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**CRITERIA FOR SELECTING
INFECTIVE AND EFFICIENT
STRAINS OF RHIZOBIUM FOR USE
IN TROPICAL AGRICULTURE**

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I. INTRODUCTION

As a general concept, tropical soils often lack available nitrogen. Of the two generally available forms of nitrogen, that fixed biologically is more economical than fertilizer N. The legume - Rhizobium symbiosis may account for 100-200 KgN/ha/yr (Norris and Date, 1976). Exploitation of this phenomenon provides an important, highly economical source of available nitrogen.

The selection of appropriate strains of Rhizobium is the first key step in effective inoculation of legumes. The most important criterion in selecting a strain is ability to form effective nitrogen-fixing nodules on the appropriate legume host, preferably under a wide range of conditions. Effectiveness, as measured by dry matter yield or the amount of nitrogen fixed, is still the fundamental criterion for selection although various other tolerances are now recognized as important.

The ability of a strain to survive in soil and compete with native strains (Means, Johnson and Erdman, 1961; Caldwell, 1968) is important to successful nodulation and nitrogen fixation. Early nodulation over a range of soil temperature and pH; ability to grow well in broth and peat (or other inoculum carrier) (Brockwell et al., 1968); tolerance to fungicides (Afifi et al., 1969; Kecskes and Vincent, 1969) and the ability to nodulate and fix nitrogen in the presence of high levels of available nitrogen (Gibson et al., 1971) are other important criteria. Discussion of these are found in several reviews (Norris and Date, 1976; Vincent, 1970; and Date, 1976).

The criteria listed above provide a general overview of important considerations in the selection of Rhizobium, per se. However, specificity

of the strain of microsymbiont and of the host genotype also deserve consideration. Some tropical legumes demonstrate a high degree of symbiotic specialization, whereas others are relatively non-specific and will nodulate promiscuously with native rhizobia (Norris and Date, 1976). Inoculation is often required with highly specific host. Strain selection is essential for preparation of effective inocula.

II. ASSESSMENT OF THE NEED FOR INOCULATION

Effective Rhizobium are essential to providing a beneficial symbiotic relationship with the host legume. In most parts of the world there is a broad range of rhizobia strains which vary in the degree of effectiveness and competitiveness. In some areas very effective and competitive strains may be the major constituents of the native rhizobial populations, but in other areas these strains may be: (1) lacking or (2) less effective and/or (3) less competitive. In the latter cases where there is no native rhizobial population or satisfactory strain, introduction of a superior strain must be made to create a greater potential for maximum yield (i.e. increase in nitrogen fixation). Many recent studies have been done which establish that inoculation with a superior strain is a method for increasing yields in legumes (Dube, 1976; Ham, Caldwell and Johnson, 1971; Subba Rao, 1975; Sundara-Rao et al., 1975; Sundara Rao, 1976). Some commercially prepared inocula have also improved yield (Able and Erdman, 1964; Chhonkar and Negi, 1971; Dube, 1976; Dunphy, 1978, Dunphy et al., 1977). Before beginning any study on improving yield (enhancing nitrogen fixation) in legumes through Rhizobium strain selection, there must be an assessment of the need for inoculation. This can be accomplished two ways:

- (1) Vincent (1970) describes a method to assess the need for inoculation by observing nodules on uninoculated legumes already growing in the test area and recording:
 - a. The proportion of plants bearing nodules.
 - b. The abundance or sparseness of nodules on the nodulated plants.
 - c. The location of nodules on the root system (crown, distal; tap-root, lateral).
 - d. The size and appearance of nodules: whether white in section (inactive; no N-fixation), pink with leghemoglobin (active, N-fixation) or with a green area to the base of a restricted red zone, indicative of previous leghemoglobin (inactive or partially active). (This last condition will have to be interpreted in relation to the stage of maturity of the nodules and conditions affecting growth of the plant and photosynthesis; indicating that this method of analysis is not absolute and certain physiological and environmental factors can effect coloration in the nodule).

- (2) Date (1976) attempts to assess the need for inoculation from another approach. In his approach he tests the success of inoculation through three treatments:
 - a. Uninoculated control plots to check for presence of natural occurring rhizobia.
 - b. Plots inoculated with potentially useful strain known to be effective in controlled-environment or glasshouse tests.
 - c. Plots inoculated with the same strain and supplied with combined nitrogen. This approach involves a three part evaluation of a particular strain. The first part assesses the need for inoculation. In the second part the competitiveness of a strain is compared to the native Rhizobium population. In the final part the ability of the strain to produce efficient nodules in the presence of available combined nitrogen is evaluated as a method of evaluating the suitability of the strain for inoculant production.

