2 ml sample and also to the thick suspension. Merthiolate is used extensively in serology as a preservative. When used in liquids at a final concentration of 1:10000, it does not interfere with serological reactions. The vials may be stored at 4°C for several weeks or kept frozen for several months.

(c) Preparing somatic antigens for the agglutination and fluorescent antibody techniques

(Key step 3)

The insoluble somatic antigens found on the surface of the cells are required. Soluble and most of the flagellar antigens are eliminated by frequent washing.

Harvest a fully grown culture from YMA-flats as before. Cells should be centrifuged, the supernatant discarded, and the pellet resuspended in filter sterilized saline, using a vortex

mixer. This sequence of centrifugation and resuspension is repeated three times and the cell concentration is adjusted to approximately 1 x 10^9 cells ml^{-1}. Transfer the suspension to a sterile serum bottle and close with a rubber septum. Insert a small gauge (about 23 gauge) needle through the septum to act as an air and steam vent. Heat the antigen for 1 h at 100°C to inactivate any remaining flagellar antigens. This is accomplished by partly immersing the serum bottle in boiling water or by subjecting it to heat in a steam bath. Add merthiolate solution to the antigen suspension after heating.

(d) Immunizing the rabbit
(Key steps 4, 5, and 6)

A variety of injection schedules have been used to produce antisera of sufficiently high titers. Three examples are given in Appendix 12. The schedule used in this chapter employs three different routes of injection.

Pipette 2 ml of antigen and 2 ml of Freund's complete adjuvant into a 50 ml beaker and emulsify by repeatedly drawing the mixture into a glass or plastic syringe (no needle attached) and expelling it through the orifice. The right consistency is reached when a drop of this emulsion does not disperse immediately in water. Freund's complete adjuvant is made from mineral oil and killed cells of *Mycobacterium tuberculosis* or *M. butyricum*. It is used to enhance the effect of the

antigen.

Inject 1 ml of the antigen-adjuvant emulsion into the thigh muscle on each hind leg of the rabbit.

After 2 weeks, give an intravenous injection of 1 ml of antigen without adjuvant.

After 4 weeks give an intraperitoneal injection of 1 ml antigen without adjuvant.

(e) Trial bleeding for titer determination
(Key steps 7 and 8)

Seven days after the last injection, conduct a test-bleed through the marginal ear vein. (Appendix 12).

Transfer the blood into a sterile screw-cap test tube. Allow the blood to clot at room temperature for approximately 2 h. Detach the blood clot from the test tube wall by moving a wooden applicator stick around the clot. Refrigerate overnight to separate the serum from the clot.

Decant clear serum into a test tube, minimizing carry over of red blood cells. Since only a very small amount of blood is obtained in the trial bleeding, centrifugation may not be practical. Determine the agglutination titer (Exercise 7).

(f) Collecting blood and giving booster injections
(Key steps 9 and 10)

If the titer is satisfactory (not less than 1:1600), bleed the rabbit from the heart by cardiac puncture using a bleeding rack as shown in Figure A.14 of Appendix 12. Obtain 30-50 ml of blood. Transfer the blood into a sterile, screw-cap test tube of 50 ml capacity.

After the blood has been clotted and refrigerated, decant the serum and centrifuge at 5,000 x g for 15 min (under refrigeration, if possible) to clear the serum of red blood cells.

Transfer the clear serum supernatant into an appropriate container for storage by freezing. Serum should be stored in 1-2 ml portions in suitable sized vials. This may not be necessary if the blood is to be processed for conjugation with fluorescein isothiocyanate (FITC). Sera from different rabbits receiving the same antigen may be pooled.

If the titer was too low in the trial bleeding (less than 1:1600), give a booster injection of 1 ml antigen subcutaneously immediately after the titer determination. Bleed 1 week later by cardiac puncture.

If more antiserum is desired, the level of immunoglobulins in the rabbit can be maintained by booster injections 7-14 days after each bleeding. However, in such a case, it would be advisable to make intraperitoneal injections of sterile saline each time after the blood has been taken to replenish the liquid level in the animal. The volume of saline injected should be equal to the volume of blood taken from the rabbit.

Note: Storage vials should also be adequately labelled to indicate rhizobial strain, serum batch-number and date. Records of injection schedules and agglutination titers determinations should be noted. Weight, age, sex and other relevant information on the animals are also usually recorded.

Requirements

(a) Culturing rhizobia for antigens

Microscope, incubator
Microscope slides, immersion oil
Inoculation loop, flame
Gram stain solutions (Appendix 3)
Wash bottle with distilled water
YMA flats in 500 ml medicine bottles or
Erlenmeyer flasks (250 ml) containing 100 ml of a defined medium (Appendix 3)
Cultures of rhizobia

(b) Preparing antigens for immunodiffusion

Centrifuge, balance, vortex mixer (optional)
Sterile glass beads (4 mm diameter, sterilized and stored in test tubes at 20 beads per tube)
Pipettes (10 ml)
Centrifuge tubes (30-50 ml capacity) with caps, rack
Saline, sterile, (membrane filtered 0.85% NaCl w/v)

(c) Preparing antigens for the agglutination and the FA technique

Centrifuge, balance, vortex mixer (optional)

Stove or bunsen burner with tripod and gauze screen, or steam bath

Centrifuge tubes 30-50 ml with caps, rack

Pipette (1 ml)

Glass beads as in (b)

Saline, sterile

Serum bottle with rubber septum, syringe needle (small gauge)

1% membrane filtered merthiolate (also called Thimerosal) solution (B.D.H., Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, N.Y., or Sigma ChemicalCorp.)

Inoculated YMA flats from (b)

(d) Immunizing the rabbit

Large towel (approximately 100 cm x 75 cm)

Small sterile beaker (50 ml capacity)

Glass syringes (10 ml capacity)

Plastic syringes (1-5 ml capacity)

Syringe needles 20 gauge, 22 gauge and 26 gauge (sterile)

Freund's complete adjuvant (DIFCO Laboratories, Detroit, Michigan, USA)

Rhizobial antigens from (c)

(e) Trial-bleeding for titer determination

Refrigerator

Large towel, scalpel, vaseline, razor blade

Cotton wool or tissue paper, alcohol (70%)

Test tubes with caps, rack

Wooden applicator sticks or thin glass rods

Requirements for titer determination (Exercise 7)

(f) Bleeding for collection and injecting boosters

Centrifuge, balance, freezer

Bleeding rack (Appendix 12)

Syringes (with 18 gauge needles)

Screw-cap test tubes (50 ml), rack

Centrifuge tubes (50 ml with caps), rack

Vials 5 ml (for storage of serum)

Merthiolate solution (Appendix 4)

Alcohol 70%, cotton wipes or soft tissue paper

Glass vials (20 ml) for bulk storage

EXERCISE 7

TO PERFORM AGGLUTINATION REACTIONS WITH PURE CULTURES OF RHIZOBIUM

The somatic agglutination reactions of whole cells with homologous antiserum are studied. The titer of the antiserum is determined using the tray, tube, and microscope-slide methods.

Key steps/objectives

1) Culture rhizobia
2) Harvest culture for antigen preparation
3) Prepare serial dilutions of antiserum and perform titration in trays, tubes, and on microscope slides
4) Read and record titers

(a) Preparation of somatic antigens from cultured cells (Key steps 1 and 2)

Obtain a young broth- or agar-slant culture of a strain from Exercise 6. Inoculate in duplicate, two YMA slopes in 500 ml flat culture bottles with inoculum from the broth or slant culture. If a broth culture is used, 1-2 ml of the broth can be squirted onto the agar surface and spread with a loop.

Under aseptic conditions, harvest the culture in saline after 3-5 days for fast-growing and 7-10 days for slow-growing rhizobia. Wash the cells three times in filter-sterilized saline by repeated resuspension and centrifugation (5000-8000 rpm). To inactivate the flagellar antigens, heat treat the antigen preparation as in Exercise 6. Finally, visually adjust the concentration of cells to approximately 1×10^9 cells ml^{-1} with sterile saline, and use the McFarland barium-sulfate standards (Appendix 6). If a photoelectric nephelometer or spectrophotometer is available, the turbidity may be adjusted more precisely.

(b) Dilution of stock antiserum
(Key step 3)

Prepare the two-fold dilutions of the antiserum as follows:

Arrange 10 test tubes (16 x 125 mm) in a row on a test tube rack. Label them 1 through 10. Pipette 9.6 ml of saline into Tube 1. Pipette 2.5 ml of saline into tubes 2-10.

Accurately pipette 0.4 ml of the stock antiserum into Tube 1. Mix the saline and serum thoroughly by sucking the serum-saline mixture into the pipette and then expelling the contents. Repeat this process five times. Expelling should be done gently to avoid frothing. This tube now contains antiserum of a 1/25 dilution.

Using a fresh pipette, remove 2.5 ml of diluted serum from Tube 1 and transfer to Tube 2. Mix well. (The dilution of the serum in Tube 2 will be l/25 x l/2 = 1/50.)

Using a fresh pipette each time, repeat the dilution down the series by transferring 2.5 ml of the diluted serum successively from the previous tube to the next until Tube 10. (Tube 10 should have a serum dilution of 1/12800).

Familiarize yourself with the identification system used for wells in the plastic agglutination tray.

(c) Performing agglutinations in microtiter trays
(Key step 3 and 4)

Start with the highest dilution (Tube 10) and a clean Pasteur pipette (calibrated to deliver 0.03 ml per drop). Place 2 drops of the diluted antiserum into well A-10 of the plastic agglutination tray. Next, using the same Pasteur pipette (after blotting the tip dry), place 2 drops of the antiserum of the next highest dilution (Tube 9) into well A-9 of the agglutination tray. Repeat until all the dilutions of the antiserum have been dispensed into the respective wells of Row-A of the agglutination tray.

Next, with a clean, calibrated Pasteur pipette, dispense 2

drops of the homologous antigen (approximately 1 x 10^9 cells ml^{-1}) into each of the wells from well A-1 through A-10. Avoid touching the antiserum in the well or the walls of the well with the tip of the antigen pipette. Discard the antigen pipette after use.

Work from the well containing the most to the least dilute antiserum. Using a clean glass applicator (a fine capillary tube sealed at both ends or a fine solid glass rod rounded and smooth at both ends), carefully stir the antigen-antiserum mixture in each well. Avoid spillage into neighboring wells. Rinse the stirring rod in a beaker of water and wipe dry with tissue paper between each well. Change the rinsing water frequently. The same stirring rod may be used for each new well.

Place 2 drops of serum of l/25 dilution into well A-11. Add 2 drops of saline with another calibrated Pasture pipette. This serves as the serum-saline control.

Place 2 drops of saline into well A-12. Add 2 drops of antigen. This serves as the antigen-saline control.

Seal all wells (A-1 through A-12) with a strip of cellophane tape. Float the agglutination tray in a water bath at 52°C for 4 h and then hold overnight in the refrigerator. Alternatively, the reaction mixture may be incubated in an

incubator at 37°C for 2 h and then transferred to a refrigerator before reading the reactions. Figure 7.1 shows the steps for the antiserum titer determination in wells. Read and record positive agglutinations at the highest dilution of the serum (Figure 7.2). Positive agglutination will appear as granular clumps with clear supernatant. Negative agglutinations are indicated by settling of the cells on the bottom of the well and turbid supernatant.

To calculate the titer, (serum titer is the reciprocal of the highest serum dilution at which positive agglutination occurs) multiply by two the highest dilution of the serum at which positive agglutination occurs. This is because equal volumes of the diluted serum and antigen were titrated in the well. (Example: if positive agglutination was detected at l/3200 dilution of the serum the true titer will be 3200 x 2 = 6400).

Further confirmation of a positive reaction can be made by gently stirring the reactants in the well with a sterile inoculating needle with a 2 mm loop. Stirring will cause the granular clumps to float in suspension. Observe with a stereo-microscope or magnifying glass and note the granular clumps suspended in a clear suspending solution. Stir the antigen-saline control and observe the turbid appearance showing no separation into granular clumps and no clear suspending solution. Flame the inoculating needle for reuse

in other wells. Flaming will remove contaminating reactants and thus prevent carry over. The antigen-saline control will help in distinguishing between positive and negative agglutinations. Record titer of antiserum and date of experiment.

(d) Performing agglutinations in tubes
(Key steps 3 and 4)

Prepare a two-fold dilution series of the antiserum as described for tray agglutinations (10 dilutions ranging from l/25 through l/12800). Remaining diluted serum prepared previously for the tray method may be used, provided both methods are done on the same day.

Arrange 24 tubes in agglutination tube racks. Tubes with an internal diameter of 5 mm and length of 60 mm are suitable. Special tubes called Dreyer tubes, if available, are preferred. Label them adequately to facilitate reading the antiserum dilution in each tube. Alternatively, arrange the tubes systematically in a tube-rack to avoid labeling.

Dispense 10 drops (0.03 ml $drop^{-1}$) of each dilution into the series of agglutination tubes set up for the titration. Set up duplicate tubes for each antiserum dilution.

Add 10 drops of each antigen (approximately 1 x 10^9 cells ml^{-1})

to each of the agglutination tubes with a clean Pasteur pipette. Avoid contact of the antigen pipette with the mouth or walls of the tubes containing the serum. Do not attempt to stir or mix the reaction mixture in the tubes. Also set up antigen-saline and antiserum-saline tubes as controls.

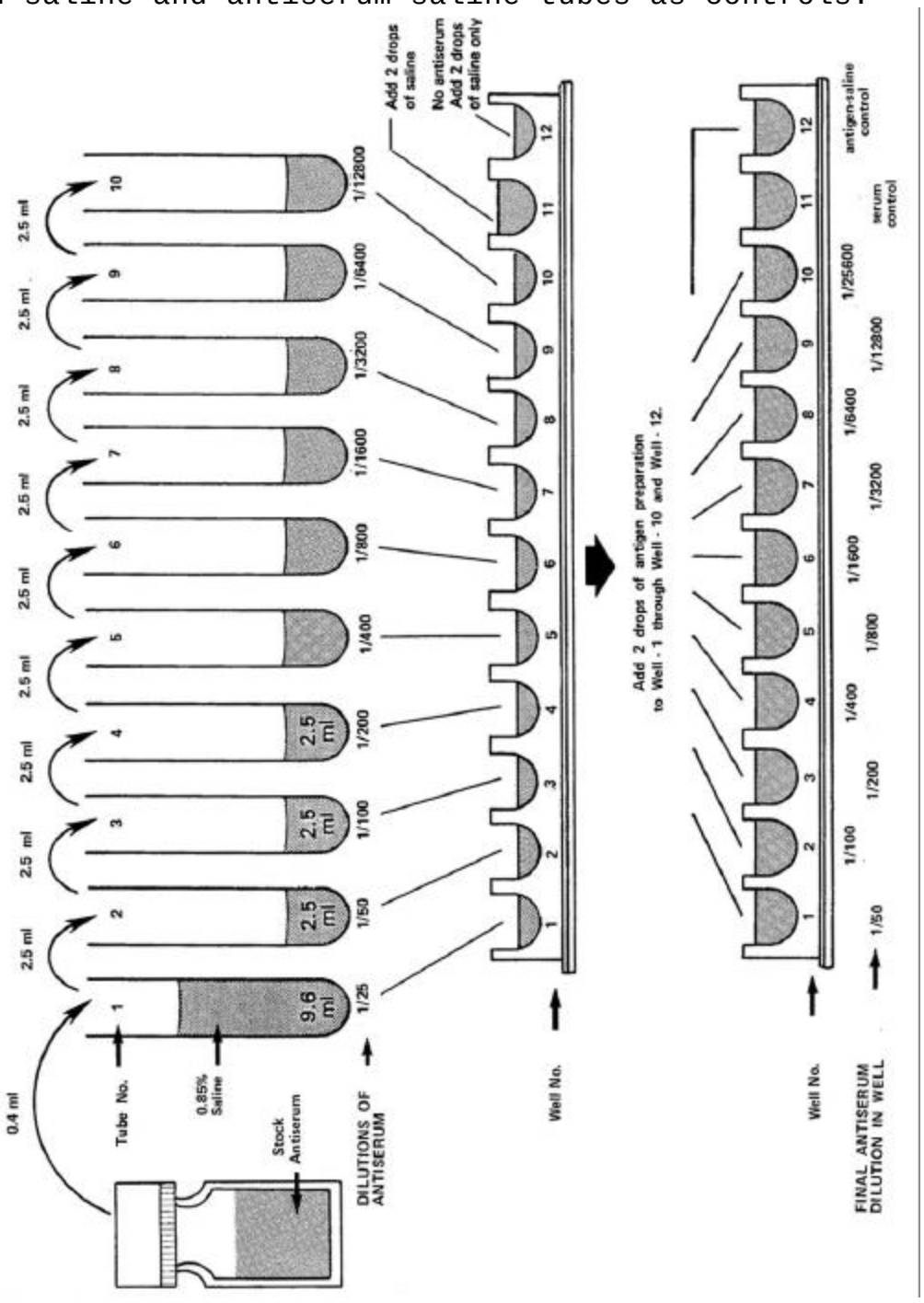


Figure 7.1. Scheme for antiserum titer determination in wells

Incubate the reaction mixtures in the tubes in a water bath at 52°C for 4 h. Alternatively, the tubes may be incubated in a water-bath at 37°C for 24 h. Read the tubes after the initial incubation. Read the tubes again after keeping them overnight in the refrigerator (4°C). Place glass beads of suitable size in the mouth of the tubes to prevent evaporation. "PARAFILM" can be substituted if glass beads are not available. Covering mouths of tubes is not necessary if the water bath is equipped with a lid/cover. The filled portion of the tubes should be immersed half-way in the water bath thereby facilitating mixing of the antigen and antiserum through convection.

Record positive agglutinations. These appear as granular clumps in a clear supernatant. When spun gently, negative tubes will produce a "wisp of smoke" effect arising from the bottom of the tube, indicating the sediment is not granular. Calculate the titer as outlined for tray agglutination.

(e) Performing agglutinations on microscope slides
(Key steps 3 and 4)

This method has the advantages of using only small amounts of

serum and giving results within minutes. There is, however, some loss of accuracy and a degree of subjectivity in interpretations. The method should not be depended on without some objective evidence (e.g. tube agglutination) to support interpretation.

Partition a microscope slide into two sections with melted vaseline or petroleum jelly. Prepare 10 slides and lay them in a row on black paper. One section is used for one dilution of the test serum and the other section for the antigen control. Label one section "T" for test-serum and "C" for antigen-control.

Using the remaining diluted serum prepared for the tray methods, place a drop (0.03 ml) of the serum with a calibrated Pasteur pipette (starting from the highest dilution) in the test-serum section of each slide.

To each drop of serum add a drop (0.03 ml) of the antigen. Also place a drop of antigen on each control section.

Add a drop (0.03 ml) of saline to the antigen in the control section of the slides.

Stir the antigen-antiserum mixtures with the loop of an inoculation needle.

Flame and cool the needle after each mixing to avoid "carry over" between tests.

Immediately after mixing, slowly rock the slide back and forth for 1-2 min. With a high-titer antiserum, the agglutination should occur quickly and should be detectable with the naked eye or a dissecting microscope.
Record positive agglutinations and calculate the titer.
Compare the results of this method with those from the tray and tube agglutination methods.

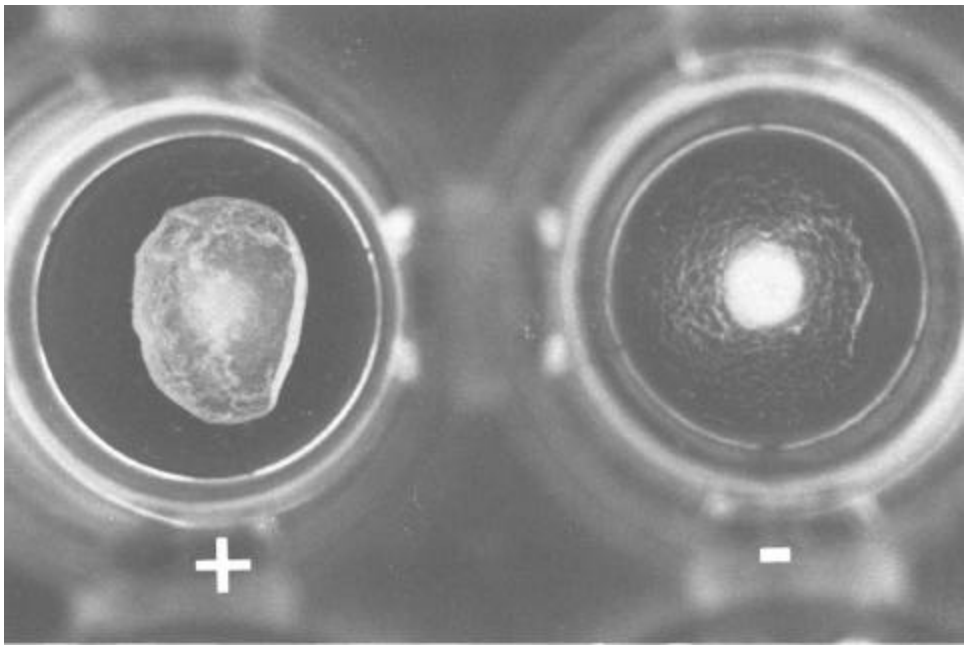


Figure 7.2. Agglutination reactions in wells of agglutination tray.

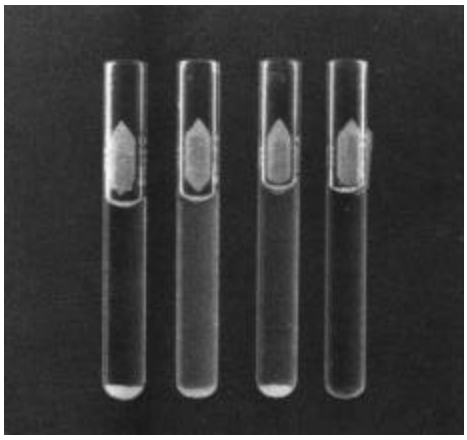


Figure 7.3. Agglutination reactions in agglutination tubes

Requirements

(a) Preparation of somatic antigens from cultured cells.

Centrifuge

McFarland barium sulfate standards (or photoelectric nephelometer)

Boiling water or steam bath

Centrifuge tubes

Test tubes

Serum vials with rubber stoppers

Sterile pipettes (1 ml) and Pasteur pipettes

Sterile glass beads

Sterile saline (100 ml)

YMA slopes in 500 ml flat culture bottles

Broth or agar slant culture of rhizobia

(b) Dilution of stock antiserum

Antiserum (1 ml) from Exercise 6

Sterile saline (200 ml)

1 ml sterile pipettes (two)

5 ml sterile pipettes (10)

Test tubes (10) and rack

(c) Performing agglutinations in microtiter trays

Plastic agglutination tray (rigid polystyrene "U" plate, Cooke Laboratory Products, Alexandria, VA, USA)

Calibrated Pasteur pipettes (0.03 ml/drop)

Glass applicator

Rubber bulb (1-2 ml capacity)

Cellophane tape

Tissue paper

Diluted antiserum from (b)

Antigen suspension (1×10^9 rhizobia ml^{-1})

Binocular dissecting microscope

Magnifying glass (hand lens)

Water bath(52°C), incubator (37°C), refrigerator(4°C)

(d) Performing agglutinations in tubes

Calibrated Pasteur pipettes

Agglutination tubes or other small tubes

Tube rack

"Parafilm"

Diluted antiserum from (b)

Antigen suspension from (c)

Water bath (52°C), incubator (37°C), refrigerator (4°C)

(e) Performing agglutinations on microscope slides

Clean microscope slides

Calibrated Pasteur pipettes

Rubber bulb

Vaseline or petroleum jelly

Diluted antiserum from (b)

Antigen suspension from (c)

EXERCISE 8

TO AGGLUTINATE ANTIGENS FROM ROOT NODULES

Identification of a particular Rhizobium strain, by direct use of the bacteroids in the nodules, is described. This direct method eliminates the time consuming steps of isolating the strain in pure culture prior to its use as an antigen in an agglutination reaction.

Key steps/objectives

1) Develop antiserum of *B. japonicum*)
2) Prepare Leonard jars
3) Prepare broth inoculum
4) Pregerminate soybean seeds
5) Plant and inoculate pregerminated soybean seeds
6) Harvest plants for nodules
7) Perform agglutinations with bacteroid antigens
8) Read and record agglutinations

(a) Developing antisera
(Key step 1)

Inoculate *B. japonicum* strain TAL 379 onto YMA-flats. Harvest culture and make antigen preparation for the development of antibodies for agglutination as described in Exercise 6.

Other strains of *B. japonicum*, for which antisera are available, may be substituted in place of TAL 379.

(b) Culturing soybean plants nodulated with a serologically marked strain of *B. japonicum*

(Key steps 2, 3, 4, 5, and 6)

Prepare Leonard jars. This should be done well ahead of experiment.

Inoculate 100 ml of YM-broth in a 250 ml Erlenmeyer flask with a loopful of TAL 379 from an agar slant. This should be initiated at least 7 days before planting the Leonard jars to give sufficient time for the growth of the culture.

Surface sterilize soybean seeds as described in Appendix 10. Plate seeds on water-agar plates for germination. Do not invert plates for soybean and other large seeded legumes. Pregerminate seeds 1-2 days before planting and inoculation in Leonard jars.

Plant three germinated soybean seeds in each Leonard jar. Inoculate each seed with 1 ml of TAL 379 broth culture. Plant four jars.

Harvest and wash the soybean nodules after 30-35 days of plant growth. Separate the nodules from the roots. Pack and seal

the washed nodules in small polyethylene bags (size 100 mm x 100 mm and 0.04 mm thickness). Use one bag per plant and label. Bags of the specified size or slightly smaller can be purchased commercially or can be made in the laboratory if the polyethylene material and a bag sealer are available.

(c) Separating bacteroid-antigens from nodules for agglutination

(Key step 7)

Fill a 1 liter beaker with approximately 500 ml of water and bring it to a boil; then control the heating source to produce gentle boiling. Immerse one bag of nodules in the boiling water for 3-5 min, then remove the bag and cool. Save the remaining bags of nodules for Exercise 9.

Cut open the plastic bag with scissors. Using forceps, transfer the nodules to the agglutination tray, one nodule per well. Have one nodule in each well, beginning at well A-1 through A-10 (refer to the well identification system on the agglutination tray). Leave wells B-1 through B-10 empty; these wells will be used for agglutinations with antigens separated in the series of wells in row A.

With a Pasteur pipette, place 6 drops (0.03 ml $drop^{-1}$) of saline into each well containing a nodule. (Excess heat-treated nodules can be stored frozen and thawed later for

use in agglutination without losing the ability of the bacteroid-antigens to agglutinate).

Gently press out (do not homogenize) the nodule contents into the saline in the wells with fine forceps or a round-ended glass rod (4 mm diameter). Gently stir the exuding nodular contents into the saline and push the resulting nodule tissue against the wall of the well. Rinse the rod and wipe dry for each nodule. Suitable flat toothpicks can be substituted for forceps or glass rods. Toothpicks are used once and discarded.

With a fresh Pasteur pipette, transfer 3 drops of the antigen from well A-1 to B-1. Rinse the pipette thoroughly with hot water by sucking the hot water into the pipette and emptying the pipette contents in another beaker. Next, transfer (with the same pipette) 3 drops from well A-2 to B-2. With alternate rinsing of the pipettes between transfers, transfer the antigen A-3 to B-3, A-4 to B-4, and so on until A-10 to B-10. (In these transfers, the same pipette is used each time as the nodules were formed by one strain, TAL 379. If the nodules were formed by strains from a mixed inoculum, each antigen transfer must be done with a fresh Pasteur pipette). Variable Finn pipettes with disposable tips are excellent substitutes for Pasteur pipettes, especially when large numbers of nodules need to be identified, since a fresh tip can be used for each nodule.

(d) Agglutinating the bacteroid-antigens with homologous antiserum

(Key steps 7 and 8)

Prepare a 1/25 dilution of antiserum TAL 379 by diluting 0.4 ml of the stock antiserum in 9.6 ml of saline.

Dispense the diluted antiserum by placing 3 drops in each of the wells B-1 through B-10. Place 3 drops of the 1/25 diluted antiserum and 3 drops of saline in well B-11 (serum control). Set up the antigen-saline control in well B-12 by placing 3 drops of antigen from well A-1 followed by the addition of 3 drops of saline. Mix the reactants in the wells with a round-ended glass applicator, starting at well B-10 and proceeding towards well B-1. Use the same applicator for mixing the contents in the wells. Rinse and wipe dry the applicator between each mixing. The contents in the wells may also be mixed by holding the plate loosely in a level position with both hands and tapping the side of the plate with a free forefinger. Avoid spilling during this operation.

Cover the tops of the wells containing the reactants with a strip of cellophane tape. This will prevent evaporation during incubation. Leave a tab of tape to assist removal.

Place the trays at 37°C for 2 h in an incubator. At the end of this time transfer the trays to 4°C in a refrigerator and

leave overnight.

Record the appearance of the positive agglutinations by comparison with the antigen-saline control. (The titer of the antiserum at which the agglutinations occurred would be 1/25 x 3/6 = 1/50.)

This method has been used on nodules of *Glycine max*, *Centrosema pubescens, Vigna unguiculata,* and *Phaseolus lunatus* with good results. These legumes have nodules of similar size. With other species of legumes, the volume of saline to be used in squashing the nodule to extract the bacteroid antigen has to be determined by trial and error. The volumes of the bacteroid-antigen and the serum in the wells also have to be determined before large numbers of nodules are identified by agglutination. The use of thick suspensions of the bacteroid antigen should be avoided because unreacted antigen produces turbidity resulting in ambiguity in the recognition of positive agglutinations. The ultimate purpose of manipulating the volumes of saline and serum to be used during the agglutination is to regulate the density of the antigen close to 1×10^{9} bacteroids ml^{-1}.

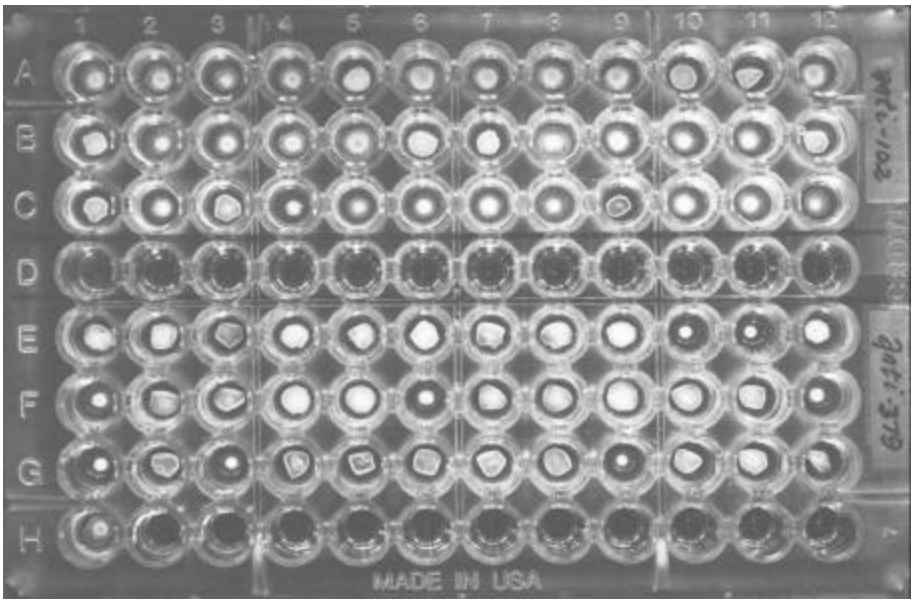


Figure 8.1. Identification of nodule bacteroids by agglutination in an agglutination tray.

Requirements

(a) Developing antisera

See Exercise 6

(b) Culturing soybean plants nodulated with serologically marked strains of *B. japonicum*

Sterilizing solutions (See Appendix 10)
Sterile water (500 ml)
Sterile 250 ml wide-mouthed flask
Soybean seeds
Water-agar plates (three)
100 ml broth culture of *B. japonicum* (TAL 379)
Sterile 5 ml pipettes
Sterilized gravel (mulch for Leonard jars)
Leonard jars (four)
Isopropyl alcohol in spray bottle
Spirit lamp, matches
Forceps
Scissors, polyethylene bags

(c) Separating bacteroid-antigen from nodules

Beaker (1 l)
Scissors, fine forceps

Sterile Pasteur pipettes (or variable Finn pipettes with disposable tips if available)

Bunsen burner

Round-ended glass rod

Sterile saline (250 ml)

Tissue paper

Agglutination tray (rigid polystyrene "U" plate)

Nodules containing bacteroids of *B. japonicum* TAL 379

Nodules containing bacteroids of *B. japonicum* TAL 378

(d) Agglutinating the antigens with homologous antiserum

Antiserum (TAL 379)

Calibrated Pasteur pipettes

Antigen from (c)

Glass applicator

Cellophane tape

Incubator (37°C), refrigerator (4°C)

EXERCISE 9

RHIZOBIAL ANTIGEN-ANTIBODY REACTIONS IN GEL BY IMMUNODIFFUSION

Immunodiffusion in gel allows for the recognition of antigenically identical strains and for differentiating between closely related nonidentical strains. Soluble and diffusible heat-stable antigens of varying molecular size are studied in this technique.

Key steps/objectives

1) Inoculate YMA flats for antigen preparation
2) Prepare gel in plastic Petri dishes
3) Harvest cultures for antigen preparation
4) Perform immunodiffusion
5) Observe and record diffusion patterns

(a) Preparing gel for diffusion
(Key step 2)

Place 100 ml of saline into a 250 ml Erlenmeyer flask. Add 0.75 g of Difco Noble agar or Oxoid Ion agar No. 2 to the flask and melt by steaming, autoclaving or heating in a microwave oven. If direct heat is applied to melt the agar, prevent charring of the agar on the bottom of the flask by constant stirring and controlling the heat. To the melted

agar, add 1 ml of a 2.5 % (w/v) solution of sodium azide (a preservative), and swirl the flask to ensure proper distribution of the sodium azide. Pipette 25 ml of the hot gel into Petri dishes kept on a level surface. Allow the agar to solidify. A total of four plates with a gel layer 4 mm in thickness should result.

Trace the outline of the bottom of a Petri dish on a sheet of white paper. Draw a hexagonal pattern of six circles (4 mm diameters) equidistant (5 mm from edge to edge) from one another in the center of the plate outline on this paper. Draw a seventh well in the center of the hexagonal pattern and shade in the circles (Figure 9.1). This pattern on the paper serves as a template for cutting out wells from the gel.

Place a Petri dish (containing gel) on the template. The pattern of circles should be visible through the gel. Cut wells into the gel using a 4 mm cork-borer. The cork-borer should be held vertically when cutting the wells, otherwise wells with oblique walls will result. Carefully remove the gel plugs with pins or other suitable implement or remove the plugs by suction using a Pasteur pipette (with a slightly bent tip) attached to a suction apparatus. (A Pasteur pipette attached to an aspirator or vacuum pump, with a "trap" in between for the gel plugs, is a suitable suction apparatus.)

It will take some practice to produce plates with seven intact

wells. A drop of molten agar may be necessary to seal off the

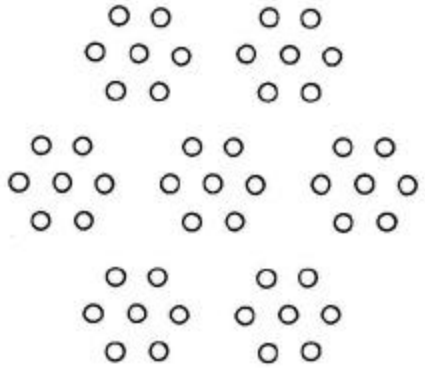


Figure 9.1. Hexagonal pattern template for Petri dishes.

bottom of the well. Sealing the well is usually not necessary with the plastic Petri dishes but is essential for glass Petri dishes. The gel plates may be refrigerated if not required for immediate use. Make four sets (one set per Petri dish) of the hexagonal pattern of wells. Three or seven sets of wells can be made per Petri dish with sufficient experience and care.

(b) Preparing antigens
(Key steps 1 and 3)

Culture the following strains of *Bradyrhizobium* sp. on YMA flats:

TAL 651 (from *Calopogonium mucunoides)*
TAL 653, 655, and 855 (from *Centrosema pubescens*)

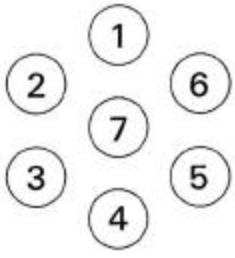
TAL 642 (from *Lablab purpureus*)

Harvest the cultures after 7 days of growth (Exercise 6) and prepare antigen suspensions for immunodiffusion. A final volume of 1.0-1.5 ml of a dense antigen suspension containing approximately 1 x 10^{10} cells ml^{-1} is desirable.

Divide the antigen suspension of each strain into two small screw-capped tubes. Small McCartney bottles are better substitutes if these are available. Heat treat one sample for 1 h at 100°C by immersing the tube in boiling water. Leave the other sample unheated (untreated).

(c) Setting up immunodiffusion reactions
(Key steps 4 and 5)

Place 2 drops (0.04 ml $drop^{-1}$) of each heat-treated antigen in their respective wells. The position of the different antigens for the diffusion is as shown in Figure 9.2.



Well number	Antigen
1 and 4	TAL 655
2	TAL 653
3	TAL 855
5	TAL 651
6	TAL 642

Figure 9.2. Well pattern for immunodiffusion.

Place the undiluted antiserum of TAL 655 in Well-7.

Similarly, set up another set of wells for immunodiffusion with untreated (unheated) antigen.

Labelling on the bottom of the Petri dishes is essential to facilitate the identification of the antigens in the wells. Orientation of the dish can be established with a single line at the 12 o'clock position and a diagrammatic record of the location of each well.

Incubate the Petri dishes at room temperature in a water-saturated atmosphere. A saturated atmosphere is necessary to prevent moisture loss from the gel. Air-tight plastic boxes can be improvised to provide this environment by placement of wet paper towels on the inside prior to closing of the boxes.

Make observations at 24 and 48 h. Record your observations in the form of drawings. Compare the diffusion patterns of the heated and unheated antigens. Interpret the diffusion patterns for reactions of identity, partial identity, and nonidentity. Heating can significantly alter the reactivity, concentration and diffusibility of the somatic antigens leading to stronger and well separated precipitin bands.

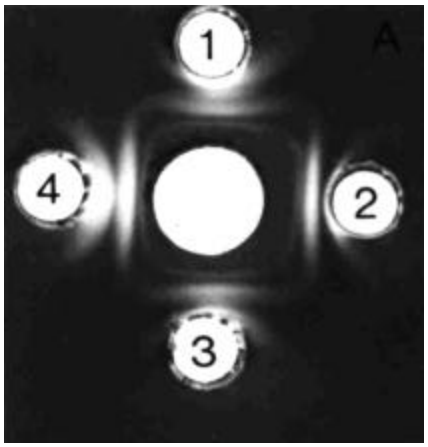


Figure 9.3. Immunodiffusion reactions showing preciption bands.

Requirements

(a) Preparing gel for diffusion

Autoclave, stove or microwave oven
Saline (100 ml)
Erlenmeyer flask (250 ml)
Sodium azide
Noble agar (purified agar) from Difco, Detroit, Michigan or Oxoid Ion agar No. 2
Plastic Petri dishes (four)
Hexagonal pattern template
Cork-borer (4 mm)

(b) Preparing antigens

Agar slant cultures of bradyrhizobia (TAL 642, 651, 653, 655, and 855) or other rhizobia
YMA slopes (five) in 500 ml flat medicine bottles
Screw-capped tubes (or small McCartneys)
Steam- or water-bath

(c) Setting up immunodiffusion reaction

Pasteur pipettes
Rubber bulbs (1-2 ml capacity)
Air tight plastic boxes (or substitute of similar

function)

EXERCISE 10

TO IDENTIFY NODULES BY GEL IMMUNODIFFUSION

In this exercise, a precise technique is described to differentiate and identify the occupant strain(s) of nodules from plants inoculated with a mixture of two serologically distinct strains. Bacteroids from nodules are used directly as antigens.

Key steps/objectives

1) Prepare Leonard jars
2) Culture strains for stock broth cultures and for antigen preparation
3) Pregerminate soybean seeds
4) Sample stock broth cultures for viable counts and prepare mixed inoculum
5) Plant and inoculate pregerminated seeds
6) Harvest nodules
7) Set up the Gelman immunodiffusion apparatus. Prepare gel in Petri dishes
8) Prepare antigen from nodules and cultured cells of inoculum strains
9) Perform gel diffusion in microscope slides and Petri dishes
10) Record precipitin bands by drawing; analyze

immunodiffusion patterns

(a) Preparing mixed-strain inocula of *B. japonicum* (Key steps 2 and 4)

Prepare two flasks each containing 150 ml of YM-broth. Inoculate one flask with *B. japonicum* strain TAL 379 str and the other flask with *B. japonicum* strain TAL 378 spc. These two flasks will provide stock cultures of each strain. Allow 7 days for maximum growth of the strains. (The two strains used in this experiment are antibiotic resistant. TAL 379 is resistant to streptomycin (str) and TAL 378 is resistant to spectinomycin (spc)). The nodules formed by these two strains will be identified by gel immunodiffusion in this exercise and by their ability to grow in YMA plates containing antibiotics in Exercise 12.

After 7 days of growth of the stock cultures, aseptically and accurately transfer 50 ml of TAL 379 str to a 250 ml sterile flask. Do the same flask transfer 50 ml of TAL 378 spc. (Use a fresh 10 ml pipette for the transfers and pipette 5 times to remove each 50 ml. Use a fresh pipette when transferring different strains). Swirl the flask to ensure a good mixture of the two strains and label this flask M.

Use the drop- or spread-plate methods (Exercise 4) to obtain viable counts of TAL 378 spc and TAL 379 str. When the viable

counts become available later, the actual ratios of the competing strains in the mixed inocula can be more accurately computed.

Set aside the remaining portions of the two stock cultures and use these as inocula for single strain inoculation.

(b) Culturing of soybean plants inoculated with a single strain and a mixture of strains of *B. japonicum*

(Key steps 1, 3, 5, and 6)

Prepare 15 Leonard jars (Appendix 11).

Surface sterilize and pregerminate 60 soybean seeds of good viability as described in Appendix 10. Allow two days for the pregermination of the seeds.

Select well-germinated seeds and plant three per jar. Inoculate each seed at sowing with 1 ml of the broth inoculum of the appropriate treatment. Plant four jars for each treatment and label adequately.

Proceed to plant another 4 jars and inoculate the seeds with 1 ml per seed of the stock culture of TAL 379 str and label. Similarly set up 4 more jars but inoculate with TAL 378 spc.

Finally, plant the remaining three jars and leave them uninoculated. These three jars serve as uninoculated

controls.

Seven days after planting, thin down to two uniform plants per jar. Harvest all treatment after 30-35 days. Carefully remove and wash the root-system of each plant. Count and record the number of nodules on the roots of each plant. Detach the nodules and pack them in small plastic bags as described in Exercise 8. Label the bags adequately for later identification of the treatments. (The remaining plants in the jars will be harvested at a later date for use in Exercise 13).

(c) Preparing nodule bacteroid-antigens
(Key step 8)

Proceed as explained in Exercise 8. Prepare nodule bacteroid-antigens from nodules of the treatments which received the mixed broth inoculum.

(d) Preparing soluble antigen from cultured cells
(Key step 8)

Inoculate one YMA flat each with TAL 379 str and TAL 378 spc. Harvest these strains after 7 days and prepare soluble antigen for immunodiffusion as described in Exercise 9.

(e) Setting up the immunodiffusion system

(Key step 7, 9, and 10)

The gel (see Exercise 9) for immunodiffusion is prepared on microscope slides. Thin (1 mm) microscope slides are especially suitable for this method using the Gelman immunodiffusion apparatus (Gelman Instrument Company, Ann Arbor, Michigan U.S.A.) described in this exercise. The various components of the apparatus include the immunoframes, immunoframe holders, rinsing tanks, and the immunodiffusion punch set. Familiarize yourself with their construction and use(s). The Gelman product numbers for the various components are given in the list of requirements.

Study the immunoframe which has been especially constructed to hold microscope slides. Each immunoframe holds six standard microscope slides, three in each of its two compartments. Each compartment is divided into three windows and one slide is centered over each window. All three slides must be placed in close contact with one another. Complete the arrangement of slides in each immunoframe and place the immunoframes on a clean and level surface. (A level surface is important to obtain gel of uniform thickness.)

With a Pasteur pipette, dispense minimal amounts of the molten gel around the edges of each slide to seal off the fine gaps at all points of contact between slides and between slides and compartment walls. (Sealing is necessary to prevent leakage

of the gel to the bottom when the melted gel is poured.)

Allow the gel to cool to obtain proper seal.

Pipette 10 ml of the molten gel into each compartment of the immunoframe. Empty the pipette beginning at one end of the compartment and proceed to the other end moving the pipette in a zig-zag motion, to evenly spread the gel over the slides. Complete layering the gel over all the slides in the immunoframes.

Allow 1 h for the gel to cool and set, in a dust-free environment. The gel should be protected from dust particles settling on its surface during the cooling process by improvising suitable covers.

On cooling and setting of the gel, mount the immunoframes onto the immunoframe-holder. It can accommodate a maximum of three immunoframes. Place the whole assembly into a rinsing tank containing approximately 80 ml of water and replace the lid. Store the rinsing tank and its contents overnight at 4◦C (refrigerator) or at room temperature (26-28◦C) to improve the setting of the gel.

Examine the immunodiffusion gel-punch set. The gel-punch consists of a die and an attached system of cutters. The arrangement of cutters on the die allows the production of two

sets of the hexagonal pattern of wells (used in this technique) on one slide at any one time. The gel punch is designed to fit the sides of the immunoframe and when the punch supports are properly mounted, the punch can be slid back and forth to desired positions.

Mount the gel punch onto the immunoframe and position it over a slide. Gently press the punch down on the gel and hold for 3-4 seconds to cut out the hexagonal patterns.

The 3 mm wells on the slides can hold 8-10 microliters of antiserum or antigen. These small volumes can be conveniently delivered with a variable volume (5-50 microliters) Finn pipette with disposable tips.

Perform the immunodiffusion with the nodule bacteroid-antigens with reference to Figure 10.1. Identify all the nodules being analyzed for strain occupancy using the scheme given in Figure 10.1.

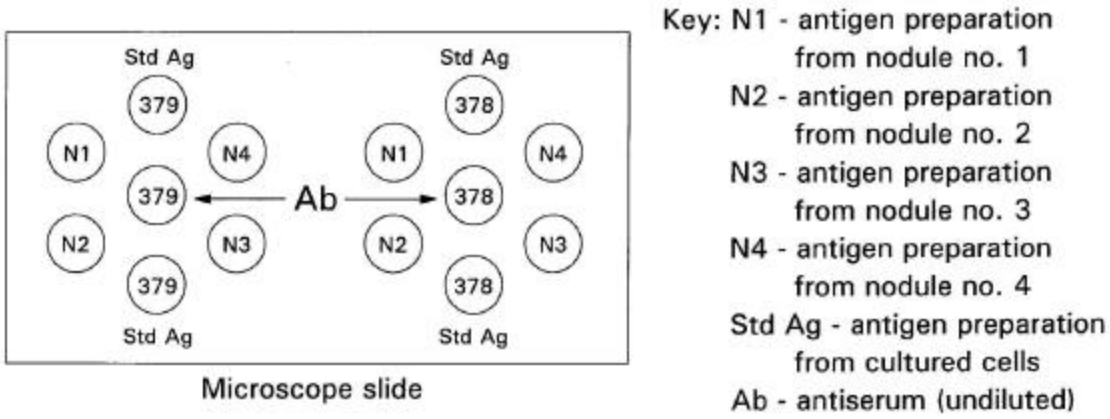


Figure 10.1. Scheme for identifying nodules from inoculated with a mixture of two strains.

Set the Finn pipette to deliver 8 microliters of the antigen or antiserum. Deliver the antigens and antisera to their respective wells in the hexagonal system according to scheme (Figure 10.1) given. (Note that each nodule formed by the mixed inoculum is identified against the antisera of the two component *B. japonicum* strains in the mixture.)

Assemble the immunoframes (housing the microscope slides) on the immunoframe-holders and incubate the assembly in a saturated atmosphere (provided by approximately 80 ml of water in the rinsing tank). Incubation at room temperature (26-28°C) allows precipitin band development between 24 to 48 hours.

In Table 10.1 record the number of nodules giving positive precipitation bands against each antiserum. Nodules giving

reactions of identity with both the antisera indicate mixed infections, i.e., the nodule contains both the strains from the mixed broth inoculum.

Use the nodule analysis data to examine whether the proportion of nodules formed by each strain was according to its representation in the mixture using Chi-square analysis.

If sufficient nodules and antisera are available, perform a parallel immunodiffusion exercise with gel prepared in Petri dishes (Chapter 11). Follow a similar scheme of nodule identification as detailed for the microscope slide method in this chapter.