This method is analogous to the experiments where one assesses the success of inoculation of a particular strain. Details of this methodology are presented in this text (Section V).

III. CRITERIA FOR SELECTION OF EFFECTIVE STRAINS

In the analysis of effective strains of Rhizobium criteria for selecting superior strains must be established. Many factors should be considered when evaluating a particular strain. One should consider the three variables which affect the Rhizobium-legume symbiosis: (1) the host, (2) the environment and, (3) the organism, itself. In most cases the environment and host legume have been already selected. The burden of improving legume yield via improved nitrogen fixation lies in the selection of effective rhizobia.

Persistence in the soil is a characteristic with which one should be concerned. Within this characteristic such categories as: (1) survival of Rhizobium while not associated with legumes, (2) the ability to multiply in the soil such that rhizobial population is capable of nodulating the legume host, and (3) the ability of the Rhizobium to persist in the soil and produce effective nodules on the roots of regenerating annuals or on new roots of perennials (Date, 1975).

Aside from these primary prerequisites for an effective strain many various secondary characteristics should be considered such as pH tolerance (Norris, 1973; Norris and Date, 1976; Roughley, 1976; Able and Erdman, 1964), ability to survive in different soil types (Balasundaran, 1976; Kumara-Rao et al, 1975; Agati and Garcia, 1940), ability to grow and nodulate in the presence of available combined nitrogen (Sundara-Rao et al, 1975; Weber, 1966; Gibson et al, 1971; Ham, Lawn and Brun, 1976; Sistachs, 1976; Allison, Goover and Minor, 1942; and Misra and Gaur, 1974).

A. Effectivity in Nitrogen Fixation

Effectivity is the primary concern of anyone who is attempting to increase legume yield through symbiotic nitrogen fixation. Effectivity as evaluated by increase in dry weight yield or total-N-content is the fundamental criterion for selecting superior strains. Effectivity is probably the easiest parameter of nitrogen fixation to evaluate. Many quantitative tests have been developed to measure the reduction of dinitrogen to ammonia. Some of these tests involve expensive equipment and highly trained personnel to operate the equipment and interpret the data. In selecting rhizobia one should use simple tests such as dry weight yield or total-N-content in evaluating a strain for effectivity. Once an effective strain has been determined, it is necessary to establish whether that strain will nodulate and produce efficient nodules in the presence of a native rhizobial population.

B. Competition for Nodule Formation

An important characteristic in strain selection is the ability of a strain to successfully compete with the less effective native rhizobia for nodule (infection) sites. The Rhizobium-legume interaction, itself, may dictate whether a superior strain will compete with the native rhizobial population. In many cases, less effective strains are more competitive than highly effective strains. It is possible to visualize that less effective native Rhizobium could saturate infection sites preventing nodulation by the applied strain. In some instances, a strain may be more efficient in one host genotype while not in another. Ideally, one should be able to introduce a highly effective Rhizobium strain to

a field where a less effective native population is present and obtain an efficient symbiosis. In the greenhouse this concept holds true, but in the field where various environmental conditions exist the selection of Rhizobium tolerant to various environmental conditions are essential to achieving maximum benefit from symbiotic nitrogen fixation.

C. Environmental Conditions and Nitrogen Fixation

The major environmental conditions which should be considered are:

1. Extremes in temperature
2. High elevation
3. Varying soil types
4. High levels of available soil nitrogen
5. Moisture levels
6. Extremes in pH

The rationale for using these environmental conditions as criteria for selection are as follows:

Extremes in temperature. The effect of temperature on symbiotic nitrogen fixation has been well documented (Gibson, 1961; 1963; 1967a; 1967b). This effect can be examined in the laboratory or in growth chambers using a tube method (see Appendix II, sections D and E). Effectivity is measured by total-N-content in these experiments. The optimum temperature for the growth of rhizobia is 28°C. Depending on the environment where the rhizobia are intended, it may be important to select rhizobia which are tolerant to higher temperatures than 28°C. The selection of high or low temperature tolerant Rhizobium should be supplemental to an overall selection of effective strains. One factor which should be considered when evaluating temperature tolerances of the

Rhizobium strains is the temperature optimum of the particular host plant which in most cases is 28°C.

High elevations. In many areas of the world it is necessary to cultivate legumes in high elevations (above 1500 m). The native populations of rhizobia (particularly Rhizobium trifolii) in high elevations are relatively ineffective for recognized cultivars (Brockwell, Bryant, and Gault, 1972). Bryant (1971) proposes that the poor persistence of white clover at high elevations (above 1500 m) is partially due to poor nodulation (Brockwell, Bryant, and Gault, 1972). In high elevations the rhizobia fail to persist and are ineffective with the legume host. If it is necessary to cultivate legumes in high elevations such as using legumes (white clover or subterranean clover) to reclaim eroded catchments, borrow pits or strip mining pits, it may be necessary to select rhizobia which persist in soil at high elevations (above 1500 m). While high elevation, in itself, probably does not influence nitrogen fixation, factors which accompany high elevation such as low temperature and low micronutrient level may limit the potential of an introduced strain.

Varying soil types. Damirgi, Frederick and Anderson (1967) showed that particular strains of Rhizobium were associated with particular soil types. This is an important criteria for selection of effective Rhizobium in that one strain of Rhizobium may be effective in one soil type and not in another. This phenomenon may be related to the pH of the soil, the organic content, or the ionic composition. It is recommended that strains be selected from the environment where they are

intended to be used and in that selection the most effective may be isolated.

High levels of nitrogen. A factor which less developed countries should be aware of is that in some tropical soils high levels of available nitrogen may exist (18ppm to 400ppm). Such levels of nitrogen are likely to inhibit nodulation (Norris and Date, 1976), while not providing nitrogen necessary for optimal plant growth. This problem could be alleviated by selecting rhizobia tolerant to high nitrogen levels, but in this case a simple agricultural practice of mulching can also alleviate this problem by removing available nitrogen (Griffith and Manning, 1949; Griffith, 1951). Mulching in this case may provide a much less expensive alternative than strain selection for nitrogen tolerant rhizobia.

Moisture levels. High moisture levels reduce the number of rhizobia in a soil population. It has been shown that certain species of rhizobia withstand desiccation in field soils (Parker, Trinick, and Chatel, 1977). While moisture stress has received some attention in the literature, a strategy for selecting rhizobia for tolerance to moisture stress remains to be postulated. This criterion should be considered when introducing new strains to a particular environment.

pH extremes. The optimum pH for rhizobia is 6.8. Most rhizobia will not grow at pH 4.0 or lower, while the upper limit is 8.5 (Date and Halliday, 1979). All types of rhizobia grow best in a buffered environment where the pH does not vary more than 0.5 pH units. pH tolerance would allow introduction of a new strain of Rhizobium to many different soils.

We have illustrated many criteria important in the selection of rhizobia. There are two primary criteria which should be considered in every strain selection: (1) Effectiveness and (2) Competitive ability. There is an additional criterion which should be considered which is the ability of a strain to survive and persist in the soil in various environmental conditions. Due to the diversity of environmental factors which may effect the symbiosis, one should select strains which can overcome environmental obstacles and provide an efficient symbiosis.

Date and Halliday (1979) present an excellent example illustrating the selection of rhizobia for growth in a particular region. They were concerned with the selection of strains which would form an efficient nitrogen-fixing symbiosis with pasture legumes in the presence of adverse environmental conditions of the tropical savannahs of South America such as low pH, limited available phosphorous and high aluminum and manganese content. The isolation of rhizobia from soil or native legumes with similar environmental stresses to that of the test area would represent the ideal method for selecting strains which could tolerate the particular environmental stress(es). As with the above example Date and Halliday isolated acid-tolerant strains which may serve to provide an efficient symbiosis in tropical acid soils. Many isolation procedures have been developed which involve the same basic methodology. The next section will describe a suitable method for isolating rhizobia, from soil or from the nodules of the plant.