Table 10.1. Identification of nodules for the mixed inoculum treatment

Ratio of TAL 378:379	No. of nodules examined	Nodule occupancy (%)			Chi-square deviation (1 df)
		TAL378	TAL379	TAL378+TAL379	

Requirements

(a) Preparing the mixed broth inoculum

Transfer chamber
Agar slant cultures of *B. japonicum* strains TAL 379 str and TAL 378 spc
YM-broth, 150 ml (two flasks)
Sterile 125 ml Erlenmeyer flasks (two)
Sterile 10 ml pipettes (five)
Sterile 1 ml pipettes (15)
Sterile calibrated Pasteur pipettes
90 ml sterile water in each of milk dilution bottles
Quarter-strength YM-broth or sterile water (9 ml in 30 ml capacity screw-cap tubes)
YMA plates

(b) Culturing of soybean plants inoculated with a single and mixture of strains *B. japonicum*

Leonard jars
Soybean seeds
Sterilizing solutions (see Appendix 10)
Sterile water
Water agar plates
Pipettes, 10 ml (three-five)
Spirit lamp, alcohol in spray bottle, matches

Forceps

Inoculant broth of TAL 379 str and TAL 378 spc

(c) Preparing nodule bacteroid antigen

See Exercise 8

(d) Preparing soluble antigens from cultured cells

Slant cultures of TAL 378 spc and TAL 379 str

YMA flats (two)

Other requirements as in Exercise 9

(e) Setting up immunodiffusion systems

Immunoframes (Gelman Product No. 51447)

Immunoframe holders (Gelman Product No. 51448)

Rinsing tanks (Gelman Product No. 51457)

Immunodiffusion punch-set (Gelman Product No. 51450)

Microscope slides without frosted ends (Approx. 1 mm thick) from Curtin Matheson Scientific Inc.

Finn pipettes (Variable Volumetrics Inc., Woburn, MA, USA)

Pasteur pipettes

EXERCISE 11

TO DEVELOP AND USE FLUORESCENT ANTIBODIES (FA)

The immunoglobulins are separated from antiserum, purified, and conjugated with fluorescent dye. The conjugate is then used to identify Rhizobium in nodules by the “direct” FA technique. A modification of this method, commonly referred to as the indirect FA technique, is described in Appendix 13. Both methods are very useful for the identification of Rhizobium strains in ecological research.

Key steps/objectives

1) Precipitate the serum globulins
2) Precipitate the serum globulins for a second and third time
3) Dialyze the serum globulins
4) Determine the protein content of the dialysate
5) Conjugate the immunoglobulins, with fluorescein isothiocyanate (FITC)
6) Purify the FA by column chromatography
7) Test the quality of the FA
8) Type nodules with the FA technique

(a) Fractionating serum globulins
(Key steps 1 and 2)

Place a 250 ml beaker filled with crushed ice onto a magnetic stirring plate. Immerse a 50 ml centrifuge tube containing 15 ml antiserum into the ice and clamp the tube to a ring stand. Drop a 12 mm (0.5 in) stirring bar into the tube. To the same ring stand, attach a 30 ml burette filled with cold 3.9 M ammonium sulfate solution. The tip of the burette should be close to the surface of the antiserum. Add 15 ml ammonium sulfate solution to the antiserum at the approximate rate of one drop per second while stirring continuously.

Allow the resulting cloudy mixture to stand overnight (or for at least 2 h) at 4°C.

Separate the globulins by centrifugation in a refrigerated centrifuge at 5,000-10,000 rpm for 30 min. Discard the supernatant and dissolve the precipitated globulins in enough saline to bring the solution back to the original serum volume (15 ml).

Repeat the precipitation and centrifugation steps twice as above, but without the intermediate step of overnight refrigeration. Instead, allow the precipitates to settle for 5 min at 4°C before centrifugation. Three precipitations are usually sufficient to render the globulins completely white and free of hemoglobin.

(b) Purifying the serum globulins

(Key step 3)

Dissolve the final precipitate in approximately 7.5 ml of saline (half of original volume) and dialyze against 2 liters of saline (adjusted to pH 8 with 0.1N sodium hydroxide) in a coldroom with frequent changes of saline until the ammonium sulfate is no longer detectable in the dialysate. Three changes of dialyzing fluid at intervals of 4, 10 (overnight) and 4 hours again, with another 4-hour run before completion is usually sufficient. Merthiolate may be added to the dialyzing fluid as a preservative at a concentration of 0.01% (w/v).

To determine the presence of sulfate, mix a few drops of the dialyzing fluid with an equal volume of a saturated barium chloride solution. If the mixture does not become cloudy, the dialysis can be considered complete.

If phosphate has been used as buffer for the dialyzing fluid, use Nessler's reagent to detect ammonium (Appendix 4) because phosphate will interfere with the sulfate precipitation.

In a small test tube, mix a few drops of the dialyzing fluid with an equal amount of Nessler's reagent. A very fine brown precipitate will form in the presence of ammonium.

(c) Determining the protein content of the dialyzate

(Key step 4)

After the globulin has been rendered free of ammonium sulfate, protein concentration is determined by the biuret test which utilizes the following reaction:

$$\text{Protein} + CuSO_4 + NaOH \rightarrow \text{Purple color}$$

The amount of purple color formed is proportional to the amount of protein present (if alkaline $CuS0_4$ is in excess). By using several levels of protein and reading the purple color at the appropriate wavelength, a standard curve can be prepared showing protein concentration versus absorption.

Make a protein standard solution using BSA (bovine serum albumin) at a concentration of 20 mg ml^{-1}. 200 mg BSA dissolved in 10 ml distilled water should be sufficient for the protein determination.

Prepare fresh biuret reagent (Appendix 4).

In 15 ml test tubes set up standard and sample dilutions according to Table 11.1.

Allow the tubes to stand for 30 min at room temperature.

Use tube no. 6 to zero the spectrophotometer at 540 nm. Read

and record the absorbance of the standards (tubes 1-5) and the unknowns (tubes 7-8).

Use the values obtained from tubes 1-6 (Table 11.1) to construct a standard curve plotting absorbance (y-axis) against mg ml^{-1} protein per tube (x-axis).

Use this curve to read off amount of protein in (mg ml^{-1}) the globulin test samples (tubes 7 and 8).

Make a new curve for each protein determination.

Usually at least one of the two unknowns will fall within the range of the curve.

Table 11.l. Schedule for total serum protein determination

Tube no.*	Biuret reagent (ml)	Water (ml)	BSA stock (ml)	% BSA (mg ml^{-1})	Absorbance (at 540nm)
1	8	1.0	1.0	2.0	
2	8	1.2	0.8	1.6	
3	8	1.4	0.6	1.2	
4	8	1.6	0.4	0.8	
5	8	1.8	0.2	0.4	
6	8	2.0	0.0	0.0	
7	8	1.2	0.8		
8	8	1.8	0.2		

- Tubes 1-6 contain BSA standards; tubes 7 and 8 contain globulin test-samples

After determining the protein concentration adjust the

dialyzed immunoglobulin solution to 10 mg^{-1} ml by adding saline.

(d) Conjugating the globulins with fluorescent dye (Key step 5)

Place 10 ml of the 1% globulin solution (a total of 100 mg protein) in a 50 ml beaker. Add 4 ml of 0.15 M sodium phosphate buffer (pH 9). (Appendix 5).

In a separate 50 ml beaker dissolve 3.0 mg of FITC in 4 ml of a 0.1 M sodium phosphate buffer pH 8 (Appendix 5) continuously stirring with a magnetic stirrer.

Add this mixture to the buffered globulin solution. For the conjugation, the ratio of FITC to globulin is 0.03 mg of FITC per mg of protein.

Adjust the pH of the FITC-immunoglobulin mixture to 9.2-9.5 with 0.1 N sodium hydroxide and increase the volume to a total of 20 ml with phosphate buffered saline (PBS) pH 7.1 (Appendix 5).

Add merthiolate solution. The merthiolate should be present at a concentration of 1:10,000 to act as a preservative. Conjugate at room temperature for 8 h or overnight with continuous mixing provided by a magnetic stirrer. Set the

stirrer at the lowest possible speed to avoid frothing. To ensure that the sample is well insulated from heat generated by the stirrer, place a thin piece of a good insulation material such as styrofoam on the stirrer and clamp the sample-container to a ring stand to elevate it slightly above the stirrer.

(e) Purifying the Fluorescent antibodies
(Key step 6)

Separate the conjugated fluorescent antibodies (FA) from unreacted FITC by column chromatography or dialysis.

For the column chromatography method, prepare a slurry of Sephadex G 25-150 or G 25-300 in PBS in a 1 liter Erlenmeyer flask. Use approximately 10 ml PBS g^{-1} dry Sephadex at this stage. The bed volume of G 25-150 Sephadex is 5 ml g^{-1} dry gel when swollen in PBS. Allow to settle and remove fine particles by decanting. Repeat until the supernatant liquid is clear.

Add merthiolate (1:10,000) and leave at room temperature for 3 h to allow the Sephadex particles to swell. Alternatively, the slurry may be heat-treated at 90°C for 1 h.

Plug a glass column approximately 2.5 x 30 cm with a small amount of glass wool and close the outflow. Add 2-3 ml of

PBS. Premeasure the slurry to fill approximately 20 cm of the column when settled. Pour the slurry into the column in one continuous flow. The volume of a packed Sephadex column should be approximately three to five times the volume of the conjugate to be purified.

Sephadex consists of tiny porous beads of cross-linked dextran (biopolymer) which swell on imbibing water. When contained in a chromatography column, the beads form a molecular sieve which will separate compounds according to molecular size. Large molecules of the conjugated immunoglobulins will meet little obstruction as they pass through the interstitial spaces between the beads and emerge with shorter elution times. The much smaller molecules of the free FITC will penetrate the lattice structure of the Sephadex, which increases the elution time.

Equilibrate the column by passing at least three column volumes of PBS through it. Control the outflow carefully so that the column bed remains covered with liquid. The column must be replaced should it run dry. Measure the pH of the outflowing eluent. Repeated rinsing with buffer or distilled water is necessary if the pH is higher than neutral.

Allow buffer to settle almost to the top of the bed without drying the bed then add the conjugate with a Pasteur pipette. Permit the conjugate to penetrate the Sephadex until the

conjugate level is slightly above column bed. Gently wash the conjugate into the column with several 2 ml increments of PBS, added with a Pasteur pipette. After all the conjugate has penetrated the Sephadex to at least 3 cm into the Sephadex, a reservoir filled with PBS containing Merthiolate (0.01%) may be connected to the top of the column to maintain a PBS filled column until the purified fluorescent antibodies have been collected.

Collect the first yellow banded fraction (FA) in a small (50 ml) beaker taking care to stop the collection when no color is seen in the eluted buffer. The unconjugated FITC fraction is seen as a slow moving diffused yellow band.

If the collected material is dilute, it may be concentrated using carbowax (polyethylene glycol). The conjugate is placed in a beaker. Then a dialysis bag containing approximately 5 g of carbowax is immersed into the conjugate and left in the cold for 4 to 8 h or until the FA solution has reached a volume of 15 to 20 ml.

An alternate way to purify FA is through dialysis. Dialyze against PBS pH 7.1 until no color is detected in the dialysate. This may take more than 36 h.

Distribute the purified FA in 1 ml volumes in labeled 2 ml screw cap vials and store in the freezer. Lyophilization is

also possible at this point if facilities are available.

Often, some particulate matter accumulates at the bottom of the containers. This should be eliminated by centrifugation or by filtration through a 0.45 micron membrane filter prior to use.

The Sephadex may be used repeatedly after thorough washing. The unconjugated FITC should be washed off the column by passing distilled water through it until no yellow color can be detected. The Sephadex may then be washed again batchwise and stored in a refrigerator.

(f) Testing the quality of the fluorescent antibody (Key step 7)

Prepare twofold dilutions of the FA in saline (or PBS) for the titer determination. Dilute the FA in the range of 1:1, 1:2, 1:4, and so forth, up to 1:32. Using a small transfer loop, make thin smears on clean microscope slides from: (a) a young liquid culture of the homologous rhizobial strain for which the FA was prepared (b) a young liquid non-homologous rhizobial culture. Use a separate slide for each dilution of FA.

Air dry and heat fix the smears by passing them rapidly over the flame of a Bunsen burner.

Cover each smear completely with 1 drop of each dilution of the FA. More FA material may be needed if the smears are too large to be covered by 1 drop. Incubate in a moisture-saturated chamber for 20 min at room temperature. A moisture-saturated chamber may be made from a large Petri dish into which a wet piece of filter paper is placed. Two glass rods are placed on the filter paper and spaced to provide a rail to support the slides. Larger incubation chambers can easily be improvised, but care has to be taken that the slides are resting level and that they are well separated from each other.

Wash off the excess FA with a gentle stream of PBS from a wash bottle or Pasteur pipette, taking care to avoid dislodging the cells in the smears. Then wash the smears by submerging the slides in saline or PBS for 20 min. Similarly, wash the smears in water for 15 min and air dry. Add a drop of mounting fluid (Appendix 5) and mount with a cover slip. Observe the smear under a UV-microscope equipped with a HBO mercury vapor light source and a suitable filter pack for FITC excitation. To ensure the validity of the results, compare the reactions with homologous and non-homologous strains of rhizobia.

The intensity of the fluorescence decreases with the higher dilutions of the applied FA. Grade each smear for the

intensity of the fluorescence using the following scale:

Grade	Fluorescence
4+	Brilliant yellow-green
3+	Bright yellow-green
2+	Yellow-green
1+	Dull-green
0	No fluorescence

Ideally, FAs should show a 4+ reaction even after they have been diluted by several twofold steps. Occasionally, FITC conjugations yield only FA of 3+ rating. The FA are diluted before use. The highest dilution which still results in an intensity of fluorescence comparable to the undiluted FA is used for strain identification. The non-homologous reaction should show no more than background fluorescence. Strains which cross react may show from 4+ down to 1+ reactions.

(g) Typing nodules using the FA technique
(Key step 8)

The soybean plants (inoculated with pure TAL 379 str, pure TAL 378 spc, and a mixture of TAL 379 str + TAL 378 spc) which had been harvested in Exercise 10 and stored in the refrigerator will be used in this exercise.

Select the nodules that resulted from the mixed inoculum treatment. (TAL 379 str and TAL 378 spc). Each nodule has to be reacted with the FA of the two component strains of the mixed inoculum. The nodules should be cleaned with water and blotted dry.

Prepare two templates on microscope slides showing twelve dots evenly spaced representing the location of 12 smears from individual nodules plus two dots for controls of pure Rhizobium cultures. The size of the dots and their spacing should be as shown in Figure 11.1.

Lay out two clean microscope slides, and using a pencil, code one with TAL 379 str and the other with TAL 378 spc.

Place each slide over one template and apply the nodule smears: Grip a nodule with a blunt tipped forceps, section it with a scalpel and make a thin smear of the cut surface on the slide marked TAL 379 over the location of the first dot on the template.

Make a duplicate smear with the same nodule at the corresponding location of the other slide marked TAL 378 spc.

Flame the forceps and scalpel. Take a second nodule and make similar smears on corresponding positions (n_2) on the other

slides. Continue until all twelve positions have been covered on each slide. Remember to completely burn off adhering particles from scalpel and forceps before each new nodule. Smears of more nodules can be made on additional slides in the manner described. Make two smears of the homologous culture for controls.

Air dry and heat fix the smears.

With a Pasteur pipette, place a drop of RhITC (rhodamine gel) (Appendix 4) on the smears. This eliminates much of the background fluorescence normally caused by nodule debris.

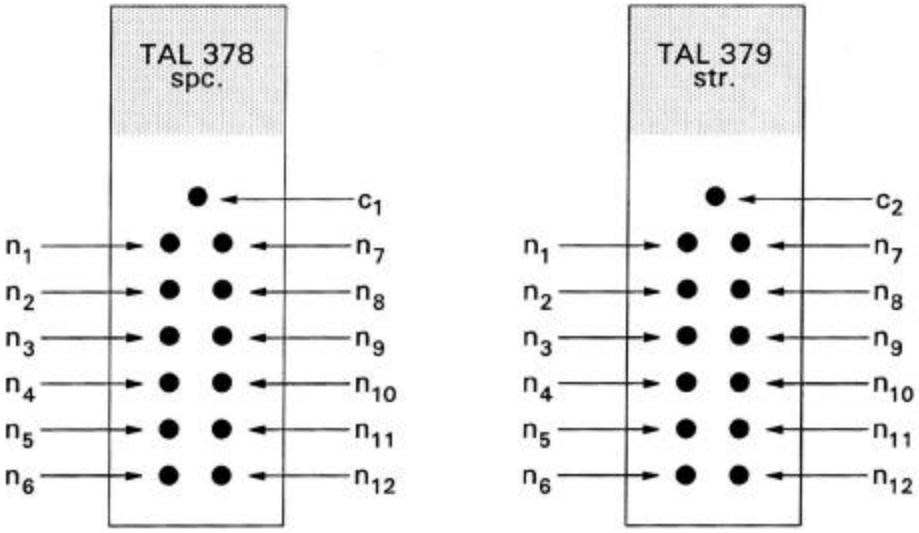


Figure 11.1. Scheme of nodule smears for strain identification by FA.

Before the rhodamine gel dries, add 1 drop of FA solution and allow to react in a moisture-saturated chamber at room

temperature. Incubate, rinse, wash, and dry the slides following the same procedures as described for the determination of the FA titer. After the smears have dried, circle the smears with a fine permanent marker or diamond pen on the reverse side of the slide. This will be helpful in locating them under the microscope.

Add sufficient mounting fluid, approximately 2 drops per slide for 12 or more smears. Place a long (4 cm) coverslip over the smears, taking care to exclude air bubbles. Observe the preparations with a UV-microscope under a 40x or 60x objective. Also, observe under a 90x or 100x objective with oil immersion.

If the microscope is equipped with a phase contrast condenser, first focus on the smear using incandescent light before observing under UV light. This will greatly reduce the fading of the smear through prolonged exposure to ultraviolet light.

A strong positive reaction is indicated by brilliant yellow green fluorescence of the smear on a dark purple background. No cells will be visible (i.e., no fluorescence) if the specific strain is not present on the smear. A mixed infection (a nodule containing both TAL 379 str and TAL 378 spc) is obvious when smears from a single nodule fluoresce with the FA-stains of both strains.
Compare results from this method with those of the other

method used.

Requirements

(a) Fractionating serum globulin

Ringstand with three clamps
Magnetic stirplate with a 12 mm (0.5 in) stirring bar
Centrifuge
Refrigerator
Balance (for centrifuge tubes)
Burette
Graduated pipette 10 ml
Two 5 ml centrifuge tubes with caps
Beaker 250 ml with crushed ice
Cold 3.9 M ammonium sulfate solution
Saline (0.85% NaCl filtered through 20 ìm filter)
Rabbit antiserum (15 ml)

(b) Purifying serum globulins

Cold-room or large refrigerator
Magnetic stirplate, 5-8 cm (2-3 in) stirring bar
Three 3 liter flasks or beakers
Two 1 ml pipettes; one 10 ml pipette
Test tube
Dialyzing tubing (20 cm)
Surgical gloves
Six liters of saline adjusted to pH 8 with NaOH

Filtered saline

Merthiolate solution (filtered, 1%)

Saturated barium chloride solution

Nessler's reagent (optional)

Gamma globulins (final precipitate from [a])

(c) Determining the protein content of the dialysate

Spectrophotometer; two cuvettes

Eight 15 ml test tubes

Test tube rack

Pipette (5 ml) two 1 ml pipettes

Distilled water

Filtered saline

Vial for dialysate

Bovine serum albumin solution (20 mg ml^{-1})

Dialyzed globulin solution from (b)

Biuret reagents

(d) Conjugating the globulins with fluorescent dye

Analytical balance, spatula, weighing paper

Magnetic stirplate; 12 mm (0.5 in) stirring bar

Ring stand with two clamps

pH meter

Two 50 ml beakers; parafilm or foil for covering

Two 10 ml pipettes, 1 ml pipettes

Merthiolate solution (1% and filtered)

Sodium phosphate buffer 0.15 M pH 9 (Appendix 4)

Sodium phosphate buffer 0.1 M pH 8 (Appendix 4)

Fluorescein isothiocyanate (FITC) (Sigma Chemical Company, P.O. Box 14508, St. Louis, MO. 63178, USA)

Phosphate buffered saline (PBS) (Appendix 4)

Sodium hydroxide solution, 0.1 N

Rabbit gamma globulin from (c)

(e) Purifying the FA

Suction pump or Aspirator

Refrigerator, freezer

Centrifuge

Balance for centrifuge tubes

Two centrifuge tubes with caps

Chromatography column (approximately 2.5 cm x 20 cm)

Glass wool

Pasteur pipette with rubber bulb

Erlenmeyer flask, 1 liter with screw cap (or large glass bottle)

Glass beaker (50-100 ml)

Two liter reservoir for PBS with connecting tubing and plug for column

Phosphate buffered saline, 2 liters containing 0.01% Merthiolate

Sephadex G 25-150 (or G 25-300) (Sigma Chemical Company)

Distilled Water

Carbowax (polyethylene glycol)

Dialyzing tubing

Membrane filter unit with filter of 0.45 m pore size

Screw cap vials for storage of FA

FITC conjugate from (d)

(f) Testing the quality of the FA

UV Microscope (instrument with epifluorescence condensor preferable)

Transfer loop, flame

Microscope slides, cover slips, mounting fluid

Incubation chambers

Rinsing tank containing PBS; rinsing tank containing distilled water

Wash bottle containing PBS, wash bottle containing distilled water

Supply of PBS (2 liters)

Test tubes, rack

Pasteur pipettes, rubber ball

Young cultures of *B. japonicum* strains TAL 378 and TAL 379

FA from (e)

(g) Typing nodules using the FA technique

UV microscope (instrument with epifluorescence condenser preferable)

Microscope slides, cover slips (long), mounting fluid, immersion oil

Inoculation loop, flame, forceps, scalpel

Incubation chambers, rinsing tanks as in (f)

Nodules containing TAL 378 spc and TAL 379 str (Chapter 12)

Pure cultures of TAL 378 and TAL 379

FA of TAL 379 and TAL 378 (diluted for use)

Rhodamine gel (optional)

EXERCISE 12

TO DEVELOP ANTIBIOTIC RESISTANT RHIZOBIA

Rhizobia bearing genetic markers are obtained through a mass selection technique. Bacterial strains contain small numbers of naturally occurring mutants which are resistant to high concentrations of certain antibiotics. This resistance may be used for the recognition of rhizobial strains.

Key steps/objectives

1) Culture rhizobia in YM broth
2) Prepare YMA plates containing antibiotics
3) Spread selected culture(s) onto appropriate antibiotic and non-antibiotic plates
4) Check for natural resistance
5) Transfer resistant colonies to YMA slants
6) Culture resistant isolates in YM broth
7) Spread broth culture(s) resistant to streptomycin onto plates containing spectinomycin (and vice versa)
8) Transfer double resistant mutants to YMA slants. Confirm resistance to streptomycin and spectinomycin; streak onto plates containing both antibiotics
9) Confirm retention of symbiotic effectiveness of resistant strain

(a) Culturing selected strains

(Key step 1)

Select strains for the development of antibiotic resistant mutants. Culture the strains in duplicate flasks containing 50 ml YM broth. Place on a shaker for 3-7 days according to the growth rates of the strains chosen.

(b) Preparing YMA plates containing antibiotics

(Key step 2)

Prepare a stock solution of streptomycin (str) with a concentration of 4 mg ml^{-1} (Appendix 3). Filter sterilize 5 ml of the stock through a sterile millipore filter of 0.20 micron pore size. Add the filtrate to 500 ml of YMA (in a 1 liter Erlenmeyer flask) kept molten in a water bath at 50°C. Mix well, but avoid vigorous shaking to minimize the formation of air bubbles. Return the flasks to the water bath for 10 min to re-equilibrate the temperature and to allow the air bubbles to dissipate from the agar. Pour the plates. These plates will have streptomycin 40 ìg ml^{-1} agar.

Similarly prepare plates containing 250 ìg ml^{-1} of spectinomycin (spc) from a stock solution containing 25 mg ml^{-1} (Appendix 3).

Also prepare YMA plates containing a mixture of both the

antibiotics in the above concentrations for use in the selection of rhizobia with resistance to two antibiotics.

Prepare an equal number of YMA plates without antibiotics. All the plates may be stored under refrigeration.

(c) Selecting spontaneous mutants with resistance to one antibiotic

(Key step 3,4,and 5)

Spread 0.1 ml of each broth culture on plates containing (a) no additives; (b) streptomycin (40 ìg ml^{-1}); and (c) spectinomycin (250 ìg ml^{-1}).

The growth rates of rhizobial strains may be retarded in the presence of antibiotics. Prepare to incubate up to 12 days but check for emerging colonies everyday after day 5.

The plates of treatment (a) which contain no antibiotics should have abundant rhizobial growth.

The plates of treatment (b) and (c) should have very little growth compared with treatment (a). Not more than 30 resistant colonies are expected since the rate of mutation is 1 in 10^5 to 1 in 10^7 with most strains of rhizobia.

Pick four colonies each from treatments (b) and (c) and

transfer to separate YMA slants (containing no antibiotics) in culture tubes. Incubate at 25-30°C for 5-9 days, then store at 4°C. These four isolates must be kept separate till the end of the selection process.

Confirm the antibiotic resistance of str and spc isolates. Streak the mutants on YMA containing antibiotics and on a control plate containing plain YMA. Incubate at 25-30°C for 5-9 days.

(d) Selecting strains of rhizobia with resistance to two antibiotics

(Key steps 6, 7, and 8)

To develop strains resistant to both streptomycin and spectinomycin, spread a 0.1 ml broth culture of a spc mutant on a plate containing streptomycin (in a similar manner, a str mutant should be spread on YMA containing spectinomycin). Incubate at 25-30°C for 5-9 days.

Check for growth of colonies on the plates containing antibiotics. Again, because of a similar mutation rate as with resistance to one antibiotic, no more than 30 colonies of spontaneous mutants with double resistance (str · spc) are expected.

Transfer four of these colonies to YMA slants (containing no

antibiotics) in culture tubes, incubate, and store. Confirm resistance to both antibiotics by streaking on plates containing both streptomycin (40 ìg ml^{-1}) and spectinomycin (250 ìg ml^{-1}) and on control plates of plain YMA. Incubate at 25-30◦C and compare growth on antibiotic and control plates.

Streptomycin, spectinomycin and streptomycin-spectinomycin resistant strains usually retain their N_2-fixing capability. Mutant strains should be compared with their parent strain in a symbiotic effectiveness test as described in Chapter 20 prior to use in ecological experiments. To be useful, mutant strains should not show significant differences in N_2-fixation from the parent strain.

Requirements

(a) Culturing selected strains

Transfer hood, incubator and shaker
Bunsen burner
Inoculation loop
Two flasks, 150 ml, containing 30 ml YM broth each
Culture of rhizobia

(b) Preparing YMA plates containing antibiotics

Filled water bath adjusted to 50°C
Suction pump or aspirator with moisture trap
Two sterile filter sterilizing units with sterile millipore filter (0.20 micron)
Three 10 ml pipettes
Wash bottle with distilled water
Stock solution of streptomycin (4 mg $ml^{-}1$)
Stock solution of spectinomycin (250 mg ml^{-1})
Sterile molten YMA, 3 l, in three 2 l or six 10 l Erlenmeyer flasks
Petri dishes, sterile

(c) Selecting spontaneous mutants with resistance to one antibiotic

Incubator, bunsen burner, transfer loop, small beaker of alcohol

YMA plates containing streptomycin (40 ìg ml^{-1})

YMA plates containing spectinomycin (250 ìg ml^{-1})

YMA plates

Spreading stick

Broth culture

Six YMA slants in culture tubes

Graduated pipette, 1 ml

(d) Selecting strains of rhizobia with multiple antibiotic resistance

Transfer or laminar flow hood, tools and incubator as in (c)

Antibiotic stock solutions and YMA plates as in (c)

Six YMA slants

Mutant broth inoculum resistant to streptomycin

Mutant broth inoculum resistant to spectinomycin

Alcohol, spreading stick

EXERCISE 13

TO IDENTIFY ANTIBIOTIC-RESISTANT MARKED STRAINS OF RHIZOBIA IN NODULES

Antibiotic resistant marked strains of rhizobia may be identified by their ability to grow on media containing antibiotics. The antibiotic marker technique is applied in ecological studies where strain identification is not possible by serology due to cross reactions of the strains, or because of unavailability of antisera. Antibiotic markers also provide useful confirmatory data.

Key steps/objectives

1) Set aside inoculated soybean plants (from Exercise 8)
2) Prepare antibiotic plates for nodule typing
3) Harvest soybean plants; clean, trim, and sterilize roots; type nodules
4) Read results
5) Compare results to those obtained by the serological methods (Exercise 9 and 10)

(a) Culturing plants inoculated with antibiotic resistant marked strain(s) of Rhizobium

(Key step 1)

Soybean plants which have been set up for Exercise 8 will also be used in this exercise. They have been inoculated separately with TAL 379 str, TAL 378 spc, and a mixture of TAL 379 str and TAL 378 spc.

Obtain the viable cell counts of the inocula as determined in Exercise 4.

(b) Preparing YMA containing antibiotics for nodule typing (Key step 2)

Prepare plates containing: a) streptomycin (40 ìg ml^{-1} YMA); b) spectinomycin (250 ìg ml^{-1} YMA); and c) plain YMA as in Exercise 12.

Draw a grid pattern on the bottom of each plate. Draw approximately 20 squares, each of which can be individually identified by letter and number (Figure 13.1). Squares with identical number and letter combinations on each of the three plates are meant to correspond to the same nodule.

(c) Typing nodules using antibiotic resistant markers. (Key step 3)

Harvest one of each inoculation treatment from Leonard jars, saved from Exercise 8.

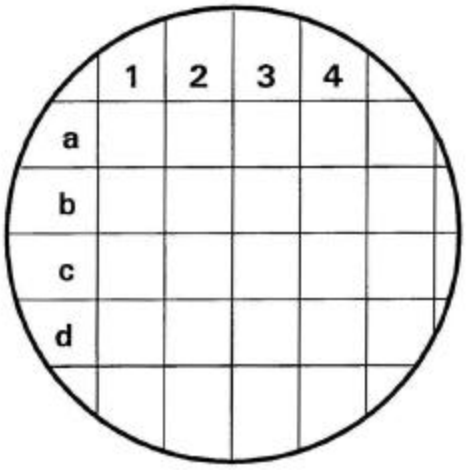


Figure 13.1. Plate with grid pattern for nodule identification by antibiotic resistance.

Detach and surface sterilize nodules as in Exercise 1.

Pick up one nodule with a pair of sterile, blunt-tipped forceps. While holding the nodule between the tips of the forceps, apply just enough pressure until the milky nodule content emerges. Spread this nodule material within its allotted square of the grid pattern on each plate.

Inoculate the plain YMA plate last to check for sufficiency of nodule inoculum. Process at least 20 nodules from each replication in this way. Flame forceps thoroughly between fresh nodules.

Alternatively, sterile toothpicks or pins may be used to

transfer bacteroids from the nodules to the plates. This method is especially useful for smaller nodules.

In another method, all non-nodulated excess root material is trimmed off with a pair of scissors and discarded. The trimmed nodulated part of the root is then sterilized and placed onto sterile filter paper in a sterile Petri dish. A sketch is made of the nodulated root system on a record sheet and the nodules assigned reference numbers. The nodules are then detached with sterile forceps, one at a time, and processed as described above, starting with the first nodule on the upper part of the root. In this way it is possible to identify not only which nodules on the plants were formed by the introduced, marked strain but also the specific location and distribution of those nodules in the root system.

Incubate the plates at 25-30°C and make daily observations. Some contaminants (bacteria & fungi) may be resistant to the levels of spc and str used. Therefore, if the nodules have not been properly surface sterilized, these contaminants may appear on the plates earlier than the rhizobia.

(d) Interpreting the growth patterns
(Key steps 4 and 5)

Five to 8 days after inoculation, inspect the plates for signs of growth. Since corresponding squares on the three different

plates have been inoculated with bacteroids from the same nodule, it should now be possible to determine which strain or strains occupied the nodule by the presence and absence of growth (Figure 13.2).

Compare the results from this exercise with those obtained in the identification by the agglutination method (Exercise 8) and the gel immunodiffusion method (Exercise 9).

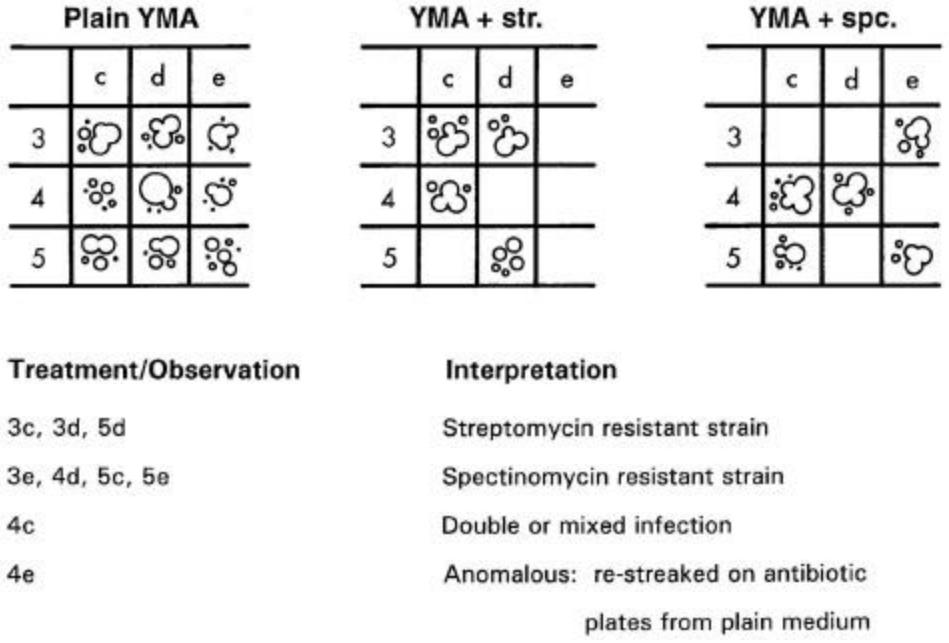


Figure 13.2. Interpreting growth patterns on antibiotic Plates.

Requirements

(a) Culturing plants with antibiotic resistant marked strains of *B. japonicum.*

Leonard jar with 2 soybean plants inoculated with TAL 379 str

Leonard jar with 2 soybean plants inoculated with TAL 378 spc

Leonard jar with 2 soybean plants inoculated with a mixture of TAL 379 str and TAL 378 spc

(b) Preparing YMA containing antibiotics for nodule typing (Exercise 12)

Plates containing YMA + streptomycin (40 ìg ml^{-1})

Plates containing YMA + spectinomycin (250 ìg ml^{-1})

YMA plates

Felt pen with permanent ink, ruler

(c) Typing nodules through the use of antibiotic resistant strains of *B. japonicum*

Incubator (25-30°C)

Requirements for sterilizing nodules (Appendix 10)

Soybean plants listed in (a)

Running water

Scissors, forceps (2)

Plates prepared in (b)

Optional: sterile toothpicks or pins

(d) Interpreting the growth patterns

Inoculated plates from (c)

EXERCISE 14

TO IDENTIFY RHIZOBIUM USING PHAGES

Bacteriophages of rhizobia (rhizobiophages) are isolated from the soil and used for identifying rhizobia. Strains of rhizobia vary in their resistance to rhizobiophages. The different patterns of susceptibility which result from exposure to a range of phages are used for strain identification.

Key steps/objectives

1) Collect soil samples from various soybean fields
2) Inoculate YM broth with TAL 379
3) Inoculate broth cultures of TAL 379 with soil from soybean fields
4) Filter broth cultures
5) Enrich phage suspensions by filtration
6) Assay filtrates for phage concentrations
7) Inoculate YM broth with rhizobial strains (A to F) to be typed
8) Spread-plate rhizobial suspensions and spot phages
9) Inspect plates for phage forming units (PFUs) and tabulate results

(a) Isolating bacteriophages

(Key steps 1, 2, 3, 4, and 5)

Collect soil samples from sites where soybeans are growing or have been grown. Obtain the soil from the rhizosphere of individual plants. Include some root material and, if possible, nodules. Collect samples from eight locations. Mix each soil thoroughly and store the samples at 4°C until use.

Sixteen 250 ml flasks, each containing 100 ml sterile YM broth, are required for each soil sample. Inoculate the flasks with *B. japonicum* strain of choice (eg. TAL 379) in batches of four, with a lag period of 1 day between each batch. This will provide cultures in the exponential growth phase when needed for subsequent phage inoculation. Incubate cultures on a rotary shaker at 25°-30°C.

When the first batch of cultures has reached its exponential phase of growth (2-4 days after inoculation), add 1 gram of soil to each flask of batch one. Make sure that each flask is inoculated with soil from a different location. Incubate for 18-20 h at 25-30°C.

Remove cells and soil by centrifugation (10,000 x g for 15 min) and filter the supernatant through a sterilized membrane filter (0.20 ìm). This filtrate contains the rhizobiophages which are small enough to pass through the filter. Add 10 ml of each filtrate to a fresh culture (second batch) of the same strain of rhizobia, incubate on a shaker for 18-20 h and again

centrifuge and filter. Repeat this procedure two more times, making certain that the filtrate is matched to the corresponding culture.

The turbidity in the bacterial cultures should diminish noticeably 8-10 h after the addition of the phage filtrate. The last filtrate is the phage suspension and should contain 10^6-10^9 phage particles. Dispense the filtrate into 20 ml tubes, add four drops of chloroform and store in the refrigerator at 4°C.

(b) Assaying for phage by the overlay method
(Key step 6)

Make tenfold serial dilutions of the phage filtrates in phosphate buffered saline (PBS at pH 7.1, Appendix 5). Add 0.1 ml of each dilution to tubes containing 2.5 ml melted YMA kept at 50°C in a water bath. Stir in 0.5 ml of a fresh culture of TAL 379 and immediately pour the YMA mixture over plates of MNA and distribute evenly. Prepare controls with only PBS and TAL 379 (no phage) added to agar and poured over YMA plates. Allow plates to stand for 10-15 min. Incubate inverted plates for 24-72 h and look for plaques (small clear zones). Count the plaques per plate. To calculate the number of Plaque Forming Units (PFU) in 1 ml of the original filtrate, multiply the number of plaques per plate by 10 and by the dilution factor. If 20 plaques were counted on a plate

containing a 10^{-5} dilution, the number of PFUs is 20 x 10 x 10^5 = 2 x 10^7 PFU/ml.

(c) Characterizing rhizobia using phages
(Key steps 7, 8, and 9)

Because of the specificity of bacteriophages for their bacterial hosts, each strain of bacterium exhibits a unique pattern of susceptibility against a large number of different bacteriophages. This unique pattern can be used to identify (phage-type) the organisms of interest.

Select several strains of *B. japonicum* and distinguish between them by their susceptibility to a range of phages. Incubate each strain in duplicate flasks of 50 ml MN broth at 25-30°C. Include strains of *B. japonicum* TAL 379 and TAL 378.

After 5-9 days incubation, spread 0.1 ml of each of the cultures to be typed over a separate MNA plate with a sterile glass spreader. Spot the surface of the bacterial lawn with a small loopful of each of the collected phage suspensions. The location of the spots should be marked on the back of the plates. Allow the plates to stand for 10-15 min, invert and incubate for 24-48 h.

Inspect the plates for a clear zone where each of the phages was spotted. Record presence (+) or absence (-) of plaques in

a table similar to the example in Table 14.1.

The susceptibility of a rhizobial strain to a range of phages can be regarded as its "fingerprint," enabling it to be recognized in ecological investigations.

Table 14.1. An example of results of Rhizobium identification by phage typing.

	Strains of Rhizobium						
Phage Isolate	A	B	C	D	E	F	TAL379
1	+			+	+		+
2		+	+	+			+
3			+	+			+
4	+				+		+
5	+		+		+		+
6	+	+	+			+	+
7			+		+	+	+
8				+	+	+	

*A, B, C, D, E and F are *B. japonicum* isolates from nodules of soybean

Requirements

(a) Isolating bacteriophages

Refrigerator, rotary shaker, centrifuge balance

Desiccator, centrifuge tubes (50 ml), rack, pipettes (10 ml)

Membrane filter units, sterile with Millipore filters of 0.20 ìm pore size

Soil samples (from four locations where inoculated soybeans are/were grown)

Digging tools, plastic bags

Erlenmeyer flasks, 150 ml, containing 50 ml MN broth each

Transfer loop, flame

Chloroform solution (1%)

Slant culture of *B. japonicum* (TAL 379)

(b) Assaying for phage by the overlay method

Incubator, water bath, rotary shaker

PBS (pH 7.1)

Pipettes, 1 ml

Tubes containing 2.5 ml liquid YMA (50°C)

Plates of MNA

Phage filtrates from (a)

Broth cultures of TAL 379

(c) Characterizing rhizobia using phages

Inoculation loop, flame

Pipettes (1 ml)

Spreaders

Erlenmeyer flasks of 125 ml containing 50 ml MNA

MNA plates

Slant cultures of a range of *B. japonicum* strains including strain *B. japonicum* strain TAL 379)

Phage isolates from (a) as well as others if available.

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SECTION C

RHIZOBIUM STRAIN SELECTION INTRODUCTION

After rhizobial strains have been isolated from nodules, they must be evaluated for their ability to form nodules and fix nitrogen with targeted legumes. The source of rhizobial strains for a strain selection program can range from local isolates, to strains already tested in other parts of the region or country, to cultures from various overseas collections. Preliminary screening is performed in the greenhouse, where numerous strains can be tested on several host varieties. If the inoculated plants form nodules and produce healthy green leaves when grown in nitrogen-free media, it can be assumed that an effective symbiosis has been established. Rhizobia selected in greenhouse trials, where conditions are usually optimal, must then be evaluated in the field. Rhizobia which adapt to the agronomic conditions under which the host legumes will be cultivated and which enhance crop production through nitrogen fixation can then be selected for inoculant production.

The legume-rhizobial symbiosis exhibits widely differing degrees of specificity. In some instances, the symbiosis is highly specific in that a particular species or strain of *Rhizobium* or *Bradyrhizobium* can form an effective symbiotic association with only one particular legume species or

variety. The temperate legumes *Trifolium, Cicer, Phaseolus, Medicago* and tropical species like *Glycine max, Leucaena*, and *Lotononis* are in this category. There are also intermediate cases which exhibit varying degrees of cross-inoculation capability as in *Centrosema, Phaseolus acutifolius, P. lunatus*, some *Desmodium* spp. and *Acacia* spp. At the opposite extreme are the promiscuous associations, in which diverse legumes may be infected by one or more of several rhizobia. This condition is more prevalent in the tropical legumes than in the temperate species. Because the earlier studies of symbiotic nitrogen fixation were initiated in temperate regions, the taxonomy of the genera *Rhizobium* and *Bradyrhizobium* were based on a host-dependent classification system which emphasizes temperate associations (see Section A). A large number of tropical rhizobia which form symbiotic associations with *Vigna, Macroptilium, Arachis, Cajanus, Lablab*, and other genera of legumes are simply labelled as the "cowpea miscellany" or *Bradyrhizobium* spp.

In some cases it is desirable to select a strain for a wide range of hosts. An example would be the *Bradyrhizobium* sp. (CB756; TAL 309) isolated from the nodule of *Macrotyloma africanum*. This strain effectively nodulates approximately 40 of the promiscuous tropical legumes. This `broad-spectrum strain' characteristic would be advantageous if this superior strain of *Bradyrhizobium* sp. were to be introduced to locations where those diverse legumes are to be grown. In a

different situation, it might be advisable to work with a very specific symbiosis to ensure infection by a particular inoculant strain that competes with native soil rhizobia. Due to these and other considerations, characterizing rhizobial associations is of utmost importance when a legume cultivar is being developed through breeding or when a legume is being introduced into a new environment.

Field evaluation of effective rhizobia is critical because the symbiosis may be affected by a number of environmental factors discussed earlier. The ability of an inoculant strain to persist in a particular environment, while in some cases competing against a resident soil population of rhizobia, is of critical importance. A combination of the above factors should be anticipated in the selection process to ensure good performance at different geographical locations. The task of introducing superior strains into soils that are already inhabited by effective rhizobia is difficult, and evaluation methods are an important key to success.

Final evaluation of the symbiosis will be based on several measurable parameters. Short term trials with Leonard jars or sterile sand culture pots can provide an adequate basis for gross comparison of strains. The shoot dry weight of plants harvested at floral initiation or after significant plant biomass accumulation is the generally accepted criterion for nitrogen-fixing effectiveness, but nodule dry weight may also

be employed. Nodule number is a less reliable indicator of strain effectiveness. The measurement of activity in the nodules by the nitrogen-fixing enzyme, nitrogenase, may also be done. This is accomplished by means of the acetylene reduction assay, which is a measure of ethylene production and indicates nitrogenase activity. However, the results of this assay should not be used to conclude on the actual amounts of nitrogen fixed. This assay requires the availability of a gas chromatograph and other rather sophisticated equipment and materials. Total nitrogen accumulation in the shoot can be measured by the Kjeldahl method. Since total nitrogen content and nodule dry weight frequently correlate well with shoot dry weight, the latter parameter provides an acceptable basis for strain comparison. The final proof of inoculation response must come from the field when the seed and nitrogen yields at harvest are determined for grain legumes or from the dry matter production for forage legumes.

EXERCISE 15

TO TEST FOR GENETIC COMPATIBILITY BETWEEN RHIZOBIA AND LEGUMES

Specificity and promiscuity in the symbioses are studied in cross-inoculation experiments. The specific requirements of certain legumes for particular rhizobia are demonstrated.

Key steps/objectives:

1) Culture strains of rhizobia
2) Prepare seedling-agar tubes and Leonard jars
3) Prepare water-agar plates
4) Select, surface sterilize, and germinate seeds
5) Plant pregerminated seeds in seedling-agar tubes and Leonard jars
6) Thin seedlings in Leonard jars
7) Inoculate seedlings in Leonard jars and tubes
8) Make periodic observations of nodulation
9) Harvest after 5 weeks
10) Evaluate results

(a) Culturing strains of rhizobia
(Key step 1)

Culture each of the *Rhizobium* spp. and *Bradyrhizobium* spp. listed in Table 15.1 in 100 ml of YM broth in 250 ml

Erlenmeyer flasks.

(b) Preparing seedling-agar tubes and Leonard jars (Key step 2)

Prepare 54 seedling-agar slants in 22 x 250 mm tubes. The composition of the seedling-agar is detailed in Appendix 3 and its preparation in Appendix 7. A simple set-up for dispensing the melted agar into the tubes is illustrated in Appendix 7 (Figure A.9).

Set up 108 Leonard jars as explained in Appendix 11. Nitrogen-free nutrient solution for use in Leonard jars is of similar composition as that used for making seedling-agar.

Table 15.1. Strains of Rhizobium and hosts according to cross-inoculation groups

TAL No.	Rhizobial species	Host legume
169	*Bradyrhizobium* sp.	*Macroptilium atropurpureum* (siratro)
169	*B.* sp.	*Vigna unguiculata* (cowpea)
182	*R.l.* bv. phaseoli	*Phaseolus vulgaris* (bean)
379	*B. japonicum*	*Glycine max* (soybean)
380	*R. meliloti*	*Medicago* spp. (alfalfa, sweet clover)
382	*R.l.* bv. trifolii	*Trifolium* spp. (clover)
1145	*R.* sp. (*Leucaena*)	*Leucaena* sp.
620	*R.* sp. (*Cicer arietinum*)	*C. arietinum* (chickpea)
634	*R.l.* bv. viceae	*Lens culinaris* (lentil)

Each treatment (rhizobial species-legume host combination and controls) in this exercise will be done in duplicate. Refer to Table 15.2 for the treatments and the various combinations to test genetic compatibility between rhizobia and legumes.

(c) Preparing germination plates
(Key step 3)

Make 300 ml of 0.75% (w/v) water-agar in a 500 ml flask and sterilize. Pour 25 ml of melted water-agar into 12 or more Petri dishes and allow to cool. Surface sterilized seeds will be pregerminated in these plates.

(d) Surface sterilizing seeds
(Key step 4)

Check percentage germination of each legume species in advance of experiment. Batches of seeds with more than 70% viability will be suitable. Select undamaged seeds for uniformity in size and color. Surface sterilize enough seeds (at least 200 of each species) to give at least 100 germinated seeds.

Surface sterilize the seeds (Appendix 10) by immersion in a 3% sodium hypochlorite solution for 3-5 min. (To prepare 3% sodium hypochlorite solution, add 10 parts of commercial

bleach [5.25% sodium hypochlorite] to 7.5 parts of water.)

Hard seed-coated species (e.g., leucaena, siratro) are scarified and sterilized simultaneously by immersion for 10 min in concentrated sulfuric acid. Drain off all excess acid prior to rinsing with sterile water. (If acid is used, the first rinse should be done quickly to prevent loss of viability of the seeds caused by the heat generated when water is added to the acid.)

Rinse seeds with six to eight changes of sterile water after surface sterilization. Allow the seeds to imbibe water by soaking for 1 h and then rinse twice. Transfer the seeds aseptically to agar plates with a spoon-shaped spatula.

Each batch of 100 seeds should be dispensed evenly in two or more (depending on seed size) water-agar plates and incubated at 25-30°C. (The large-seeded species, e.g., *Phaseolus* and *Cicer* may need more water-agar plates.) Invert the plates containing the small-seeded species to provide straight radicles that are much easier to handle in later steps of the exercise.

(e) Planting and inoculating
(Key steps 5, 6, and 7)

Soybean, cowpea, bean, chickpea, lentil, and leucaena seeds

will be planted in Leonard jars. Make three well-spaced holes in the rooting medium to a depth that will accommodate the pregerminated seeds 1 cm below the surface. Pick up well germinated seeds with sterile forceps and place one seed in each hole with the radicle entering first. (Proper orientation of the radicle during planting is important to ensure proper emergence of the shoot and establishment of the seedling.) After placement of the seed, inoculate (1 ml per seed) with the rhizobial culture and cover the hole with the rooting medium. If vermiculite is used as the rooting medium, autoclaving will cause swelling and loosening of the vermiculite. This leads to poor anchorage of the root. Therefore, gentle compacting of the vermiculite will be required before planting/sowing of the seeds. Firmness of the rooting medium can be restored by pressing it down with the bottom (sterilized by flaming) of a 125 ml Erlenmeyer flask.

After planting and inoculation are completed, add sterile gravel over the surface of the rooting medium. Set up 18 jars for each species.

Siratro, clover, and alfalfa will be cultured on agar slants in tubes. Select and plant one seedling on the agar surface. Observe the usual aseptic precautions, taking care to sterilize the hands with 70% alcohol, flame sterilizing the inoculating loop and mouth of the tube etc. when transferring the seedling. Using an inoculating loop, pick up the

pregerminated seedlings and transfer them into the tubes. The seedling radicles should be 0.5-1.0 cm long and straight. After planting, tubes should be kept in a slant position for the radicles to adhere to the agar surface for at least 2 h. Dispense 1 ml of culture over the roots of the seedlings in the agar slants. Use a fresh pipette for each new rhizobial species or strain. Aluminum foil wrapped around the lower part of the tubes will shield the roots from light and heat. Seedling-agar tubes need to be placed in suitable wooden racks and kept in a growth chamber (environmental growth chamber or in a temperature controlled greenhouse) for proper seedling development.

Thin plants in the Leonard jars to two uniform plants per jar after 5 days. Excise the shoot of the unwanted plant aseptically using scissors. Avoid disturbing the rooting medium during thinning. To facilitate proper inoculation, carefully clear (with a sterile glass rod) the rooting medium around the root of the plant, to a depth of 1 cm. Dispense drops of rhizobial culture (totaling 1 ml) into the cleared area around the root. Dispense 1 ml of rhizobia culture over the roots of the seedlings in the agar slants. Use a fresh pipette for each strain of rhizobia. Place the inoculated jars on the benches in the green house.

(f) Observing periodically and harvesting
(Key steps 8 and 9)

Examine the plants over a period of 5 weeks. Note color and growth. Replenish tubes and Leonard jars with sterile water as required. At the end of the fifth week, excise the tops and determine their dry weight (dry for 48 h at 70°C). Remove roots from the jars and tubes and wash them free of rooting medium. Where nodules are present, describe nodule shape, size, pigmentation, and distribution.

(g) Evaluating the experiment
(Key step 10)

Note cross-inoculation groups as recorded in Table 15.2 and the ineffectiveness and effectiveness of each rhizobial species-legume combination. Effectiveness will be apparent from the green coloration of the plant and abundant nodules that are red/pink when sliced open.

Table 15.2. For recording presence (+) or absence (-) of nodules in each rhizobia/legume combination.

Legume / Rhizobia	soybean	cowpea	bean	lentil	leucaena	chickpea	alfalfa	clover	siratro
B. japonicum TAL 379									
Bradyrhizobium sp. TAL 169									
R. l. bv. phaseoli TAL 182									
R.l. bv. viceae TAL 634									
Rhizobium sp. (leucaena) TAL 1145									
Rhizobium sp. (chickpea) TAL 620									
R. meliloti TAL 380									
R.l. bv. trifolii TAL 382									
Uninoculated									

Requirements

(a) Culturing strains of rhizobia

Slant cultures of rhizobia
YM broth in flasks

(b) Preparing seedling-agar tubes and Leonard jars

Seedling-agar slants
Leonard jars

(c) Preparing media for germination

Agar-powder, Petri dishes, 500 ml flasks
Balance

(d) Surface-sterilizing seeds

Seeds of cowpea, bean, soybean, alfalfa, clover, leucaena, siratro, chickpea, and lentils
3% sodium hypochlorite solution or other sterilants (Appendix 10)
Concentrated sulfuric acid
Sterile water
Sterile flasks or beakers
Incubator

(e) Planting and inoculating

Pregerminated seeds of the various species

Leonard jars

Seedling-agar slants, wooden racks, growth chamber

Aluminum foil

Alcohol, forceps

Sterile pipettes (1 ml), cultures of rhizobia

(f) Observing periodically and harvesting

Sterile water

Scissors, paper bags or envelopes

Drying oven (70°C)

Scalpels or razor blades

EXERCISE 16

TO SCREEN RHIZOBIA FOR NITROGEN FIXATION POTENTIAL

The nitrogen fixation potential of a number of strains of pure cultures of *Bradyrhizobium japonicum* in symbiotic association with soybean is compared. The most effective strains in this exercise will be compared later in potted field soil.

Key steps/objectives

1) Prepare Leonard jars
2) Culture rhizobia
3) Prepare water-agar plates
4) Sterilize and plate seeds for germination
5) Plant and inoculate seedlings in Leonard jars
6) Observe progress of experiment
7) Harvest experiment
8) Analyze data

(a) Experimental design and treatments

The experiment is set up as a Randomized Complete Block Design (RCBD) with three blocks or replications (Figure 16.1). There are 14 inoculation treatments, a plus-nitrogen control with no inoculation, and a non-inoculated control with no nitrogen. The plus-nitrogen control will contain 70 ppm N applied as a

0.05% KNO_3 (w/v) solution. The nitrogen is added to the nutrient solution in the reservoir of the Leonard jar assembly.

(b) Preparing Leonard jars
(Key step 1)

A total of 48 Leonard jar assemblies will be required. Prepare the jars as explained in Appendix 11.

(c) Culturing the rhizobia
(Key step 2)

Each of the 14 strains of *B. japonicum* to be evaluated is cultured for 5-7 days prior to planting. Grow the rhizobia in 100 ml Erlenmeyer flasks containing 20 ml of yeast-mannitol broth. Incubate these at room temperature (25-30°C) on a rotary
shaker for 5-7 days.

(d) Surface-sterilizing the seeds
(Key step 3 and 4)

Check the germination (percentage viability) of the soybean seeds and surface sterilize a sufficient number of uniform, undamaged seeds to give about 200 germinated seeds. Sterilize by immersing seeds in 3% sodium hypochlorite solution for 3-5

minutes as described in Appendix 10. Germinate the seeds by plating on sterile water- agar (0.75% [w/v]) and incubate at room

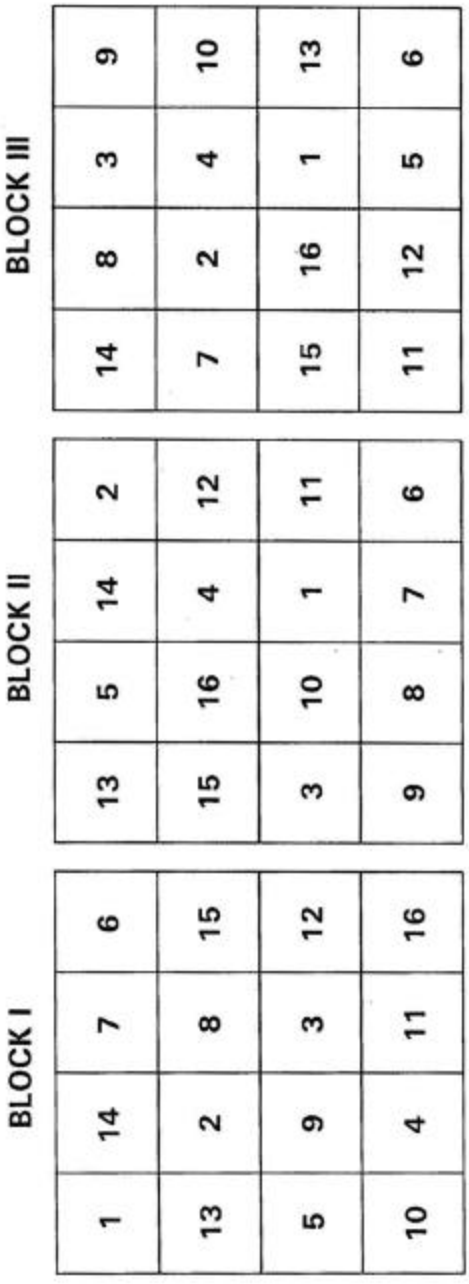

Figure 16.1. An example of a randomized complete block design experiment.

temperature (25-30°C) until the radicles are 0.5 - 1.0 cm long. Avoid overcrowding agar plates with the seeds. (Contact between seeds in an overcrowded plate increases the risk of cross-contamination from a partially sterilized seed to neighboring seeds. Uncrowded plates [approximately 25-30 seeds] produce more uniform and better germination due to better availability of moisture.)

(e) Planting and inoculating of seeds
(Key step 5 and 6)

Follow the method for planting and inoculating the seeds described in Exercise 15. Plant three, well-germinated seeds in each jar. Plant three jars per treatment. Label the jars and indicate block (replicate) assignment. Group the treatments according to block assignment and keep them separated.

Remove all Leonard jars of Block I to the growth room (or glasshouse) bench. Randomize the placement of the jars within Block I.

Similarly randomize the placement of the Leonard jars of Block II and Block III.

Make daily observation of the experiment. Five to ten days after planting, thin to two uniform plants per jar. Begin by thinning down the controls first. Excise the shoot of the unwanted plant with sterilized scissors. Bear in mind that growing conditions such as temperature and light intensity during this experiment must be in the range to which the species are adapted. Excessive temperatures are particularly damaging and can severely impair the infection process, nodule development, and nodule function.

Plants may "green-up" gradually at the time that nodules begin to function, delivering fixed nitrogen for plant metabolism. Plants inoculated with ineffective strains of rhizobia, and also the uninoculated controls, will remain yellow (chlorotic) and stunted.

(f) Harvesting the plants
(Key step 7 and 8)

Harvest the plants after 30 days. To minimize errors during harvest, the stem should be cut at the point of cotyledon attachment. This point is marked by a scar on the stem. These scars are not visible in some species. The stem should then be cut at the level of the growth medium. Place the plant shoots in labeled paper bags. Dry to constant weight at 70◦C for 2 days. Each bag should contain the plant shoots from only one jar. (Paper envelopes may be substituted for

smaller plants, e.g., *Centrosema, Trifolium, Desmodium,* etc.)

Roots and adhering rooting medium are dislodged into a coarse sieve. Wash the rooting medium from the roots using a gentle stream of water. Describe the nodule distribution mentioned in Appendix 1 (e.g., prolific tap-root nodulation; occasional nodules on lateral roots and distant from the tap-root; large numbers of small nodules; small number of large nodules). Detach the nodules, count them, determine their total fresh weight, and place them in vials or aluminum foil weighing-boats for drying. Dry the nodules to constant weight at 70◦C for 2 days. (Nodule harvest from each Leonard jar must be treated individually as in the case of the shoots.) Do not pool nodules of the three replicates of any one treatment into a single vial.

Determine dry weight of shoots and of nodules for all treatments.

Perform an analysis of variance on the dry weight data (shoots and nodules) using the method as described in Appendix 17.

Plot the mean shoot weight (Y-axis) against the mean nodule dry weight (X-axis). Determine the correlation coefficient (r) of the plot and test the significance of r at the 5% and 1% levels of confidence.

Draw the "best" regression line on your plot after determining the regression equation for the regression line.

Shoot weight and nodule weight are usually highly correlated, thus shoot weight is used routinely as an indicator of relative strain effectiveness.
Other parameters that are highly correlated with shoot weight are total nitrogen of shoot and nodule dry weight.
Nitrogenase activity (acetylene reduction) may not easily correlate unless done under very controlled conditions.

Requirements

(a) Experimental design and treatments

No special requirements

(b) Preparing Leonard jars

48 Leonard jars

(c) Culturing the rhizobia for testing

Agar-slant cultures of *B. japonicum*
Yeast-mannitol broth
Shaker

(d) Surface-sterilizing the seeds

Soybean seeds
Sodium hypochlorite solution (3%) or commercial bleach (Chlorox)
Water agar plates
Incubator

(e) Planting and inoculating of seeds

Broth cultures of *B. japonicum* from (c)

Pregerminated seeds

Sterile pipettes (1 ml) or Pasteur pipettes

Alcohol lamp and matches

Forceps, glass rods, and alcohol spray bottle

0.05% KNO_3 (w/v) solution

Bench space in greenhouse

(f) Harvesting the plants

Scissors, paper envelopes or bags

Coarse sieve, vials or aluminum foil weighing boats

Drying oven (70°C)

Weighing balance

EXERCISE 17

SELECTING EFFECTIVE STRAINS OF RHIZOBIA IN POTTED FIELD-SOIL

Strains of rhizobia previously screened in Leonard jars are evaluated further in potted field soil. The effectiveness of mixed and single strain inocula are compared. Infective native rhizobial populations in field soil are determined.

Key steps/objectives

1) Culture rhizobia
2) Collect soil from test field
3) Prepare soil, determine pH and total N content
4) Pot the soil
5) Determine water holding ability (field capacity) of soil
6) Apply fertilizer
7) Plant and inoculate surface sterilized seeds
8) Thin seedlings to desired number
9) Inspect for nodulation and perform MPN counts
10) Water and observe plants
11) Harvest plants, examine nodulation
12) Analyze data

(a) Designing the experiment and treatments

The experimental design is a randomized complete block with

three blocks as in Exercise 16. There are 18 treatments: 15 inoculated (14 single strain inoculations and one treatment receiving a mixed broth inoculum comprising the three best strains selected in Leonard jars from Exercise 16); a plus-N control without inoculation; and two sets of non-inoculated controls. At 2 weeks the extra set of the non-inoculated controls is removed for inspection for nodulation by native rhizobia. If nodulation is observed in the non-inoculated controls, initiate MPN counts of the native population using the soil set aside for this purpose. Pots are sown with eight seeds and four plants are maintained for the experiment upon thinning.

(b) Preparing the inoculum
(Key step 1)

All of the 14 cultures of *B. japonicum* used in Exercise 16 are evaluated in soil. Inoculate each strain into 70 ml of yeast-mannitol broth contained in 125 ml Erlenmeyer flasks. Allow strains to grow for 5-7 days to reach maximum turbidity (approximately 1×10^9 cells ml^{-1}). To prepare the mixed inoculum, pipette 10 ml of the fully grown broth culture of each of the three best strains into a clean 125 ml Erlenmeyer flask. Use a fresh pipette for each strain. Mix the contents thoroughly by swirling.

(c) Choosing the site for collecting soil

The ideal site for soil collection is the one where the field experiment (which follows the pot experiment) is to be conducted. The site soil should be low in nitrogen. The native rhizobial population should be less than 10^3 rhizobia per g soil; no previous history of inoculation and cultivation with the intended legume; no water-logging or salinity problems.

In practice, these prerequisites may not be met in the chosen site. This, however, should not deter experimentation with a particular soil.

(d) Collecting, preparing, and potting field soil
(Key steps 2, 3, and 4)

With a steel spade or other suitable implement, obtain field soil from a depth of 10-15 cm. Soil samples should be taken randomly within a soil type. Collect and transport the soil (approximately 150 kg) in strong plastic bags to a clean room. Spread large pieces of clean cardboard on the floor and cover with thick, clean, plastic sheets or tarpaulins. Empty the bags of soil onto the plastic to pool all the collected soil. Spread the soil and allow it to air dry. Mix the soil thoroughly and remove debris (e.g., stones, roots, leaves, etc.) Break lumps with a wooden mallet. Sift the soil using a 5 mm mesh screen. Take a sample to determine the soil pH

using a pH-meter. If the soil is acid, add lime to bring the pH to 6.0-6.5. Mix the soil and lime thoroughly and allow to equilibrate for at least 7 days. During the equilibration period, cover the soil with a plastic sheet. Use one of the methods shown in Appendix 16 to calculate the amount of lime needed to adjust the pH level of the soil.

Obtain strong PVC (polyvinylchloride) pots. Pots of 15-16 cm diameter, and 18 cm height with a capacity of just over 3 l and with at least one hole on the bottom, are suitable for potting. Pots should be clean. Plastic bags of suitable size and thickness will be used as inner liners for the pots. Punch holes (1 cm diameter) in the bottom of the bags to allow for drainage. Position the bags in the pots and fold the open end of the bag over the rim of the pot.

Pots of the recommended size will hold approximately 2.4-2.7 kg of a soil high in organic matter. Tropical soils with less organic matter but occupying a similar volume will be heavier. Weigh 2.4 kg of soil in each plastic bag and place in the pot. (Any coarse balance is suitable for weighing the soil, as high precision is not required.) Gently tamp the pots on the floor to compact the soil. Soil in all pots must be tamped down to occupy nearly the same volume to achieve similar bulk density.

Set aside 250 g of soil in a refrigerator (4◦C) for MPN count

of the native rhizobial population following the method described in Exercise 5.

(e) Adjusting moist field soil to field capacity
(Key step 5)

A soil moisture content at field capacity is suitable for most plants. Since the field capacity varies with different soils, determine the field capacity for the soil under investigation. At sowing and during initial phase of seed germination and seedling establishment, the soil moisture should be maintained at field capacity for better plant performance. Determine the field capacity of the moist field soil using the simple method described in Appendix 21.

(f) Applying fertilizer
(Key step 6)

The fertility of the soil must be adjusted to optimal levels to obtain good growth of the plants. The following fertilizer treatments are recommended. Rates per pot have been calculated on the basis of 2.4 kg-soil per pot.

Phosphorus, P

100 kg P ha^{-1}; applied as 500 kg ha^{-1} triple super-phosphate (TSP*); 529 mg pot^{-1} (or 468 mg KH_2PO_4 pot^{-1}).

Potassium, K

200 kg K ha^{-1}; applied as 382 kg ha^{-1} KCl; 404.2 mg pot^{-1} (K_2SO_4 may also be used)

Magnesium, Mg

5 kg Mg ha^{-1}; applied as 50 kg ha^{-1} $MgS0_4.7H_2O$; 53.3 mg pot^{-1}

Zinc, Zn

10 kg Zn ha^{-1}; applied as 46.8 kg ha^{-1} $ZnSO_4.7H_20$; 49.5 mg pot^{-1}

Molybdenum, Mo

1.0 kg Mo ha^{-1}; applied as 1.76 kg $(NH_4)_6$ $Mo_70_{24}.H_20$ ha^{-1}; 1.95 mg pot^{-1}

Nitrogen, N (for N-control pots)

100 kg N ha^{-1}; applied as 222 kg ha^{-1} urea, $CO(NH_2)_2$; 219 mg pot^{-1} 25% of N is applied at planting and the remaining 75% at 3 weeks.

Prepare the fertilizers (except the insoluble triple superphosphate) in the form of solutions and pipette them on to the soil surface and allow to dry. Add the triple superphosphate. Mix the soil in each pot thoroughly to ensure uniform distribution of the nutrients (mixing is easily achieved by removing the bag of soil from the pot and massaging).

(g) Planting and inoculating the seeds

(Key steps 7 and 8)

At the planting rate of eight seeds per pot, a total of 24 seeds are needed for each treatment in triplicate. A grand total of 408 seeds are needed for all the 17 treatments. From a batch of seeds with good germination, select 500 seeds and surface sterilize as in Appendix 10. Allow the sterilized seeds to imbibe water for 1 h. Give the seeds a final rinse and plant the seeds at a depth of 2 cm. Inoculate each seed with 1 ml of the culture, following the method described in Chapter 19. Label the treatments and assign block numbers.

Water the soil in the pots to field capacity using the data from Step 2. Add sterilized coarse sand mulch to control contamination.

Randomize the pots on the greenhouse bench.

When plants are 5 days old, thin to four uniform plants per pot as described in Exercise 15.

(h) Inspecting non-inoculated control plants for nodulation by native rhizobia

(Key step 9)

When plants are 3 weeks old, remove the extra set of non-inoculated controls to inspect for nodulation by native rhizobia. Carefully remove the plastic bag containing the

plants from the pot and place it in a shallow basin. Slit the bag open. With a gentle stream of water wash the roots. Examine for nodulation. Similarly observe the remaining two pots set up for inspection.

If nodules are present, make preparations for performing the MPN count of rhizobia in the soil set aside for this purpose in (d). The count may be done in growth-pouches or Leonard jars following the method described in Exercise 5.

Weigh 100 g of the soil, dilute it in 900 ml of sterile water and prepare a fourfold dilution series ranging from 4^1 - 4^{10} dilution. Inoculate each dilution in quadruplicate. A fourfold series gives more precision than a tenfold series, especially for soils when populations are less than 1×10^3 rhizobia per g soil. Note that the starting sample has been diluted 1:10. More details on the method and calculations are given in Exercise 5.

(i) Watering the pots and making periodic observation (Key step 10)

During active growth and fixation, legumes will use a considerable amount of water each day. During this period, the pots need to be watered regularly. Water the pots more than once each day if needed.

Weigh sample pots showing vigorously growing plants to determine the volume of water needed to replace the water lost. If there are large differences in plant growth, pots should be watered to weight on a pot by pot basis. Measure out the required volume of water in a measuring cylinder and pour into the pot without excessively disturbing the soil. Keep plants well watered and make growth observations periodically as in Exercise 15.

(j) Harvesting the experiment

(Key steps 11 and 12)

Harvest the plants at 35 days. Determine dry weight of shoots and nodules for all treatments. Analyze yield data as in Exercise 15.

Requirements

(a) Designing the experiment and treatments

No special requirements

(b) Preparing the inoculum

Transfer chamber
Agar slant cultures of rhizobia
Yeast-mannitol broth
Shaker
Erlenmeyer flasks
Pipettes

(c) Choosing the site for collecting soil

Soil analysis data

(d) Collecting, preparing, and potting field soil

Steel spade
Strong plastic bags, plastic sheets, cardboard
Wooden mallet
1 cm mesh screen
pH meter
Lime ($CaCO_3$)

PVC pots and plastic bags (inner liners for pots)

Balance for weighing potted soil

(e) Adjusting moist field soil to field capacity

Determine field capacity (Appendix 21)

(f) Applying fertilizer

Weighing balance

Triple superphosphate, potassium chloride, zinc sulfate, ammonium molybdate, urea, lime, magnesium sulfate, potassium phosphate

Pipettes (1 ml and 10 ml)

(g) Planting and inoculating seeds

Seeds, sodium hypochlorite solution (3%), sterile water

Sterile empty beakers

Sterile pipettes, forceps, marker pens

Balance, water

Scissors, alcohol lamp, matches

(h) Inspecting non-inoculated control plants for nodulation by native rhizobia

Tap water, scissors, shallow basin

EXERCISE 18

TO VERIFY THE NITROGEN-FIXING POTENTIAL OF GLASSHOUSE SELECTED SOYBEAN RHIZOBIA IN THE FIELD ENVIRONMENT

Strains of rhizobia, previously selected in potted field soil, are evaluated in the field environment so as to further identify the most effective strains for inoculant production. The effectiveness of a multi-strain inoculant is compared with single-strain inoculants.

Key steps/objectives

1) Select rhizobial strains and prepare the inoculants
2) Prepare the field and apply fertilizers
3) Inoculate the seeds and plant
4) Determine the number of rhizobia on the inoculated seeds
5) Inspect the field and weed as necessary
6) Harvest at 50% flowering (early harvest)
7) Harvest for grain yield (final harvest)
8) Analyze the data

(a) Setting up the experiment

Set up the experiment as a randomized complete block with four replications (Fig. 18.1). Set up eight treatments; six inoculated (five single-strain and one multi-strain); a

plus-nitrogen; and non-inoculated control without nitrogen.

Field Dimensions: A field area of 360 m^2 (24 m x 15 m) is required. Make rows 7.5 m in length and 0.5 m apart. Each treatment plot is flanked by an uninoculated guard (border) row along each side, with two center harvest rows (see Figure 18.2). The area of each plot is 11.25 m^2 (0.001125 ha). The area harvested for grain yield is 3.75 m^2.

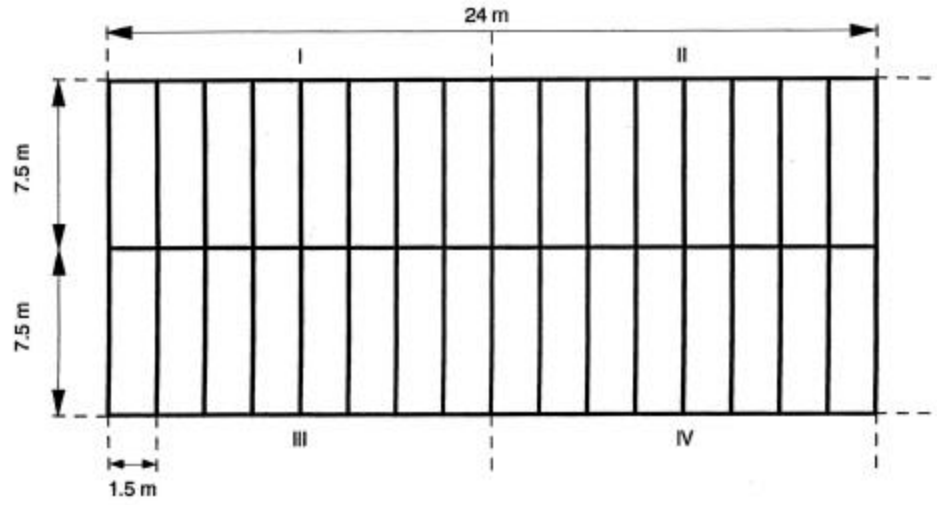

Figure 18.1. Field layout and dimensions.

Choice of rhizobial strains: Use five of the best strains, according to their order of ranking in Exercise 17. From this group select three serologically distinct strains for the preparation of the multi-strain inoculant for use in this exercise and later in Exercise 19.

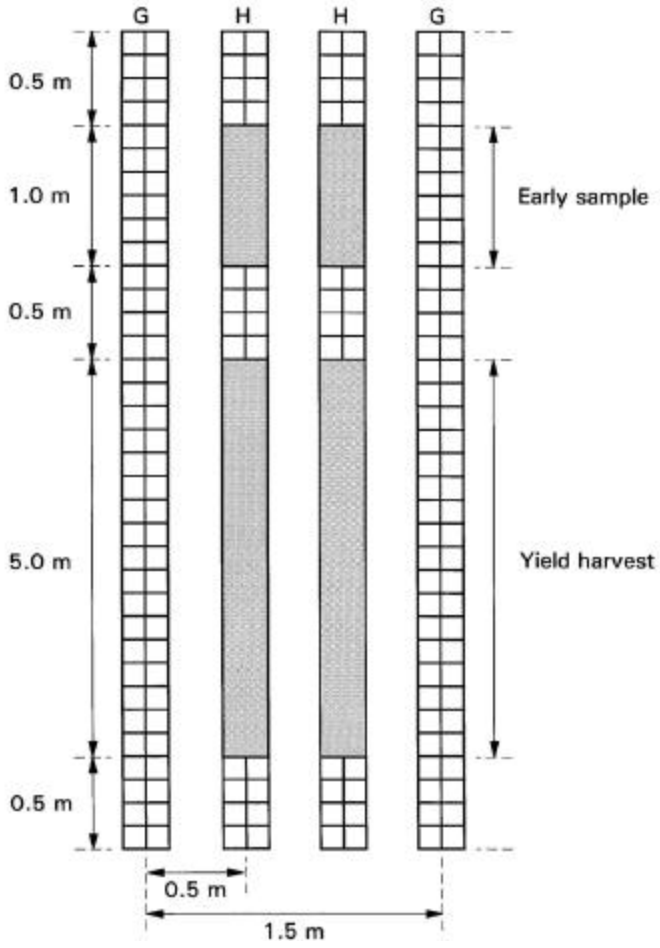

Figure 18.2. Field layout and dimensions.

(b) Selecting rhizobia for the experiment
(Key step 1)

Serologically distinct and/or antibiotic resistant labeled rhizobia may be selected using methods described in Section B.

If selection of serologically distinct strains is not possible (because of cross-reactions amongst the strains chosen), a multi-strain inoculant can still be prepared but may not be

suitable for studying aspects of strain ecology (competition, persistence, etc.) in the soil by serological methods. Antibiotic labeling offers an alternative to the use of serologically distinct strains. However, the antibiotic labeling method is suggested as more reliable only when each of the component strains in the multi-strain inoculant has double antibiotic resistance labels. Single-label strains may also be used but with caution.

Since three strains are used in the multi-strain inoculant, the process of labeling (multilabeling) and identification of the strains may become too involved, especially when more antibiotics are needed in the development of the resistant strains. Moreover, the labeled strains need to be confirmed for retention of symbiotic effectiveness (Leonard jar screening, Exercise 16) when compared with the parent strains prior to their use in the inoculants.

(c) Preparing inoculants
(Key step 1)

In this experiment, inoculate the seeds (except controls) with peat cultures.

Prepare the peat inoculants of the five strains following procedures described in Exercise 21 using gamma-irradiated or autoclaved peat. In preparing the multi-strain inoculant,

grow the 3 chosen strains of rhizobia separately. Aseptically mix equal volumes of each strain in a sterile Erlenmeyer flask. Use this mixture to inoculate the peat.

Prepare the inoculants in advance of the experiment and allow them to mature for at least 2 weeks at 25-30°C. Determine and record the quality of each inoculant (number of viable rhizobia per g peat) by plate counts (Chapter 5 or 6). After the 2 weeks of curing, the inoculants may be stored up to 4 weeks in a refrigerator (4°C).

(d) Preparing seeds for inoculation and planting
(Key steps 3 and 4)

A planting distance of 3.7 cm between seeds is optimal for good soybean yields. Based on this planting distance, approximately 203 seeds are needed per 7.5 m row. Since there are two inoculated rows per plot and four replications, a total of 1624 seeds will be needed for each inoculated treatment. Count or weigh 2000 seeds for each treatment to make allowances for losses and for samples to be taken for determining the number of rhizobia per seed at planting. The seed numbers should be converted to weight measures for convenience. Weigh out the seeds for each treatment in clean plastic bags and label accordingly.

For soybean, 10 g of peat-based inoculant and 3.0 ml of gum

arabic for 100 g of seed are recommended for experiments. Inoculate the seeds as described in Exercise 23.

Inoculate the seeds just before planting. Keep the seeds in their plastic bags and in a cool place away from direct sunlight.

Set aside 20 seeds of each inoculated treatment and with minimum delay determine the number of rhizobia per seed (inoculation rate) as described in Exercise 23.

(e) Preparing the field
(Key step 2)

Conduct the experiment in the field site from where soil was previously collected for Exercise 17. Drive posts into the soil at the four corners of the field to indicate the boundary of the experimental site. Clear and remove all surface vegetation and treat the field with herbicide. Plow the field after sufficient time has been given for the herbicide to take effect in killing the weeds. Remove large rocks, plant roots, and other forms of debris. Till the soil to break up lumps and prepare a smooth, firm seed bed. Alternatively, the sowing may be done without plowing. This will minimize disturbance to the soil and release of soil nitrogen.

Mark the plots and designate treatments for the different

plots (Treatment should be randomized in advance of planting and recorded.)

(f) Controlling cross-contamination by modifying irrigation methods

(Key step 2)

Rhizobia are soil bacteria and are easily spread when soil borne or in soil suspension. Surface overflow resulting from heavy rains and the flood-irrigation method may cause serious cross-contamination. In this particular exercise, where several different strains of rhizobia are tested, the methods of irrigating the field may be modified to control heavy cross-contamination.

Surface overflow resulting from heavy rains and the flood-irrigation method may cause serious cross-contamination.

Cross-contamination from rainwash may be controlled by the preparation of elevated seed beds (bunds). This method will result in the creation of shallow ditches between the seed beds (rows). Alternately, an elevated plot with a surrounding ditch would be suitable for areas of heavy rains. Elevated plots may be preferred over elevated rows, as the latter are more susceptible to erosion. Rain water can be efficiently drained away during heavy rains if the rows are prepared so as to follow the general inclination of the slope if the slope is

not too great.

In locations of very low rainfall where irrigation water is obtained from canals or rivers, flood irrigation is frequently practiced (Egypt, Sudan, etc.). In this situation, ditches between rows are preferred as they deliver water more efficiently to the roots of plants growing on elevated rows than to rows of plants on an elevated plot. However, if plots are not elevated, irrigation by flooding the entire surface of the plot may be done. This would require the construction of an elevated bund around each treatment plot to prevent water flow from one plot to neighboring plots. Water must be controlled to flow only from the main stream into the plot. Backflow into the mainstream must be prevented. Successive irrigation by channeling water from one plot to neighboring plots must be prevented.

Sprinkler and drip-irrigation methods may be used if these are available.

(g) Applying fertilizer
(Key step 2)

Fertilize the field soil to optimize conditions for growth. Follow levels of fertility as recommended for the potted soils (Exercise 17).

Lime the soil to pH 6.0-6.5. The quantity of lime may vary from 500-10,000 kg ha^{-1} (depending on the soil and its initial pH) to bring about appreciable changes in the soil pH. Apply the lime 2 weeks prior to the application of the other fertilizers. Use the lime requirement data from Appendix 16.

To facilitate the application of the fertilizers, each of the four blocks is fertilized individually by broadcasting. The rates per block (90 m^2) are as follows: triple superphosphate, 4.5 kg; potassium chloride, 3.44 kg; zinc sulphate, 0.42 kg; ammonium molybdate, 0.016 kg; magnesium sulfate 0.45 kg.

Weigh out the fertilizer quantities in containers (plastic bags or buckets) of adequate size and apply by broadcasting. The smaller quantities, for example, zinc sulphate, ammonium molybdate and magnesium sulfate, may be mixed with an inert carrier (e.g., sand) and broadcasted or sprayed on. Do not apply the urea with the other fertilizers as this is applied at planting only to the plus-N controls. Till in the fertilizers soon after application.

The field is ready for planting one day after the application of the fertilizers.

(h) Planting the experiment
(Key step 3)

Make furrows 7.5 m long, 0.5 m apart, and four per plot and 3-3.5 cm in depth. Make furrows for only a few plots at a time so that open furrows are not subjected to drying out from prolonged exposure in the sun.

Irrigate the experimental site just enough to moisten the soil the evening prior to the day of sowing, if the soil is dry. Irrigate again immediately after sowing the trial.

A straight 2 m long wooden stick with 3.7 cm graduations, placed alongside the furrow, is a useful guide for even placement of seeds.

Plant the controls and guard rows first and cover the seeds on completion of each row.

Prevent contamination of the seeds by sterilizing your hands when handling each batch of seeds inoculated with a different strain. Hands are easily sterilized by thorough washing with soap and water followed by swabbing with alcohol after the hands are dry.

Apply urea, only to the plus-nitrogen controls, at the rate of 0.23 kg urea per plot with 25% (58 g per plot) at planting and the remaining 75% (174 g per plot) at 4 weeks. Weigh out 58 g each of urea in four bags, one for each of the four replicates.

Make a furrow 4-5 cm deep, parallel to and 4-5 cm away from the planted row. Evenly distribute the urea with your hands. Cover the furrows immediately after application. Exposure of the urea will result in hydrolysis and loss of N (as ammonia) to the atmosphere.

(i) Monitoring the trial and harvest
(Key steps 5, 6, and 7)

Inspect the field frequently for plant damage by disease and insect pests. Take appropriate measures to control these pests. Weed the plots whenever necessary.

Make frequent observations of plant growth and color. Note treatments with early signs of N fixation.

Record the time taken for 50% of plant population to initiate flowering. Make an early harvest at this time.

The area of the plots for early harvest and harvest for grain yield are indicated in Figure 18.2. Harvest plants for dry matter yield. Observe nodule size, color, and distribution on the root. Obtain the fresh and dry weight of nodules.

If facilities are available, perform the acetylene reduction assay to determine nitrogenase activity as described in

Appendix 15.

Record time for the plants to reach maturity. Process the plants for determining grain yield (dried to 5-6% storage moisture). Express grain yield on a kg ha^{-1} basis.

(j) Analyzing the data
(Key step 8)

Analyze the data from the early harvest for correlation (Appendix 18) tops vs. nodule weight; tops vs. nodule numbers; tops vs. nitrogenase activity (if available); nodule weight vs. nitrogenase activity. In addition, perform a correlation analysis to correlate total nitrogen with all the parameters measured.

Rank the strains according to nitrogen fixing potential, and compare your data with that from the Leonard jars and potted-soil experiments.

Compare the performance of the multi-strain inoculant with single-strain inoculants. What could be the advantage of a multi-strain inoculant? Rank the data obtained for grain yield. Does ranking of strains according to dry matter production at early harvest and at grain yield agree?

Requirements

(a) Setting up the experiment

Measuring tape (50 m)
Field site (24 m x 15 m)
Five best rhizobial strains from Exercise 17

(b) Selecting strains for the experiment

Serologically distinct or antibiotic labeled strains of rhizobia

(c) Preparing inoculants

Agar slant cultures from (a)
Five Erlenmeyer flasks (250 ml) each containing 100 ml YM broth
Six sterile plastic syringes (50 ml); six sterile needles (3/4 in, 18 gauge)
Six bags of peat (50 g per bag) autoclaved or irradiated
Sterile 10 ml pipettes
Incubator
Quality check of inoculants (materials as in Exercise 21)

(d) Inoculating the seeds

Soybean seeds, balance, plastic bags

Peat inoculants, gum arabic solution, 10 ml pipette with wide-bore tip (for pipetting gum arabic solution)

Samples of inoculated seed

(e) Preparing the field

Field area

Four wooden posts for marking field perimeter

Herbicide(s) and spraying equipment

Plowing and tilling machinery

Other field preparation accessories

(f) Controlling cross-contamination by modifying irrigation methods

Suitable field design to control cross-contamination

(g) Applying fertilizer

Magnesium sulfate 0.45 kg x 4 blocks = 1.8 kg

Triple superphosphate 4.5 kg x 4 blocks = 18 kg

Potassium chloride 3.44 kg x 4 blocks = 13.76 kg

Zinc sulfate 0.42 kg x 4 blocks = 1.68 kg

Ammonium molybdate 0.016 kg x 4 blocks = 0.064 kg

Balance, plastic bags or plastic buckets

Tiller or hoes

(h) Planting the experiment

Inoculated and non-inoculated soybean seeds from (d)
Irrigation water
Metric tape, hoes or suitable equipment for making furrows
Planting guide for even placement of seeds
Soap, water, clean rags, alcohol in spray bottle
Urea for N-controls
Covered container to keep seeds

(i) Monitoring the trial and harvest

Insecticides and spraying equipment
Weeding tools, hoes
Scissors/snips, paper bags, aluminum weighing boats
Coarse sieve
Drying oven (70°C)
Balance

(j) Analyzing the data

Calculators and statistical tables
Statistical assistance

EXERCISE 19

TO INVESTIGATE THE IMPORTANCE OF OPTIMAL SOIL FERTILITY IN THE RESPONSE OF A LEGUME TO INOCULATION WITH RHIZOBIA*

This experiment is designed to compare inoculation response in unamended soil (except for liming) and in soil fertilized to optimal levels. Inoculation response is evaluated using the three basic treatments: (l) inoculated; (2) plus nitrogen without inoculation; and (3) no nitrogen without inoculation. Each of these three treatments is set up at two different fertility levels. A multi-strain peat-based inoculant is used to study the effect of soil fertility on the competition of rhizobia for nodulation.

Key steps/objectives

1) Prepare the mixed inoculant
2) Prepare the field and apply fertilizers
3) Inoculate the seeds and sow
4) Determine the number of rhizobia on the inoculated seed
5) Inspect the field and weed
6) Harvest at 50% flowering
7) Harvest for grain yield
8) Analyze yield and nodule identification data

*The experiment described in this exercise is based on the

design used in the International Network of Legume Inoculation Trials (INLIT) promoted by the NifTAL Project, Department of Agronomy and Soil Science, College of Tropical Agriculture and Human Resources, University of Hawaii.

(a) Setting up the experiment

Experimental design and treatments:

The experiment is designed as a randomized complete block with four replicates. There are three basic treatments: (1) inoculated; (2) plus nitrogen without inoculation; and (3) no nitrogen without inoculation. Each of these basic treatments are set up at two fertility levels giving a total of six treatments. Peat inoculant containing a mixture of three strains of rhizobia are used to inoculate seeds. The randomized treatments and field layout are indicated in Figure 19.1.

Field dimensions:

Plants are raised in plots of 7.5 m x 2.4 m (0.0018 ha). The rows are 60 cm apart with four rows per plot. A total field area of 0.0432 ha (28.8 m x 15 m) is needed for the experiment. Details of a plot showing areas reserved for early sampling and grain yield determinations are presented in Figure 18.2.

(b) Preparing the mixed inoculant and inoculating the seeds (Key step 1)

The inoculants are prepared in advance of the experiment. The three antigenically distinct strains selected in Exercise 18 are used here.

Culture each of the three strains separately in 150 ml of yeast-mannitol broth in Erlenmeyer flasks. Inoculate fully grown broth cultures into 50 g of gamma-irradiated or autoclaved peat as described in Exercise 21. Each package of the peat should be inoculated with only one strain.

Incubate the bags for 2 weeks at 25-30°C. At the end of the maturity period, determine the quality of the peat inoculants of the different strains. Aseptically remove 1 g samples in duplicate from each bag and plate serially diluted samples as described in Exercise 21.

Refrigerate the inoculant bags immediately after sampling. Immediate refrigeration for 2-3 weeks or longer will maintain the original population at sampling without significant changes.

From the quality check (plate-counts) of inoculants, determine the number of rhizobia per g inoculant for the three strains.

From this information, determine the weights of the inoculants to be mixed to give a 1:1:1 ratio as shown in the following example.

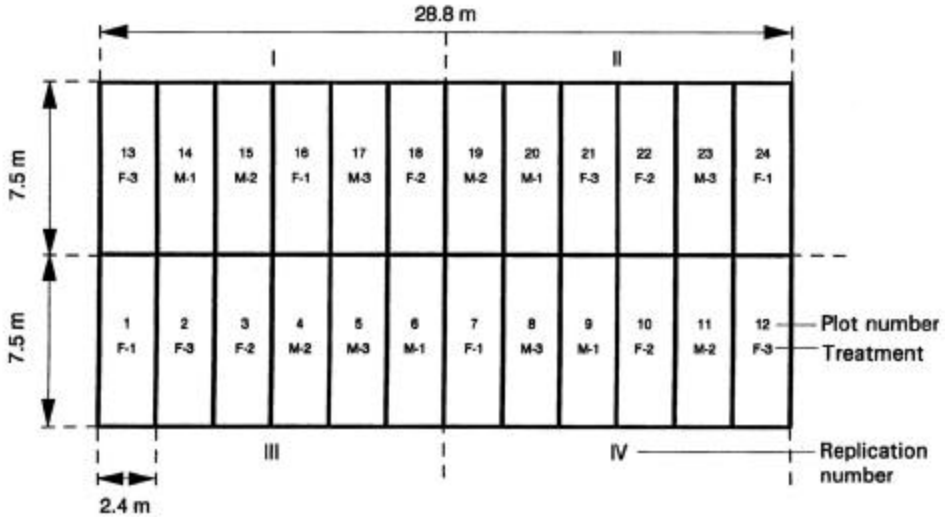

Figure 19.1. Field layout and dimension.

The various treatments are randomized. Farm fertility and maximal fertility plots are indicated by F and M respectively. Treatment details for each plot are given in Table 19.1.

In a quality check of three strains, the number of viable rhizobia per g of each inoculant were as follows:

Strain A 1.8×10^9

Strain B 2.6×10^9

Strain C 3.4×10^9

To establish a 1:1:1 ratio of A, B, and C in a mixture,

determine factors that will convert strains A and B to 3.4 x 10^9 rhizobia per g peat. Start with the strain that has the highest count of rhizobia/g.

For strain A, conversion factor = (3.4/1.8) = 1.9

This means that 1.9 g of peat inoculant of strain A will contain 3.4 x 10^9 rhizobia.

For strain B, conversion factor = (3.4/2.6) = 1.3

This means that 1.3 g of peat inoculant of strain B will contain 3.4 x 10^9 rhizobia.

Therefore, a peat inoculant mixture containing 1.9 g of inoculant A, 1.3 g of inoculant B, and 1 g of inoculant C will result in a 1:1:1 ratio of the three component strains.

Alternatively, if equal weights of the inoculants of the three strains were mixed, a ratio of 1:1:1 will not be established. The ratio would then be 1.8:2.6:3.4, or approximately 2:3:3. Competition of the three strains may still be studied using this approximated ratio.

Calculate the weight of seed (as described in Exercise 18) for the four rows of each inoculated plot and for all the inoculated treatments in the experiment using a planting

distance of 3.7 cm between seeds. With this information and the recommended inoculation rate of 10 g of mixed peat inoculant per 100 g of soybean seed, calculate the total weight of inoculant needed for all the inoculated treatments. Alternatively, 1 g of mixed inoculant may be used to inoculate 100 g of seed to achieve a lower inoculation rate.

Mix the inoculants one day ahead of planting. Remove the bags of inoculants from the refrigerator. Weigh out calculated amounts into a sterile 1 liter beaker. Mix the inoculants thoroughly with a spatula. (Observe aseptic techniques throughout the preparation.) After mixing, cover the beaker with aluminum foil and refrigerate immediately.

Inoculate the seeds (Exercise 23) just before planting. Save seed samples and determine the number of viable rhizobia on the seeds at sowing as described in Exercise 23.

(c) Choosing a site and preparing the field
(Key step 2)

A field site having soil conforming to the description as outlined in Chapter 21 will be suitable. A farmer's field, if available, is preferred.

The fertility status of the site soil has to be determined. If facilities are available, analyze soil samples for: free

nitrate; extractable P; exchangeable K, Ca, and Mg; exchangeable Al and Mn, if soil pH is below 5.2 and 5.6 respectively; soil pH; organic matter.

Collect soil samples and determine the population of native soybean rhizobia using the MPN method (Exercise 5).

Prepare the field as outlined in Exercise 18. The field preparation may need modifications to control cross-contamination resulting from heavy rain-wash or from flood-irrigation method.

(d) Applying fertilizers
(Key step 2)

Since two fertility levels are used, namely the farmer's fertility without amendments (F) and maximal fertility (M), each treatment plot has to be marked to facilitate recognition during fertilizer applications. This is especially important as each plot is fertilized individually.

Mark boundaries by driving in short stakes at the four corners of each plot. About 6-9 inches of the stake should remain exposed to allow for good visibility and for ready recognition of plot boundaries. Erect a sign-board of suitable size in front of each treatment plot indicating its treatment according to the field layout presented in Figure 19.1.

Lime the soil in the F plots only if the pH is below 5.4. Lime all M plots to pH 6.0-6.5. Allow the soil to equilibrate for at least two weeks after the lime application. Determine the lime requirement of the soil using one of the methods described in Appendix 16.

The fertilizer recommendations for maximal fertility are similar to rates used in Exercises 17 and 18. The amount of fertilizer applied per plot is as follows: triple superphosphate, 0.9 kg; potassium chloride, 0.69 kg; zinc sulfate, 0.08 kg; ammonium molybdate, 0.0033 kg; magnesium sulfate, 0.09 kg; and urea, 0.373 kg.

Weigh out the quantities of the fertilizers in plastic bags and assign the treatment labels. Mix smaller quantities of the fertilizers (zinc sulphate, magnesium sulfate and ammonium molybdate) with an inert carrier (e.g., sand) to facilitate application by broadcasting or by spraying.

Apply the fertilizers individually to all plots of the various treatments (Table 19.1). Till the fertilizers into the soil after broadcasting. The nitrogen (urea) is applied as side-dressing in furrows at sowing (0.09 kg $plot^{-1}$) and after 4 weeks from emergence (0.28 kg $plot^{-1}$).

(e) Planting the experiment

(Key steps 3 and 4)

Inoculate the soybean seeds as described in Exercise 23. Set aside about 100 inoculated seeds for determining number of rhizobia per seed. (Use only 20 randomly selected seeds from this sample for the determination.)

Table 19.1. Summary of treatments

Fertility Levels	Fertilizers applied[a]						
	P	K	Zn	Mo	Ca	N	Inoculation
M-1	+	+	+	+	+		
M-2	+	+	+	+	+	+	
M-3	+	+	+	+	+		+
F-1[b]							
F-2						+	
F-3							+

a. + and - indicate application and no application respectively.

b. Ca is added as calcium carbonate if pH is below 5.3-5.4

Make furrows 7.5 m long, 0.6 m apart, and 3-3.5 cm deep. Plant the soybean seeds at approximately 35 seeds per M row doing the uninoculated treatments first. Thin rows evenly to 27 plants per M row at two weeks. Irrigate the field. Take precautions against cross-contamination during sowing (see Exercise 18).

Note that in the experiment in Exercise 18, a single

uninoculated guard row was common to two adjacent plots. In this experiment, each plot does not share common guard rows with adjacent plots. Each plot in this experiment has four rows (two guard and two harvest rows) and the guard rows are of the same treatment as the harvest rows.

After sowing, side-dress the recommended amount of urea in furrows of the plus-N controls and irrigate the field if necessary.

(f) Monitoring the trial and harvest
(Key step 5)

Carry out regular field inspections, weeding and harvesting activities as described in Exercise 18.

(g) Harvesting nodules for strain identification
(Key steps 6 and 7)

Since the seeds of the inoculated treatments were coated with a mixed peat inoculant containing equal proportions of three antigenically distinct strains, the nodules may be harvested and processed for establishing nodule identity. Data from nodule identification can then be analyzed for strain competition for nodulation at the two fertility levels.

Nodules for strain identification are obtained by harvesting

at 50% flowering. Obtain plants from each inoculated plot (F-3 and M-3). Excavate plants only from the sample rows of each plot. Select the plants randomly.

Wash the roots to clean off soil and adhering debris. Pick the nodules from the roots. Count and pool all the nodules of the two plants obtained from the same plot to obtain the plot sample. Similarly obtain plot samples of nodules from all the F-3 and M-3 plots.

Identify at least 30 nodules at random from each inoculated plot sample using one of the serological methods described in Section B. Store the rest of the nodules by: (1) desiccation over silica gel; (2) by freezing; or (3) after oven drying at 70°C, storing in small plastic bags or vials. Large batches of nodules require much time for identification. If nodulation is poor (less than ten nodules per plant) identify all nodules from each plot sample. Small nodules (diameter 1 mm or less) may not contain sufficient antigen for identification against three antisera using the agglutination or gel-diffusion techniques. However, small nodules are better identified using the fluorescent-antibody technique as this method requires only antigen smears from the nodule.

Apply the Chi-square (X^2) method to determine from the data whether or not the frequencies observed in the nodule identification depart significantly from the expected

frequencies of 1:1:1 for the three strains at each fertility level.

If there is a significant departure from the expected ratio of 1:1:1, the data indicate competition. Is the competition pattern the same at both fertility levels?

(h) Analyzing the yield data
(Key step 8)

The harvest data at 50% flowering and from grain yield will reveal valuable information on the importance of fertility to ensure a good response to inoculation with effective strains of rhizobia.

Using the harvest data, carry out statistical analysis; determine the treatment giving the highest yield. The Duncan's New Multiple Range Test is suggested for preliminary analysis of the data.

The data may be more rigorously treated by other statistical approaches to detect significant interactions in the treatments. Such analysis may require expert statistical assistance.

Requirements

(a) Setting up the experiment

Measuring tape (50 m)
Field site (28.2 m x 15 m)

(b) Preparing the mixed inoculant and inoculating the seeds

Transfer chamber
Agar slant cultures of antigenically distinct strains used in Exercise 18
Three Erlenmeyer flasks (250 ml) each containing 150 ml YM broth
Three sterile plastic syringes (50 ml), three sterile needles (3/4 in, 18 gauge)
Three bags of peat (50 g per bag) autoclaved or irradiated
Incubator, refrigerator
Quality check of inoculants (materials as in Chapter 27)
Sensitive balance (to weigh peat)
Coarse balance (to weigh seeds)
Beaker (1 liter), spatula, aluminum foil
Gum arabic solution, pipettes (wide-bore tip), plastic bags

(c) Choosing a site and preparing the field

Farmer's field or alternative site

Soil analysis data

Soil sample for MPN counts

Wooden posts to mark field perimeter

Herbicide(s) and spraying equipment

Plowing and tilling machinery

Suitable field drainage to control cross-contamination

Other field preparation accessories

Lime

(d) Applying fertilizers

Wooden stakes (boundary markers)

Sign-boards for all M and F plots

Triple superphosphate 0.9 kg x 12 plots = 10.8 kg

Potassium chloride 0.69 kg x 12 plots = 8.3 kg

Zinc sulphate 0.96 kg x 12 plots = 11.5 kg

Ammonium molybdate 0.0033 kg x 12 plots = 0.04 kg

Magnesium sulfate 0.09 kg x 12 plots = 1.1 kg

Urea 0.0373 kg x 8 plots =0.3 kg

(e) Planting the experiment

Irrigation

Inoculated and non-inoculated soybean seeds from (b)

Metric tape, hoes or other suitable equipment for making

furrows

Planting guide for even placement of seeds

Urea for N-controls

(f) Monitoring the trial and harvest

As in Exercise 18

(g) Harvesting nodules for strain identification

Source of water

Hoe for digging, coarse sieve

Glass or plastic vials (for storage of nodules, Appendix 2), marker pens

Plastic bags

Freezer space (or silica gel in vials)

(h) Analyzing the yield data

Calculator and statistical tables

Statistical assistance

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SECTION D

INOCULATION TECHNOLOGY INTRODUCTION

The agricultural benefits possible from use of selected, high-nitrogen fixing strains of rhizobia can be realized only when farmers obtain and properly use high-quality inoculants on their legume seeds or soil before planting. Technology on growing rhizobia, preparing inoculants with suitable carrier materials, and distributing viable inoculants to farmers is essential. This section is concerned with inoculant production and use.

Culture of Rhizobia

Rhizobia are easy to grow in the laboratory. These bacteria are aerobic and also microaerophilic. They require aeration which may be provided by using a mechanical shaker or by bubbling sterile air through the medium. Rhizobia grow best at 25-30°C. The medium must supply energy, a source of nitrogen, certain mineral salts, and growth-factors. Most commonly used is a yeast extract mannitol mineral salts medium, but if cost or availability is a concern, sucrose or glycerol may be substituted.

Rhizobia grows best at 25° to 30°C. Vessels or fermenters vary in size from a few milliliters up to several thousand liters.

Incubation time required will vary with size of seed or starter inoculum. Large inocula decrease the incubation time needed to attain the 5 X 10^8 to 1 X 10^9 rhizobial cells per milliliter considered necessary, particularly when using a non-sterile carrier material.

The broth culture should be checked frequently for purity and any abrupt change in pH which indicates contamination. Prior to incorporation with the carrier material (peat, coal, etc.) the culture should be checked serologically (agglutination, FA etc.) with its homologous antiserum.

Incorporation into Carrier

The properties of a good carrier material are: No toxicity to rhizobia, good moisture absorption capacity (for example not more than 15% moisture in most peats prior to addition of culture), suitable pH (6.5-7.0), fine particle size for better adherence to seed (70 to 100% through 200 mesh screen), free of lump-forming materials, and in ample quantities at moderate cost.

Non-availability of peat in some countries has prompted trial of a wide range of substitutes, e.g., coal, charcoal, bagasse, filter-mud, ground plant residues, and combinations of these with soils. None has proven as consistent in its ability to afford adequate survival of rhizobia as peat.

Extraction, drying, and milling of peat are the most capital intensive aspects of inoculant production. The dried peat is milled to 100-200 mesh and neutralized to pH 6.5-7.0, preferably, with precipitated calcium carbonate. Both sterilized and non-sterilized peats are used in commercial production systems. Sterile peat (gamma-irradiated at 2.5 – 5.0 megarads or autoclaved) is generally accredited with better rhizobia survival characteristics than non-sterile peat. Heat sterilization of some peats has been found to produce undesirable changes and to release toxins. Sterilization by gamma radiation is preferred.

Under commercial conditions in the United States, quality-tested broth cultures are incorporated, one liter per kilogram of peat, and packaged in thin gauge (0.05 mm) polyethylene bags. Bags of this specification permit gas exchange while minimizing moisture loss from the inoculant. Inoculants are also produced by aseptic injection of quality tested broth cultures in packages of presterilized peat.

Inoculants are matured for about 2 weeks at 25-30◦C to attain maximum numbers of around 10^9 - 10^{10} cells/g of inoculant. Thereafter, most inoculants are maintained under refrigeration (4^oC). Some inoculants have better survival at 26^o – 28^oC. The final moisture content of the peat inoculant should be 40-60% on a wet weight basis for inoculants produced with

presterilized peat. A lower moisture content (30-40%) is preferred for better rhizobia survival in non-sterile peat. Batches of inoculants are usually sampled for a check on their quality at the time of leaving the production plant. This is done by the direct plate count in the case of inoculants based on sterile carriers. When non-sterile carrier material is used, inoculant quality can only be tested adequately by a plant infection test which lasts for about three weeks and which conflicts with a need to distribute the inoculant quickly. Inoculants must bear an expiration date and have an absolute minimum of 106 viable rhizobia/g and 108 viable rhizobia/g for non-sterile and presterilized peat, respectively, at that time. In the United States credit for returned inoculants that have passed their expiration date is an essential facet of inoculation technology acceptance by farmers and is the standard policy of the more reputable inoculant producers.

Soil and Seed Inoculation

The essence of legume inoculation is the placement of such a large population of a highly effective nitrogen-fixing rhizobia that is compatible with the host legume variety in close proximity to the emerging radicle that the majority of the nodules which form contain the introduced rhizobia.

It is important to assess the need to inoculate a particular

legume at a specific site. Sometimes an adequate population of effective native strains will ensure ample nodulation without inoculation. Alternatively, an inoculant strain may not survive in adequate numbers or be sufficiently competitive against the native rhizobia, and yield benefits are unlikely from inoculation.

The most common means of introducing rhizobia to the soil is as seed-applied inoculant. In its simplest (and least satisfactory) form, peat inoculant is mixed with water to form a slurry and mixed with the seeds. Better results are obtained when the inoculant is coated on the seed with an adhesive. An adhesive increases the amount of inoculant that will adhere to the seed. A good inoculant adhesive must be nontoxic to the rhizobia and provide protection during planting and in the soil. Gum Arabic has these properties, but is expensive to farmers and not readily available at many locations. Other adhesives used successfully include methyl ethyl cellulose, sucrose solutions, and vegetable oils. An additional coating of calcium carbonate, rock phosphate or other pelleting material can enhance the success of inoculation. This is often done when adverse weather conditions prevent immediate sowing of inoculated seeds, as protection against insects in the soil, when the soil is hot and dry or very acidic, or as protection against pesticides.

EXERCISE 20

TO PRODUCE BROTH CULTURES IN SIMPLE GLASS FERMENTORS

Glass fermenters are set up in a laboratory and used for the small scale production of broth cultures. The broth cultures are monitored periodically for cell number and contamination during growth.

Key steps/objectives

1) Initiate starter broth cultures
2) Assemble small fermentor units
3) Sterilize fermentors
4) Become familiar with operation details
5) Inoculate the fermentors
6) Take broth samples periodically for cell count and check for contamination
7) Test for contamination
8) Perform total counts and optical density measurements
9) Perform viable counts by the spread plate method on the presumptive test media
10) Perform agglutination tests with the homologous antisera

(a) Inoculating starter cultures
(Key step 1)

Prepare four 50 ml flasks or tubes each containing 25 ml of YMB. Obtain slant, or lyophilized bead preserved cultures of bradyrhizobia (e.g., *B. japonicum* TAL 102) and fast growing rhizobia (e.g., *Rhizobium* sp. TAL 1145 from *Leucaena leucocephala*). Inoculate two flasks with each rhizobial strain and aerate at 25-30°C. These will serve as "starter" cultures for inoculating the YMB in the fermentors.

(b) Assembling simple fermentors
(Key steps 2 and 3)

Set up two fermentors (one for each strain) as shown in Figure 20.1. The main fermentation vessel is a slightly modified 4 l Erlenmeyer flask with a sampling port (glass tubing 4 mm ID) fitted close to its base. Fill each fermentor with 2-3 l of YMB. Connect the cotton packed filters to prevent the entry of contaminants via the air lines. All rubber stoppers and tubings must be autoclavable. Insert the large rubber stopper which holds the air inlet and outlet tubes with their respective filters, firmly into the neck of the flask.

Connect the air inlet tube to an aquarium pump. Activate the pump and check the air inlet and outlet filters for air resistance. Air should flow freely through both filters while bubbling through the broth and simultaneously aerating and agitating the medium. The cotton in the filters should be packed uniformly but loosely. Overpacking the air inlet

filter can cause resistance to incoming air and lead to poor aeration. Overpacking of the outlet filter can lead to poor air escape and pressure build-up in the fermentor.

Disconnect the fermentor from the pump and prepare it for autoclaving. Make sure that the stopper which holds the air tubes is still firmly seated. The air supply system must be well protected to prevent entry of contaminants. Wrap the top of each flask with a wide band of non-absorbent cotton and secure it with a string. Then, add a protective wrapper of aluminum foil (Figure 20.2). Close the air inlet tube with a clamp at the spot indicated in Figure 20.1 to prevent the broth from leaving the flask due to pressure build up in the flask during autoclaving. Pressure relief during autoclaving occurs through the air outlet tube which must be left open. The filters should remain connected to the fermentor during autoclaving. To provide a convenient place for them, make an oversized wire ring to fit snugly around the neck of the fermentor vessel and twist it to obtain an eyelet or loop on each side. Each filter may then also be fitted with a piece of wire ending in a small hook. Hook the filters onto the eyelet (Figure 20.2). Sterilize the assembly for 40 min, if it contains approximately 2 l of broth. Adjust the sterilization time according to the volume of liquid; increase time by 10 min for each additional liter.

After the fermentor has cooled, remove the clamp from the air

inlet tubing. Connect the air supply to check for proper

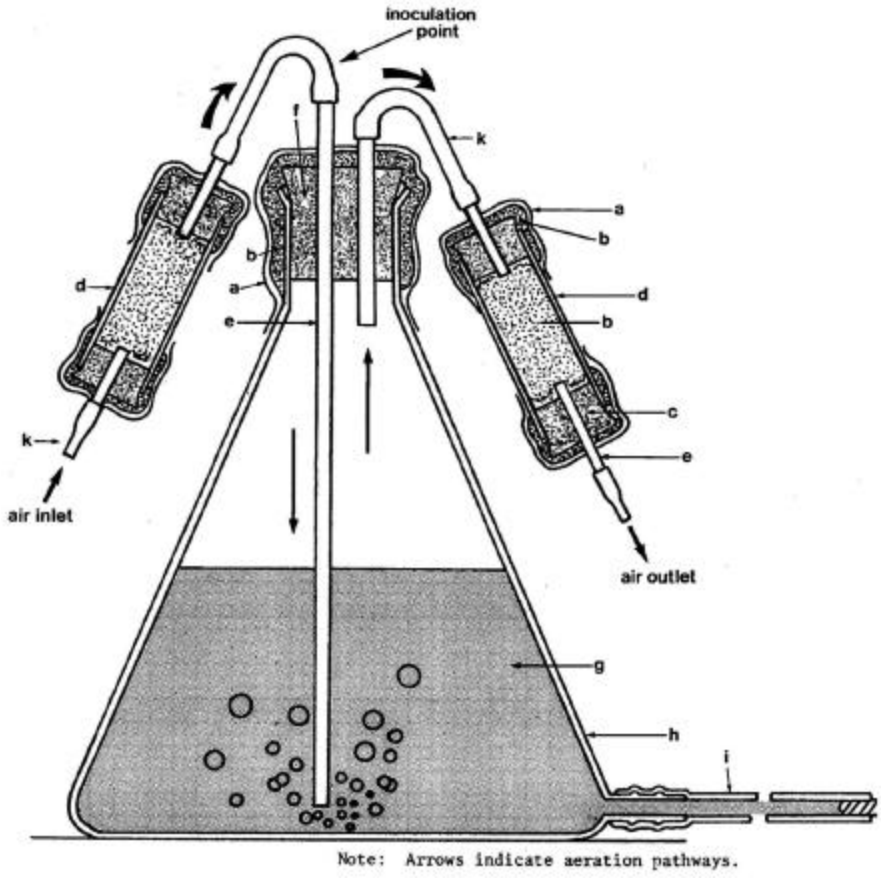

Figure 20.1. Scheme of simple fermentor unit a - Aluminum foil; b - Non absorbent cotton; c - Autoclavable stopper; d - Filter unit; e - Glass tubing; f - Wire ring; g - Growth medium; h - Flask; i - Sampling tube; j - Plug; k - Latex tubing; l - Hose clamp; m - Aquarium pump; n - Wire hook

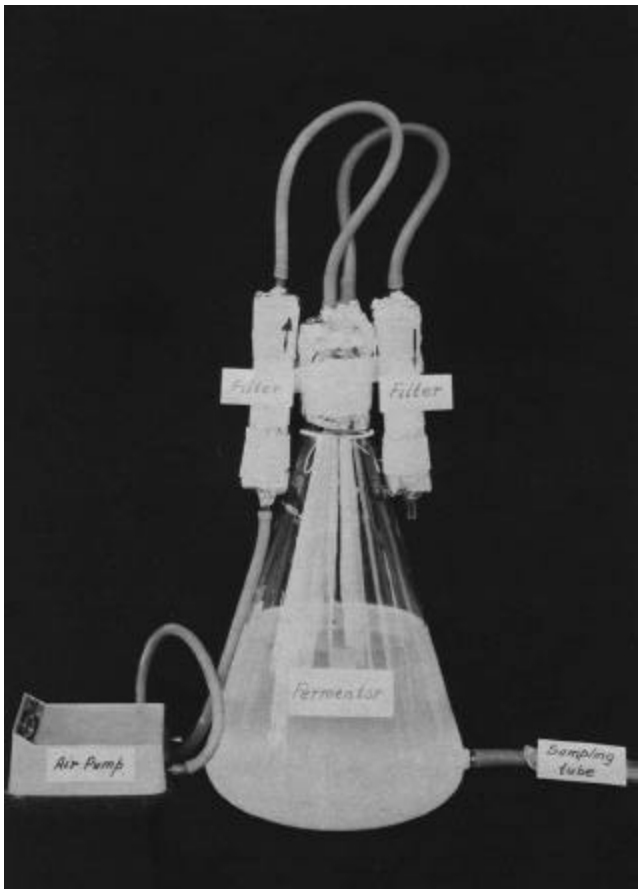

Figure 20.2. Simple fermentor in operation.

aeration once again and for leaks in the system.

Various types of air systems have been used to aerate small fermentors including compressors, compressed air in tanks, aspirators, and aquarium pumps. The latter have been very satisfactory for small units and are inexpensive, silent, and dependable.

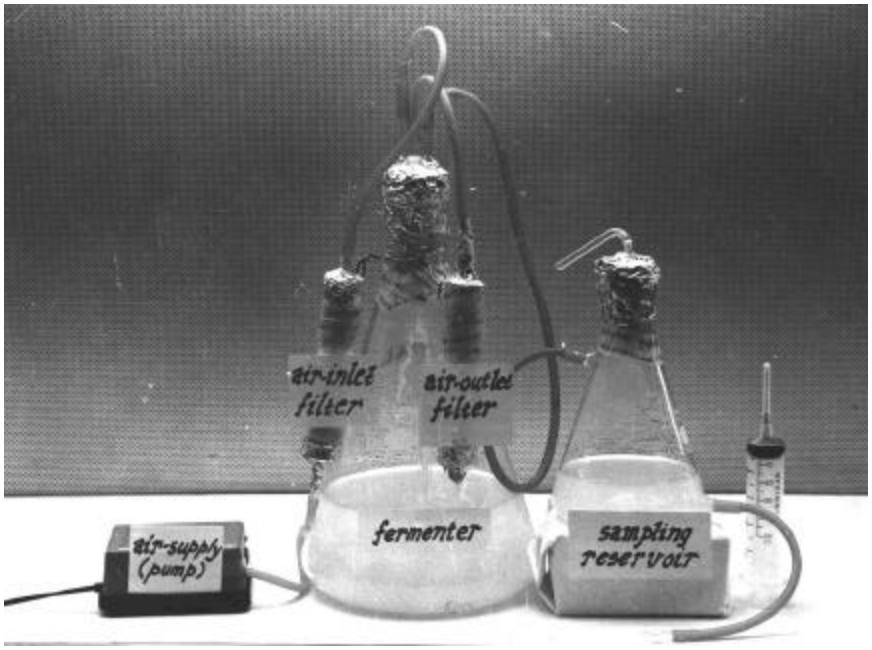

Figure 20.3 Modified fermenter.

Although a pressure relief valve may be desirable, it is not really necessary. Most aquarium pumps generate only low pressure, sufficient however, for several (four) fermentor units which may be connected to one aquarium pump using a manifold.

(c) Operating the glass fermentors

(Key step 4)

General operation: If, after autoclaving, the fermentor has been inspected and found to function properly, it is ready for inoculation with the starter culture. If an aquarium pump is used, and more than one fermentor is attached, adjust the air to achieve an equal flow to each fermentor. For other air supply systems, adjust the air flow on the bypass which may be installed between the pump and the air inlet filter.

The glass fermentor is inoculated through the latex air inlet, just above the main stopper tubing, with a sterilized syringe fitted with an 18 G needle. Care must be taken that no contaminants are introduced. Twenty ml of the starter culture are removed aseptically from its flask. The air inlet tubing is swabbed with 70% alcohol (or 3% hydrogen peroxide) about one inch above its connection to the glass tube. The needle is inserted downwards into the tubing and the culture is injected. The airstream will facilitate speedy entry and incorporation of the starter inoculum into the YMB. The culture is incubated at 25-30°C under continuous aeration.

Sampling procedures: Aseptically, with a sterile syringe, withdraw culture broth from the fermentor through the sampling

tubing attached to the sampling port. Swab the tubing with 70% alcohol or 3% hydrogen peroxide. Insert the needle into the sterilized portion of the tubing and withdraw the desired amount of culture broth. For quality control purposes (such as Gram stain, pH measurements, optical density measurements, the total count, and plate counts), 5-10 ml of culture are sufficient and may be withdrawn by using a 5 or 10 ml syringe fitted with a 22 gauge needle.

For injection of the broth culture into bags of sterile carrier (peat), 40 ml samples are usually withdrawn with a sterile 50 ml syringe fitted with a 18 gauge needle. Alternatively, an automatic motorized syringe equipped with a 16 gauge needle may also be used to withdraw broth culture if large numbers of bags are to be injected.

In a modified system, a 1 l collection flask is connected to the fermentor as shown in Figure 20.3. This collection flask should be autoclaved together with the fermentor. It is connected to the fermentor via a tubing attached to a sampling tube running through the stopper on top of the unit and into the broth culture. A sampling port at the bottom of the fermentor is not needed in this case. The broth culture is forced into the sampling flask by temporarily closing off the air outlet of the fermentor while the pump is running.

(d) Producing broth inoculum

(Key steps 5, 6, 7, 8, 9, and 10)

When the starter cultures have reached the end of their log phase of growth (7 days for a slow-growing rhizobia and 5 days for a fast-growing rhizobia, respectively), they are ready to be used for inoculating the fermentor.

Inoculate one fermentor with *B. japonicum* TAL 102 and the other with the fast-growing *Rhizobium* sp. TAL 1145.

Take a 10 ml sample from each fermentor at the end of the growth period of each strain and conduct the following tests:

1) pH tests: A contamination problem is usually evident when the pH of the broth decreases toward acidity especially with slow-growing, alkali-producing rhizobia (e.g., *B. japonicum*). However, with fast-growing, acid producing rhizobia (e.g., *Rhizobium* sp. from *L. leucocephala*) the pH test is less helpful since most contaminants are usually acid producers. Test the broth pH of the slow-growing rhizobia by adding two drops of bromthymol blue (0.5% w/v in alcohol) in 1 ml broth. With most strains tested, the pH does not change during mass culturing. A yellow coloration indicates acidity (presence of contaminants) and a green to blue coloration alkalinity (absence of contaminants).

2) Gram stain (Exercise 3)

3) Peptone glucose test (Exercise 3)

4) Total count with Helber or Petroff-Hausser counter (Exercise 4)

5) Optical density measurement (Exercise 4)

6) Spread plate count on YMA containing Congo Red and on YMA containing BTB (Exercise 4)

7) Agglutination with the homologous antiserum: This should be done just before harvesting when the culture has no less than 1×10^9 cells per ml. Dilute 2 ml of the cell suspension with 2 ml saline. Mix well and heat in boiling water for 30 min. After cooling, pipette 0.5 ml into an agglutination tube and add 0.5 ml of a 1:50 dilution of the homologous antiserum which should have a titer of at least 800. Perform the agglutination test as described in Exercise 7.

The broth cultures may be incorporated into carrier material when the total count indicates a cell concentration of more than 1×10^9 cells per ml and purity of culture has been established.

Requirements

(a) Inoculating starter cultures

Transfer chamber
Platform shaker
Inoculation loop, flame
Erlenmeyer flasks or screw capped tubes of 50 ml capacity containing 25 ml YMB each

(b) Assembling simple fermentors

Large autoclave, aquarium pumps or compressor
Cork borer, small glass file, bunsen burner
For each fermentor: Erlenmeyer flask, 4 l, (This flask is modified by the addition of an outflow tube (ID 4 mm) at its base. These modified flasks are not available commercially, but any glass blower should be able to attach the short 3-5 ml glass tube.)
#12 autoclavable stopper
Glass tubing, inside diameter (ID 4 mm), approximately 120 cm
Glass tubing, (ID 30 mm), two pieces of 10 cm length for making air filters. Barrels of 50 ml syringes may be cut to size and used instead.
Rubber stoppers #4, autoclavable, four pieces
Hose clamps, two, air by-pass (T-piece with short latex

tube and clamp)

Surgical rubber tubing (ID 4 mm), approximately 150 cm

Glasswool, cotton wool, non-absorbent, aluminum foil

Sampling tubes (ID 4 mm)

YMB (2-3 l per fermentor)

(c) Operating the glass fermentors

Syringes (30 ml), sterile with 18G needles

70% alcohol, cotton swabs or tissue paper

Broth culture of *B. japonicum* TAL 102

Broth culture of *Rhizobium* sp. TAL 1145

Syringes, sterile (10 ml); 22G needles

Test tubes, sterile (for samples)

(d) Producing broth inoculum

Spectrophotometer; cuvettes, transfer chamber

Antisera of TAL 102 and TAL 1145

Plates of peptone glucose agar

Plates of YMA containing BTB

Plates of YMA containing Congo Red

Syringes (20-30 ml); 22G needles

Test tubes, sterile (for samples)

Pipettes, sterile (1 ml); pipettes, sterile (10 ml)

Tubes containing 9 ml sterile diluent, rack

Pasteur pipettes, sterile, calibrated

Solution of Bromthymol Blue (0.5% w/v in ethanol)

Materials and supplies for Gram stain (Appendix 3)

EXERCISE 21

TO PREPARE A RANGE OF CARRIER MATERIALS AND PRODUCE INOCULANTS

Carriers for rhizobia are prepared from various materials such as peat, charcoal, and lignite. These carriers are used for the production of granular and powdered inoculants. The quality of these inoculants is tested and compared.

Key steps/objectives

1) Select and dry carrier materials
2) Grind carrier materials
3) Sift carrier materials and select suitable particle sizes for granular and powdered inoculants
4) Neutralize carrier materials
5) Determine water holding capacity of carriers
6) Package the carrier materials
7) Sterilize the carriers
8) Examine the carriers for sterility after sterilization
9) Inoculate carriers with broth cultures from fermentors
10) Plate peat cultures for quality control
11) Inoculate plants for the plant infection count
12) Test strain identity serologically
13) Record and tabulate results. Compare carrier treatments
14) Apply quality standards

(a) Milling inoculant carrier materials
(Key steps 1, 2, and 3)

Carrier materials are chosen to fill criteria set forth in the introduction to this section. For this exercise select peat, charcoal and lignite, or three other carrier materials if these are not available. Work with each carrier individually. Weigh 5 kg of each carrier and grind it in a hammer mill. Thoroughly clean the hammer mill with a brush or with a jet of air from a compressor before grinding the next carrier.

Stack up a set of sieves in series: 16 mesh (1 mm), 42 mesh (355 ìm), 100 mesh (150 ìm), and 200 mesh (75 ìm). Place this series of sieves on a collecting pan, and clamp the stack and collecting pan to a sieve shaker. Add the milled carrier to the uppermost sieve and activate the shaker for 60 min. Collect the fraction caught on the 42 mesh sieve and the fraction caught in the pan. The remainder should be returned to the mill and ground again. Particles of 16-42 mesh are used for the preparation of granular carriers (soil implants); particles of 200 mesh and finer, make carriers suitable for seed coating.

(b) Preparing and characterizing inoculant carriers
(Key steps 4, 5, and 6)

The pH of an inoculant carrier should be around 6.5-7.0. In a 400 ml glass beaker suspend 10 g of the carrier into 90 ml water. Stir the mixture on a magnetic stirrer while monitoring the pH with the electrode of a pH meter. If the pH is lower than 6.5, gradually add precipitated, powdered calcium carbonate ($CaCO_3$) until a pH of 6.5 has been reached. Record the amount of ($CaCO_3$) needed to neutralize 10 g of the carrier. Add a corresponding amount to the remaining carrier e.g., if 0.25 g were needed to neutralize 10 g of carrier in the water suspension, add 2.5 g of $CaCO_3$ to every 100 g of dry carrier. Mix well by hand. Repeat the same procedure for all carriers.

The water (moisture) holding capacity of a carrier determines the maximum amount of liquid inoculum that can be added to it. Carriers vary greatly in their water holding capacity.

Before the water-holding capacity can be measured, the inherent moisture level in the carrier must be determined. This may be done most conveniently on a moisture balance. Use a drying oven if a moisture balance is not available. Weigh 10 g accurately on a foil or glass weighing dish and place it into the oven at 70°C for 24 h. Weigh and return to the oven. Another weighing at 48 h will confirm the endpoint of moisture loss.

Use the formula below to calculate the inherent moisture

content on the dry weight basis.

$$\text{Moisture content} = \frac{(W_1 - W_2) \times 100\%}{W_2}$$

W_1 = Weight of carrier before drying

W_2 = Weight of carrier after drying at 70°C

Proceed to determine the moisture-holding capacity of the carrier. Weigh 100 g of oven dried carrier material into a 500 ml beaker. Add water with continuous stirring, until the carrier appears to be saturated. Add additional water to produce a thin slurry. Transfer this slurry to a pre-weighed measuring cylinder which has a drainhole on its bottom covered by a sieve. Allow the water to drain overnight, then weigh the measuring cylinder with the contents. Give the moisture holding capacity on the dry weight basis of the carrier. For example, if 100 g of predried carrier can hold 120 ml of water, its moisture holding capacity is 120%.

The amount of inoculum broth to be added to the carrier must be well below the carrier's moisture holding capacity as the resulting inoculum should be friable in texture. It is, however, desirable to add the largest amount possible while still retaining the desirable texture. A high moisture level is necessary because moisture is lost during storage, and the survival of rhizobia in a carrier is affected by low moisture levels.

Proceed to determine the desirable amount of moisture to be added to the carrier by a trial and error method. Prepare six bags (polyethylene 127 x 178 x 0.076 mm) of each neutralized carrier (50 g per bag). To the first bag, add an amount of water which is approximately 5 ml less than the carrier's moisture-holding capacity. If this moisture holding capacity is 60 ml (or 120%) add 55 ml. To the next bag, add 5 ml less (50 ml). Continue until each successive bag has received 5 ml less than the preceding one. Thus, bag #6 will receive 30 ml of water. Seal the bags with a bag sealer and incorporate the water into the carrier by kneading. Knead or massage the bags thoroughly until all moisture has been absorbed and the carrier/water mixture appears to be homogenous.

Examine the bags for total absorption of the water. Check for dry areas in the carrier which can usually be recognized, as unwetted carrier has a lighter color.

Allow the six treatments to equilibrate for two h, then cut the bags open and sample a few grams of each bag with your hand. A suitable carrier/water mixture should feel moist, but not soggy. It should crumble in your hand (i.e., be friable) and it should not be sticky. From each representative carrier select that treatment which has absorbed a maximum amount of water while still retaining friability. Record the carrier:water ratio and use this information to calculate the

recommended moisture level for each carrier. The recommended moisture level is usually given in percent calculated on the wet weight basis of the final preparation. The inherent moisture level of the carrier must of course be taken into consideration. The total moisture content of the inoculant is the sum of the weights of broth culture and inherent moisture of the carrier. Thus, a 90 g package of inoculum with a moisture content of 50%, made from a carrier with an inherent moisture level of 10%, contains 45 g of dry carrier, 5 g of inherent moisture and 40 g of broth inoculum.

Determine the moisture holding capacity of all the carriers used (powdered and granular), then prepare them as outlined in Table 21.1. A similar table may be made for the granular carriers. Record the moisture holding capacities in the last column of the table.

Gamma-irradiation (5 megarads) is preferred for peat sterilization over autoclaving. Gamma-irradiated peat is used here in one treatment only since irradiated peat is often unavailable. It serves as a standard because its properties as carrier material for various strains of rhizobia are well known. It is regularly used for inoculant production at NifTAL. It is packaged and sealed in 127 x 178 mm polyethylene bags of 0.076 mm thickness. Weigh 50 g portions of all other carriers into 127 x 178 mm x 0.076 mm autoclavable (polypropylene) bags. Add 1 ml of water per bag.

Make an incomplete heat seal leaving the bags slightly open. Autoclave the bags in a foil covered tray. After the bags are cool, completely heat-seal in a sterile hood.

Table 21.1. Carrier types, treatments, and quantities required for inoculant preparation and evaluation using finely milled carriers.

Carrier	Sterilization Treatment	Carrier Quantity	Recommended Moisture Level
Peat	gamma-irradiated	4 bags x 50 g	50%
Peat	autoclaved	4 bags x 50 g	*
Peat	autoclaved	2 trays x 1 kg	*
Peat	not sterilized	2 trays x 1 kg	*
Charcoal	autoclaved	4 bags x 50 g	*
Charcoal	autoclaved	2 trays x 1 kg	*
Charcoal	not sterilized	2 trays x 1 kg	*
Lignite	autoclaved	4 bags x 50 g	*
Lignite	autoclaved	2 trays x 1 kg	*
Lignite	not sterilized	2 trays x 1 kg	*

* To be determined.

For the bulk preparations, place 1 kg of neutralized carrier into each of four autoclavable trays approximately 46 x 46 cm

wide and x 10 cm deep. Spread into an even layer and cover with aluminum foil. Set aside two of these trays as non-sterilized treatments. Autoclave the other two trays at 121°C and 15 psi for 1 h. Allow to cool in the autoclave over night.

After sterilization, test a representative sample of each autoclaved carrier for sterility as described in Exercise 22.

(c) Producing inoculants
(Key Steps 7, 8 and 9)

Prepare inoculants following the treatments and replications as outlined in Table 21.1.

Obtain the fermentor cultures of *B. japonicum* (TAL 102) and *Rhizobium* sp. (TAL 1145) which were produced in Exercise 20.

Use broth culture of TAL 102 to inoculate each 1 kg portion of the autoclaved carriers in trays. Add broth culture according to recommended moisture levels determined in section (b) of this chapter. Use your gloved hands to mix the broth into the carrier until its consistency becomes uniform. (Tools are not needed for mixing, but the hands should be covered with sterile gloves to minimize contamination.) Replace the foil cover and allow the inoculant to mature at 25-30°C for 2 weeks. Repeat this procedure with TAL 1145 using the second

autoclaved tray of each carrier.

Similarly, prepare inoculants by hand-mixing the untreated (nonsterile) bulk carriers with broth cultures of TAL 102 and TAL 1145.

The presterilized carrier materials in sealed bags are aseptically injected with the suitable amount of broth culture with a sterile 50 ml syringe fitted with a sterile 18 gauge needle as follows:

Withdraw the desired amount of broth culture from the outlet tubing of the glass fermentor as described in Exercise 20. Sterilize a small area in a corner of the carrier bag with 70% ethanol. Puncture the bag in the sterilized area and insert the needle carefully to avoid piercing the opposite wall of the bag. Inject the desired amount of inoculum aiming the tip of the needle toward the center of the bag.

Seal the puncture hole with plastic labeling tape and write on it the treatment number, the strain used, and the date of preparation. Work the broth into the peat by kneading the bags until the liquid inoculum has been uniformly absorbed by the carrier. Incubate at 25-30°C for 2 weeks. Obtain inoculants prepared earlier and stored for 6 months at room temperature. One bag of each will be used in (d).

(d) Testing the quality of the inoculants
(Key steps 10, 11, and 12)

Rhizobia in the various treatments are expected to reach their maximal population 2 weeks after inoculation. Determine the number of viable rhizobia in all treatments.

Inoculants prepared in bagged gamma-irradiated and autoclaved carriers are not expected to contain many contaminants. The usual recommended counting technique is the drop-plate method (Exercise 4). Make serial dilutions of duplicate samples of the 2-week-old inoculants and those stored for 6 months. Plate dilutions ranging from 10^{-4} to 10^{-7} on YMA + Congo Red and on YMA + BTB. If proper aseptic procedures are not fully observed, contaminants may be accidentally introduced during the injection of the broth culture and during serial dilution and plating. Such contaminants will usually be detectable on these indicator media and their number should also be reported.

The hand-mixed inoculants, especially those based on nonsterilized carriers, can be expected to contain contaminants and the plant-infection count will be necessary for a reliable determination. Plate counts on indicator media may be used to give a measure of the contaminants.

Set up the plant-infection (MPN) count in growth pouches using

Leucaena leucocephala as host for TAL 1145 and soybean for TAL 102 (Exercise 5). Plate dilutions of sterile and nonsterile based carriers from 10^{-4} - 10^{-7}. Plate both the 2-week-old inoculants and those stored for 6 months from (c).

(e) Collecting, recording and analyzing the data
(Key step 13 and 14)

Determine the number of viable rhizobial cells in the various carrier treatments as described in (d). Transform the data to $\log_{10}$ and calculate the mean for the replications. Organize the data in the format as shown in Table 21.2.

The experiment with each rhizobial strain is a factorial involving three carriers (peat, charcoal, and lignite), two carrier forms (powdered and granular) and three carrier sterility conditions. Assistance may be needed for statistical analysis of the data.

Table 21.2. Multiplication of *B. japonicum* (TAL 102) in inoculants prepared from various carriers and under different sterility conditions.

	Log_{10} no. rhizobia per g moist inoculant					
	Peat		Charcoal		Lignite	
Carrier Treatment	Powdered	Granular	Powdered	Granular	Powdered	Granular
Autoclaved in						

polypropylene bag					
Autoclaved in polypropylene trays					
Untreated in polypropylene trays					
Irradiated in polyethylene bags	not done	not done	not done	not done	not done
Mean					

Compare the 2-week-old inoculants and note the decline in cell numbers. Compare the different treatments and decide whether the number of cells in the 6-month-old inoculants is sufficiently high to comply with minimum standards of quality. Minimum standards are given for the date of expiration, usually 6 months after manufacture. The minimum standards vary in different countries. In Canada, 10^6 viable rhizobia per g of peat are acceptable. In the USA, there is no federal regulation for quality of legume inoculants. Some of the states, however, have their own standards, as do the inoculant manufacturers. Australia, like NifTAL, requires a minimum of $1x10^9$ viable rhizobia per g at expiration. These inoculants are produced with irradiated peat.

Examine the results critically and contemplate the following questions:

Are all treatments well above the NifTAL minimum standard for inoculants at expiration?

Which level of sterility contributes to the highest cell population?

How are the carriers affected by the sterilization measures with respect to their ability to support high cell populations?

Why are different counting methods suggested for different levels of sterility?

Compare bulk sterilization of carriers in trays with bag sterilization and explain the advantages and disadvantages of each method.

Requirements

(a) Milling inoculant carrier materials

Hammer mill with collecting tray, bag, or bucket
Screen shaker equipped with a 16 mesh, 42 mesh, 100 mesh and 200 mesh screens (60 x 60 cm or larger)
Balance 1 - 5000 g capacity
Unground dried peat 5 kg
Unground charcoal 5 kg
Unground lignite 5 kg
Scoop or small shovel
Brush or air jet from compressor for cleaning hammer mill
Other locally available carrier material (e.g., filter mud, bagasse, coir dust to replace some or all the above mentioned carrier materials if these are not available (5 kg of each)
Aluminum foil, large roll
Trays to contain 1 - 5 kg of carrier material

(b) Preparing and characterizing inoculant carriers

Transfer chamber
Balance, toploading 0.1 g - 100 g capacity
Magnetic stirrer and 1 inch stirring bar
pH meter
Moisture balance or drying oven
Bag sealer

Autoclave

Glass beakers, 500 ml

Bottle of pH 7 buffer solution

Beakers (50 ml) for pH meter calibration

Autoclavable trays approximately 46 x 46 x 10 cm

Weighing dishes (metal or glass)

Measuring cylinder (250 ml) with drainhole and sieve

Packaged gamma-irradiated peat

Pipettes, 1 ml, sterile, one canister

Pipettes, 5 ml

Measuring cylinders, 50 ml

Aluminum foil, large roll

Scissors

Alcohol in spray bottle

Dilution tubes each containing 9 ml water

Rubber bulbs, 1 ml (for pipetting)

Calibrated, sterile Pasteur pipettes

Bottle of distilled water, 1 l

Carrier materials from (a)

YMA plates containing Congo Red

YMA plates containing BTB

Polypropylene and polyethylene bags (127 x 178 x 0.076 mm wall thickness)

Precipitated calcium carbonate

(c) Producing inoculants

Incubator

Broth culture of *B. japonicum* TAL 102 and *Rhizobium* sp. TAL 1145 approximately 13 - 15 l

Four bags each of autoclaved, powdered carriers prepared from three different materials e.g. peat, charcoal and lignite from (b)

Four bags each of autoclaved granular carriers of the same materials also from (b)

Four 50 g bags of neutralized powdered gamma irradiated peat packaged in polyethylene bags (127 x 178 x 0.076 mm thickness)

Carrier material in trays as prepared in (b)

Powdered and granular peat, two batches of 1 kg each, autoclaved

Powdered and granular peat, two batches of 1 kg each nonsterile

Powdered and granular charcoal, two batches of 1 kg each autoclaved

Powdered and granular charcoal, two batches of 1 kg each nonsterile

Powdered and granular lignite, two batches of 1 kg each autoclaved

Powdered and granular lignite, two batches of 1 kg each nonsterile

Three bags each of inoculants of *Rhizobium* (eg. TAL 1145) and *Bradyrhizobium* (eg. TAL 102) made from each of the carriers listed above and stored for 6 months at 26°C.

One package of surgical gloves

Box of 50 ml plastic syringes, sterile

Syringe needles, sterile 18 gauge

Alcohol, 70%

Tissue paper

Labeling tape

(d) Testing the quality of the inoculants

Plates of YMA + Congo Red

Plates of YMA + BTB

Plates of YMA + Brilliant Green

Serological pipettes (1 ml) sterile, glass spreaders

Calibrated Pasteur pipettes, sterile

Dilution bottles with 99 ml sterile diluent

Test tubes containing 9 ml sterile diluent

Test tube racks

Wrist action shaker (optional)

Balance, spatula weighing paper

Growth-pouch racks, growth-pouches

Plant nutrient solution, sterile

Seeds of *Glycine max* and *Leucaena leucocephala*

Bottles of sterile water

Chlorox or hydrogen peroxide for seed sterilization

Concentrated sulfuric acid

Erlenmeyer flasks, 500 ml capacity

Microscope slides, cover slips, mounting fluid

Box of flat toothpick

(e) Collecting, recording and analyzing the data

Plants of *G. max* and *L. leucocephala* from (d)

Plates with bacterial colonies from (d)

EXERCISE 22

TO PREPARE INOCULANTS USING DILUTED CULTURES OF RHIZOBIA AND PRESTERILIZED PEAT

The production capacity of small-scale inoculant production plants using presterilized peat can be increased by using diluted liquid cultures of rhizobia. In this exercise, fully grown cultures are diluted in water and other diluents of different formulations prior to incorporation into presterilized peat in packages or in polypropylene trays. The multiplication of rhizobia in the inoculants is studied.

Key steps/objectives

1) Culture *Rhizobium* sp. and *Bradyrhizobium* sp.
2) Make culture dilution flasks
3) Prepare diluents in dilution flasks
4) Prepare and package peat
5) Sterilize peat in packages and polypropylene trays
6) Prepare YMB + peat blanks and check for sterility
7) Examine YMA Congo Red plates plated with YMB-peat blanks
8) Perform viable counts on late log phase cultures
9) Prepare diluted cultures
10) Inject diluted cultures into peat
11) Mix diluted cultures with autoclaved peat in trays and package

12) Perform viable counts on inoculants at two weeks

13) Perform viable counts on inoculants at eight weeks

14) Record and analyze the data

(a) Culturing rhizobia in YMB

(Key step 1)

Prepare 500 ml YMB in each of two 1 liter Erlenmeyer flasks. Inoculate one flask with *Bradyrhizobium* sp. (e.g., *B. japonicum* TAL 102) and the other with *Rhizobium* sp. (e.g., TAL 1145 from *Leucaena leucocephala*). Both rhizobia should have antisera available for strain recognition and confirming purity (by serology) to be done later in the exercise. Incubate the inoculated flasks at 25-30°C on a shaker. To obtain late log phase cultures, allow the fast- and slow-growing rhizobia to grow for 4 to 7 days, respectively. At the end of the specified growth period, check the purity of the culture by Gram stain and by serology (simple tube agglutination or by the FA technique as described in Section B).

(b) Making a culture dilution flask and its operation

(Key step 2)

The culture dilution flask is basically a 2 l Erlenmeyer flask modified by a short glass-tubing outlet at the base of the flask as shown in Figure 22.1. Seek the assistance of a

skilled glass-blower for fitting the glass tubing to the base of the flask.

Five culture dilution flasks are required per rhizobial strain (four for diluents and one for the undiluted culture as control, see Table 22.1).

Attach a piece of surgical latex tubing of suitable size to the glass tubing outlet of each dilution vessel. Close the open end of the latex tubing with a plug made from a short piece of glass rod. Add appropriate diluent, cover the flask, and sterilize the entire unit by autoclaving.

To dilute the culture, aseptically introduce (with a pipette or a hypodermic plastic syringe fitted with a 3.5 cm and 14 gauge needle) the fully grown culture via the mouth of the culture dilution flask. Swirl the flask to ensure proper mixing and dilution of the culture in the diluent.

Withdraw the diluted culture for inoculation with a sterile plastic syringe as described for the fermentor in Exercise 20.

(c) Preparing the diluents
(Key step 3)

The late log phase cultures of each strain are diluted in 20% (v/v) solutions of yeast mannitol broth (YMB), yeast sucrose

broth (YSB), yeast-water (YW) and distilled (or deionized)

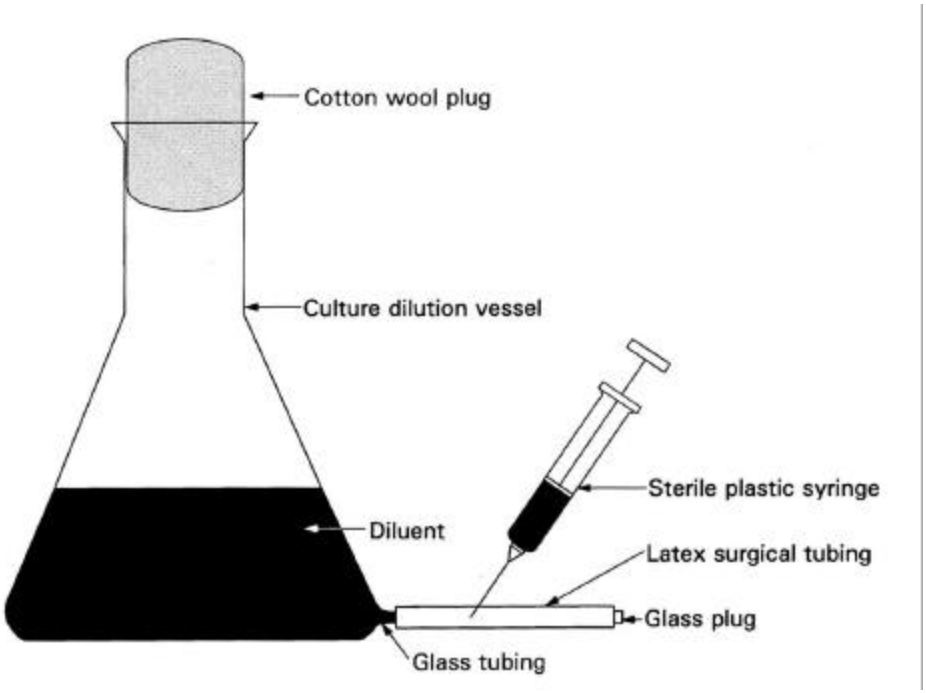

Figure 22.1. Apparatus for diluting cultures of rhizobia.

water. YSB has the same ingredients as YMB (Appendix 3) except that sucrose (10 g/l) is substituted for mannitol. YW is prepared by dissolving 0.4 g of yeast extract (Difco Labs, Michigan, USA) in one liter of distilled or deionized water.

Accurately prepare 500 ml of 20% YMB, YSB, and YW by mixing 100 ml of full strength media with 400 ml of distilled (or deionized) water in the culture dilution flasks. Prepare each diluent in duplicate since two strains will be used. Sterilize the diluents by autoclaving in the dilution flask.

Also, fill two 2 l Erlenmeyer flasks with 750 ml of distilled

(or deionized) water each, and sterilize by autoclaving. These will be used for the bulk inoculants.

(d) Preparing packaged presterilized peat and checking for sterility

(Key steps 4, 5, 6, and 7)

Packages containing 40 g of neutralized peat (pH 6.5-6.8) in 3 mil thickness (0.003 in or 0.076 mm) polyethylene and in autoclavable polypropylene bags are needed.

Prepare 62 bags of peat in polyethylene bags and heat seal after exclusion of all air from the bags. Expose the peat in polyethylene bags to gamma-irradiation (2.5-5.0 megarads). Alternatively, prepackaged irradiated peat is produced commercially and can be purchased from some commercial inoculant producers.

Similarly, package 40 g of neutralized peat in 62 autoclavable polypropylene bags (127 x 178 x 0.076 mm). Pipette 1 ml of water into each bag. (Inclusion of water during autoclaving is necessary for proper sterilization.) Follow the procedure described in Appendix 19 on using polypropylene bags for autoclaving carriers.

Autoclave the peat in the polypropylene bags for 45-60 min at 121°C and 15 psi. Allow sufficient time for the autoclave to

cool before removing the autoclaved bags. (Rapid release or loss of pressure from the autoclave after sterilization should be avoided).

Check the sterility of the treated peat by setting up peat + YMB blanks. To set up these blanks, aseptically inject 30 ml of sterile YMB into peat in two polyethylene and two polypropylene bags. Massage the bags to ensure proper incorporation of the YMB into the peat. Incubate the bags at 25-30◦C for one week.

At the end of the incubation period, aseptically remove a 10 g sample from each bag and transfer into 90 ml of sterile water in dilution bottles. Prepare serial dilutions from 10^{-1} to 10^{-4}. Plate 0.1 ml of each dilution in duplicate on plates of glucose peptone agar and YMA + Congo Red. Check the plates daily for 7 days for signs of growth and appearance of microorganisms which survived the sterilization.

If there is growth at any dilution, the sterilization was not complete. (It is not unusual to get growth of contaminants e.g., Actinomycetes, from peat samples which were previously irradiated and stored for a long time.) If there is growth, note the different types of colony morphology produced by the survivors. Make wet amounts of colonies picked from the plates and observe under phase contrast microscopy to establish cell morphology of the survivors (e.g., bacteria,

filamentous fungi, yeasts, etc.).

Only sterile peat is recommended for inoculant production by the dilution procedure. However, inoculants have been prepared from peat with surviving contaminants, as long as the contaminants were not detectable at dilutions higher than 10^{-2}. Irradiation sometimes does not provide absolute sterility, but the dilution method still produces high-quality inoculants in irradiated peat carriers.

(e) Preparing presterilized peat in polypropylene trays (Key step 5)

Obtain two sturdy trays (46 x 46 x 10 cm) made of autoclavable polypropylene. Place 1 kg of neutralized peat in each tray and spread it out to give a layer of even thickness. Cover the tray with aluminum foil. Autoclave both batches of peat at 121°C and 15 psi for 60 min. Allow the autoclave to cool before removing the trays of sterilized peat. Leave the peat to cool in the trays overnight. Do not remove the aluminum foil cover.

(f) Preparing diluted cultures of rhizobia (Key steps 8 and 9)

The various diluents prepared in step (c) are used for diluting the late log phase cultures of TAL 102 and TAL 1145.

Perform serial dilutions for viable counts (Exercise 4) of the late-log-phase cultures of TAL 102 and TAL 1145. Plate on YMA + Congo Red. Use the drop- or spread-plate methods. (Late log phase cultures may have 1-5 x 10^9 cells ml^{-1}).

Immediately after performing viable counts with the undiluted culture, accurately pipette 1 ml of the broth culture of TAL 102 into 500 ml of the 20% YMB in the dilution-flask to obtain a diluted culture. (The diluted culture will contain approximately 2-10 x 10^6 cells ml^{-1}, based on the assumption that the original undiluted culture had at least 1-5 x 10^9 viable cells ml^{-1}. The dilution factor is better estimated at a later stage after actual viable counts of the undiluted culture are obtained.)

Complete the preparation of diluted cultures of TAL 102 with YSB, YW and water as diluents.

Similarly, prepare diluted cultures of TAL 1145 using the various diluents in the dilution flasks.

(g) Preparing inoculants with presterilized peat
(Key step 10)

Aseptically, with a 50 ml plastic syringe, inject 30 ml of the diluted culture into each package of autoclaved peat and 40 ml

in the irradiated peat. Inoculate the bags as summarized in Table 22.1.

Table 22.1. Protocol for preparing inoculants of TAL 102 and TAL 1145 with the various diluents and sterilized peat.

	Packages of sterilized peat needed per strain		
Treatment	gamma-irradiated	autoclaved	Total ml of diluted culture needed strain^{-1}
water	6	6	420
YMB (20%)	6	6	420
YSB (20%)	6	6	420
YW (20%)	6	6	420
control*	6	6	420

* consists of undiluted late log phase cultures

Massage or knead the inoculated bags to work the inoculum into the peat. Label the bags to indicate the appropriate treatment and the date. Incubate the packages at 25-30°C.

(h) Preparing inoculants with presterilized peat in polypropylene trays

(Key step 11)

Add 10 ml of the late log phase culture of TAL 102 to the 750

ml of sterile water (from step c). Swirl the flask to ensure proper dilution. (The diluted culture will contain approximately 1.33-6.67 x 10^7 cells ml^{-1} based on the assumption that the original undiluted culture had 1-5 x 10^9 cells ml^{-1}.) Add this diluted culture to the autoclaved peat in the tray.

Work the diluted culture into the peat by hand-mixing. Sanitized disposable polyethylene or latex gloves must be worn during the mixing. Hand-mixing without wearing gloves results in high levels of contaminants. Continue mixing until the culture is absorbed by the peat. Break up any lumps that may result during the mixing.

Immediately after mixing, weigh out approximately 70 g quantities of the peat inoculant into polyethylene bags and heat seal. Label the packages to indicate treatments and date. Incubate the bags at 25-30°C.

Repeat the procedure to prepare inoculants of TAL 1145.

Best results are obtained if the mixing and packaging of the inoculants are done in simple but clean rooms (e.g., 5 x 3 x 3 m). Rooms of this size can be kept clean and disinfected regularly.

(i) Determining multiplication of the rhizobia in peat

inoculants prepared aseptically

(Key steps 12 and 13)

The inoculants produced as described in step (g) are most unlikely to contain significant numbers of contaminants as they were prepared aseptically by injecting the diluted cultures into presterilized (irradiated and autoclaved) peat.

Determine the multiplication of the rhizobia in these inoculants at two and eight weeks of storage. Use three replications of each treatment at each period of enumeration.

Enumerate the rhizobia in these inoculants by the drop- or spread-plate methods (see Exercise 4). Plate dilutions ranging from 10^{-4} to 10^{-7}.

(j) Determining the multiplication of the rhizobia in the peat inoculants prepared by hand-mixing in trays

(Key steps 12 and 13)

Enumerate the rhizobia in these inoculants at two and eight weeks, using three replications of each treatment.

The inoculants produced in step (h) will contain contaminants, since the mixing of the culture and peat was done without full aseptic precautions. Multiplication of the rhizobia in these inoculants may be determined by plate counts, but more

reliably by the plant infection technique (see Exercise 5).

Establish ahead of time seedlings of *L. leucocephala* and soybean for TAL 1145 and TAL 102, respectively, in growth pouches. (Growth tubes with seedling agar or NifTAL-tubes may also be used for *Leucaena*.)
Following the recommendations given in Exercise 5, 50 seedlings will be needed for enumerating the rhizobia in each bag of inoculant. Since three replications of each strain treatment are being enumerated, 150 seedlings of each host are needed. Pregerminate *Leucaena* seeds after acid scarification/sterilization (see Appendix 10).

Prepare serial dilutions of the inoculant ranging from 10^{-2} to 10^{-10}. Spread-plate dilutions 10^{-5} to 10^{-7} on YMA + Congo Red and YMA + Brilliant Green for plate counts (Exercise 4). Record the contamination on the plates and quantify if possible. Inoculate 10^{-1} to 10^{-10} dilutions onto plants in growth pouches or in tubes.

(k) Collecting, recording and analyzing the data
(Key steps 14)

Determine the number of viable rhizobia in the inoculants prepared by the various diluent formulations, sterilization and method of preparation. Transform the data to $\log_{10}$ and calculate the mean for the replications. Organize the

transformed data in the form of Tables 22.2 and 22.3.

Determine the number of rhizobia in the inoculants prepared in step (h) by the MPN method.

Table 22.2. Multiplication of *B. japonicum* (TAL 102) in inoculants prepared with diluted cultures and presterilized peat.

	Log_{10} no. of rhizobia g-1 moist inoculant			
	gamma-irradiated peat		autoclaved peat	
Diluent	2w	8w	2w	8w
water	_____	_____	_____	_____
YMB (20%)	_____	_____	_____	_____
YSB (20%)	_____	_____	_____	_____
YW (20%)	_____	_____	_____	_____
undiluted culture control	_____	_____	_____	_____

Table 22.3. Multiplication of *B. japonicum* (TAL 102) and *Rhizobium* sp. (TAL 1145) in inoculants prepared by mixing diluted cultures and autoclaved peat in trays

	Log_{10} no. of rhizobia g^{-1} moist inoculant

Enumeration method	TAL 102		TAL 1145	
	2 w	8 w	2 w	8 w
Plant infection (MPN)	_____	_____	_____	_____
YMA = Congo Red	_____	_____	_____	_____
YMA + Brilliant Green	_____	_____	_____	_____

Analyze statistically for differences in the various diluent treatments for both strains and enumeration methods as indicated in Table 22.3.

Since many biological, chemical, and physical factors influence the multiplication and survival of rhizobia in carriers, examine the data and contemplate the following questions.

Did the inoculants produced with diluted cultures reach maximum populations compared to the undiluted culture control?

How did water perform as a diluent in comparison to other diluents?

Compare the practicality and inoculant quality of the aseptic method of inoculant preparation in pre-packaged carriers to that of mixing diluted cultures with autoclaved peat in trays.

Can you confidently recognize colonies formed by rhizobia on plates in the presence of colonies formed by other microorganisms during plate counts? How did these plate counts agree with the values obtained by the plant infection technique?

Requirements

(a) Culturing rhizobia in YMB

YMA-slant cultures of *B. japonicum* (TAL 102) and *Rhizobium* sp. (TAL 1145).
Two 1 l flasks each containing 500 ml sterile YMB
Shaker
Gram stain reagents (Appendix 3)
Antisera of TAL 102 and TAL 1145 for agglutination (Exercise 7) or for FA (Exercise 11)

(b) Making culture dilution-flask and its operation.

Ten 2 l Erlenmeyer flasks
Glass tubing
Surgical latex tubing

(c) Preparing the diluents

Distilled or deionized water (500 ml)
500 ml each of 20% YMB, 20% YSB and 20% YW
Ten culture dilution flasks
Two 2 l Erlenmeyer flasks

(d) Preparing packaged presterilized peat and checking for sterility

Neutralized peat (approximately 8 kg)

Autoclavable polypropylene bags, (approximately 65 pieces)

Polyethylene bags, (approximately 150)

Bag sealing machine

Facilities for irradiating peat

Sterile YMB; sterile 50 ml plastic syringes fitted with 3.5m and 14 gauge needles

Incubator

Pipettes and milk dilution bottles containing sterile water

Plates of peptone glucose agar (PGA)

Plates of YMA + Congo Red

Phase contrast microscope

(e) Preparing presterilized peat in polypropylene trays

Two autoclavable polypropylene trays

Aluminum foil

(f) Preparing diluted cultures of rhizobia

Plates of YMA + Congo Red

Two culture dilution-flasks each containing 20% YMB

Two culture dilution-flasks each containing 20% YSB

Two culture dilution-flasks each containing 20% YW

Two culture dilution-flasks each containing sterile water

Late log phase cultures of TAL 102 and TAL 1145

(g) Preparing inoculants with presterilized peat packages

Five sterile 50 ml plastic syringes fitted with 3.5 cm and 14 gauge needles

Packages of gamma-irradiated and autoclaved peat

Diluted cultures from step (f)

(h) Preparing inoculants with presterilized peat in polypropylene trays

Two trays of autoclaved peat

Two flasks each containing 750 ml of sterile water from step(c)

Late log phase cultures of TAL 102 and TAL 1145

Two sterile 10 ml pipettes

Sanitized disposable polyethylene or latex gloves

Spatula and weighing balance

Sealing machine

(i) Determining the multiplication of the rhizobia in peat inoculants prepared aseptically.

Plates of YMA + Congo Red and YMA + Brilliant Green

Serological pipettes (1 ml), calibrated Pasteur pipettes, milk dilution bottles with 90 and 99 ml diluents, test

tubes containing 9 ml of sterile diluent

Balance, spatula, weighing paper

Wrist-action shaker

(j) Determining the multiplication of the rhizobia in peat inoculants prepared by hand-mixing in trays .

Requirements as in (i)

Seedlings (7 days old) of soybean and *Leucaena*

Growth pouches, N-free plant nutrient solution

(k) Collecting, recording and analyzing the data.

Calculators, statistical tables

Statistical assistance

EXERCISE 23

TO TEST THE SURVIVAL OF RHIZOBIA ON INOCULATED SEEDS

Survival of rhizobia on seeds is related to the number of cells applied, seed coat toxicity, and the method of application. Various seed inoculation methods are compared in this exercise.

Key steps/objectives

1) Prepare inoculants
2) Prepare adhesives
3) Coat and pellet seeds and glass beads
4) Plate count rhizobia for viable numbers on seeds and beads
5) Record and analyze results

(a) Preparing inoculants for seed inoculation
(Key step 1)

Prepare sterile carrier based inoculants for a *B. japonicum* strain (e.g. TAL 102) as described in Exercise 21.

One week after inoculating the peat, inoculate two 50 ml batches of YM broth with TAL 102. Incubate at 25-30°C on the shaker for 7 days. Set this broth culture aside in the

refrigerator after maximum turbidity has been reached. This broth culture is to be used as a liquid inoculum for seed coating.

(b) Preparing adhesives

(Key step 2)

The adhesives (stickers) used in this exercise are water, a 40% solution of gum arabic, a 5% solution of methyl ethyl cellulose (Cellofas A), a 15% sucrose (household sugar) solution, and vegetable oil. Water and oil do not require special preparations.

To prepare the gum arabic solution, heat 100 ml of distilled water to near boiling. Regulate the heat to prevent boiling. Add granular gum arabic in small (1-2 g) lots while continuously stirring the mixture. Each lot should be completely dissolved between additions until a total of 40 g have been added. The recommended gum arabic has the graininess of normal household sugar. Unlike the powdered form which is also frequently used, it dissolves easily and without clumping. The solution should be clear and straw colored. Add 2.5 g of precipitated calcium carbonate to the gum arabic solution if the pH is acid. The pH of the neutralized solution should not be lower than 6.0. Stir again until it has been evenly dispersed. Refrigerate until needed.

Prepare the Cellofas A solution by dissolving 5 grams in 100

ml distilled water, adding it in small increments while stirring the solution. Heating may not be necessary.

(c) Inoculating and pelleting seeds
(Key step 3)

Inoculate soybean seeds in batches of 100 g with inoculant preparations indicated in Table 23.1. Also inoculate a control group of 400 glass beads, 4 or 5 mm in size, using the same inoculation techniques as in treatment 7 of the table. If seeds from other legume species are chosen for this exercise, refer to Appendix 20 for recommended amounts of adhesive and inoculant. The glass beads should be approximately the same size as the seeds. Containers, spatulas, and glass rods should be sterile. Glass beads should be well washed with detergent, rinsed with distilled water, and oven dried. Seeds are not surface sterilized for this exercise.

Four inoculant coating methods are shown in Table 23.1. Direct coating (Treatment 1), the slurry method (Treatments 2-6), the two-step method (Treatments 7-9), and seed pelleting (Treatments 4 and 8) are used in combination with the slurry method and the two-step method, respectively.

Direct coating is self explanatory. Place seeds into a 1 liter flask; add 2 ml of inoculant broth and shake for

approximately 1 min until all seeds are uniformly wetted. Spread seeds on clean paper and allow to dry.

The slurry method is most commonly used by farmers. It is the

Table 23.1. Amount of stickers, inoculant and lime used for various inoculation and pelleting methods

Treatment No.	Sticker	Inoculant	Pellet	Inoculation Method
1	_____	broth (2.0 ml)	_____	direct coating
2	water (1.5 ml)	peat (0.5 g)	_____	slurry
3	gum Arabic (1.5 ml)	peat (0.5 g)	_____	slurry (with adhesive)
4	gum Arabic (2.0 ml)	peat (1.0 g)	$CaCO_3$ (20 g)	slurry (with adhesive + lime)
5	sucrose (1.5 ml)	peat (0.5 g)	_____	slurry (with adhesive)
6	Cellofas A (1.5 ml)	peat (0.5 g)	_____	slurry (with adhesive)
7	gum Arabic (1.0 ml)	peat (1.0 g)	_____	2-step
8	gum Arabic (2.0 ml)	peat (1.0 g)	$CaCO_3$ (20 g)	2-step (with lime)
9	vegetable oil (1.0 ml)	peat (1.0 g)	_____	2-step

most economic method, using less sticker and inoculant.

Immediately before use, mix sticker solution and the peat inoculant at a ratio of 1:3. Place seeds into a 500 ml beaker and add approximately 2 ml of the slurry to the seeds using a small measuring spoon. Stir continuously until the seeds are uniformly coated. Spread seeds on clean paper to dry.

The two-step method is especially useful when large numbers of rhizobia must be applied to the seed. Approximately ten times as many rhizobia can be bound to the seed as compared to the slurry method. In this method, the sticker and the inoculant are applied to the seed separately. In the first step the seeds are uniformly coated with the sticker. In the second step, the inoculant is added to the sticky seeds.

Place the pre-weighed seeds into a plastic bag. Add the sticker and then inflate the bag. Twist the bag shut to trap as much air as possible inside the bag. Swirl the bag for at least one min or until all the seeds are uniformly wet. Open the bag, add the inoculant, reinflate the bag and shake gently. Stop as soon as the seeds are uniformly black. Stop at this stage as prolonged shaking will break down the coating. Again, dry the seeds on clean paper.

Gauging the correct quantity of sticker solution is important in this method and is based more on experience than any specific recipe. Satisfactory coating will not occur if there is too little or too much gum.

Seed pelleting is used to provide the inoculant with additional protection for survival.

Immediately after seed coating add calcium carbonate to the sticky seeds in the plastic bag. Inflate the bag and gently shake for 1 min or until all seeds are uniformly white. Dry on clean paper.

The glass beads are included as a control since their surface is relatively inert. Comparison of the various seed inoculation treatments with glass bead controls will help in the detection of significant effects of toxic seed coat diffusates.

Divide each treatment of inoculated seeds and glass beads into two batches of equal size. Store one batch at 4°C (batch A) in the refrigerator and the other batch (batch B) at room temperature (25-30°C). Petri dishes are recommended as storage containers.

(d) Determining the number of viable rhizobia on seeds (Key steps 4 and 5)

The number of rhizobia on the seeds and glass beads of each treatment will be determined at 0, 1, 2, 3, 6, and 9 days after inoculation.

On each plating day, remove 20 seeds from batch A of each treatment (stored in the refrigerator) and 20 seeds from batch B of each treatment (stored at room temperature). Make four subsamples of five seeds from each.

If all treatments shown in Table 23.1 are chosen, (nine treatments-including the control-at two temperatures divided into four subsamples) the total number of samples to be plated on one day will be 9 x 2 x 4 = 72.

Transfer each subsample into a screw-capped test tube containing 5 ml of sterile diluent. Shake the test tubes vigorously for 5 min to wash the inoculum off the seeds. One ml of the resultant suspension will contain the rhizobia derived from one seed. Make a serial dilution from 10^{-1} to 10^{-5} from each subsample as described in Exercise 4.

Plate 0.1 ml of each dilution by the spread plate method on YMA plates containing Brilliant Green (1.25 ì/ml) and on YMA plates containing Congo Red (25 ì/ml). The Brilliant Green will suppress fungal growth while Congo Red will aid in detecting contaminants.

Count the rhizobial colonies and express the results as number of viable rhizobia per seed basis. Also, convert viable rhizobia per seed to per cent of 0 day viability. Enter both these data side by side. Organize the results of all counts as in Table 23.2.

Calculate the standard deviation for replicated samples using the following formula:

$$s^2 = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{r}}{r - 1}$$

Where s^2 = standard deviation
x = numbers counted
r = number of replicates

Example: The counts of four replicates of a given dilution are:

$$x = 1.5 \times 10^5;\ 2.4 \times 10^5;\ 1.8 \times 10^5;\ 2.0 \times 10^5$$
$$\log_{10} x = 5.18;\ 5.38;\ 5.25;\ 5.30$$
$$r = 4;\ (r - 1) = 3$$
$$\Sigma(x^2) = (5.18)^2 + (5.38)^2 + (5.25)^2 + (5.30)^2 = 111.429$$
$$(\Sigma x)^2 = 445.632$$
$$\frac{(\Sigma x)^2}{r} = \frac{445.632}{4} = 111.408$$
$$s^2 = \frac{111.429 - 111.408}{3} = \frac{0.021}{3} = 0.007$$

$$\underline{s^2 = 0.007}$$

Calculate the standard deviation for each treatment and tabulate the results.

Differences are significant if they exceed the standard deviation by a factor of two.

Plot two graphs using the mean counts of each treatment

(a) Mean log of viable rhizobia per seed (Y-axis) against time (X-axis). This graph will show which treatment permits the largest number of cells to be applied to the seed and also which treatment allowed the longest survival of the applied inoculum.

(b) % viable rhizobia per seed (Y-axis) against time (X-axis).

This graph will indicate the percent decline of the applied inoculum in relation to the initial number of viable cells.

Table 23.2. Percentage viability of rhizobia per seed as affected by different methods of inoculation.

Treatment Number	Viable rhizobia per seed after (days)					
	0	1	2	3	6	9
1						

2
3
4
5
6
7
8
Glass beads
(control)

Requirements

(a) Preparing inoculants

Transfer chamber
Platform shaker; incubator; refrigerator

Inoculation loop; bunsen burner
Requirements for Gram stain (Appendix 3)
Syringe 50 ml, sterile; needle, 18G sterile
Erlenmeyer flasks (20) 250 ml capacity
YMB (2 liters)
Plates of YMA and BTB; plates of YMA and Congo Red
Solution of BTB (0.5% in alcohol)
Spreader; beaker of alcohol (95%), spray bottle of alcohol (70%)
Sterile peat, sealed bag of 50 g

Pipettes, 1 ml, sterile; test tube, sterile

(b) Preparing adhesives

Refrigerator, balance,
Hot plate (unit which includes a magnetic stirrer if possible); stirring bar
Beakers, 100 ml capacity
Weighing paper; spatula
Calcium carbonate (precipitated powder)
Methyl ethyl cellulose (Cellofas A); gum arabic; sugar
Distilled water

(c) Coating and pelleting seeds

Refrigerator, balance
Spatula, bunsen burner, weighing paper
Glass stirring rods; glass beads (400)
Pipettes 5 ml, pipettes 10 ml (wide mouth)
Beakers 100 ml
Plastic bags (2 l capacity)
Distilled water; alcohol in spray bottle
Gum arabic solution from (b), Cellofas A solution from (b), sugar solution
Calcium carbonate powder
Paper towels (to place coated seeds on for drying)
Petri dishes

Peat inoculant from (a), broth culture from (a)

Soybean seeds (800 g)

(d) Determining the number of viable rhizobia on seeds

Transfer chamber

Incubator, bunsen burner, refrigerator

Pipettes, sterile 1 ml

Tubes (20 ml) screw-capped with 5 ml sterile diluent

YMA plates containing Brilliant Green

YMA plates containing Congo Red

Coated seeds from (c)

REFERENCES AND RECOMMENDED READING

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APPENDIX 1

CHARACTERISTICS OF THE SUBFAMILIES OF LEGUMES (AFTER PURSEGLOVE 1978)

Caesalpinioideae

This subfamily has 152 genera and nearly 2,800 spp. of trees and shrubs, rarely herbs, mostly tropical and subtropical and most numerous in tropical America. Lvs. nearly always alternate, pinnate or bipinnate; stipules paired, mostly deciduous; stipels mostly absent. Fls. zygomorphic, often showy, usually hermaphrodite; sepals 5 or 4 by union of 2 upper sepals, mostly free, sometimes much reduced when 2 bracteoles which are large and calyx-like cover bud; petals 5 or fewer with upper petal innermost in bud; stamens 10 or fewer, free to variously connate, dehiscing lengthwise or by terminal pore; ovary superior, 1-locular, 1-many ovules, style simple. Fr. a legume or indehiscent and drupaceous. Seeds sometimes arillate, rarely with endosperm.

Mimosoideae

This subfamily has 56 genera and about 2,800 spp. of trees and shrubs, very rarely herbs, mainly confined to the tropics and subtropics and more numerous in the southern hemisphere. Lvs. usually bipinnate, rarely once pinnate, sometimes reduced to phyllodes; stipules present, sometimes spinelike. Fls. actinomorphic, small, usually sessile and massed in

cylindrical spikes or globose heads; sepals usually 5, mostly valvate and united to form a toothed or lobed calyx; petals same number as sepals, valvate, free or connate; stamens often numerous, free or monadelphous; anthers small, versatile, often with apical gland, dehiscing longitudinally; ovary 1-locular superior, style usually filiform, stigma small and terminal. Fr. dehiscent or indehiscent, sometimes a lomentum.

Papilionoideae

According to the International Rules of Botanical Nomenclature, it would appear that the correct name for this subfamily is either Faboideae or Lotoideae. It is sometimes designated Papilionatae.

This subfamily has about 480 genera and 12,000 spp. of trees, shrubs, herbs, and climbers, generally distributed throughout the world, with the more primitive woody genera mostly in the tropics and the more advanced herbaceous genera more common in the temperate regions. Due to the very distinctive structure of the flower, members of this subfamily are very homogeneous and easy to recognize.

Lvs. usually alternate and mostly compound, pinnate, trifoliate or digitate; stipulate; stipules often present at base of individual leaflets. Fls. zygomorphic and typically papilionaceous; mostly hermaphrodite; calyx tubular and usually 5-toothed; petals 5, imbricate with descending

aestivation; upper (adaxial) petal exterior, usually largest, forming standard (vexillum); 2 lateral petals more or less parallel with each other forming wings (alae); and lowest 2 petals interior, usually joined by lower margins, to form keel (carina), which enclosed stamens and ovary. Stamens usually 10, monadelphous (all united by filaments) or diadelphous with 9 united by filaments and with upper or vexillary stamen free; rarely all stamens free; mostly all perfect; anthers 2-locullar, usually dehiscing lengthwise by slits. Ovary superior, of 1 carpel, usually 1-locular, sometimes with false septa; ovules 1-many on ventral suture. Fr. usually a legume or pod, splitting along dorsal or ventral sutures or both; sometimes indehiscent; occasionally jointed and breaking into 1-seeded segments. Seeds usually without endosperm.

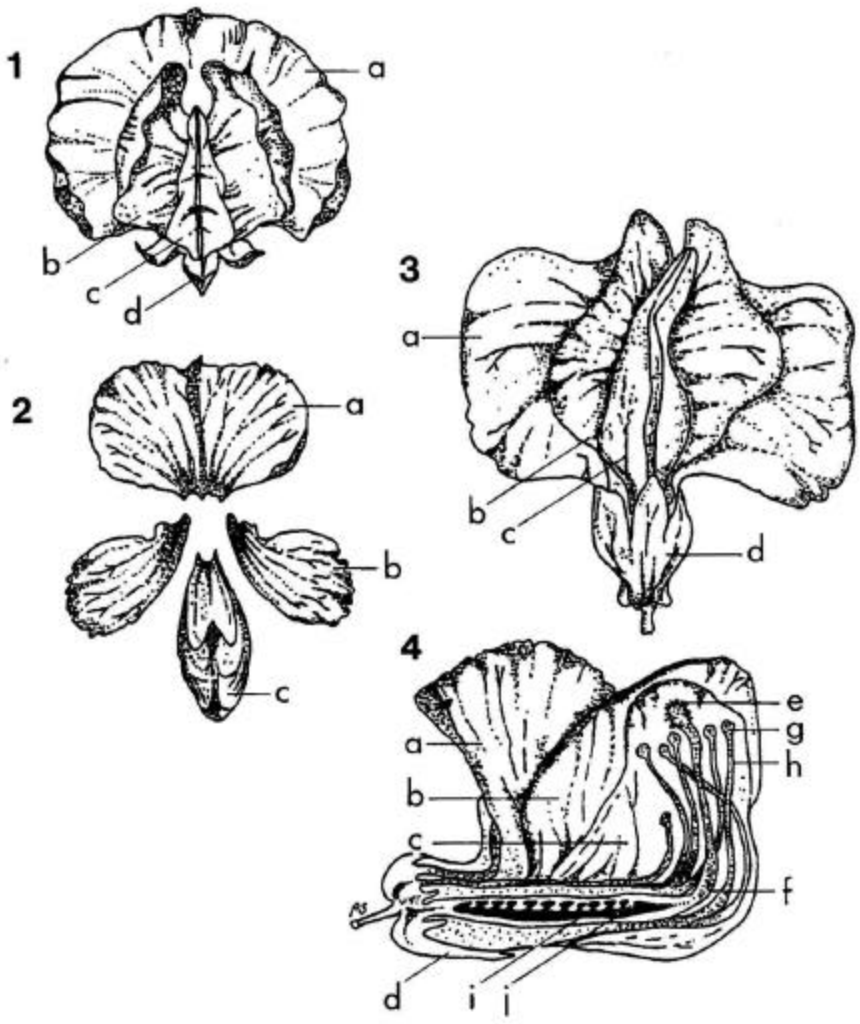


Figure A.1. Subfamily Papilionoideae. 1. Front view of flower of *Pisum sativum* (pea); 2. petals of *P. sativum*; 3. flower of *Psophocarpus tetragonolobus* (winged bean) from below; 4. flower of *Psophocarpus tetragonolobus* in longitudinal section. a-posterior or standard petal; b-lateral petal; c-keel petals (carina); d-sepals; e-stigma; f-style; g-anther; h-filament; i-ovary wall; j-ovule.

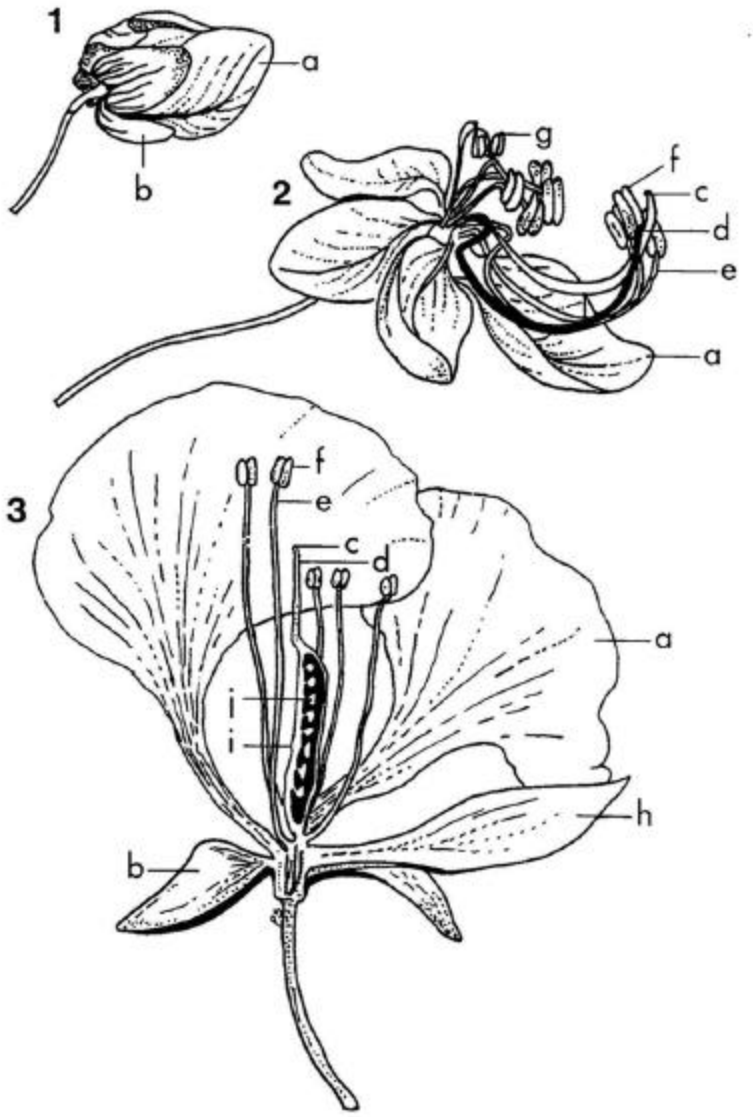


Figure A.2. Subfamily Caesalpinoideae. 1. bud of *Cassia* sp; 2. flower of *Cassia* sp; and 3. longitudinal section through flower of *Delonix regia* (Flame of the Forest of Poinciana). a-petal; b-sepal; c-stigma; d-style; e-filament; f-anther; g-anther of staminoid; h-posterior or standard petal; i-ovary wall; j-ovule.

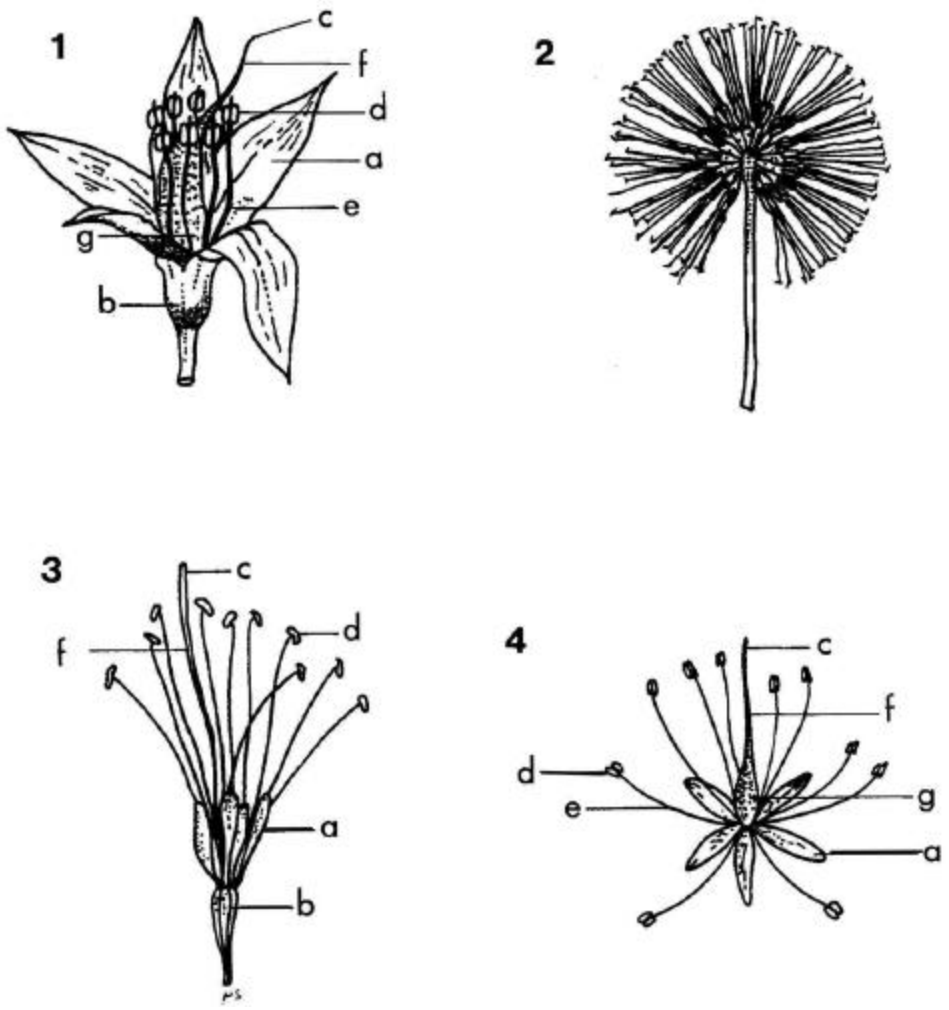


Figure A.3. Subfamily Mimosoideae. 1. Floret of *Adenanthera pavonina*; 2. inflorescence (globose head) of *Leucaena leucocephala* in longitudinal section showing arrangement of florets on torus; 3. floret of *L. leucocephala* (side view); 4. floret of *L. leucocephala* (top view). a-petal; b-sepal; c-stigma; d-anther; e-filament; f-style; g-ovary.

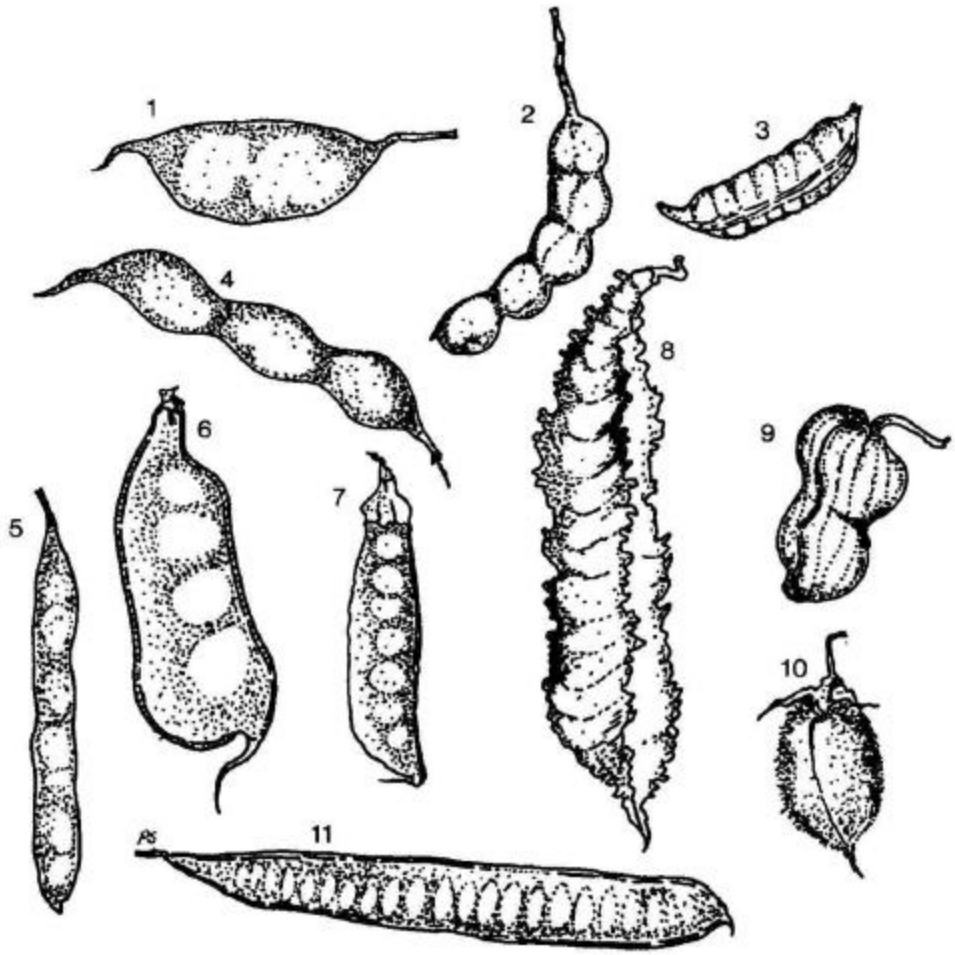


Figure A.4. Legume pods. 1. *Strongylodon lucidus*; 2. *Tamarindus indica*; 3. *Acacia farnesiana*; 4 *Parkinsonia aculeata*; 5. *Prosopis pallida*; 6. *Lablab purpureus;* 7. *Pisum sativum*; 8. *Psophocarpus tetragonolobus;* 9. *Arachis hypogaea*; 10. *cicer arietinum*; 11. *Leucaena leucocephala*.

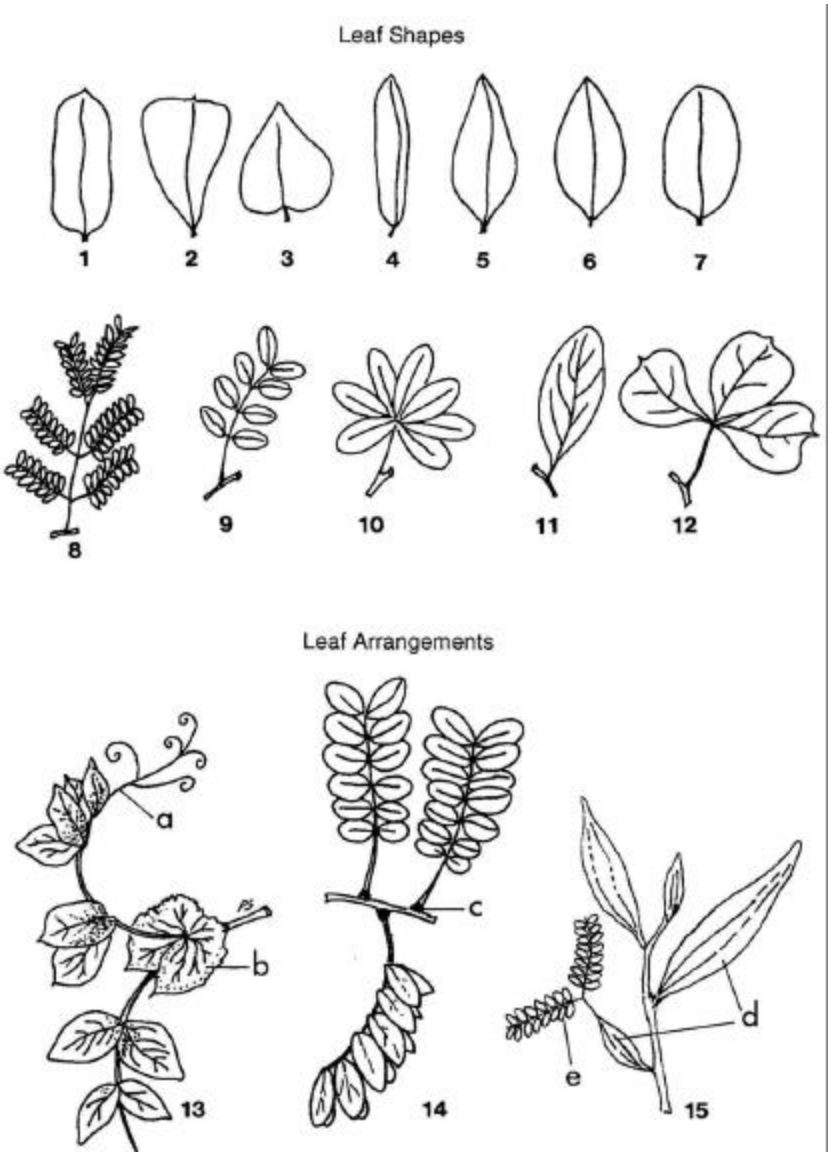


Figure A.5. Leaves of legumes and associated structures. Leaf shapes: 1. oblong; 2. cuneate; 3 cordate; 4. linear; 5. lanceolate; 6. ovate; 7. oval. Leaf arrangements: 8. bi-pinnate; 9. pinnate; 10. palmate; 11. simple; 12. trifoliate; 13. branch of ***Pisum*** showing 5-branched tendril (a) and stipule (b); 14. bi-pinnate leaf showing position of

pulvinus (c); 15. *Acacia* seedling showing simple phytolodes (d), and true compound leaves (e).

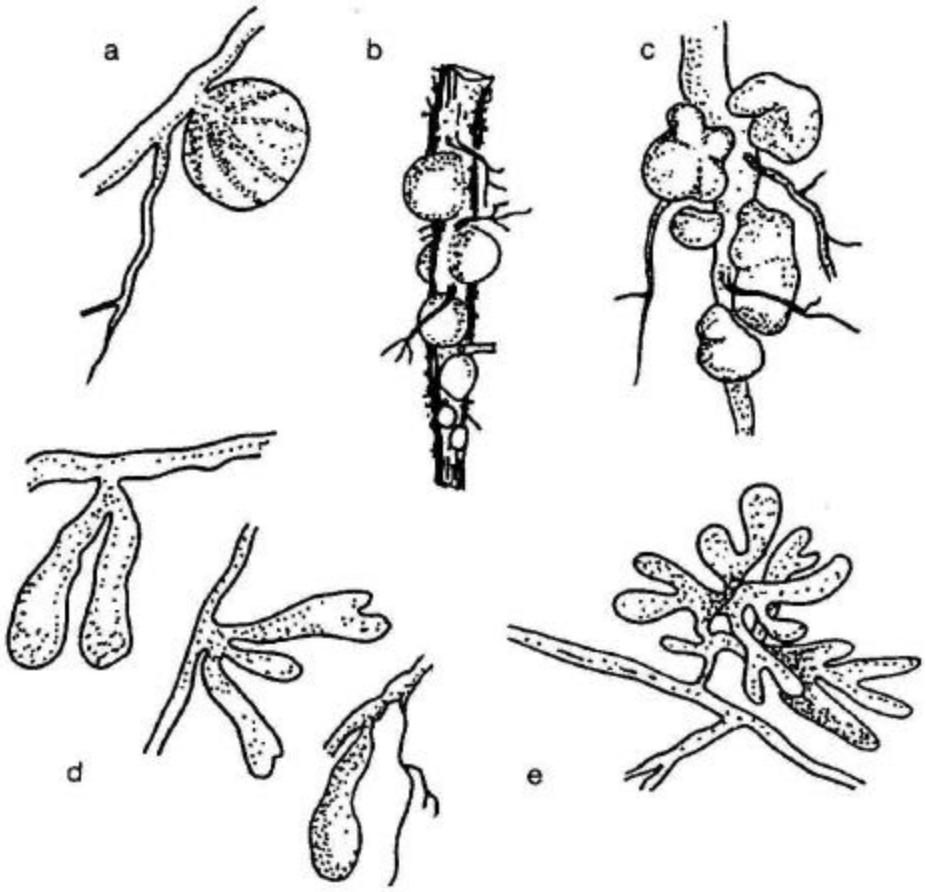


Figure A.6. Some representative shapes of leguminous nodules. Spherical: a. globose and streaked, e.g., *Glycine max, Calopogonium*, and *Vigna radiata*; b. peanut (*Arachis hopogaea*); c. semi-globose with smooth surface, e.g., *Vigna unguiculata* and *Psophocarpus*. Finger-like forms: d. elongate and lobed, e.g., *Leucaena* and *Mimosa*. Fanshaped: e. coralloid, e.g., *Crotalaria* and *Calliandra*.

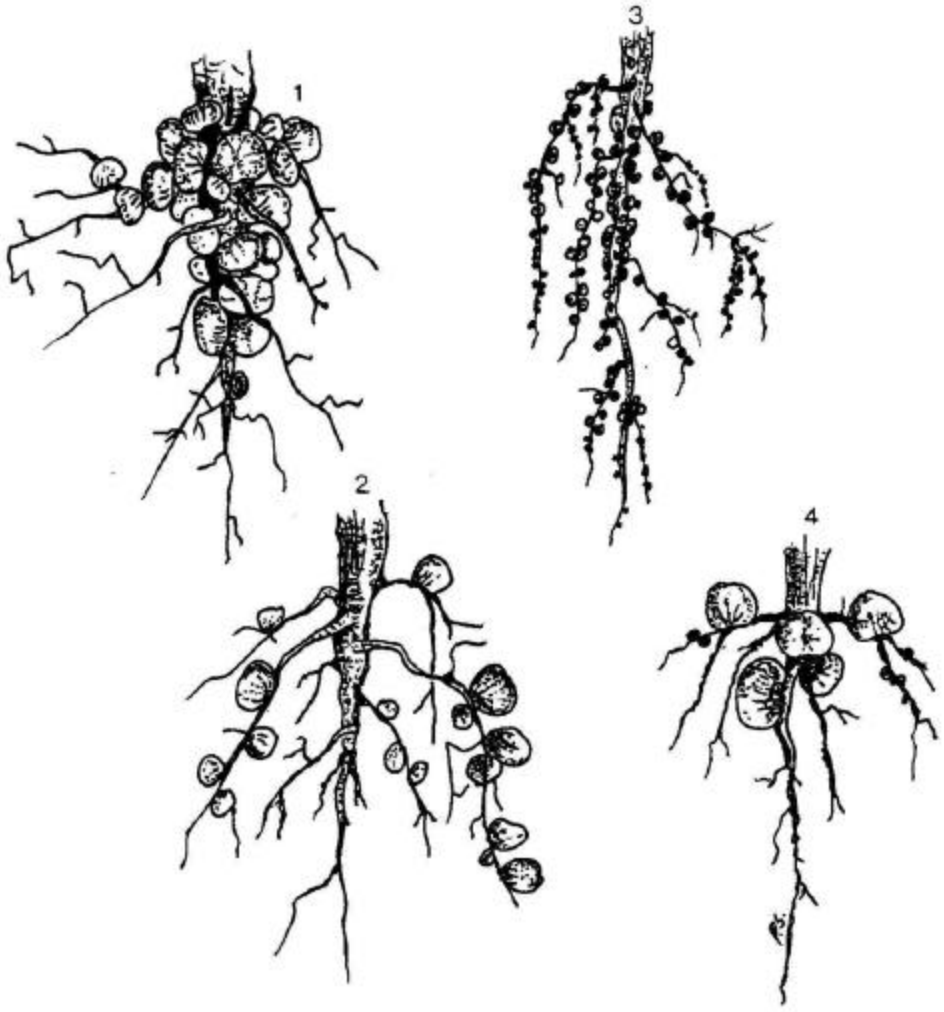


Figure A.7. Some examples of nodule distribution on roots. 1. prolific tap-root nodulation; 2. occasional nodules on laternal roots and distant from the tap-root; 3. large number of small nodules; 4. small number of large nodules.

APPENDIX 2

THE NODULE PRESERVATION VIAL

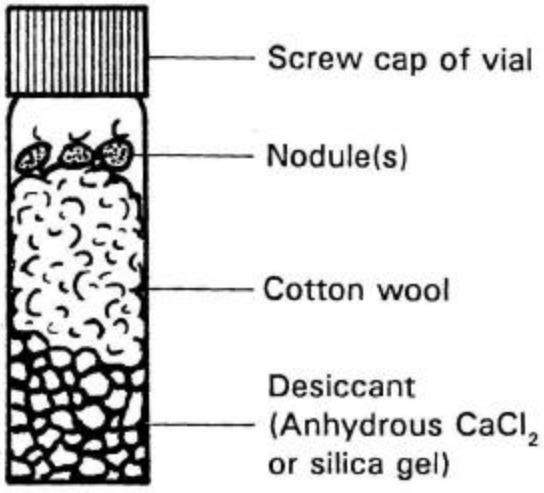


Figure A.8 Nodule preservation vial

The apparatus diagrammed above is a convenient method for nodule collection/preservation during field trips. Nodules collected this way can last 6-12 months, though recovery of the rhizobia during isolation may vary depending on the legume species.

Selection of plant(s) to sample nodules

Nodules should be collected from healthy, green plants. Such plants (if nodulated) usually have large nodules with pink/red interiors which may indicate effective fixation.

Excavate plants carefully and remove adhering soil particles. Excise each nodule from the roots, leaving a small piece of root attached. Place the nodules (at least five) in the vial and cap tightly.

For tree legumes, seedlings are the best source of nodules.

APPENDIX 3

MEDIA AND STAINING SOLUTIONS

Yeast Mannitol Broth (YMB)

Constituents:

Mannitol	10.0 g*
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast Extract	0.5 g
Distilled Water	1.0 liter

*This amount has been used traditionally, however more recent findings (H. Keyser, unpublished) show that 1 g l^{-1} is sufficient for most rhizobia.

Preparations:

- Add mannitol and salts to 1 l distilled water
- Dissolve under continuous stirring
- Adjust pH to 6.8 with 0.1 N NaOH
- Autoclave at 121°C for 15 min.

Yeast Mannitol Agar (YMA)

Constituents:

Yeast Mannitol Broth	1 liter
Agar	15 g

Preparation:

- Prepare YMB
- Add agar, shake to suspend evenly, autoclave.
- After autoclaving, shake flask to ensure even mixing of melted agar with medium.

Glucose Peptone Agar

Ingredients per liter:

Glucose	5 g
Peptone	10 g
Agar	15 g

Preparation:

- Dissolve glucose and peptone in 1 liter distilled water
- Add 10 ml BCP stock solution* to achieve a BCP concentration of 100ìg ml l^{-1} (Prepare BCP stock solution by dissolving 1 g BCP in 100 ml ethanol)
- Add agar and suspend evenly
- Autoclave at 121°C for 15 minutes

Fermentor Broth (Burton, 1967)

Constituents per liter:

Mannitol	2.0 g

Sucrose	10.0 g
Tripotassium phosphate (K_3PO_4)	0.2 g
Monopotassium phosphate (KH_2PO_4)	0.4 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2 g
Sodium chloride (NaCl)	0.06 g
Calcium carbonate ($CaCO_3$)	0.2 g
Calcium sulphate ($CaSO_4.H_2O$)	0.04 g
Yeast Extract	0.5 g
Ammonium phosphate [$(NH_4)_2HPO_4$]	0.1 g
Water	1000 ml

Micronutrient – Stock Solution (Burton)

Constituents:

Boric Acid (H_3BO_3)	2.78 g
Manganese sulphate ($MnSO_4.7H_2O$)	1.54 g
Zinc sulphate ($ZnSO_4.7H_2O$)	0.21 g
Sodium molybdate (Na_2MoO_4)	4.36 g
Ferric chloride ($FeCl_3.6H_2O$)	5.00 g
Cobalt sulphate ($CoSO_4.6H_2O$)	0.004 g
Lactic acid (88%)	580 ml
Distilled water	420 ml

*Addition of 1.0 ml per liter of medium gives: boron 0.5 ìg; manganese 0.5 ìg; zinc 0.05 ìg; molybdenum 1.0 ìg; iron 100 ìg and cobalt 0.0005 ìg per liter (or parts per million).

- Dissolve mannitol, sucrose, yeast extract and salts in 1 liter distilled water
- Add 1 ml of micronutrient stock solution
- Autoclave at 121°C for 15 min.

Bergersen's defined medium for preparation of Rhizobium for antiserum production

Constituents:

K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$CaCl_2 \cdot 6H_2O$	0.1 g
$FeCl_3 \cdot 6H_2O$	0.01 g
Sodium glutamate	1.10 g
Mannitol	10.00 g
Agar	15.00 g
Water	1 liter

Dispense known volumes into bottles, autoclave and add 1 ml of Biotin-thiamine solution per liter.

a. Dissolve 0.1 g thiamine and 0.025 g biotin in 1 liter distilled water.

b. Dispense 2 ml quantities via sterile Seitz or Millipore filter into small bottles (dispense 50 and discard remainder

of solution).

c. Store in freezer and dispense aseptically into autoclaved medium at 1 ml/liter.

DYES INCORPORATED IN MEDIA

Bromthymol Blue (BTB)

Stock solution: 0.5 g/100 ml ethanol
Add 5 ml stock/liter YMA
Final concentration of BTB: 25 ppm.

Congo Red (CR)

Stock solution: 0.25 g/100 ml
Add 10 ml stock/liter YMA
Final concentration of CR: 25 ppm.

Bromcresol Purple (BCP)

Stock solution: 1 g/100 ml ethanol
Add 10 ml stock per liter peptone glucose agar.
Final concentration: 100 ppm.

Brilliant Green (BG)

Stock solution: 125 mg/100 ml ethanol

Add 1 ml stock to 1 liter of YMA before autoclaving

Final concentration of BG: 1.25 ppm.

YMA with antibiotics

Streptomycin (str)

Stock solution: 400 mg str/100 ml water (4 mg str/ml)

Add 5 ml str stock/500 ml YMA to make plates containing 40 g str/ml.

10 ml str stock/500 ml YMA for plates containing 80 µg str/ml.

Spectinomycin (spc)

Stock solution: 1.25 g spc/50 ml water (250 mg spc/ml)

Add 5 ml spc stock to 500 ml YMA for plates with 250 µg spc/ml.

Add 10 ml spc stock to 500 ml YMA for plates with 500 mg spc/ml.

Autoclave YMA together with magnetic stirring bar in an Erlenmeyer flask. Add filter sterilized antibiotics after the agar has cooled below 80^{o}C. Mix well and pour after bubbles

resulting from mixing have dispersed.

Fahraeus C- and N-free Madium*

$CaCl_2$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.12 g
KH_2PO_4	0.1 g
$Na_2HPO_4 \cdot 2H_2O$	0.15 g
Ferric citrate	0.005 g
*Mn, Cu, Zn, B, Mo	traces
Distilled water	1000 ml

PH after autoclaving is 6.5

Sterilize at 121^{o}C for 20 minutes.

Seedling Agar (Jensen, 1942)*

$CaHPO_4$	1.0 g
K_2HPO_4	0.2 g
$MgSO_2 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.1 g
Water	1.0 liter
Agar	15.0 g
Microelements[a]	1.0 ml (Gibson 1963)*

[a] From stock containing: 0.5% B; 0.05% Mn; 0.005% Zn; 0.005% Mo; and 0.002% Cu.

*Taken from Vincent 1970

Seedling Agar Slants

Autoclave seedling agar at 121°C for 15 minutes and dispense equal volumes into tubes (tube size depends on plant species). An appropriate amount of molten agar is dispensed so that after solidifying in inclined tubes, a 5-10 cm long agar face is presented for seedling growth.

SOLUTIONS FOR GRAM STAIN (Vincent, 1970)

Solution I: Crystal violet solution

Crystal violet	10 g
Ammonium oxalate	4 g
Ethanol	100 ml
Water (distilled)	400 ml

Solution II: Iodine solution

Iodine	1 g
Potassium iodide	2 g
Ethanol	25 ml
Water (distilled)	100 ml

Solution III: 95% Ethanol

Solution IV: Counterstain

2.5% Safranin in ethanol	10 ml
Water (distilled)	100 ml

Carbol Fuchsin Stain

Basic fuchsin	1 g
Ethanol	10 ml
5% phenol solution	100 ml

The fuchsin stain should be diluted 5-10 times with distilled water before use.

Preparation of Yeast Water

Fresh starch-free cakes of yeast are preferred in making yeast-water. Suspend 100 g of yeast in 1,000 ml of water and boil slowly or steam for 3 to 4 hours, replacing the water lost regularly. Allow the cooled suspension to stand until yeast cells have settled (usually 10 to 12 hours) to the bottom. Siphon off the clear, straw-colored liquid; adjust the liquid to pH 6.6 to 6.8 with sodium hydroxide; bottle and autoclave for 30 to 40 minutes at 121°C. Following sterilization, the yeast water may be stored at room temperature.

Dried yeast may also be used in making yeast-water. One kg of dry yeast is equivalent to about 2.5 kg of wet yeast. Suspend 40 g of dry yeast in one liter of water. Boil, decant, bottle, and sterilize in the same way as described for fresh yeast. One hundred ml of yeast-water should contain about 75 mg of nitrogen.

Yeast extract powders prepared by spray-drying aqueous autolyzed yeast preparations are available in many countries. When these are available, about 0.5 g per liter of the dried preparation is used to replace yeast-water. Dry preparations are convenient and usually satisfactory.

The media containing yeast may foam excessively when aerated vigorously in fermentor vessels. Foaming can be controlled by adding a small amount of sterile white mineral oil or silicone emulsion.

Preparation of Soybean Water

Grind 100 g soybean seeds to a course flour and place in 1000 ml of water. Boil slowly for 2 hours replacing the lost water regularly. Allow to cool and centrifuge at 5000 rpm. Remove the supernatant, autoclave, and store. For rhizobia media, use 100 ml per liter. Nitrogen sources can also be prepared from other grain legume seeds in the same way.

Table A.1. N-free Nutrient Solution (Broughton and Dillworth, 1970).

Stock Solutions	Element	M	Form	MW	g/l	M
1	Ca	1000	$CaCl_2 \cdot 2H_2O$	147.03	294.1	2.0
2	P	500	KH_2PO_4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgSo_4 \cdot 7H_2O$	246.5	123.3	0.5
	K	250	K_2SO_4	174.06	87.0	0.5
	Mn	1	$MnSO_4 \cdot H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	.5	$ZnSO_4 \cdot 7H_2O$	287.56	0.288	0.001
	Cu	.2	$CuSO_4 \cdot 5H_2O$	249.69	0.100	0.0004
	Co	.1	$CoSO_4 \cdot 7H_2O$	281.12	0.056	0.0002
	Mo	.1	$Na_2MoO_2 \cdot 2H_2O$	241.98	0.048	0.0002

For each 10 liters of full strength culture solution, take 5.0 ml each of solutions 1 to 4, then add to 5.0 liters of water, then dilute to 10 liters. Use 1 N NaOH to adjust the pH to 6.6-6.8. For plus N control treatments, KNO_3 (0.05%) is added giving an N

concentration of 70 ppm.

APPENDIX 4

REAGENTS

Biuret Reagent

In 500 ml distilled or deionized water, dissolve:

Copper sulfate ($CuSO_4.5H_2O$)	1.5 g
Sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$)	6.0 g

To this mixture, add 300 ml of CO_2-free 10% NaOH slowly under continuous stirring.

Add CO_2-free H_2O to make this reagent up to 1 l and store in a tightly screw capped polyethylene or glass bottle in the cold.

Nessler's Reagent

In 15 ml of distilled water, dissolve:

Mercuric chloride	1.0 g
Potassium bromide	5.0 g
Sodium hydroxide	2.5 g

Dilute to 100 ml, refrigerate. Allow to sit in the

refrigerator for 5 days. Use the upper clear solution only, or filter.

Preparation of Gelatin-Rhodamine Isothiocyanate (RhITC) Conjugate

1. Prepare a 2% gelatin solution.
2. Add 1N NaOH dropwise until pH reaches 10-11.
3. Autoclave for 10 min at 15 psi and 121°C.
4. After cooling add rhodamine isothiocyanate (RhITC) dissolved in a minimum volume of acetone to provide 8 ìg of dye per one mg of gelatin. Remove residues by filtration through a 45ìm membrane filter.
5. Allow conjugation to proceed overnight with gentle stirring.
6. The conjugate is separated from unreacted RhITC by gel filtration on Sephadex G-25, using PBS pH 7.1 (alternatively the preparation could be dialyzed against PBS pH 7.1 until no further color is detected in the dialysate).
7. Add merthiolate to the conjugate (1:10,000) and distribute the conjugate in small volumes into screw-cap tubes and store at -20°C. Alternatively, the bulk of the conjugate could be freeze-dried and stored in a desiccator. When needed, the desired amount of the dry sample should be reconstituted in distilled water.

Mounting Medium (taken from Kawamura, 1969)

Buffered glycerol or elvanol is commonly employed. Fluorescence fades in a short time (about 30% overnight and then more gradually) in glycerol but remains for a longer time in elvanol. The fluorochrome of the rhodamine series dissolves in elvanol, however, and therefore it cannot be used except with FITC-labeled antiserum. The pH of the buffered glycerol is normally 7.0 to 7.5. However, we have used it at a pH of 8.5 with good results.

Buffered glycerol solution

0.5 M carbonate buffer (pH 9.5)	l vol.
Glycerine (reagent grade, free of autofluorescence)	9 vol.

The two reagents are mixed thoroughly (with a magnetic stirrer). The final pH should be 8.5.

Elvanol (Elvanol-buffered glycerine mixture)

Elvanol (polyvinyl alcohol, 51-05 grade)	1 vol.
0.5 M carbonate buffer (pH 9.0)	4 vol.

The two reagents are mixed with a magnetic stirrer for 16 h. One volume of reagent grade glycerine is mixed with two

volumes of the above mixture. The final mixture is stirred again with a magnetic stirrer for 16 h, centrifuged for 60 min at 12,000 rpm and the pH of the supernatant corrected to 8.5.

The final product should be kept in an air-tight container. It is best stored in tubes and kept in the dark. It will harden under the cover glass and fix it firmly.

APPENDIX 5

BUFFERS (FROM CONRATH 1972)

0.1 M Phosphate Buffer

1. Prepare stock solutions.

   (a) 0.2 M solution of monobasic sodium phosphate ($NaH_2PO_4.H_2O$) Dissolve 27.8 g in 1000 ml of distilled water.

   (b) 0.2 M solution of diabasic sodium phosphate Dissolve 52.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml of distilled water.

2. Mix x ml of (a) with y ml of (b), according to the following table, and dilute to a total of 200 ml with distilled water.

Phosphate buffered saline (0.01 M pH 7.1) (Used in FA purification)

Mix 330.0 ml of a with 670.0 ml of b, dilute with saline (8.5 g of NaCl per liter of distilled water) to 20 liters. Add Merthiolate at a concentration of 0.01% (200 ml of a 1% solution to 20 liters PBS).

Table A.2. Schedule for preparation of phosphate buffers.

a	b	pH	a	b	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	87.0	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

0.15 M Phosphate Buffer

1. Preparation of stock solutions

(a) 0.15 M NaH_2PO_4

Dissolve 2.0702 g in 100 ml of distilled water

(b) 0.15 M Na_2HPO_4

Dissolve 2.1294 g in 100 ml of distilled water

(c) 0.15 M KH_2PO_4

Dissolve 2.0413 g in 100 ml of distilled water

2. Phosphate buffer, pH 5.6

Mix 13 ml of (b) with 187 ml of (a). (Adjust pH if necessary)

3. Phosphate buffer, pH 6.4
   Mix 32.2 ml of (b) with 67.8 ml of (c). (Adjust pH if necessary)

4. Phosphate buffer, pH 7.2 - 7.3
   Mix 24 ml of (c) with 76 ml of (b). (Adjust pH if necessary)

<u>0.15 M Phosphate Buffer, pH 8.0</u>

1. Dissolve 10.6 g of anhydrous Na_2HPO_4 in about 450 ml of distilled water

2. Adjust the pH to 8.0 by the dropwise addition of 1 N hydrochloric acid; then dilute to 500 ml with distilled water

3. Check pH occasionally

<u>0.15 M Phosphate Buffer, pH 9.0 (Used in the conjugation of FA)</u>

Same as above without the addition of hydrochloric acid (Used for FA conjugation)

APPENDIX 6

(a) McFARLAND NEPHELOMETER BARIUM SULFATE STANDARDS (FROM LENETTE *ET AL.,* 1974)

1. Prepare 1% aqueous barium chloride and 1% aqueous sulfuric acid solutions.

2. Add the amounts indicated in Table A6.1 to clean dry ampoules. Ampoules should have the same diameter as the test tube to be used in the subsequent density determinations.

3. Seal the ampoules and label them.

Table A.3. Preparation of McFarland nephelometer barium sulfate standards.

Tube	Barium chloride 1% (ml)	Sulfuric acid 1% (ml)	Corresponding approx. density of bacteria (million/ml)
1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1,200
5	0.5	9.5	1,500
6	0.6	9.4	1,800
7	0.7	9.3	2,100
8	0.8	9.2	2,400
9	0.9	9.1	2,700
10	1.0	9.0	3,000

(b) TURBIDITY ADJUSTMENT OF THE BACTERIAL SUSPENSION

For bacterial agglutinations, the cell suspension is usually adjusted to approximately $1x10^9$ cells/ml. In the McFarland standards, tubes 3 and 4 will have approximately $9.0x10^8$ ($1x10^9$) and $1.2x10^9$ cells/ml respectively. The arbitrary selection of these two densities will yield satisfactory results for many systems.

With dust-free saline in a tube (blank) similar in diameter to the standards, set the nephelometer to a low nephelometric unitage. Read the corresponding unitage on tubes 3 or 4.

With approximately 8 ml of saline in another clean tube, add the turbid washed suspension of rhizobial cells dropwise with a Pasteur pipette until a turbidity is reached which is slightly lower than the corresponding standard chosen. Place the tube in the nephelometer and adjust the turbidity to the required unitage by further additions of the turbid rhizobial suspension.

If a nephelometer is not available, the turbidity is adjusted to fall between tubes 3 and 4 by visual comparison.

APPENDIX 7

PREPARATION OF SEEDLING-AGAR SLANTS FOR CULTIVATING SMALL SEEDED LEGUMES

Small seeded legumes can be cultured enclosed in tubes if these plants are to be used for the authentication of rhizobia or for enumerating rhizobia by the plant-infection technique. One of the limitations of strain evaluation in enclosed tubes is that a tube environment restricts growth conditions and proper differentiation of the plant. A nitrogen-free nutrient solution is solidified with agar for slant preparation or without agar for NifTAL-tubes.

(a) Tubes 250 mm x 25 mm (Figure A.9) are required. Tubes are stoppered with cotton plugs sufficiently loose to allow good air exchange and simultaneously filter off contaminants.

(b) A total of 1.62 l of the N-free nutrient solution is needed for 54 tubes at the rate of 30 ml per tube. For convenience, divide the nutrient solution into manageable volumes in beakers or Erlenmeyer flasks prior to the addition of the agar powder. (Example: It is convenient to have 500 ml of the N-free nutrient solution in a 1 l container as this will greatly facilitate stirring when the agar is being melted or dispensed). Add 1.5% (w/v)

agar to the N-free nutrient solution (24.3 g of agar powder will be needed for 1.62 l of N-free nutrient solution). Melt the agar either by steaming in an autoclave or by direct heating over a bunsen-flame. If direct heating is used, the mixture must be constantly stirred over gentle heat to prevent charring of the agar on the bottom on the container.

(c) Dispense the melted agar in 30 ml portions into the tubes and plug. To facilitate dispensing of the agar, a simple set-up is illustrated in Figure A.9 which is adequate for approximate volumes. Arrange tubes in suitable metal baskets and autoclave at 121°C for 30 min. To make slants, support the tubes at an angle as illustrated.

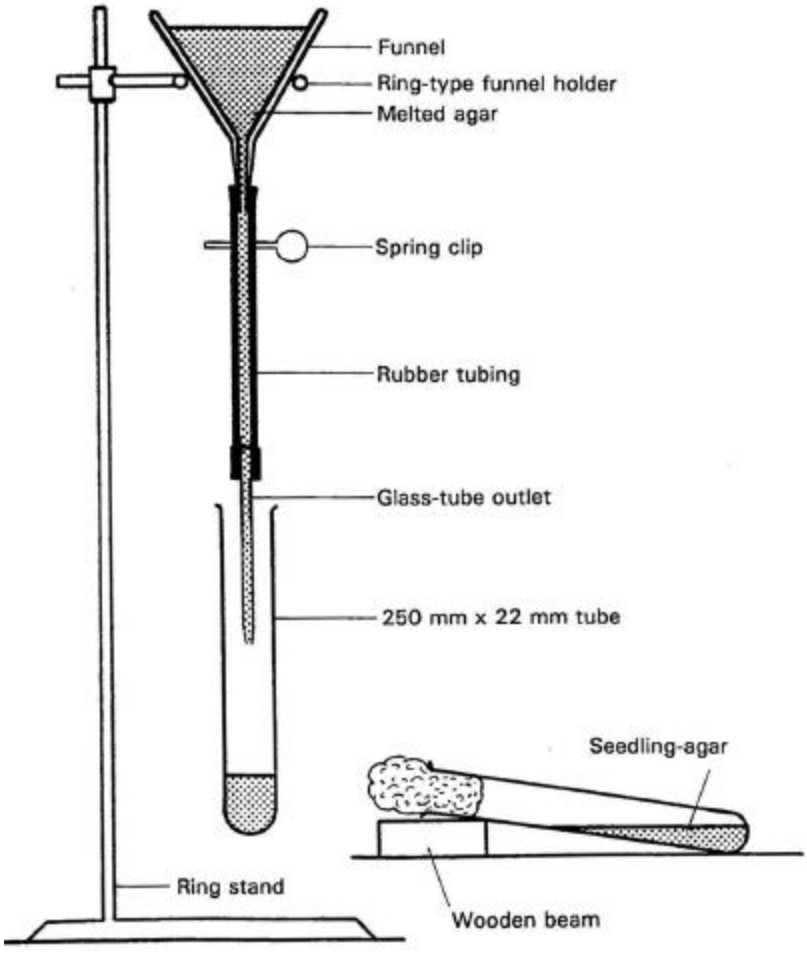


Figure A.9. Simple set up for dispensing seedling agar into tubes and forming slants.

APPENDIX 8

BUILDING A RACK FOR GROWTH POUCHES

In an effort to keep growth pouches standing upright, researchers have improvised different types of racks. Gramophone record holders have frequently been used for this purpose.

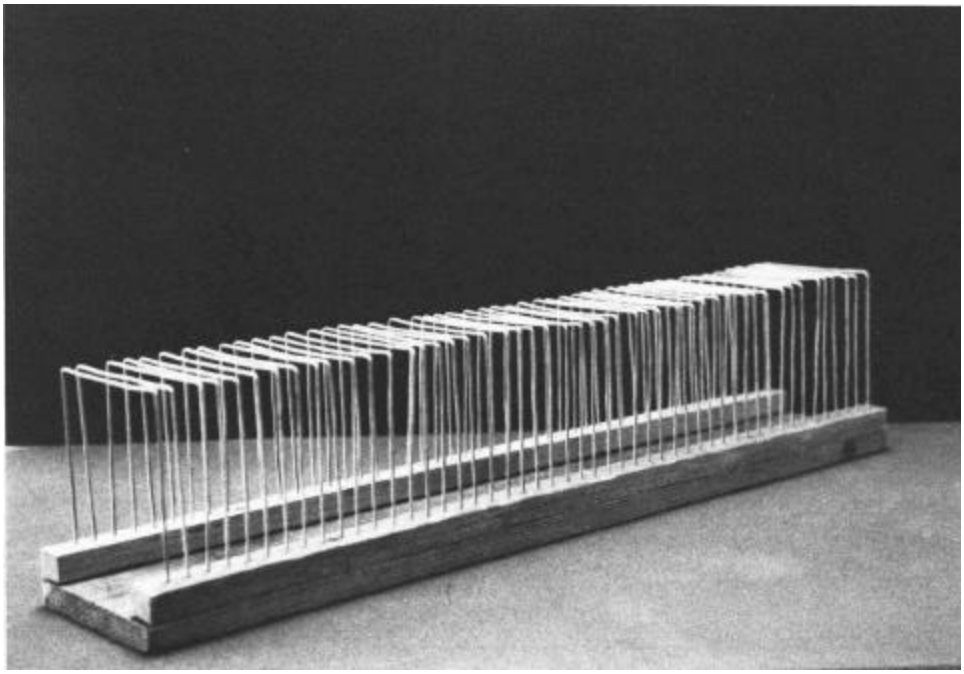


Figure A.10. Improvised rack for growth pouches

More suitable racks may be built from galvanized or stainless steel wire of at least 14 gauge and a wooden board as shown in Figure A.10. The spacing between the wire frames should be 1-1.5 cm.

Tools needed are: a drill with a bit of a slightly smaller diameter than the wire, wire cutter, small vise, and a hammer.

APPENDIX 9

RECOMMENDATIONS OF HOSTS AND GROWTH SYSTEMS FOR AUTHENTICATION

Choice of the legume for the authentication (Table A.4) depends very much on the specificity of the host. Most temperate and tropical legumes nodulated by fast-growing, acid-producing rhizobia are usually specific and would require the parent host. In most instances, the host dependent classification for rhizobia may serve as a useful guide for selecting the legume for use in authentication. If the legume from which the presumptive isolate is made is identified and its cross-inoculation group is known, but no seeds of the parent (homologous) host are available, the cross-inoculation group should be consulted to select an alternative (heterologous) host. However, this is sometimes difficult as with the pink *Bradyrhizobium* sp. from *Lotononis bainesii*, which requires only the parent host as there seems to be no substitute. Most known tropical legumes are nodulated by the slow-growing, alkali-producing rhizobia (*Bradyrhizobium*), in which case a "guinea-pig" legume like *Macroptilium atropurpureum* (siratro) can be confidently used for authentication. Over 90% of bradyrhizobia will nodulate siratro.

The choice of the growth system (Table A.4) will depend on the seed size of the host selected for authentication, and the

size of the plant. Some small seeded species e.g. *Vigna aconitifolia, Macrotyloma uniflorum*, etc., produce plants of an unsuitable size for tubes, but manageable in growth-pouches. If the size of the plant is known, most small-seeded species can be cultured in tubes or growth-pouches in growth (environmental) chambers. It is important to bear in mind that there are legumes that will not nodulate easily in tubes or pouches, resulting in a false negative authentication. Chickpea (*Cicer arietinum*) and *Leucaena retusa* are notable examples. For these species, authentication must be done in Leonard jars. The environment (growth chamber or greenhouse) where the authentication is done must be absolutely clean and adequately constructed to keep out insects and other sources of contamination.

Table A.4. Recommended Hosts and Growth Systems for Authentication of Presumptive Isolates.

Parent Host	Type of Rhizobia*	Host for Authentication	Growth System
Phaseolus vulgaris *P. coccineus*	f, ac	parent hosts	growth-pouches or Leonard jars
P. acutifolius	s, al	parent host	growth-pouches or Leonard jars
Medicago spp. *Melilotus* spp. *Trigonella* sp.	f, ac	parent hosts or *Medicago sativa*	tubes or growth-pouches
Trifolium spp.	f, ac	parent hosts	tubes or growth-pouches
Pisum spp. *Vicia* spp. *Lens culinaris*	f, ac	parent hosts or *Vicia faba*	tubes, growth-pouches or Leonard jars
Glycine max	f, ac & s, al	parent host	growth-pouch or Leonard jars
Lupinus spp. *Ornithopus* sp.	s, al	parent hosts	growth-pouches or Leonard jars
Sesbania spp.	f, ac	parent hosts	growth-pouches or Leonard jars
Leucaena leucocephala *L. diversifolia*	f, ac	parent hosts or *L. leucocephala*	tubes or growth-pouches
L. retusa	f, ac	parent host or *L. leucocephala*	Leonard jars
Lotononis bainesii	s, al	parent host	tubes or growth-

Parent Host	Type of Rhizobia*	Host for Authentication	Growth System
Phaseolus vulgaris *P. coccineus*	f, ac	parent hosts	growth-pouches or Leonard jars pouches
Cicer arietinum	f, ac or neutral	parent host	Leonard jars
Calliandra spp.	f, ac & s, al	parent host	tubes, growth-pouches or Leonard jars
Acacia senegal	f, ac	parent host	growth-pouch or Leonard jar
Acacia auriculaeformis *A. mearnsii* *A. albida* *Arachis hypogaea* *A. glabarata* *Alysicarpus vaginalis* *Cajanus cajan* *Calopogonium mucunoides* *Canavalia* spp. *Stylosanthes* spp. *Aeschenomene* spp. *Macrotyloma* spp. *Glycine wightii* (syn. *Neonotonia wightii*) *Voandzeia subterranea* *Desmodium* spp. *Centrosema* spp. *Crotalaria* spp. *Clitoria* spp. *Lablab purpureus* *Cyamopsis tetragonoloba* *Psophocarpus tetragonolobus* *Vigna* spp. *Phaseolus lunatus* *Zornia* spp. *Pacyrrhizus* spp. *Sphenostylis macrocarpa* *Macroptilium* spp.	s, al	parent hosts or Siratro (*Macroptilium atropurpureum*)	tubes, growth-pouches or Leonard jars

*In the above list, **f**, **ac** and **s**, **al** indicate fast-growing, acid-producing and slow-growing, alkali-producing respectively.

APPENDIX 10

SURFACE STERILIZATION OF SEEDS

Surface sterilization of legume seeds is dependent on the purpose and nature of the experiment. Authentication, strain selection and the enumeration of rhizobia by the plant-infection technique require legumes to be raised from surface sterilized seeds to ensure strict microbiological control.

Sterilants frequently used for surface sterilizing seeds are solutions of sodium hypochlorite (2.5% commercial bleach), acidified mercuric chloride (0.2%), hydrogen peroxide (3%), or concentrated sulfuric acid. Only hard-coated seeds are treated with concentrated sulfuric acid which scarifies (softens) the seed coat besides effecting surface sterilization.

Selected seeds must be of good viability (more than 70%), clean, and free of damage. Treated seeds (pesticides, fungicides or insecticides) must be rinsed quickly in water then dried on paper towels.

Method (a): Sterilization with mercuric chloride, sodium hypochlorite, or hydrogen peroxide solutions.

1. Place seeds in Erlenmeyer flask (wide-mouthed and previously sterilized by autoclaving). Cover the

mouth of the flask with half of a sterile Petri dish. The space taken up by the seeds should be about 25% of the volume of the flask as too many seeds will affect the efficiency of the sterilization. The Petri dish cover should be kept in place throughout the operation.

2. Rinse the seeds in 95% alcohol for 10 seconds to remove waxy material and trapped air. Drain off the alcohol.
3. Add mercuric chloride, sodium hypochlorite or hydrogen peroxide solutions in sufficient volumes to immerse the seeds completely. Swirl the contents gently to bring the seeds and sterilant into contact. After 3-5 min, drain off the sterilant.
4. Rinse with at least six changes of sterile water. Observe aseptic procedures throughout the rinsing. After the sixth rinse, pour in sufficient water to submerge the seeds and leave in the refrigerator for 4 h for the seeds to imbibe. (Some seeds e.g. the California black-eye variety of *Vigna unguiculata* should not be allowed to imbibe in water as the cotyledons fall apart.)
5. After 4 h, rinse the seeds with two or more changes of water and plate the seeds in 0.75% (w/v) water agar in Petri dishes. (Seeds can easily be scooped out of the flask with long spoons to transfer the seeds onto the agar.) Evenly spread the seeds on

the agar and avoid over-crowding. About 20-100 seeds are recommended per plate and this is dependent on the size. Large Petri dishes are needed to plate species with large seeds (e.g. *Canavalia* spp., *Vicia faba*). Large seeded species are more conveniently germinated in sterile (autoclaved) vermiculite. The vermiculite is moistened and sterilized one day in advance. Obtain a 5-10 cm layer of horticultural grade vermiculite in a shallow autoclavable polypropylene tray. Moisten the vermiculite by alternate additions of water and mix. Cover the tray with aluminum foil and sterilize by autoclaving for 15 min. Allow the vermiculite to cool overnight. Remove the foil in a laminar flow hood or other clean environment. Make furrows in the vermiculite with a sterile spatula. Sow the seeds in furrows and cover with vermiculite. Replace the aluminum foil cover.

6. Incubate at 25-30°C. Invert the plates for small seeded-species (clovers, medics, siratro, etc.) with seed diameters 3 mm and less. Inverting the plates allows the development of straight radicles from the seeds.

Method (b): Sterilization with concentrated sulfuric acid.

1. Place seeds in sterile Erlenmeyer flask and cover with half a sterile Petri dish as in method (a).

2. Add just enough acid to coat the seeds. Allow sterilization and scarification to proceed for 10 min. Drain off excess acid.
3. Add sterile water in sufficient volume to dissipate the heat generated by the exothermic reaction. Rinse and pour out the water. The first rinse should be done quickly to avoid killing the seeds by the heat. Continue rinsing the seeds with another five changes of water.
4. Leave the seeds (with some water) overnight in the refrigerator to imbibe. Rinse with two changes of sterile water.
5. Plate the sterilized seeds on water agar and incubate at 25-30°C or germinate in sterile vermiculite as described in method (a).

Methods of seed sterilization for the various leguminous species are shown in Table A.5.

Table A.5. Methods of seed surface sterilization for various legume species.

Legume Species (Common Name)	Sterilization		Recommended Germination Medium**
	Method* (a or b)	Sterilant	
Arachis hypogaea (peanut, groundnut)	a	peroxide/ bleach	v.

Legume Species (Common Name)	Sterilization Method* (a or b)	Sterilant	Recommended Germination Medium**
Glycine max (soybean)	a	peroxide/ bleach	v.
Cicer arietinum (chickpea)	a	peroxide/ bleach	v.
Lens culinaris (lentil)	a	peroxide/ bleach	w.a.
Lupinus spp. (lupines)	a	bleach/ peroxide	v./w.a.
Vigna unguiculata (cowpea)	a	bleach/ peroxide	v.
Canavalia sp. (jackbean)	a	bleach/ peroxide	v.
Phaseolus lunatus (lima bean)	a	peroxide/ bleach	v.
Phaseolus acutifolius (tepary bean)	a	peroxide	w.a./v.
Voandzeia subterranea (bambara groundnut)	a	bleach/ peroxide	v.
Phaseolus vulgaris (bean)	a	bleach/ peroxide	v.
Phaseolus coccineus (scarlet runner bean)	a	bleach/ peroxide	v.
Vigna mungo (green gram)	a	peroxide/ bleach	w.a.
Vigna radiata (urd bean)	a	peroxide/ bleach	w.a.
Vigna angularis (adzuki bean)	a	peroxide/ bleach	w.a.
Vigna umbellata (rice bean)	a	peroxide/ bleachq	w.a.
Vigna aconitifolia (mat or moth bean)	a	peroxide/ bleach	w.a.
Pisum spp. (pea)	a	peroxide/ bleach	v.
Centrosema pubescens (centro)	b	acid	w.a.

Legume Species (Common Name)	Sterilization Method* (a or b)	Sterilant	Recommended Germination Medium**
Clitoria ternatea (butterfly pea)	b	acid	w.a. (invert plates)
Cajanus cajan (pigeon pea)	b	acid	v./w.a.
Sesbania sp.	b	acid	w.a.
Medicago spp. (medics)	a	peroxide	w.a. (invert plates)
Trifolium spp. (clovers)	a	peroxide	w.a. (invert plates)
Glycine wightii	a	acid	w.a. (invert plates)
Pachyrrhizus spp. (yam bean)	b	acid	v.
Psophocarpus tetragonolobus (winged bean)	b	acid	v.
Lotononis bainesii (lotononis)	a	peroxide	w.a. (invert plates)
Desmodium spp.	b	acid	w.a. (invert plates)
Lotus spp.	a	peroxide	w.a. (invert plates)
Stylosanthes spp.	b	acid	w.a. (invert plates)
Leucaena spp.	b	acid	w.a.
Macroptilium atropurpureum (siratro)	a	acid	w.a. (invert plates)
Calopogonium mucunoides (calopo)	b	acid	w.a. (invert plates)
Pueraria phaseoloides (tropical kudzu)	b	acid	w.a. (invert plates)
Acacia spp.	b	acid	w.a. (invert plates)

* Method "a" refers to seed surface sterilization using sodium hypochlorite (bleach) or hydrogen peroxide (peroxide); Method "b" refers to seed surface sterilization and scarification using con. H_2SO_4. The sterilants are

indicated in order of preference though both can be used in surface sterilization.
** Recommended medium "v" refers to vermiculite; "w.a." refers to water agar.

APPENDIX 11

PREPARATION OF LEONARD JARS

The modified Leonard jar assembly (Figure A.11) consists of a 700 ml capacity beer bottle with the lower portion cut off. This bottle is inverted into a heavy glass jar (reservoir), 1 l minimum capacity. The mouth of the bottle should be 2-3 cm above the base of the reservoir. The growth medium (sand or vermiculite) in the bottle is irrigated by a centrally positioned cotton wick running the length of the bottle and extending out of the mouth and into the reservoir containing the nutrient solution. Various types of wick material have been used with Leonard jars, e.g. braided cotton lantern wicks, cotton rope, strands from cotton mop heads, coiled cotton wool, braided or twisted nylon rope. New wick materials should be tested for their ability to conduct water and their compatibility with plants. Generally, a 12 mm cotton rope is adequate and easy to obtain.

Place approximately 50 cm of wick material in the bottle with about 10 cm extending out of the mouth. A small amount of absorbent cotton stuffed into the neck of the bottle will aid in securing the position of the wick, and prevent the growth medium from settling in the reservoir. Wick material of cotton rope should be boiled in water and squeezed dry prior to use. This removes air trapped in the wick and improves

water conductivity.

While holding the wick in a central position, fill the bottle with growth medium (well-washed river sand or horticultural grade vermiculite). Pack the medium to minimize air spaces. Sand is easier to pack when dry. For vermiculite, it is more convenient to pack when wet. The vermiculite should be soaked overnight and the water drained off prior to packing into the bottles.

Position the bottle in the reservoir. The bottle should fit firmly on the rim of reservoir. Moisten the growth medium in the bottle by adding 150-200 ml of the N-free nutrient solution. Allow the nutrient solution to saturate the medium and the excess to drain into the reservoir. Fill the reservoir with 800 ml of the nutrient solution. Use 1600 ml if the reservoir has a 2 l capacity. Wrap the bottle and jar assembly with white or brown moisture-proof paper and secure with rubber bands at critical points along the jar. Tape may also be used. Aluminum foil wrapping may be used if it is inexpensive and available. Cap the open end of the bottle with either aluminum foil or wrapping paper. Hold the assembly by the reservoir when moving it.

Sterilize the complete assembly and nutrient solution by autoclaving for 1.5-2.0 hours at 121°C and 15 psi. For convenience, cool the assembly in the autoclave overnight.

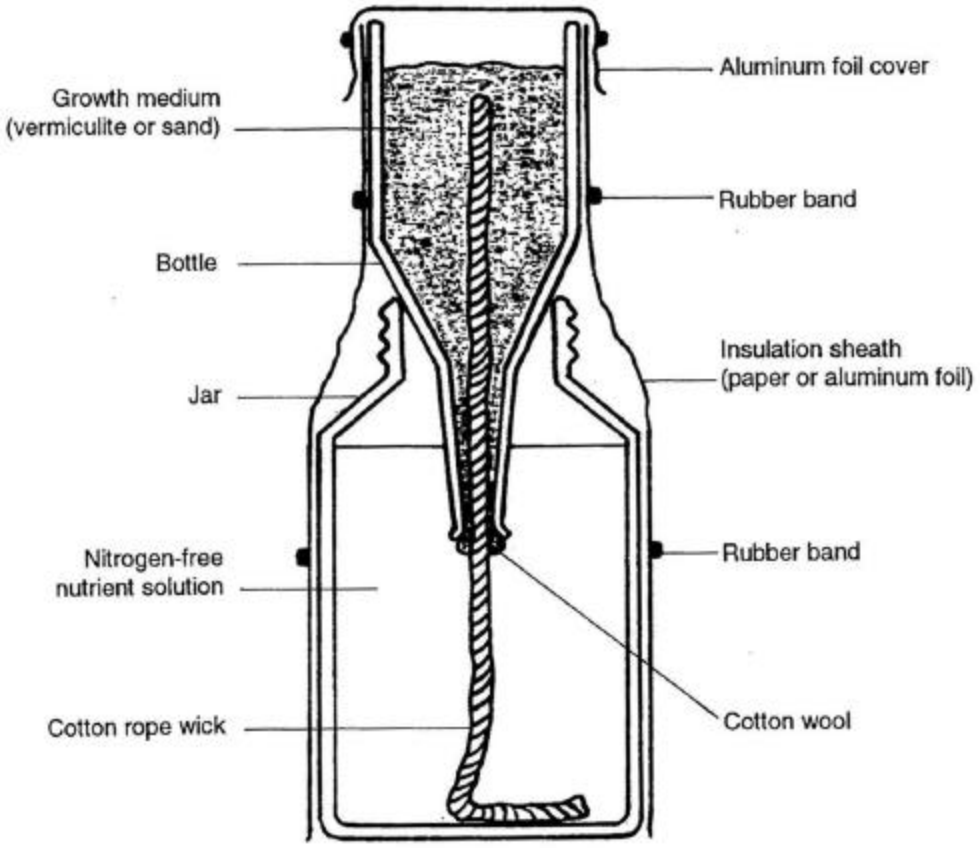


Figure A.11. The Leonard jar

APPENDIX 12

INJECTING AND BLEEDING RABBITS

Rabbits used for antiserum production should be healthy and 6-12 months old. Label each animal with an ear tattoo or tag. Maintain individual records for each rabbit of all treatments to which the rabbit is subjected.

During ear (intravenous) injections, intramuscular injections, and trial bleedings, the rabbit must be restrained (immobilized). The recommended method is to roll the rabbit tightly into a large towel. The fore and rear limbs must be well secured by the towel. For intraperitoneal injections, the animal may be strapped to a rack or held on its side by another person. During cardiac puncture, a bleeding rack is used to hold the rabbit on its back (Figure A.12). Another approach is to sedate the rabbit with an injected tranquilizer such as Rompun [(Xylazine), Haver-Lockhart Bayvet Division, Cutter Laboratories, Inc. Shawnee, Kansas, USA]. The use of ether or chloroform should be avoided.

The following schedules have been used successfully for antisera development in rabbits.

Schedule 1: (Schmidt, Bankole, and Bohlool, 1968)

DAY	PROCEDURE
1	Inject 0.5 ml intravenously (IV)
2	Inject 1.0 ml IV
3	Inject 1.5 ml IV
7	Inject 1.5 ml IV
8	Inject 2.0 ml IV
9	Inject 2.0 ml IV and 2.0 ml subcutaneously (SC)
16	Test bleeding and titer determination
18	Cardiac bleed (30-50 ml)
25	Inject 2.0 ml SC
32	Cardiac bleed (30-50 ml)
39	Inject 2.0 ml SC
46	Cardiac bleed (30-50 ml)

Schedule 2: (Dudman, 1964)

DAY	PROCEDURE
1	Inject 1 ml of mixture of equal parts culture suspension and Freund's complete adjuvant (IM)
28	1 ml IV (antigen alone)
30	Bleed from ear 10-20 ml
32	Bleed from ear 10-20 ml
34	Bleed from ear 10-20 ml

Schedule 3: (Somasegaran, unpublished)

DAY	PROCEDURE
1	Inject SC 1 ml of emulsion of equal parts of antigen suspension and Freund's complete adjuvant
14	1 ml IV (antigen suspension alone)
28	Test bleeding and titer determination
30	Cardiac bleed (30-50 ml)
37	Inject 1.0 ml IV
44	Cardiac bleed (30-50 ml)

An intramuscular injection (IM) is used to start the immunization schedule (Exercise 6). Immobilize the rabbit by rolling it tightly into a large towel. Free one of the rear legs, and use alcohol to swab a small area of the skin covering the thigh muscle. Insert the needle about 1.5 cm into the muscle and inject. A large gauge needle (20G) is recommended to introduce the emulsion quickly and reduce the animal's discomfort.

Subcutaneous (SC) booster injections are usually given to maintain the antibody titer. Inject the antigen under the skin in the shoulder area. Use a 3-5 ml syringe fitted with a 22 gauge needle.

Intravenous injections are given into the marginal ear vein of one ear. Expose the vein by shaving a small section of the ear with a razor blade. Swab the shaved area with alcohol (70%) and inject the antigen with a 1-2 ml syringe fitted with a narrow gauge (25G) needle. If the schedule calls for several consecutive injections, make the first injection at the distal end of the ear. Progress toward the base of the ear with each successive injection.

For test bleeding, extract blood from the ear not used for injections. Shave a small area along the marginal ear vein, and swab the area with alcohol (70%). To prevent blood from spreading into the fur, apply vaseline around the area to be nicked. Use a scalpel with a small pointed blade (#11) and make a small nick in the vein. Collect 1-2 ml of blood in a test tube. Stop the bleeding by applying light pressure to the injury with the thumb and forefinger. If additional bleedings are found necessary, progressively nick the ear closer to its base.

Alternatively, blood may be drawn from the marginal ear vein with a 1-2 ml syringe equipped with a 26G needle.

There are various methods of extracting larger volumes of blood from rabbits. Among those frequently practiced are the cutting of the jugular vein, ear bleeding with the help of a vacuum, and cardiac puncture. Cardiac puncture (Figure A.13)

is recommended here because it is fast and efficient. The rabbit is tied to the inclining bleeding rack. The area above the sternum is shaved and swabbed with 70% alcohol. The blood is extracted with a large syringe (50 ml) fitted with a 18G needle and emptied into a sterile screw capped tube. About 50 ml blood can be taken from a ten to twelve pound rabbit without endangering the animal's life.

A bleeding rack may be built by nailing two wooden rails to a board (Figure A.12) and elevating one side with a wooden support to provide an incline of approximately 12°. The distance between the rails should be 4-6 cm, depending on the neck size of the rabbits used. The rabbit's head is held by the rails at the upper end, while the legs are tied to a cleat at the lower end.

The Bellco rabbit bleeding apparatus (Figure A.13) is another convenient means of obtaining large quantities of blood from a rabbit. Bellco's instructions provide the following information:

Equipment required: Vacuum pump (or line), a sharp razor blade, receptacle (culture tube or flask with appropriate size rubber stopper), short piece of heavy rubber or plastic hose for attachment to vacuum line.

The ear of the animal is disinfected, a single slit is

made through the marginal ear vein, and the ear is inserted into the large opening of the apparatus. The vacuum line is opened gradually until a vacuum lock is obtained on the head of the animal. Immediately the blood begins to flow in a steady stream. As much as 50 ml can be obtained in one minute without any sign of trauma to the animal.

The entire rabbit bleeding apparatus is autoclaved, the ear of the rabbit is treated with a disinfectant, and only one tube is used for each animal.

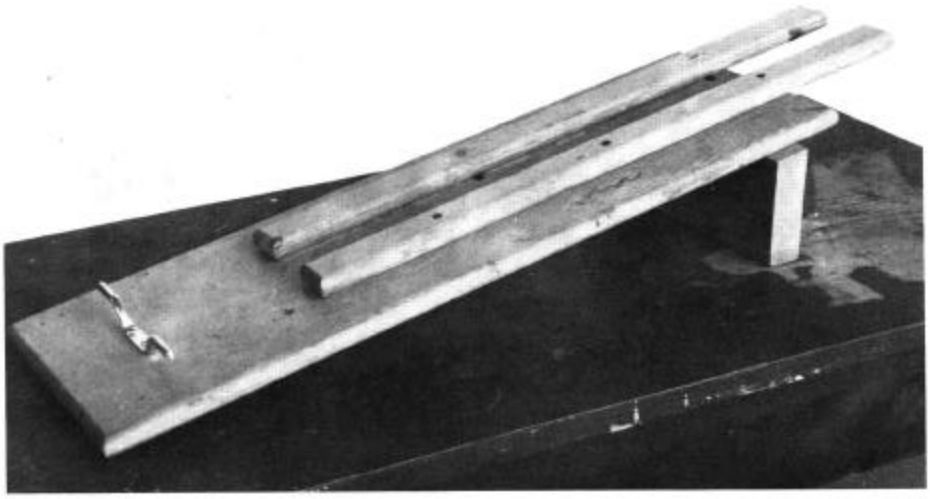


Figure A.12. A bleeding rack

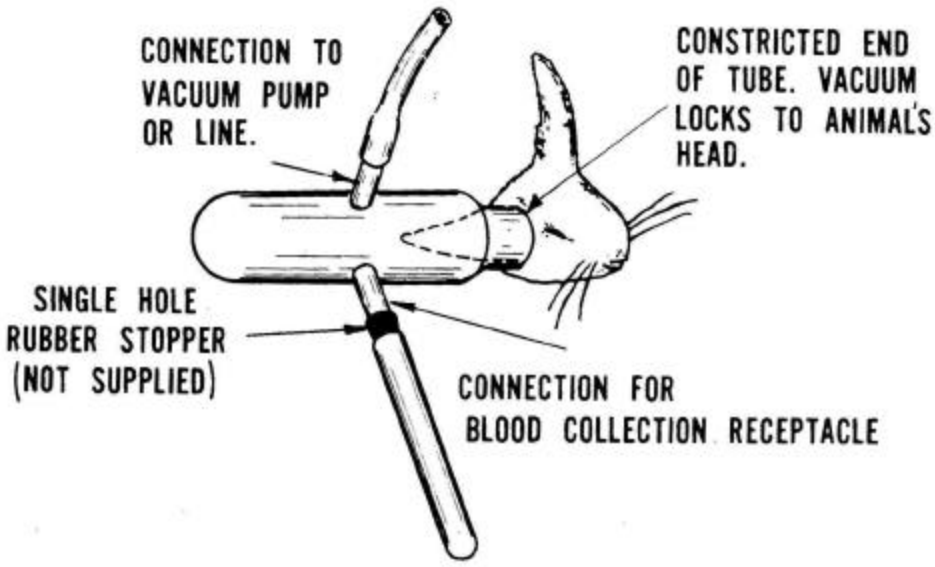


Figure A.13. The BELLCO #5640-1111 rabbit bleeding apparatus as shown on the manufacturer's instruction

sheet.

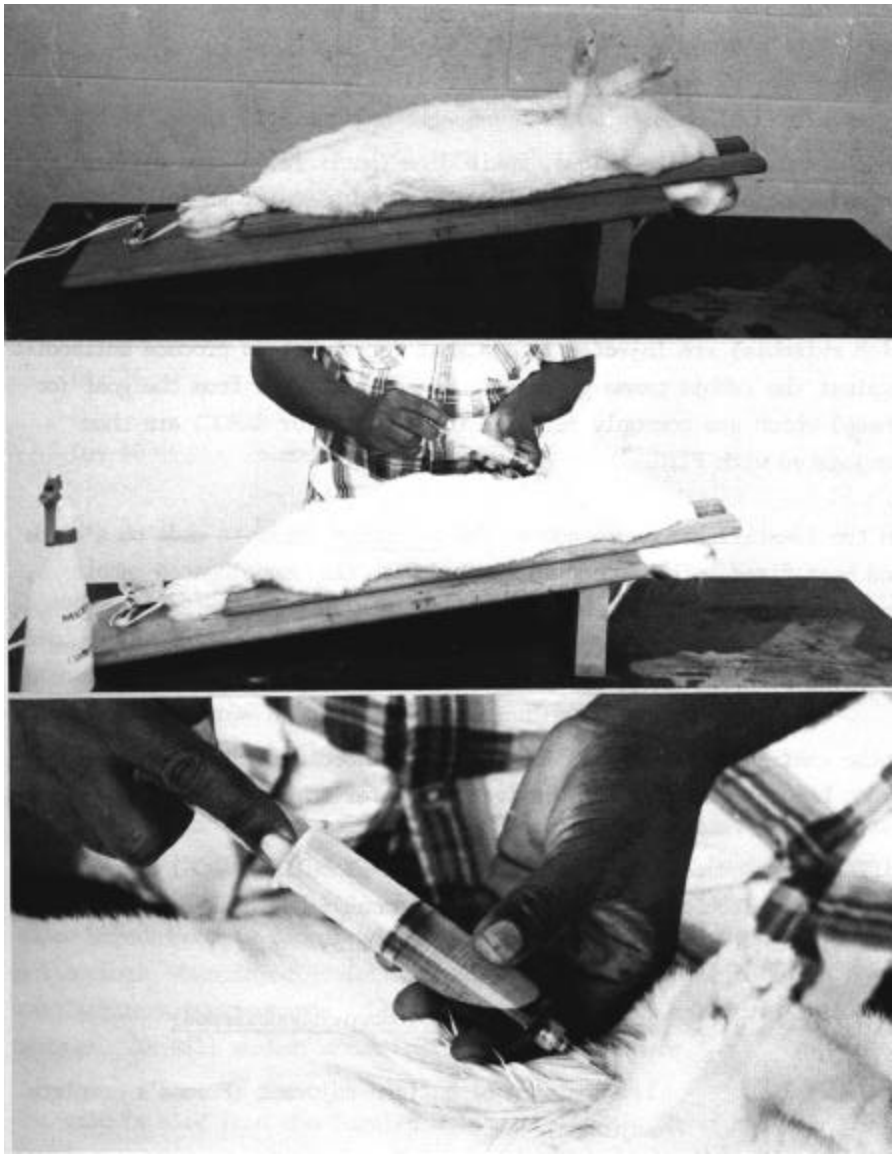


Figure A.14. Collecting blood from a rabbit by cardiac puncture:

a) Rabbit secured to bleeding rack

b) Drawing the blood

c) Close-up of draw

APPENDIX 13

THE INDIRECT FA TECHNIQUE

The indirect FA technique uses antibodies (antisera) of rabbit and goat (or sheep). The specific antiserum for the rhizobial strain is produced as described in Exercise 6, but the antiserum is not conjugated with FITC. Purified gamma-globulins from a rabbit (not immunized previously with rhizobia) are injected into a goat as antigen to produce antibodies against the rabbit gamma-globulin. The antibodies from the goat, commonly referred to as GARGG (Goat-Anti-Rabbit Gamma Globulin), are then conjugated with FITC.

In the identification procedure, rhizobial cells are smeared on a slide and heat fixed. This smear is reacted with the unconjugated rabbit antiserum specific for the rhizobial strain. After reaction, unreacted rabbit antiserum is washed off. This is followed by staining with the GARGG (or SARGG from sheep).

While GARGG is available commercially (e.g., from Difco Laboratories) some investigators prefer to produce their own.

NifTAL has produced GARGG successfully using a 1% solution of purified rabbit gamma-globulin as antigen. The following

injection schedule has proven successful (Intramuscular - IM, Intravenous - IV, Intraperitoneal - IP, and Subcutaneous - SC).

Injection Schedule (Hoben, unpublished)

Day 1	1:1 emulsion of antigen: Freund's complete adjuvant - 20 ml IM (10 ml into each thigh muscle)
Day 14	1:1 emulsion of antigen: Freund's incomplete adjuvant - 4 ml IM (2 ml into each thigh muscle) Antigen - 2 ml SC (1 ml above each shoulder) Antigen - 2 ml IP Antigen - 2 ml IV (optional)
Day 28	1:1 emulsion of antigen: Freund's incomplete adjuvant - 4 ml IM (2 ml into each thigh muscle) Antigen - 2 ml SC (shoulders) Antigen - 2 ml SC (1 ml into each hip region)
Day 33	Trial bleeding
Day 34	Blood collection
Day 40	Blood collection

Day 54 First booster injection (same as day 28).

Booster injections can be made on 28 day cycles. Injection and blood collections may be continued beyond day 34. The blood may be collected 6 days and 12 days after each set of booster injections. The booster injections follow the same protocol as day 28. Complete adjuvant should only be used in the beginning of the immunization. Incomplete adjuvant should be given on subsequent injection days.

One person is required to hold the animal down, while another gives the injections. A tranquilizer, such as Rompun (made by Bayer Leverkusen) is recommended to subdue the animal during blood collection. When the tranquilizer is injected intramuscularly according to the manufacturers instructions, the animal will fall asleep within 5-15 minutes, and awaken after two hours.

The goat is bled from the jugular vein as follows: shave the appropriate area on the neck and locate the vein by touch. Press the thumb of your left hand onto the vein. This will block the blood flow and enlarge the vein just above your thumb. Swab this area with 70% ethanol and insert a sterile 20 gauge needle (holder-needle assembly for use with Vacutainer glass tubes) into the jugular vein. Place the Vacutainer glass tube into the holder and collect the blood. Keep exchanging Vacutainers glass tubes until the desired

amount is collected. A 50 pound goat can safely deliver 300 ml in one bleeding.

The blood is handled as described in Exercise 6 and the resulting antiserum is checked for quality by immunodiffusion (Exercise 9) as follows:

Dilute the goat (sheep) antiserum in twofold steps from 1:2 to 1:32. Using the hexagonal immunodiffusion pattern, place the different dilutions into the outer wells and the antigen (1% rabbit globulin solution) into the center well. If sufficient antibodies are present in the serum, strong precipitin bands will be produced at dilutions of 1:4 or higher. Antisera of acceptable quality are then conjugated with FITC (Exercise 11).

The indirect FA technique eliminates the need for conjugating rabbit antisera. It is considered more sensitive than the direct FA technique. The indirect method can be used with any rhizobial antisera produced in a rabbit, even those with low titer which are not suitable for conjugation. It differs from the direct method mainly by the inclusion of the additional reaction step, while most of the procedures detailed in Exercise 11 for the direct technique remain the same.

Since nonspecific fluorescence may occasionally occur with the indirect method, a control smear treated only with conjugated

GARGG should be included.

The staining is done as follows:

1. Make a thin smear and heat fix.

2. Cover the smear with 1:10 diluted rabbit antiserum and incubate for 20 minutes.

3. Briefly wash off the excess antiserum with PBS.

4. Cover the smear with diluted FITC conjugate of goat anti-rabbit globulin and incubate for 20 minutes. (A suitable dilution of the conjugated GARGG to be used is determined by its staining titer.)

5. Wash off excess FITC conjugate and place in PBS for 20 minutes.

6. Complete the washing process by placing the slide in distilled water for 10 minutes.

7. Air dry, mount, and observe under the UV microscope.

APPENDIX 14

ADDITIONAL EXPLANATIONS TO THE CALCULATION OF THE MOST PROBABLE NUMBER (MPN)

Example:

Determine the number of *B. japonicum* cells contained in 1 g of a 100 g bag of inoculant made from nonsterile peat.

1) Dilute the 100 g of inoculant in 900 ml water.
2) Make a tenfold dilution series (Table A14.3)
3) Set up plants in quadruplicates as described in Chapter 6 and inoculate each plant with 1 ml of the dilutions.
4) Record nodulation (+ or -).
5) Beside each dilution, write the number of nodulated (+) units.
6) Add the total of the nodulated units assuming the results shown in Table A.6.
7) Note that number of replications, n = 4; dilution steps, s = 10; number of nodulated units, (+) = 21; lowest dilution in the series, d = 10^{-1}.
8) Use Table A.10 which is calculated for tenfold dilutions and locate 21 (for 21+ units) in column n = 4.
9) Find the most likely number (m) in column s = 10

corresponding to 21 in the n = 4 column. The most

Table A.6. Evaluation of soybean inoculant prepared from nonsterile peat.

	NODULATION				
	---------Replications--------				NUMBER OF NODULATED UNITS
DILUTION	I	II	III	IV	
10^{-1}	+	+	+	+	4
10^{-2}	+	+	+	+	4
10^{-3}	+	+	+	+	4
10^{-4}	+	-	+	+	3
10^{-5}	-	-	+	+	2
10^{-6}	+	-	+	-	2
10^{-7}	-	-	+	-	1
10^{-8}	+	-	-	-	1
10^{-9}	-	-	-	-	0
10^{-10}	-	-	-	-	0
				Total	21

likely number is m = 3.1 X 10^4.

10) Multiply most likely number with the reciprocal of lowest dilution used in the series (d = 10^1).

(3.1×10^4) X (10^1) = 3.1×10^5

The peat inoculant contained 3.1 X 10^5 rhizobia per gram. Since the original sample was diluted 1:10 (100 g peat in 900 ml sterile water) and aliquot (v) used for inoculation was 1 ml, the actual

calculation should be:

$$X = \frac{m \times d}{V} = \frac{3.1 \times 10^4 \times 10^1}{1} = 3.1 \times 10^5 \text{ rhizobia } g^{-1} \text{ inoculant}$$

Determing the most probable number in soil

The MPN count is often used to determine the number of rhizobia present in soil. Whereas a tenfold dilution series with two or four replicates is sufficient for most peat inoculants, which usually have a relatively high number of rhizobia (>10^8 cells g^{-1}), a fourfold or even twofold dilution series with replications in quadruplicate is usually chosen for soil. The smaller dilution steps provide a more precise estimate when less than 10,000 cells of rhizobia per gram soil are expected. The first sample of the series, however, is frequently diluted tenfold or a 100-fold.

Example: 100 grams of field soil were diluted in 900 ml of sterile water. A quadruplicate dilution series was prepared ranging from 4^{-1} to 4^{-9}. Aliquots of 2 ml were used for the inoculations.

Use Table A.9 for four fold dilutions in this appendix. In column (n = 4), find 25 for 25 + units.

Table A.7. Determination of the population of native cowpea-rhizobia in field soil using sirato as the trap host.

	NODULATION				
	---------Replications---------				Number of Nodulated units
Dilution	I	II	III	IV	
10^{-1}	+	+	+	+	4
4^{-1}	+	+	+	+	4
4^{-2}	+	+	+	+	4
4^{-3}	+	+	+	+	4
4^{-4}	+	+	+	-	3
4^{-5}		+	+	+	3
4^{-6}	+	+			2
4^{-7}	+				1
4^{-8}					0
4^{-9}	-	-	-	-	0
				Total	25

n = 4; s = 10; d = 10^{-1}; + units = 25

m = 1.6 X 10^{3}

d (lowest dilution) = 10^{-1}

v = 2 ml

$$X = \frac{1.6 \times 10^{3} \times 10^{1}}{2} = 8000$$

The most probable number of rhizobia in the field soil was

8000 cells per gram.

Table A.8. Number (M) of rhizobia estimated by the plant infection count (After Vincent 1970): A. Two-fold dilutions: (A=2)

Positive tubes		Dilution steps (s)			
n=4	n=2	s=10			
40	20	>520			
39					
38	19	520			
37		370			
36	18	290			
35		220			
34	17	180			
33		140	s=8		
32	16	120	>130		
31		95			
30	15	78	130		
29		65	93		
28	14	54	72		
27		45	55		
26	13	37	45		
25		31	35	s=6	
24	12	26	29	>33	
23		21	24		
22	11	18	19	33	
21		15	16	23	
20	10	13	13	18	
19		11	11	14	
18	9	8.9	9.3	11	
17		7.4	7.7	8.9	s=4
16	8	6.3	6.4	7.4	>8.3
15		5.2	5.4	6.0	
14	7	4.4	4.6	4.9	8.3
13		3.7	3.8	4.1	5.9
12	6	3.2	3.2	3.4	4.6
11		2.6	2.6	2.7	3.4
10	5	2.2	2.2	2.3	2.8
9		1.8	1.9	1.9	2.2
8	4	1.5	1.5	1.6	1.8
7		1.2	1.3	1.3	1.4
6	3	1.0	1.0	1.0	1.1
5		0.79	0.79	0.81	0.97
4	2	0.60	0.60	0.62	0.66
3		0.42	0.43	0.43	0.46
2	1	0.27	0.27	0.27	0.29
1		<0.2	<0.2	<0.2	<0.2
0	0				
Approx range Factor, 95%		2000	500	120	30

fiducial limits	n=2	2.7
(x,÷)	n=4	2.0

Calculated from Table VIII[2] of Fisher and Yates (1963).

Table A.9. Number (M) of rhizobia estimated by the plant infection count (After Vincent 1970): B. four-fold dilutions; (A=4)

Positive tubes		Dilution steps (s)			
n=4	n=2	s=10			
40	20				
39		$>2.0\times10^{5}$			
38	19	2.0×10^{5}			
37		1.2			
36	18	8.1×10^{4}			
35		5.5			
34	17	3.8			
33		2.6	s=8		
32	16	1.8	$>1.3\times10^{4}$		
31		1.3			
30	15	9.1×10^{3}	1.3×10^{4}		
29		6.3	7.9×10^{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	$>7.9\times10^{2}$	
23		8.0×10^{2}	8.0×10^{2}		
22	11	5.6	5.6	7.9×10^{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	7.1×10^{1}	7.1×10^{1}	7.2×10^{1}	$>5.0\times10^{1}$
15		5.0	5.0	5.1	
14	7	3.5	3.5	3.5	5.0×10^{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	8.9×10^{0}	8.9×10^{0}	8.9×10^{0}	9.6×10^{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		7.2×10^{-1}	7.2×10^{-1}	7.2×10^{-1}	7.2×10^{-1}
2	1	4.4	4.4	4.4	4.4
1					0
0	0	$<4.4\times10^{-1}$	$<4.4\times10^{-1}$	$<4.4\times10^{-1}$	$<4.4\times10^{-1}$

Approx. range	5×10^{5}	3×10^{4}	2×10^{3}	1×10^{2}
Factor for 95% fiducial limits	n=2 4.0			

(x,÷) n=4 2.7

Calculated from Table VIII[2] of Fisher and Yates (1963).

Table A.10. Number (M) of rhizobia estimated by the plant infection count (After Vincent 1970): C. Ten-fold dilutions; (A=10)

Positive tubes		Dilution steps (s)			
n=4	n=2	s=10			
40	20	>7x10^{8}			
39					
38	19	6.9			
37		3.4			
36	18	1.8			
35		1.0			
34	17	5.9x10^{7}			
33		3.1	s=8		
32	16	1.7	>7x10^{6}		
31		1.0			
30	15	5.8x10^{6}	6.9		
29		3.1	3.4		
28	14	1.7	1.8		
27		1.0	1.0		
26	13	5.8x10^{5}	5.9x10^{5}		
25		3.1	3.1	s=6	
24	12	1.7	1.7	>7x10^{4}	
23		1.0	1.0		
22	11	5.8x10^{4}	5.8x10^{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	5.8x10^{3}	5.8x10^{3}	5.9x10^{3}	
17		3.1	3.1	3.1	s=4
16	8	1.7	1.7	1.7	>7x10^{2}
15		1.0	1.0	1.0	
14	7	5.8x10^{2}	5.8x10^{2}	5.8x10^{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	5.8x10^{1}	5.8x10^{1}	5.8x10^{1}	5.9x10^{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	5.8x1	5.8x1	5.8x1	5.8x1
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		<0.6	<0.6	<0.6	<0.6
0	0				
Approx. range		10^{9}	10^{7}	10^{5}	10^{3}
Factor, 95% fiducial limits*	n=2	6.6			

(x,÷):		n=4	3.8						

Calculated from Table VIII[2] of Fisher and Yates (1963).

Table A.10. Number (M) of rhizobia estimated by the plant infection count (After Vincent 1970): C. Ten-fold dilutions; (A=10)

Positive tubes		Dilution steps (s)			
n=4	n=2	s=10			
40	20	$>7x10^8$			
39					
38	19	6.9			
37		3.4			
36	18	1.8			
35		1.0			
34	17	$5.9x10^7$			
33		3.1	s=8		
32	16	1.7	$>7x10^6$		
31		1.0			
30	15	$5.8x10^6$	6.9		
29		3.1	3.4		
28	14	1.7	1.8		
27		1.0	1.0		
26	13	$5.8x10^5$	$5.9x10^5$		
25		3.1	3.1	s=6	
24	12	1.7	1.7	$>7x10^4$	
23		1.0	1.0		
22	11	$5.8x10^4$	$5.8x10^4$	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	$5.8x10^3$	$5.8x10^3$	$5.9x10^3$	
17		3.1	3.1	3.1	s=4
16	8	1.7	1.7	1.7	$>7x10^2$
15		1.0	1.0	1.0	
14	7	$5.8x10^2$	$5.8x10^2$	$5.8x10^2$	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	$5.8x10^1$	$5.8x10^1$	$5.8x10^1$	$5.9x10^1$
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	5.8x1	5.8x1	5.8x1	5.8x1
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		<0.6	<0.6	<0.6	<0.6
0	0				
Approx. range		10^9	10^7	10^5	10^3
Factor, 95% fiducial limits*		n=2	6.6		
		n=4	3.8		

(x,÷):	n=4	3.8

Calculated from Table VIII[2] of Fisher and Yates (1963).
*Cochran; Biometrics, 1950, 6, 105.

APPENDIX 15

THE ACETYLENE REDUCTION METHOD FOR MEASURING NITROGENASE ACTIVITY

The nitrogenase enzyme-complex is responsible for biological nitrogen fixation in the root nodules of legumes. Nitrogenase is synthesized by bacteroids in the nodules and also the reduction of molecular nitrogen to NH_3 takes place within the cytoplasm of the bacteroids. The enzyme-complex consists of two distinct protein components with iron atoms common to both and molybdenum present in only one of the two components. Both the Fe-protein and Mo-Fe-protein are essential for nitrogenase activity. During the reduction process, molecular nitrogen is converted to NH_3 through a series of steps involving enzyme(s) and ATP. Though molecular nitrogen is the natural substrate for nitrogenase, other triple bonded "nitrogen-analogues" like acetylene ($HC{\equiv}CH$), cyanide ($H\text{-}C{\equiv}N$), nitrous oxide ($N{\equiv}N\text{-}O$) and methyl isocyanide ($CH_3\text{-}N{\equiv}C$) can also undergo reduction mediated by the nitrogenase complex. Because of its lack of toxicity and easy availability, acetylene is frequently used to assay for nitrogenase activity. In the assay procedure, root nodules of legumes are exposed to 5-25% of acetylene-in-air mixture and incubated at 25°C-30°C. The ethylene (C_2H_4) produced by the reduction of the acetylene is measured by gas chromatography. Although acetylene reduction is a sensitive method for assaying

nitrogenase activity, the reduction information may not be translated into nitrogen fixed because of frequent theoretical disagreement in the stoichiometry of the two reduction processes.

The Gas Chromatograph

A gas chromatograph with a hydrogen Flame-Ionization-Detector (FID) is usually used in the assay. A stainless steel column, 3 m long and 1 mm in diameter, is filled with molecular sieve material, usually Porapak media (produced by Waters Associates Inc., Farmingham, Mass., U.S.A.). Porapak is a porous polymer composed of ethylvinylbenzene cross-linked with divinylbenzene to form a uniform structure of distinct pore size. It is available in bead form with different mesh sizes. Porapak N of 100-200 mesh size allows good separation of C_2H_2 and C_2H_4 using N_2 as carrier gas.

A column temperature of 50-70°C with a carrier gas flow rate of 50 ml min^{-1} is used for routine work. Air and hydrogen gas are adjusted to flow at the rates of 300 ml min^{-1} respectively.

Different gases have different retention times in the column, therefore the gas chromatograph-recorder will trace out the peaks in order of emergence. A gas mixture containing CH_4, C_2H_2 and C_2H_4 will have the trace pattern illustrated in Figure A15.1. It is important for the operator to become familiar with the acetylene and ethylene peaks traced out on the gas

chromatograph recorder chart.

Source of Acetylene

Acetylene is available commercially in cylinders. Very pure acetylene (99%) is available in small cylinders for analytical work. Small amounts for laboratory work can be conveniently prepared by reacting calcium carbide with water. About 15 ml of water is used for each gram of calcium carbide. A simple apparatus for generating acetylene is shown in Figure A.15. Acetylene generated this way contains very minute quantities of phosphine, ethylene and methane.

Calibration of the gas chromatograph

In the calibration process, exact amounts of ethylene have to be injected into the gas chromatograph and the peak heights measured. The concentration of ethylene giving a particular peak height is computed. A calibration curve is obtained by plotting peak height (Y-axis) against ethylene concentration (X-axis). The calibration curve should be linear and pass through the origin.

1. Obtain a small volume of the 99% pure ethylene for the calibration.
2. Dilute the pure ethylene as follows:
   Determine the true volume of a 1000 ml volumetric flask. Fill the flask completely with water to the mouth. Without trapping any air, carefully place a "Suba-seal" (W. Freeman & Co., Led., Barnsely,

Yorkshire) or a long, sleeved rubber stopper for serum bottles (Wheaton Scientific, Millville, New Jersey) to contain the water. Invert the flask to detect air trapped during the placement of the seal or stopper. Repeat filling and sealing if too much air is trapped. Pour the water into a measuring cylinder and record the volume.

3. Flush out the flask with N_2 and seal again. Remove 1 ml of the air from the sealed flask with a syringe. Then inject 1 ml of pure ethylene into the sealed flask and allow to stand for 10 min at room temperature to equilibrate.
4. With a 1 ml plastic syringe, pierce the rubber seal, remove 1 ml of the diluted ethylene from the flask, and inject it into the gas chromatograph. Measure the height of the ethylene peak from the trace. Inject two more 1 ml samples to check for reproducibility of the peaks. Note column temperature of the gas chromatograph.

Calculations of the calibration

Suppose the diluted ethylene (now referred to as the standard) was equilibrated at 23°C and 756 mm Hg pressure. Convert these values to NTP using the gas law relationship:

$$\frac{P_1V_1}{T_1} = \frac{P_2V_2}{T_2}$$

P_1 = 760 mm Hg: V_1 = unknown; T_1 = 273°K

P_2 = 756 mm Hg: V_2 = 1 ml; T_2 (273° + 23°)K
therefore, volume of ethylene, V_1 at NTP

$$= 1 \text{ X } (765/760) \text{ X } (273/296)$$
$$= V_1 = 0.9174 \text{ ml}$$

According to molar volume, 1 mole of C_2H_4 at NTP will occupy 22.4 liter (22.4 x 10^3 ml).

Therefore, 0.9174 ml $C_2H_4 = \frac{0.9174}{22.4 \text{ X } 10^3}$ mole

$$= 0.041 \text{ X } 10^{-3} \text{ mole}$$
$$= (0.041 \text{ X } 10^{-3}) \text{ X } 10^9 \text{ nmole}$$
$$= 4.1 \text{ X } 10^4 \text{ nmole}$$

The accurate volume of the completely fixed volumetric flask (1 liter) was 1038 ml. Only 1 ml of the pure ethylene was diluted in the atmosphere of the flask.

Therefore, 1 ml of the diluted ethylene = $\frac{4.1 \text{ X } 10^4 \text{ nmole}}{1098 \text{ ml}}$

$$= 39.499 \text{ nmole}$$

When 1.0 ml of the diluted sample was injected into the gas Chromatograph, an ethylene peak height of 75 divisions (on the recorder chart paper) at x64 attenuation was produced.

Assume that 1 division on the recorder chart paper is equal to 1 Flame-Ionization-Detector (FID) unit. Therefore, (75 x 64) FID units = 39.499 nmole.Therefore, 1 FID unit at x1 attenuation

$= \frac{39.499}{75 \text{ X } 64} = 0.0082$ nmole

$$\underline{1 \text{ FID unit} = 0.0082 \text{ nmole} = 8.2 \text{ X } 10^{-3} \text{ nmole}}$$

From the standard ethylene preparation, inject in duplicate 0.2,0.4, 0.6, 0.8 and 1.0 ml of the gas. Measure the peak heights corresponding to these volumes and n moles of the ethylene. Plot the calibration curve (i.e., peak height vs n moles ethylene).

Assaying for nitrogenase activity with nodulated roots

To bring acetylene and nitrogenase into contact, the nodules must be contained in a suitable air-tight vessel into which acetylene can be introduced. After a specified incubation period, samples are withdrawn and analyzed for ethylene produced with a gas chromatograph.

Calibrate the gas chromatograph with the pure ethylene standard. This should be done very much in advance of bringing in incubated nodule samples for gas analysis.

Prepare incubation vessels from 1 liter Nalgene PVC wide mouth bottles (or equivalent) for incubation of the nodulated roots. Carefully drill a 15 mm hole in the center of the cap of each bottle and fit a rubber septum (serum bottle flange-type stopper) of appropriate size to give a leak-proof fit. If metal caps are used, caps should have rubber liners to prevent leaks.

Carefully excavate whole plants from the field or from Leonard jars. Cut off the tops at the point of the scar left by the

cotyledons. Place the tops into labelled bags to be dried for dry weight determination. Remove as much of the soil or growth medium adhering to the roots as possible before placing it into the incubation vessel. Retrieve and include any nodule(s) which becomes detached during the excavating or cleaning operations.

Do not wash the roots to clean them as wetting decreases the nitrogenase activity significantly. A wet nodule probably traps the acetylene on the surface of the nodule by slight solution in water, thus making less acetylene available to the nitrogenase in the nodule. If the root system becomes wet, the nodules should be dried by blotting prior to being placed in the bottle. Nodulated roots from solution culture experiments should be treated similarly.

With a 50 ml plastic syringe (Beckton-Dickinson, Rutherford, New Jersey) fitted with an 18G needle and 1.5 inches long, remove 5 or 10% of the atmosphere in the incubation vessel. Replace this with a corresponding volume of acetylene. Record the time when incubation is initiated. Allow the incubation to proceed for 30-45 minutes with periodic shaking of the bottles in between to permit good contact between the nodules and the acetylene.

At the end of the incubation, shake the bottle, withdraw a 1 ml gas sample through the septum, and inject into the gas

chromatograph. Duplicate the injections and note the attenuation. Other details can be indicated against each trace on the recorder chart paper.

Remove the nodulated roots from the incubation vessels after gas samples have been removed for analysis. Wash the roots and pick the nodules. Obtain the fresh weight of nodules after blotting dry, and finally, oven dry the nodules at 70°C.

Calculate the nitrogenase activity from the information provided by the gas chromatograph as shown in the following example.

Example: Nodulated roots of two soybean plants were placed in a 1000 ml incubation vessel (PVC wide-mouthed bottle). After the cap of the incubation vessel was secured tightly, 50 ml (5%) of the air was withdrawn from the incubation vessel (via the rubber septum in the cap) with a 50 ml plastic syringe and replaced with 50 ml of C_2H_2. After 30 min incubation, 1 ml of the gas sample was withdrawn with a 1 ml syringe and injected into the gas chromatograph. A peak height of 40 divisions and x32 attenuation was produced. Calculate the C_2H_4 produced by the nodules. (Use values of the standard calculated previously from the calibration of the gas chromatograph.)

Calculations:

Peak height = 40 divisions; Attenuation = x32

Incubation time = 30 min; volume injected = 1 ml

Total FID units = 40 x 32 = 1280

From the calibration 1 FID unit = 8.2×10^{-3} nmole C_2H_4

Therefore, 1280 FID units = $(8.2 \times 10^{-3}) \times (1280)$ nmole C_2H_4

Since the volume of the incubation vessel was 1000 ml, then the total volume of C_2H_4 produced = $(8.2 \times 10^{-3}) \times (1280) \times (1000)$ n moles

= 10496 nmoles

$= \frac{10496}{1000} = 10.496$ µmoles

10.496 ìmoles of C_2H_4 were produced by 2 soybean roots in 30 minutes.

Therefore ìmoles C_2H_4/plant/hour $= \frac{10.496}{2} \times \frac{60}{30} = 10.496$

General formula for calculating nitrogenase activity:

$$\text{nitrogenase activity} = \frac{\text{ethylene produced}}{\text{time(h)} \times \text{number of plants}}$$

Plot nitrogenase activity on the Y-axis and nodule (fresh or dry) weight on the X-axis. Plot a similar graph, but with dry weight of plant tops on the X-axis. Process both sets of plots statistically and obtain the coefficient of correlation (Appendix 18) for each of the two plots.

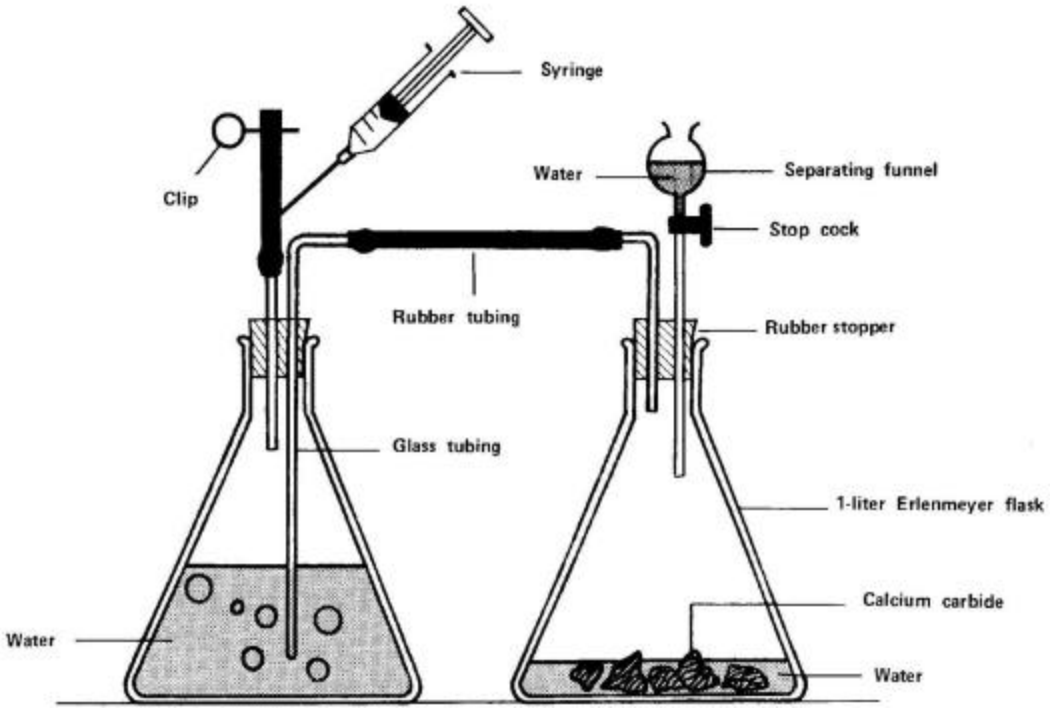


Figure A.15. A simple apparatus for generating small amounts of acetylene (C_2H_2) in the laboratory

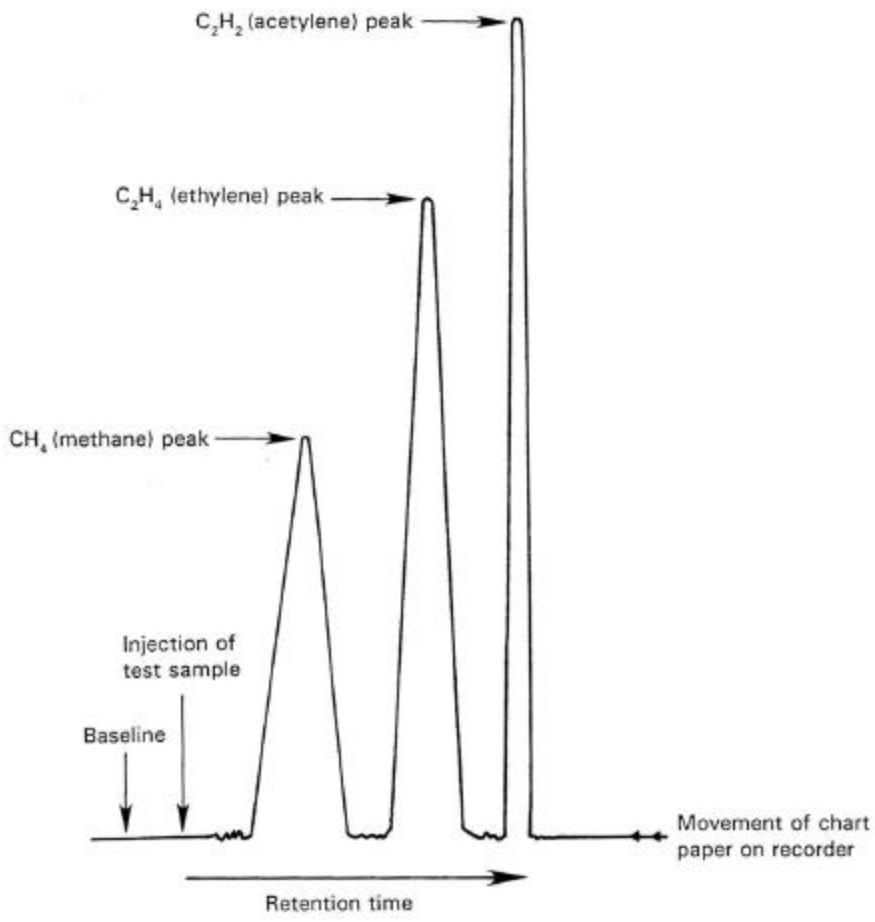


Figure A.16. Trace pattern from an injection of a gas mixture containing CH_4, C_2H_2 and C_2H_4 showing the sequence of emergence of the different peaks. (Adapted from Postgate 1971)

APPENDIX 16

METHODS FOR DETERMINING LIME REQUIREMENTS OF ACID SOILS
(REPRODUCED WITH PERMISSION FROM CHAPMAN AND PRATT, 1961)

Lime requirement of acid soils

Many procedures have been developed for measuring the lime requirement of soils, defined as the amount of lime needed to bring the pH value from its present value to any given pH value. Two methods are described here. The first method is the most reliable, but requires more time and equipment, and involves a direct titration with calcium hydroxide. The second method, developed by Shoemaker (1959), depends on the depression in pH of a buffer solution when soil is added. It is rapid and involves a greater error, but can be used in the lime requirement estimation of large numbers of samples in relatively little time.

Calcium Hydroxide Titration

Reagents - Calcium hydroxide solution. Add 1 g of calcium oxide or 1.5 g of calcium hydroxide per l of carbon dioxide-free water used. Mix and let stand protected from air until the excess has settled. Siphon off the solution. Store in a bottle protected from the carbon dioxide of the air.

Procedure - Place 10 g of acid soil in each of seven 100 ml beakers and add 0, 5, 15, 20, 30, 40, and 50 ml of calcium hydroxide solution to beakers 1 through 7 respectively. Add sufficient water to make each sample to a soil to water ratio of 1:5. Let stand for 3 days and determine the pH value of the soil-water suspension. Plot the pH against the milliequivalents (me) of calcium added per 100 g of soil and determine the amount of lime needed to bring the pH to the desired level. One me of calcium per 100 g is equal to 100 pounds of lime per acre, assuming the lime is mixed with 2,000,000 pounds of soil.

Remarks: - This method can be used if only a few samples are to be analyzed. If, however, there are large numbers, the space and time limitations become too great and the faster method described in the next section can be used.

Three days are required for the reaction of calcium hydroxide with acid soil to come to an approximate equilibrium. Actually, about 97 percent of the reaction is complete in this time and the true equilibrium is attained after many days.

Buffer Method

Reagents: - Buffer solution. Dissolve 1.8 g of p-nitrophenol, 2.5 ml of triethanolamine, 3.0 g of potassium chromate, 2.0 g of $Ca(OAc)_2$ $2H_20$, and 40.0 g of $CaCl_2.2H_20$ in approximately 800

ml of distilled water. Adjust the pH to 7.50 using hydrochloric acid or sodium hydroxide solutions, and dilute to 1 l. Best results are obtained if 10-20 l are prepared at one time. If protected from carbon dioxide, this reagent will remain stable for 6 months or more. When titrated with hydrochloric acid, 50 ml of buffer should require 2.6-2.7 me to bring the pH to 3.5 and the titration curve should be a straight line between pH 7.5 and 3.5.

Procedure: - Weigh 10.0 g of soil and transfer to a 125 ml Erlenmeyer flask. Add 20 ml of buffer solution and shake for 10 min. Transfer to a 50 ml beaker and use a pH meter to determine the pH value. The lime requirement is proportional to the depression in pH of the buffer. The lime requirement can be determined from the data in Table A.11, or the data in Table A.11 can be plotted and the lime requirement obtained by reading from the pH vs. lime requirement line.

If the pH of the soil-buffer suspension is greater than approximately 6.5, as is found with some highly acid, sandy soils, repeat the procedure using 50 g of soil and 20 ml of buffer, then divide the obtained lime requirement by 5. This modification gives better accuracy for poorly buffered soils of low lime requirement.

The answer is obtained in terms of tons of pure calcium carbonate per 2,000,000 pounds of soil to bring the pH to

6.5. Appropriate corrections must be made for variations in depth of mixing of lime or in bulk density of soils. A 6.5-inch depth of soil over an acre in area will have 2,000,000 pounds of dry soil if the bulk density is 1.35.

Table A.11. Lime requirement scale for buffer method.

Soil Buffer pH	Lime Requirement	Soil Buffer pH	Lime Requirement
	tons $CaCO_3$*		tons $CaCO_3$
6.7	1.6	5.7	7.6
6.6	2.2	5.6	8.2
6.5	2.8	5.5	8.9
6.4	3.4	5.4	9.5
6.3	4.0	5.3	10.1
6.2	4.5	5.2	11.0
6.1	5.2	5.1	11.7
6.0	5.8	5.0	12.4
5.9	6.4	4.9	13.2
5.8	7.0	4.8	14.0

*Tons of pure calcium carbonate per 2,000,000 pounds of soil or per acre if it is mixed with 6.5 inches of soil having a bulk density of 1.35.

APPENDIX 17

ANALYSIS OF VARIANCE FOR A RHIZOBIUM STRAIN SELECTION EXPERIMENT

The data in Table A.12 presents the dry weight (g) of plant tops from a strain selection experiment for soybean (*G. max* var. Jupiter). The experiment was a Randomized Complete Block Design (RCBD), with 3 blocks and 16 treatments (14 inoculated + 2 controls). Each treatment was replicated once within each block. Each treatment-plot was a Leonard jar unit with two soybean plants. The plant tops were harvested at 32 days and oven dried at 70°C. The strains of *Bradyrhizobium japonicum* have been ranked according to dry weight.

Summary of calculations for the analysis of variance for the strain selection experiment.

No. of treatments = k = 16

No. of blocks = b = 3

No. of replicates per treatment per block = n = 1

Calculate the Grand Total (GT) by adding up all the treatment totals:

$GT = T_1 + T_2 \ T_3$ ----- $+ T_k$

= 31.09 + 28.85 + 28.04 ----- + 20.07

= 344.83

Table A.12. Data from a strain selection experiment for soybean.

TREATMENTS	Dry Weight of Plant Tops (g) BLOCKS B1	B2	B3	Treatment Total (T)	Treatment Means (x)
TAL 102	9.66	10.60	10.83	31.09	10.36
TAL 379	9.36	9.00	10.49	28.85	9.62
TAL 206	8.41	9.44	10.19	28.04	9.35
TAL 435	8.61	9.23	8.22	26.06	8.69
TAL 411	9.20	8.19	8.46	25.85	8.62
Allen 527	8.11	8.82	8.62	25.55	8.52
TAL 211	8.83	6.32	9.14	24.29	8.10
TAL 487	6.27	8.67	8.35	23.29	7.76
CB 1795	6.79	8.17	5.70	20.66	6.89
TAL 650	6.95	5.83	6.83	19.61	6.54
TAL 649	6.55	4.82	8.10	19.47	6.49
TAL 860	6.00	4.83	6.54	17.37	5.79
TAL 183	6.11	3.46	5.51	15.08	5.03
TAL 378	5.39	4.46	5.07	14.92	4.97
Control*	1.53	1.30	1.80	4.63	1.54
Control**	8.41	7.83	5.83	20.07	6.36
	114.18	110.97	119.68	344.83	116.36

* Uninoculated
** 70 ppm N

Calculate the Grand Mean (X) by adding up all the treatment means:

$$X = x_1 + x_2 + x_3 \text{ ----- } x_k$$

$= 10.36 + 9.62 + 9.35 \text{ ----- } + 6.36$

$= \underline{116.13}$

Calculate the Correction Factor (CF)

$$CF = \frac{(GT)^2}{bkn} = \frac{(344.83)^2}{3 \times 16 \times 1}$$

$= \underline{2477.2444}$

Calculate the total sum of Squares (SS)

$SS = Ó x^2 - CF$

$= 9.66^2 + 10.60^2 + 10.83^2 \text{ ----- } + 5.83^2 - 2477.244$

$= \underline{247.8507}$

Calculate the Treatment Sum of Squares (SST)

$$SST = \frac{T^2}{bn} - CF$$

$$= \frac{31.09^2 + 28.85^2 ... + 4.63^2 + 20.07^2}{3 \times 1} - 2477.2444$$

$= \underline{217.4785}$

Calculate the Block Sum of Squares (SSB)

$$SSB = \frac{B^2}{kn} - CF$$

$$= \frac{114.18^2 + 110.97^2 + 119.68^2}{16} - 2477.2444$$

$= \underline{2.4253}$

Calculate the Error Sum of Squares (SSE)

$$SSE = SS - (SST + SSB)$$

$$= 247.8507 - (217.4785 + 2.4253)$$

$$= \underline{27.9469}$$

Prepare the Analysis of Variance according to Table A.13.

Table A.13. Analysis of Variance

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio
Treatments	SST	k-1	$\frac{SST}{(k-1)}$	$\frac{SST}{SSE} \times \frac{(bkn-k-b+1)}{(k-1)}$
Blocks	SSB	b-1	$\frac{SSB}{(b-1)}$	$\frac{SSB}{SSE} \times \frac{(bkn-k-b+1)}{(b-1)}$
Error	SSE	bkn-k-b+1	$\frac{SSE}{(bkn-k-b+1)}$	
Total	SS	bkn-1		

Using the above formulations, substitute with actual figures from the calculations and prepare the Table A.14.:

Table A.14. Analysis of Variance

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio (calculated)	F-Ratio (tabular 5%)
Treatments	217.4785	16-1=15	$\frac{217.4785}{15} = 14.4986$	$\frac{14.4986}{0.9316} = 15.5$	2.01
Blocks	2.4253	3-1=2	$\frac{2.4253}{} = 1.2126$	$\frac{1.2126}{} = 1.30$	3.32

			2	0.9316
Error	27.9469	48-16-3+1=30	$\frac{27.9469}{30}$ =	0.9316
Total	247.8507	48-1=47		

Use of the F-distribution

The statistic F is a ratio of two variances and these variances are the 'mean squares'. To identify the F-distribution, the degrees of freedom (df) of each variance needs to be specified. The degrees of freedom of two variances may be represented as df_1 and df_2, where df_1 is the number of degrees of freedom in the numerator and df_2 is the number of degrees of freedom in the denominator.

From the calculations in the table of analysis of variance for Treatments, $F(df_1, df_2) = F(15,30)$. From a F - distribution table, the critical value for F(15,30) with p = 0.05 is 2.01. Enter this tabular value into the table.

Similarly for Blocks, the critical value for F(2,30) with p = 0.05 is 3.32. Enter this tabular value into the table.

Since the calculated F-ratio for treatments is greater than the tabular value of F at the 5% level, the results indicate significant differences between the strains of ***B. japonicum*** in their nitrogen-fixing effectiveness.

The calculated F-ratio for blocks is less than the tabular value indicating the "blocking" of the experiment did not create any significant disuniformity in the aeration, light, or other environmental factors in the greenhouse.

Calculate the Least Significant Different (LSD)

$$LSD_{0.05} = t_{0.05}\sqrt{\frac{2s^2}{n}}$$

Where $t_{0.05}$ =The tabular value of t for degrees of freedom for error at the 5% probability level

s^2 = Mean square for error

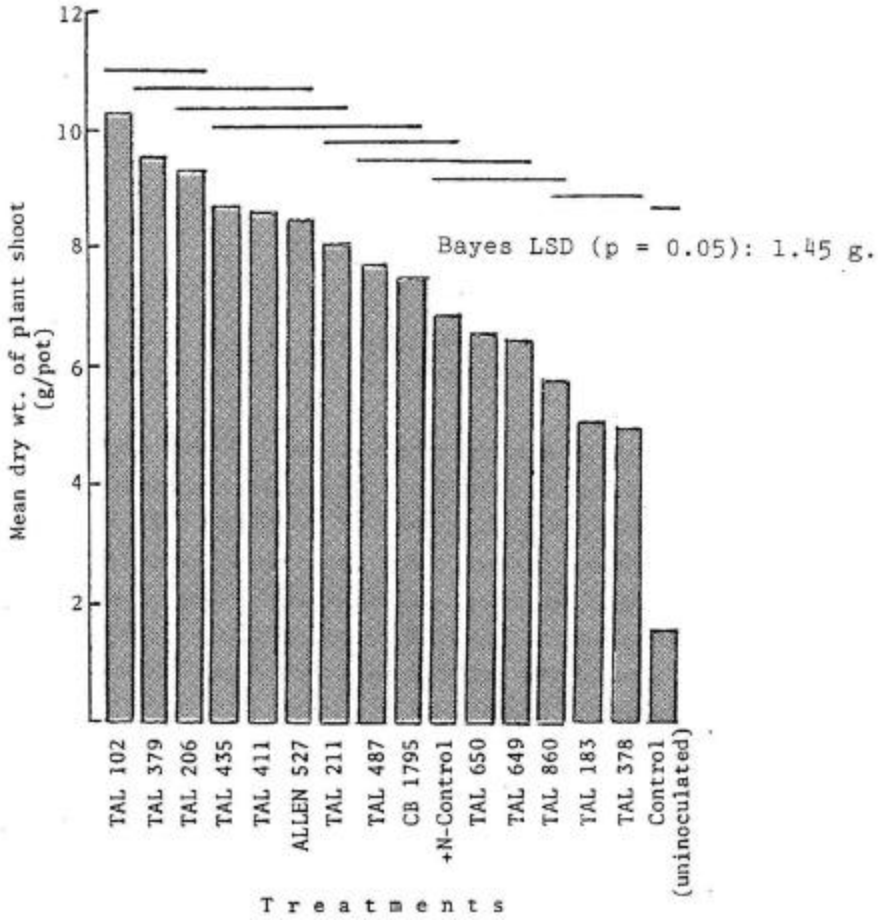
n = Number of replications

$$LSD_{0.05} = 2.042\sqrt{\frac{2 \times (0.9316)}{3}}$$

= 2.042 x 0.79

= 1.60 g

The LSD is used to compare values of two adjacent means. A pair of means which differ by more than the LSD is considered significantly different at the probability level of t employed. If comparison between means not adjacent to each other in a ranked array are made, the Duncan's Multiple Range test should be used. However, this test requires the computation of the Bayes LSD whose value may differ from the LSD as calculated above. The calculation of the Bayes LSD is not presented here but its use is illustrated in Figure A.17.



Means not joined by the same line differ at p = 0.05 as given by Duncan's New Multiple Range Test.

Figure A.17. Effect of various strains of *B. japonicum* on the dry weight of shoots of soybean (*G. max* var.

Jupiter)

APPENDIX 18

COMPUTING THE COEFFICIENT OF CORRELATION (r) TO SHOW THE RELATIONSHIP BETWEEN SHOOT AND NODULE WEIGHTS IN A RHIZOBIUM STRAIN SELECTION EXPERIMENT

The data in Table A.15 presents the dry weights (g) of the plant tops and nodules from a rhizobial strain selection experiment for cowpea (*Vigna unguiculata*). The experiment was a Randomized Complete Block Design with three blocks and 11 treatments (nine inoculated and two controls). Each treatment was replicated once within each block. Each treatment-plot was a Leonard jar with two cowpea plants. The plants were harvested at 30 days and the tops and nodules oven-dried at 70°C for 2 days. The plus-N control will be omitted from the correlation analysis.

Construct a new table containing the following data sets of plant tops (x) and nodules (y), as in Table A.16.

The number of pairs (n) excluding the plus N control is 10.

Calculations:

Calculate the Mean of x = M_x

$$M_x = \frac{\sum x}{n} = \frac{14.21}{10} = 1.421$$

Calculate $M_x^2 = (1.421)^2 = 2.019$

Table A.15. Dry weights of tops and nodules from a rhizobial strain selection experiment.

Rhizobia	Host of Isolation	Dry weight(s)	
		plant tops	nodules
TAL 173	*Vigna unguiculata*	2.29	0.21
TAL 651	*Calopogonium mucunoides*	2.08	0.23
TAL 209	*Vigna unguiculata*	1.71	0.29
TAL 309	*Macrotyloma africanum*	1.67	0.26
TAL 1147	*Desmodium intortum*	1.67	0.22
TAL 22	*Phaseolus lunatus*	1.63	0.18
TAL 310	*Dolichos biflorus*	1.30	0.19
TAL 647	*Pueraria phaseoloides*	0.97	0.15
TAL 379	*Glycine max*	0.75	0.13
Control (uninoculated)	--	0.14	0.00
Control (+ 70 ppm N)	--	4.03	0.00

Compute the Standard Deviation (SD) for x:

$$SD_x = \sqrt{\frac{\Sigma x^2}{n} - M_x^2} = \sqrt{\frac{23.94}{10} - 2.019} = 0.616$$

Similarly compute the SD_y after determining M_y and M_y^2:

$$M_y = \frac{\Sigma y}{n} = \frac{1.86}{10} = 0.186$$

$$M_y^2 = (0.186)^2 = 0.0345$$

$$SD_y = \sqrt{\frac{\Sigma y^2}{n} - M_y^2} = \sqrt{\frac{0.4054}{10} - 0.0345}$$

$$SD_y = 0.077$$

Table A.16. Data sets for use in the computational formula for r.

x	x^2	y	y^2	xy
2.29	5.244	0.21	0.044	0.4809
2.08	4.326	0.23	0.053	0.4784
1.71	2.924	0.29	0.084	0.4959
1.67	2.788	0.26	0.068	0.4342
1.67	2.788	0.22	0.048	0.3674
1.63	2.657	0.18	0.032	0.2934
1.30	1.690	0.19	0.036	0.2470
0.97	0.941	0.15	0.023	0.1455
0.75	0.563	0.14	0.017	0.0975
0.14	0.019	0.00	0.000	0.0000

Óx = 14.21 $Óx^2$ = 23.94 Óy = 1.86 $Óy^2$ = 0.4054 Óxy = 3.0402

The correlation coefficient:

$$r = \frac{\frac{\Sigma xy}{n} - (M_x \cdot M_y)}{SD_x \cdot SD_y}$$

From the table:

$$\frac{\Sigma xy}{n} = \frac{3.0402}{10} = 0.3040$$

Therefore:

$$r = \frac{0.3040 - (1.421 \times 0.186)}{(0.616 \times 0.077)} = \frac{0.3040 - 0.2643}{0.0474}$$

$$r = \frac{0.0397}{0.0474} = 0.8375\ (0.84^{**})$$

** Denotes significance of r at 1%.

To test the significance of r at the 5% (p = 0.05) and 1% (p = 0.01) significance levels consult a table giving the values of the correlation coefficient. The significant value of r depends on the degrees of freedom (df) as with the F-test. Since the data used in the correlation is paired, the df = n-2.

Whenever r is equal to or greater then the appropriate significant value, regardless of whether r is positive or negative, we can conclude that r is significant at the level of probability being used.

From the table of correlation coefficients, for df n=8, r is 0.632 at p = 0.05 and 0.765 at p = 0.01. Since r calculated from the data (r = 0.84) is greater than the tabulated value at both levels of significance, we conclude that r is highly

significant. From the viewpoint of the top and nodule dry weights, the highly significant value of r indicates that there is a linear relationship between the dry weight of the plant tops and the nodule dry weight under the growth conditions of the experiment.

Once a relationship between the dry weights of tops and nodules has been established, the data can be represented graphically. The best straight line (the regression line) can then be drawn through the points after obtaining the regression equation describing the line. This line is easily transferred to the graph by drawing a line through any pair of points on it, preferably chosen as far apart as possible.

The equation for the regression line for a predicted value of y is given by:

$$y = \left[\frac{r \cdot SD_y}{SD_x}\right] x - \left[\frac{r \cdot SD_y}{SD_x}\right] M_x + M_y$$

Since r = 0.84; SD_y = 0.077; SD_x = 0.616; M_x = 1.421 and M_y = 0.186;

$$y = \left[\frac{0.84 \times 0.077}{0.616}\right] x - \left[\frac{0.84 \times 0.077}{0.616}\right] 1.421 + 0.186$$

$$y = 0.105x + 0.037$$

By substituting into the regression equation, when x = 2, then y = 0.246 and when x = 0.5 then y = 0.089. These two points

determine the regression line shown in Figure A.18.

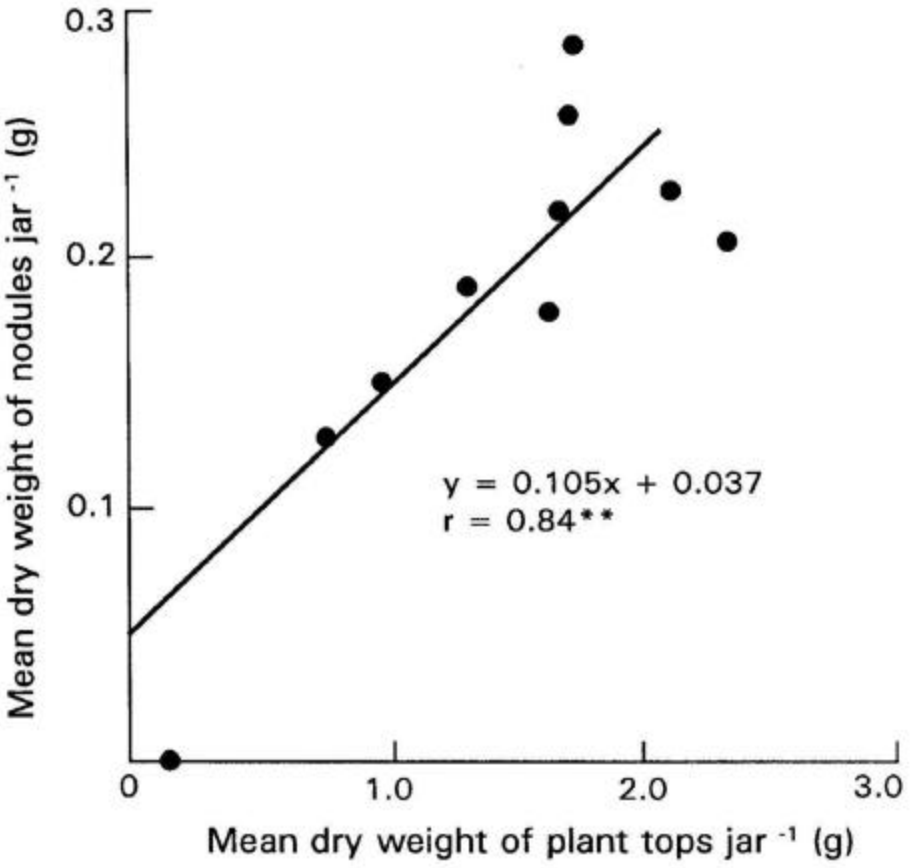


Figure A.18. Relationship between dry weights of nodules and plant tops in cowpea (*Vigna unguiculata*)

APPENDIX 19

A BRIEF DESCRIPTION OF INOCULANT CARRIER PREPARATION

Detailed operational procedures on the preparation of carriers is given by Roughley in “The Preparation and Use of Legume Seed Inoculants,” Plant and Soil, 1970, 32:675-701. The basic principles are summarized below.

Inoculum carriers may be prepared in the laboratory from peat, soils, or other materials high in organic matter. Peat is usually wet when harvested. It is drained, strained, and shredded then dried with forced air at a temperature not exceeding 100^{o}C. Higher drying temperatures should be avoided as it may cause development of toxic substances which may be harmful to rhizobia. The peat is then ground in a hammer mill to a particle size of 10 – 40 microns. Neutralization is achieved by adding calcium carbonate during mixing in a drum. The calcium carbonate may also be added later by injecting it together with the broth into the carrier sealed in a bag.

Any carrier may be produced similarly from organic matter. Its water holding capacity should be determined by adding a little water at a time until the desired consistency has been reached. The amount of calcium carbonate needed for neutralization should also be experimentally determined by adding a little at a time until a neutral point is reached.

Carriers may be heat sealed into thin gauged (1.5 mil) polyethylene or polypropylene (3.0 ml) bags. If sterilization is desired, the sealed polyethylene bags may be gamma-irradiated at 5 Mega rads. Autoclaving is possible with polypropylene bags which are heat resistant. (Polypropylene bags containing 50 g of peat are usually autoclaved for, 60 minutes at 15 lbs pressure, and 121^{o}C). Polyethylene, if not thicker than 1.5 mil, permits air exchange which is thought to be necessary to keep the rhizobia viable. Polypropylene is not permeable to air. Inoculant bags made from this material are usually perforated or sealed with a small cotton plug in the seam to allow for gas exchange.

High density polypropylene is autoclavable and can be used as a container for sterilizing the carrier. However, certain precautions need to be taken when using this material for this purpose.

Complete sealing is avoided after the carrier has been placed in the bags. Instead, the open end of the bag is folded down and held in place by a paper clip to allow steam to enter the bags during autoclaving. Also, bags are placed in wire baskets or in trays with perforated bottoms. Bags should be arranged upright with sufficient space between them to allow for steam circulation. After autoclaving, the bags are allowed to cool in the autoclave. The bags are then sealed with a bag sealer in a laminar flow hood or in a simple

transfer hood (Appendix 21). The paper clip is removed just prior to sealing.

Alternatively, about three quarters of the open end of the bag is sealed off after placing the carrier in the bag. The remaining unsealed portion is folded down and held by a paper clip. The sealing is completed after autoclaving.

APPENDIX 20

SEED INOCULATING PROCEDURE

This procedure allows the highest possible numbers of rhizobia to be applied to each seed. Recommendations for quantity of sticker (gum Arabic) and inoculant for given weights of seeds are as follows:

Table A.17. Suitable quantities of peat inoculant and sticker for inoculating legume seeds.

Legume species	Seed Weight (g)	Peat Inoculant (g)	Gum Arabic Solution (ml)
Arachis hypogaea	100	10	4.0
Centrosema pubescens	20	3	0.75
Cicer arietinum	100	7	3.5
Cajanus cajan	100	8	3.5
Desmodium intortum	10	4	1.5
Glycine max	100	10	3.0
Lens culinaris	50	5	2.0
Leucaena leucocephala	50	10	3.0
Medicago sativa	5	4	0.4
Phaseolus vulgaris	100	8	2.5
Stylosanthes guianensis	5	2	0.2
Vicia faba	100	7	3.5
Vigna radiata	100	9	3.5
Vigna unguiculata	100	8	3.5

The procedure is based on inoculation of batches of seed of the weights given in the table above. Place the weighed batch of seed in a polyethylene bag (of approximately 30 X 50 cm size) and add the volume of gum indicated in Table A.17. Shape the neck of the bag so as to permit it to be inflated and then clasp it closed. Gently shake the bag for at least

60 seconds coating all the seeds thoroughly with the gum so that they appear wet.

Open the bag and add the quantity of peat-based inoculant specified in the table. Reinflate the bag and gently shake the seeds with the peat until they are coated and appear uniformly black. Stop at this stage because prolonged shaking will break down the coating.

Immediately empty the seeds from the bag onto clean paper, spread out the seeds, and allow to air dry. Do not dry in direct sunlight.

Repeat with additional batches, using a fresh bag each time, until the quantity of seed required for the experiment has been inoculated.

Mix the inoculated seed from the various batches. The seeds are now ready for planting.

Note: The proportions of gum and inoculant have been tested with sees varieties in the NifTAL seed collection and consistently give well-coated seeds. Varieties with different seed size will require slightly different quantities of gum and inoculant. There is no substitute for trial runs to perfect your inoculation technique prior to coating seeds

that will be planted in the experiment.

APPENDIX 21

DETERMINING FIELD CAPACITY OF FIELD SOIL

Field capacity may be explained as the amount of water the soil will hold in semi-equilibrium in contact with dry soil.

The field capacity of a field soil needs to be known so that the same soil in the pots can be maintained at similar field capacity during plant growth. Serious differences in the water-status of the potted soil, especially during active plant growth can lead to large errors.

Drill a hole on the bottom of a 250 ml plastic cylinder. The hole allows air to escape when the cylinder is being filled with soil. The measuring cylinder chosen must be sufficiently transparent for making observations. Cylinders of glass or Perspex are also suitable.

Fill the cylinder with samples of soil used in the pots. Tamp the cylinder to a similar consistency as that in pots.

Pour tap water gently into the cylinder until two thirds of the volume of the soil in the cylinder is wetted (see Figure A.19). This is accomplished by pouring the water to be absorbed into the soil in small volume increments. Continue the addition of water until the migrating wet-front indicates

wetting two thirds the volume of soil in the cylinder. The migrating water-front can be observed through the wall of the measuring cylinder.

Allow the column to equilibrate in the laboratory for 48 hours.

Mark off, on the cylinder, the middle 5 cm of the column (See Figure A.19). With a metal spatula (with flat end bent 90 degrees to form a scoop) remove and discard soil until the top of the 5 cm mark is reached. Continue removing the soil, but this time do not discard the soil. Collect the soil in a metal weighing boat. Continue removing the soil until the lower 5 cm mark. Then weigh the soil on the weighing boat and dry the soil in an oven at 110^{o}C, to constant weight. Calculate percent moisture in the soil.

Obtain another sample of soil of the same soil weight (20-30 g) and dry at 110^{o}C to constant weight. This sample gives the moisture content of the soil not adjusted to field capacity.

The following examples illustrates the use of the weight measurements to determine the field capacity. The following assumptions are made to simplify the calculations.

1) The moisture content of the potting soil at field capacity = 25%.

2) The moisture content of moist soil (soil not adjusted to field capacity) = 15%.

3) The weight of moist soil in pots = 2.4 kg.

4) The dry weight of field soil = 2400 X 0.85 g.

The dry weight of adjusted soil = W X 0.75 g.

Use the following equation to calculate the total weight of adjusted soil:

$$2400 \times 0.85 = W \times 0.75;\ W = 2720\ g$$

Add 320 g of water (2720 g – 2400 g) adjust the moisture level of 2400 g of soil from 15% to 25%.

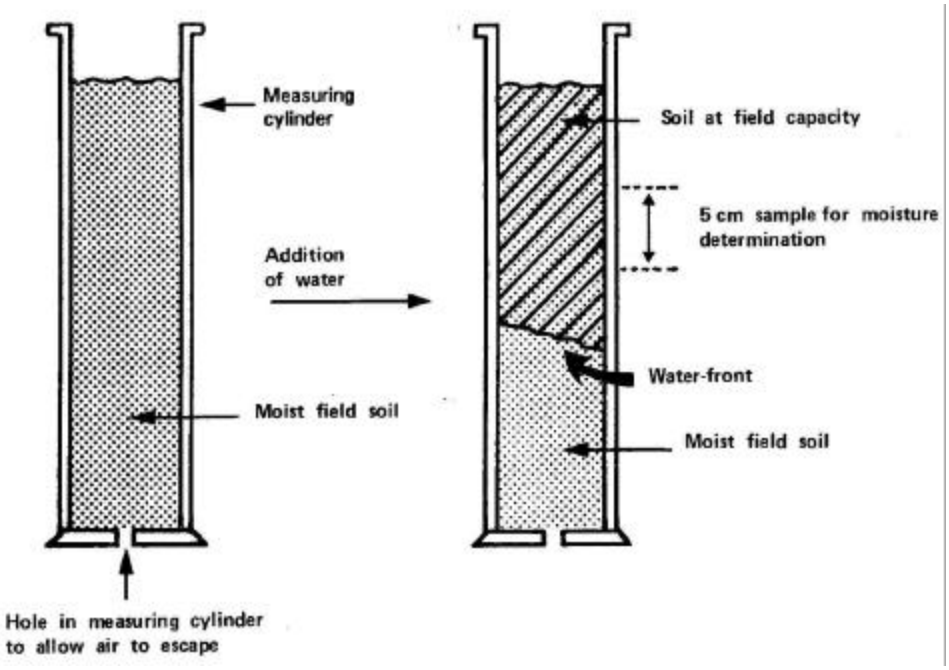


Figure A.19. Determining field capacity of field soil.

APPENDIX 22

THE SIMPLE TRANSFER CHAMBER

A simple transfer chamber for aseptic work may be constructed from the materials listed in the following text, and according to the plans in Figures A.20, A.21 and A.22.

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.

The Bunsen burner is inserted into the base of the chamber through a hole, allowing approximately 1 inch of the tip of the burner to protrude into the chamber. 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 the air inside the chamber resulting in a unidirectional warm air current. This warm air current exits through the open front, preventing entry of contaminants.

When using this chamber, the following instructions should be followed:

(1) Open the hinged door and wipe the interior thoroughly

with an antiseptic such as 70% ethanol. Allow the ethanol to dry.

(2) Turn on the gas and light the burner. The flame should be blue and adjusted to no more than 6 cm in height.

(3) Close the hinged door and wait 20 minutes before using the chamber.

When through working in the chamber, turn off the flame and disconnect the gas line on the outside of the chamber. This is an important safeguard to prevent the possibility of gas leaking into and filling the chamber, which could result in an explosion the next time the burner is lit. Such an explosion is not only theoretically possible but has happened when proper precautions were not observed. With correct practice and precautions, this transfer chamber can produce good results.

Components of the Simple Transfer Chamber

(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 cm diameter hole for bunsen burner.

(3) Top: made of plywood.

(4) Reinforcement: made of hardwood or plywood; serves as anchor for the door.

(5) Door: made of plate glass with hardwood frame; is attached to the reinforcement plate via hinges.

(6) Two sides: made of plywood and glass.

(7) Eight wooden moldings: to hold glass for window and door.

(8) Eight wooden moldings: to hold glass for the back window.

(9) Sixteen wooden moldings: to hold glass for the side windows.

(10) Four wooden legs: 10 cm high.

The plywood used should be 2 cm thick with a smooth finish on both sides. The chamber should be painted with oil based epoxy paint, leaving a hard, smooth coat.

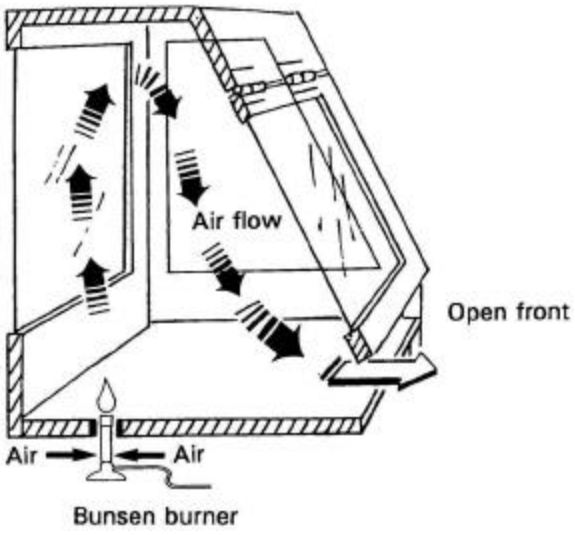


Figure A.20. Cross section of Chamber Illustrating Working Principle.

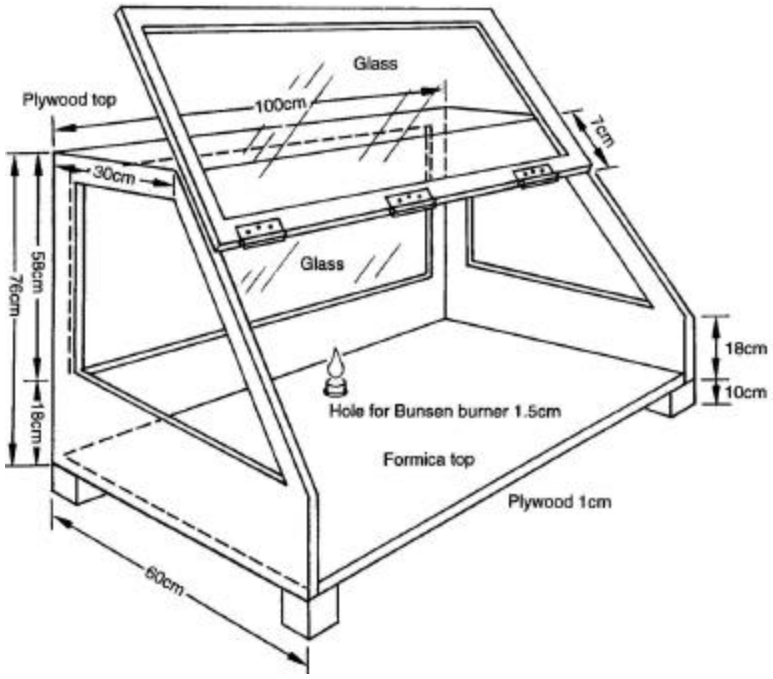


Figure A.21. Simple Transfer Chamber.

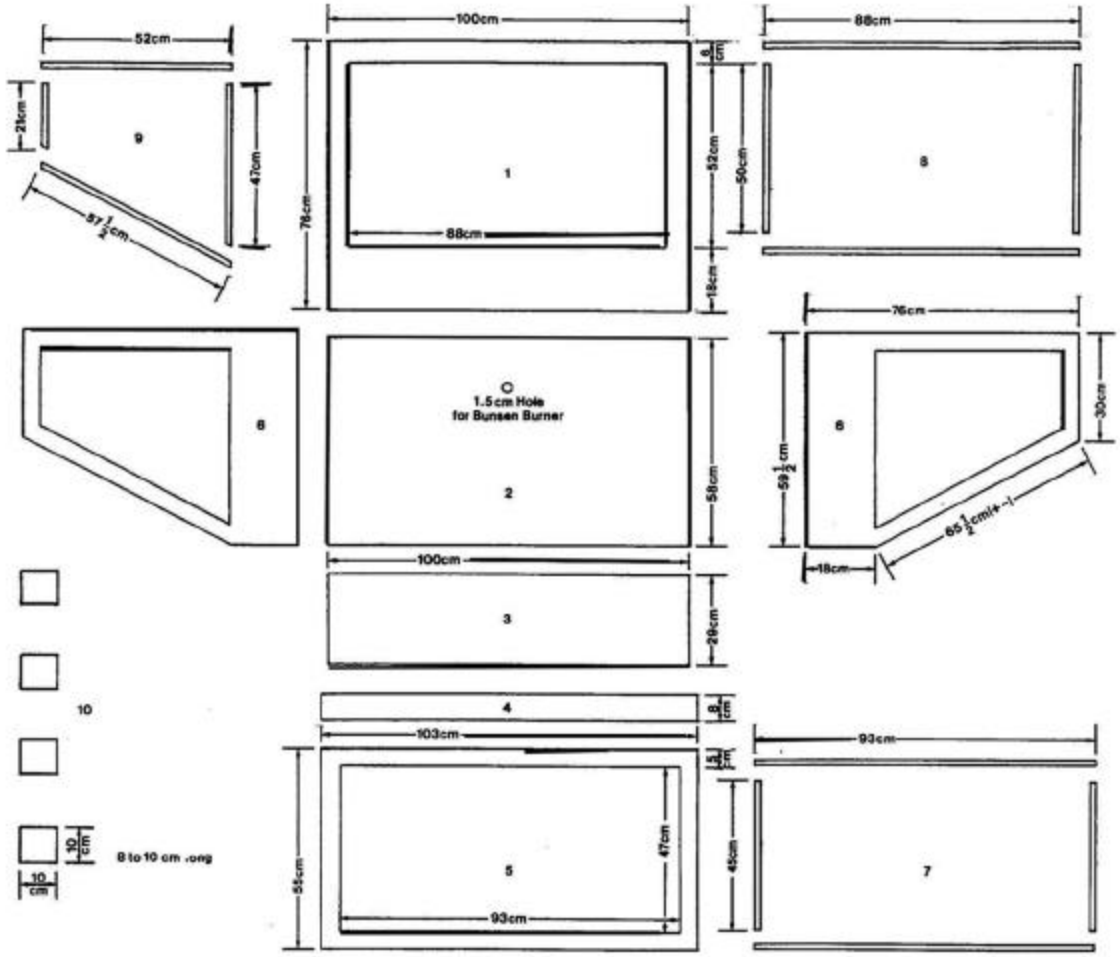


Figure A.22. Components of Simple Transfer Chamber.

APPENDIX 23

FREEZE DRYING CULTURES OF RHIZOBIA

Freeze drying or lyophilizing is a method of stabilizing materials of biological origin. This is one of the preferred methods for the long-term storage of cultures of microorganisms. Cultures of rhizobia remain viable for many years when freeze dried and vacuum sealed in glass ampoules.

Summary of the Process

Freeze drying allows moisture to be removed from the material without concurrent biological changes. This is done by removing moisture under a vacuum. To prevent frothing as air is withdrawn when the initial vacuum is applied, the culture is either prefrozen or subjected to centrifugation. In the former case, the ice will change directly from the solid to the vapor stage. In the latter, the temperature of the suspension falls as the water vapor is removed until it freezes and further drying occurs by sublimation.

During freeze drying, the ice does not evaporate simultaneously from all parts of the material, but continuously from the outer boundary until only a dry cake is left, resembling the original sample in size and shape.

Freeze drying equipment may come with a variety of accessories. The essential components of freeze drying apparatus are: a vacuum chamber to hold the material to be freeze dried or a manifold to which ampoules or a vacuum vessel can be attached; a water trap; and a vacuum pump. A vacuum gauge is usually connected to the system between the water trap and the vacuum pump. The water vapor which evolves during freeze drying is captured in the water trap, thus prevented from entering the pump. Water traps may be chambers to which drying agents have been added, such as phosphorus pentoxide, or they may be refrigerated condensers with compressors capable of cooling temperatures below -40°C.

Evaporation may be hastened by heating the materials to be freeze dried. The action of the vacuum will keep the material frozen as long as it contains water. The rate and efficiency of the flow of water vapor from the material to the condenser chamber is directly related to the vapor pressure differential; that is, the vapor pressure of the frozen material minus the vapor pressure of the condenser chamber. Since vapor pressure and material temperature are inversely related, it is desirable to have a condenser temperature of about -40°C to -50°C, and a material temperature as high as possible without causing a meltback of the material. For cultures in ampoules, room temperature is usually sufficient.

Freeze drying is carried out in two stages. During the

primary stage, 90%-95% of the moisture is removed. After the secondary stage, approximately 1% of the moisture remains. The retention of a small amount of moisture is essential for the survival of bacteria. This is achieved by suspending the cells in a medium which will not permit complete removal of moisture. At NifTAL a mixture of peptone (5%) and sucrose (10%) is used. Since a high fatality rate occurs even under these conditions, highly concentrated cell suspensions are used.

It is often convenient to use one machine for the first stage and another machine for the second stage of freeze drying as the setup for each stage is different. Ampoules are constricted with an ampoule constrictor after the first stage of drying. This permits easier sealing under vacuum after the second stage has been completed. The ampoules are tested with a high frequency tester to assure successful sealing, then stored in the dark in a metal drawer cabinet at room temperature.

Practice of Freeze Drying

The practice of freeze drying may vary from laboratory to laboratory. The methods described below are performed at NifTAL.

a) Preparation of Cotton Plugs

Cotton plugs are used to plug ampoules. No. 0 dental cotton balls (Richmond Dental Cotton Co., NC, USA) may be used for this purpose. Prior to use they are placed into 100 ml beakers, covered with aluminum foil, and sterilized by autoclaving. This is followed by a 1 h drying period at 80°C in a dry air oven.

b) Preparation of Labels

Paper and ink must be compatible (nontoxic) with the rhizobia and resistant to moisture. Whatman #1 filter paper, purchased in large sheets, and ordinary typewriter ink of vegetable base are suitable. An IBM computer equipped with a printer is used for typing the labels with the strain identification number and date. Only one identification number is printed at one time. The labels are cut manually to measure 4 x 25 mm. A margin of 10 mm is left on one side. This empty margin will later be touching the bottom of the ampoule thus preventing the written part from being submerged and rendered unreadable when the cell suspension has been added.

c) Preparation of Ampoules

Freeze drying ampoules of 0.5 ml capacity, inner diameter of 6 mm and 100 mm length, are purchased from Edward's High Vacuum, W. Sessex, UK. They are checked for defects such as cracks

and pinholes, then soaked in 10% HCl overnight. They are then rinsed in tap water at least six times or until the pH of the last washing is neutral indicating complete removal of the acid. This is followed by three rinses with deionized water and drying in the oven. Labels are added to the ampoules with forceps. The ampoules are then placed into a 250 mm beaker, covered with aluminum foil and autoclaved. The now sterile ampoules are dried in an oven at 80°C for 1-2 h.

d) Preparing Freeze Drying Medium

A solution is made in distilled water containing 5% peptone and 10% sucrose. The peptone/sucrose solution is dispensed in 2 ml portions into snap top culture tubes and sterilized by autoclaving.

The inclusion of 10% sucrose or another sugar in the freeze drying medium will automatically cause it to retain 1% moisture after dehydration. This will improve the viability of the suspended organism. Total desiccation would result in death of all bacteria.

e) Growing and Harvesting Cultures for Freeze Drying

Only authenticated cultures should be selected for freeze drying. They should be tested again for purity by streaking them out on YMA plates containing Congo Red and plates

containing BTB as well as by Gram stain. If antisera are available, they should be used as an additional check for strain identity and purity of culture.

After these tests, the cultures are grown on YMA slants in 50 ml culture tubes at 25-30°C. They should be harvested a few days after their log phase of growth. All work should be done under strict aseptic conditions in a transfer chamber.

Two ml of the previously prepared peptone/glucose medium are added to each slant culture. The growth is gently dislodged with an inoculation loop and then transferred into a 10 ml vial. In the case of large batches, the growth from several slants is pooled in a 50 ml culture tube. The suspension is emulsified on a vortex mixer and immediately transferred to the freeze drying ampoules. The cell suspension should contain approximately between 5×10^9 to 1×10^{10} cells per ml. Usually 6-8 ml are sufficient for 30-40 ampoules.

f) Filling the Ampoules

For this operation, stringent aseptic conditions cannot be over-emphasized. The work should be carried out on a laminar flow chamber that has been cleaned with an antiseptic such as 70% ethanol, and, if possible, irradiated with ultraviolet light for 20 min before use. As an additional precaution, wearing a disposable face mask and sterile surgical gloves is

recommended.

To avoid a "mix-up" and/or cross-contamination, only one strain should be handled at a time. Sterile cotton plugged Pasteur pipettes with long, fine capillaries and equipped with a rubber suction bulb of 1 ml capacity are used to transfer the cell suspensions to the ampoules. Eight drops of suspension, delivered by a Pasteur pipette with a 16 gauge tip will equal a volume of approximately 0.2 ml of material. If each ampoule receives 0.2-1 ml, the actual number of cells per ampoule are: $0.2 \times 5 \times 10^9 = 1 \times 10^9$ cells. This is a sufficiently large number for survival.

Loading the ampoules requires a steady hand and practice. Contamination of the upper portion of the ampoule with the cell suspension should be avoided as this will cause charring during the constriction process.

If large batches of ampoules are to be filled, a repetitive Cornwall syringe (available through Scientific Products, Co., USA) of 1 ml capacity is recommended.

After filling, use a sterile glass rod to push a sterile cotton plug into the center of each ampoule. A second sterile cotton plug is used to close the opening. The ampoules are then loaded into a paper towel lined VirTis vacuum jar (available through Scientific Products, Co., USA). The jar

holds approximately 50 ampoules.

Ideally, freeze drying should be carried out at this stage without delay. We frequently store filled and plugged ampoules contained in a vacuum jar in a freezer overnight, without ill effect to the survival of the cultures.

g) Primary Freeze Drying

We use a LABCONCO No. 12 freeze dryer (Lab Con Co Corporation, Kansas City, MO, USA) for the first stage of lyophilization. It is equipped with a large 48 port manifold, a freeze bath, a condenser chamber, and a heavy duty vacuum pump. The machine has two compressors, one for the freeze bath and the other for the condenser. A McLeod manometer is used to monitor the vacuum.

On the night before use, the freeze bath is filled to approximately the 10 cm level with methanol, and its condenser is activated. The bath will reach a temperature of -40°C on the following morning. Vacuum jars containing ampoules may then be placed in the freeze bath. The condenser chamber is closed, and its compressor turned on. The condenser temperature usually drops to -40°C in 20 min. The vacuum pump may then be activated. Fifteen minutes later, the vacuum gauge should indicate a reading below 0.1 torr. The vacuum jars containing the frozen ampoules may then be removed from

the freeze bath and attached to the manifold. This should be done quickly to prevent thawing of the ampoules and a subsequent bubbling over of the suspensions. Sufficient time should be allowed for the vacuum to re-establish itself between the attaching of each jar. The paper towel liner in the jar will help to prevent a thawing of the material. As an additional precaution, the jars may be further insulated by wrapping them in paper bags for an initial 30 min or until the evaporating water is cooling the suspensions in the ampoules effectively. Freeze drying is continued for approximately 6 h. The primary drying is completed when the pressure gauge shows a reading of 1.3×10^{-1} mbar or below.

h) Prior to Secondary Freeze Drying

The ampoules are constricted at approximately 6 cm as measured from the bottom. The constriction should be done in equal distance from each of the two cotton plugs to avoid charring, which may have a toxic effect on the culture.

Constrictions may be done manually over a finely adjusted propane plus oxygen flame. This is a learned skill which requires practice. The ampoule is rotated slightly below the tip of the blue flame so the flame passes over the horizontally held tube but not below it. The rotating is continued until the walls of the heated area have constricted and thickened and the inner diameter is not more than 2 mm.

At this point, the ampoule is removed from the flame and pulled out until the inner diameter measures a little less than 1mm.

At NifTAL, most ampoules are constricted on an Edwards Ampoule constrictor (Edwards High Vacuum). This machine performs beautifully on a propane plus air flame, provided both the retaining wheels are slightly adjusted from paralleled to toed-in position during the process, and the flame is properly adjusted. Constricting one ampoule requires approximately 1 min.

i) Secondary Freeze Drying

An Edward's Modulyo freeze dryer is used at NifTAL for the second stage of freeze drying. This unit is equipped with a double manifold which can hold 96 ampoules, a condenser chamber, and a two stage vacuum pump. Pressure is measured by a built-in Pirani gauge.

The condenser is switched on until a temperature of -50°C has been reached. Then, the vacuum pump is activated and freeze drying is continued for 12-18 h to reduce the moisture level in the ampoules to 1%. At the completion of freeze drying, the reading on the Pirani gauge should show a pressure of 0.01 torr or less. The ampoules are then sealed with a twin jet torch (Figure A.23). This is done by heating both sides of

the constriction simultaneously (Figure A.24), and pulling gently at the bottom of the ampoule with a slight twist until the constricted area has sealed and is disconnected from its upper end which remains on the freeze dryer. The freeze dryer may then be switched off and air permitted to flow slowly into the chamber. The drain should be opened to remove the condensed water.

The ampoules are checked for presence of leaks before storage. This is done with an Edward's T2 HF ampoule tester which is a high frequency probe. At discharge, a properly sealed ampoule will display a blue flame. Ampoules without vacuum seals will show no color. The spark tester should be used only briefly on each ampoule as each discharge may kill a certain number of bacteria.

j) Storing the Freeze Dried Cultures

Ideally, lyophilized cultures of rhizobia should be stored at 4°C and in the dark.

Optimal storage conditions are not always available and storage at room temperature and away from light is an accepted alternative. At NifTAL, cultures are stored within a steel cabinet in an air conditioned room held at 20°C.

k) Opening of Ampoules

Ampoules containing freeze dried bacteria culture should be opened in an aseptic environment. A mark is filed on the ampoule at about the middle of the cotton wool plug, and a red hot glass rod is applied to the mark. The ampoule should then crack at the marked area. Care should be taken in opening the ampoule slowly so that the onrushing air will filter through the cotton plug without drawing it into the ampoule.

Often the heated glass rod will not cause the desired crack at the mark. In such a case, two layers of sterile tissue paper are wrapped around the ampoule and minimal pressure is applied to break open the ampoule at the file mark. This method is especially recommended for ampoules which do not contain cotton plugs.

The cotton plug is removed with forceps and discarded as culture may be adhering to it. It should be replaced with a new sterile cotton wool plug.

The contents of the ampoule is rehydrated with 0.5 ml sterile water. Since the number of surviving cells may be low, attempts are made for maximum recovery. A loopful is streaked out on a YMA plate containing Congo red and on another plate containing BTB. The label which may contain a large number of cells is transferred to another YMA plate.

The remainder of the culture is then removed with a sterile Pasteur pipette and added to 50 ml YM broth contained in a 125 ml Erlenmeyer flask. Broth and plate cultures are then incubated at their optimal temperatures.

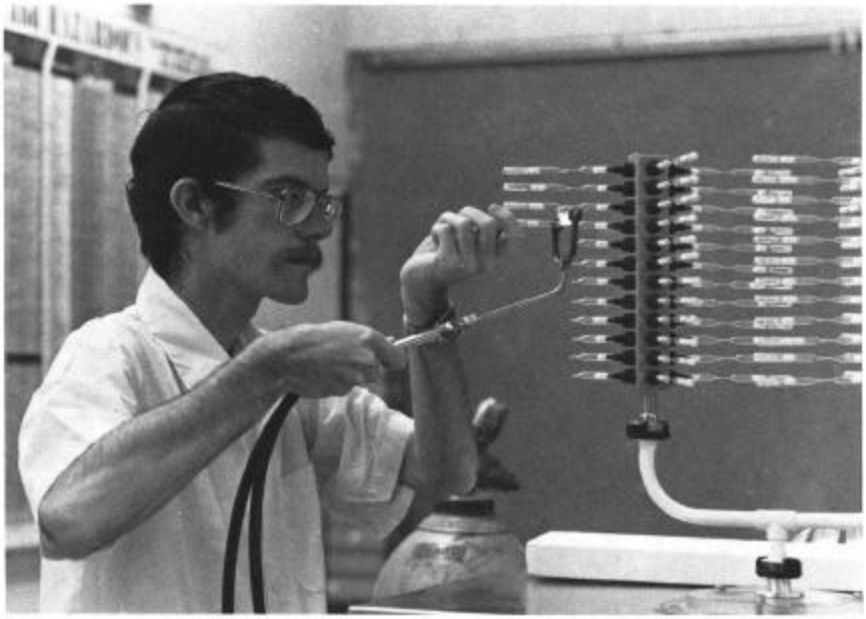


Figure A.23. Sealing ampoules

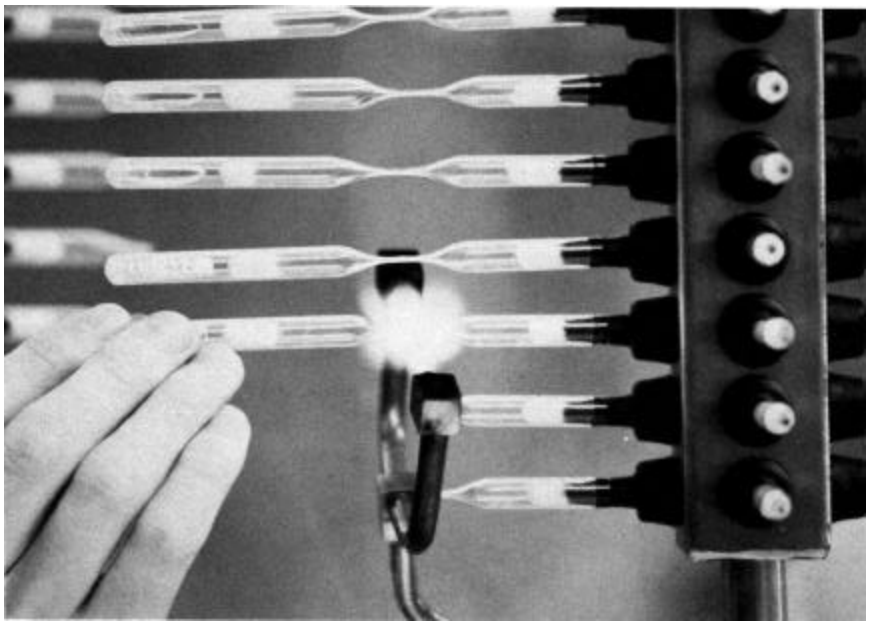


Figure A.24. Sealing ampoules (close-up)

APPENDIX 24

SOURCE OF RHIZOBIA STRAINS

The NifTAL Rhizobia Germplasm Resource is a comprehensive collection of rhizobia for numerous legumes (tropical and temperate) and is maintained at the NifTAL Center. All strains cited in the various exercises of this book are available on written request addressed to: Curator, Rhizobia Germplasm Resource, NifTAL Center and MIRCEN, University of Hawaii, 1000 Holomua Road, Paia, Hawaii 96779, USA.

The INLIT strains of rhizobia are also available. INLIT is an acronym for NifTAL's **I**nternational **N**etwork of **L**egume **I**noculation **T**rials in which response to inoculation with rhizobia on 18 species of economically important legumes were tested worldwide. A set of three effective and antigenically distinct strains of rhizobia tested in the INLIT are listed in Table A24.1. Because each strain in the group of three rhizobia recommended for each legume is antigenically distinct, serological methods of strain identification can be used to study competition, persistence and other ecological aspects.

There are also other laboratories/institutions which maintain collections of rhizobia:

Dr. Carlos Batthyany
Nitrosoil, Florida 622, 4 Piso
Buenos Aires,
ARGENTINA

Rhizobia for Tropical Legumes

Dr. R. J. Roughley
Australian Inoculants Research and Control Service
Horticultural Research Station
P.O. Box 720
Gosford, N.S.W. 2250
AUSTRALIA

AIRCS Strains

Dr. R. A. Date
CSIRO, Div. Tropical Crops and Pastures
Mill Road, St. Lucia
Queensland 4067
AUSTRALIA

Rhizobia for Tropical Legumes

Dr. F. Bergersen
Microbiology Section
CSIRO, Div of Plant Industry
Canberra, ACT 2600
AUSTRALIA

Rhizobia for Clovers, Medics and other Temperate Species

Prof. J. R. Jardim Freire
Rhizobium MIRCEN
IPAGRO
Caixa Postal 776
90000 Porto Alegre Do Sul
BRAZIL

Rhizobia for Tropical Legumes

Dr. D. J. Hume
Crop Science Dept.
University of Guelph
Guelph, Ontario N1G 2W1
CANADA

Rhizobia for Pea, Lupin,Alfalfa and Soybean

Dr. John Day
Soil Microbiology Dept.
Rothamsted Experimental Sta.
Harpenden, Herts. AL5 2JQ
UNITED KINGDOM

Rhizobia for Clovers, Alfalfa, Peas, Beans, and other Temperate Legumes

Plant Diseases Division
D.S.I.R.
Private Bag
Auckland,
NEW ZEALAND

Rhizobia for Clovers, Alfalfa, and Lupin

Dr. Peter van Berkum
USDA CCNFL
Bldg. 001 Rm 309 BARC-W
Beltsville, MD 20705
USA

Rhizobia for Soybean and Temperate Legumes

Dr. O. P. Rupela
Senior Microbiologist
Legumes Program
ICRISAT
Pantancheru, A.P. 502 324
INDIA

Rhizobia for Chickpea, Pigeon Pea, and Peanut

More addresses of institutions which have rhizobia collections can be found in Skinner, F.A., E. Hamatova and V.F. McGowan, 1983. *In*: World catalog of ***Rhizobium*** Collections. V.B.D. Skerman (ed.). World Data Center for Microorganisms at the University of Queensland, Brisbane, Australia.

Table A.18. Legumes and recommended strains of rhizobia

LEGUMES	RHIZOBIA*	TAL#	OTHER DESIGNATION(S)
Arachis hypogaea	B	1000	TAL 1000
		169	Nit 176A22(Nitragin)
		1371	T-1; Nit 8All(Nitragin)
		1127	IHP 38

LEGUMES	RHIZOBIA*	TAL#	OTHER DESIGNATION(S)
Cajanus cajan	B	1132	IHP 195
		569	MAR 472
Centrosema pubescens	B	651	UMKL 44
		655	UMKL 09
		1146	CIAT 590
Cicer arietinum	R	620	IHP 3889; CB1189
		480	UASB 67
		1148	Nit 27A3 (Nitragin)
Desmodium intortum	B	569	MAR 472
		1147	CIAT 299
		667	CIAT 13; MAR 471
Glycine max	B	102	USDA 110
		377	USDA 138
		379	CB 1809; USDA 136b
Lens culinaris	R	634	Nit 92A3 (Nitragin)
		638	I-2
		640	I-11
Leucaena leucocephala	R	82	TAL 82
		1145	CIAT 1967
		582	CB 81
Medicago sativa	R	380	SU 47
		1372	POA 116
		1373	POA 135
Phaseolus lunatus	B	22	TAL 22
		169	Nit 176A22 (Nitragin)
		644	CIAT 257
Phaseolus vulgaris	R	182	TAL 182
		1797	CIAT 899
		1383	CIAT 632
Pisum sativum	R	634	Nit 92A3 (Nitragin)
		1236	ALLEN 344
		1402	Nit 128C75 (Nitragin)
Psophocarpus tetragonolobus	B	228	TAL 228
		1021	Nit 132B13 (Nitragin)
		1022	Nit 132B14 (Nitragin)
Stylosanthes guianenis	B	309	CB 756
		310	CB 1024
		658	CIAT 71
Vicia faba	R	1397	Nit 175F9 (Nitragin)
		1399	Nit 175F12 (Nitragin)
		1400	Nit 175F16 (Nitragin)

LEGUMES	RHIZOBIA*	TAL#	OTHER DESIGNATION(S)
Vigna mungo	B	441	UPLB M6
		420	THA 301
		169	Nit 176A22 (Nitragin)
Vigna radiata	B	441	UPLB M6
		420	THA 301
		169	Nit 76A22 (Nitragin)
Vigna unguiculata	B	209	TAL 209
		173	Nit 176A30 (Nitragin)
		658	CIAT 71

*B = ***Bradyrhizobium***; R = ***Rhizobium***. Each group consists of three antigenically distinct strains of rhizobia.