IV. ISOLATION, PURIFICATION AND MAINTENANCE OF RHIZOBIA

Soil: Rhizobia may be isolated from the soil directly through selective media or indirectly through plant-infection techniques. Many selective media have been developed (Graham, 1964 and Pattison and Skinner, 1974), but these media have failed to make much of an impact in the literature as an effective isolation method and are impractical (Parker, Trinick and Chatel, 1977). The most practical method of isolating rhizobia from soil involves the indirect determination of rhizobia in the soil via the plant-infection technique (Vincent, 1970 and Brockwell, 1963), or nodulation-dilution frequency tests (Tuzimura and Watanabe, 1961 and Brockwell et al., 1975). These methods are essentially the same. The experimental method involves diluting soil samples and mixing the diluted samples with surface sterilized seeds. The rhizobia are then isolated from the nodules. There are various methods of isolation of rhizobia from nodules; (Allen, 1951 and Date and Halliday, unpublished manuscript; see page 11 and Vincent, 1970, see Appendix VII) provide a satisfactory method. The nodules are homogenized and plated on a suitable rhizobia media containing a fungal growth inhibitor such as cycloheximide (Actidione) at 0.002% (Appendix I).

Native legumes. Isolation of rhizobia from native legumes is the most practical method of obtaining different strains for a culture collection. Strains isolated through this method provide one with a myriad of strains usually varying in nitrogen-fixing potential. There is a distinct methodology which should be observed when isolating rhizobia from nodules; this methodology should also be applied to isolation of rhizobia in the

plant-infection techniques mentioned previously. Some recommendations are as follows: (Vincent, 1970).

1. Extreme care should be taken in removing the root system such that the nodules are not damaged.
2. The undamaged nodules should be cleansed and surface sterilized to prevent confusion from rhizobia on the outside of the nodule and those on the inside.
3. The healthy (undamaged) nodules should be used immediately or stored at a low temperature if they are not used at that time.

A general method representing an easy accurate method of isolation from the nodules of leguminous plants is listed below:

Several nodules from a single sample can be taken through their process as groups.

- (1) Wash the nodules in tap water to remove gross soil contamination and transfer to tissue paper to dry.
- (2) Immerse the nodules briefly (5-10 sec.) in 95% ethanol. Pass across to the next container of 0.1% acidified mercuric chloride (HgCl_2 , 1g; HCl , 5 ml; water, 1 liter) and leave for 4 minutes.
- (3) Transfer nodules with alcohol flamed forceps to the first of the containers of sterile water to rinse off surface sterilant. Repeat in the next five containers, leaving nodules in the last one for at least one hour to imbibe in sterile water.
- (4) Nodules are then transferred to a separate sterile spotting plate (one nodule plus one drop of sterile water per well). Each nodule is crushed in the points of a pair of forceps to give turbid suspension in each well. Forceps should be flamed in alcohol after each nodule.
- (5) A loopful of each suspension onto a yeast mannitol agar plate (See Appendix I). Alternatively, nodules can be transferred to a sterile petri dish, crushed individually between the points of the forceps, transferring a drop of suspension

directly to the agar plate and then streaking as before. Avoid transferring any fragment of the squashed nodule onto the prepared agar plate.

- (6) Incubate the plates at 28°C and check for growth typical of Rhizobium along the streak lines. Well isolated single colonies should be picked off and restreaked on clean plates to obtain pure rhizobia cultures. It is possible that more than one typical colony type may appear on the plate streaked from a single nodule and each of these should be taken to pure culture and held for characterization.

A. Purification Techniques

The absolute purification of rhizobia relies on the positive identification of the particular species and strain. A simple method of purification of Rhizobium would be the use of selective medium, which would exclude all other organisms, but as previously mentioned selective media are not practical. Since there is no selective medium, it is necessary to identify the organism or determine whether it is the particular Rhizobium you want. Rhizobium have various characteristics which are important in distinguishing them from other organisms. There are tests which utilize these characteristics and help determine whether Rhizobium or contaminants are present.

Gram stain: This is a stain which divides all bacteria into two classes; they either react positively or negatively. Rhizobium are gram-negative (clear red) under light microscope. Contaminants conversely are gram-positive and appear dark violet. The gram stain is definitely not an absolute test in that there are many gram-negative organisms in nature. It, however, is a simple test and can establish the presence of a gram-positive contaminant. Methodology for this test is listed in Appendix VIII.

Ketolactose test: (Bernaerts and Deley, 1963). This reaction involves the oxidation of lactose to form 3-Ketolactose. This reaction only occurs

with Agrobacterium spp. and is diagnostic for them. Since Agrobacterium spp. are a common nodule contaminant, it is a good practice to test for 3-Ketolactose in nodule isolates. To test for 3-Ketolactose one can apply an isolate to an agar plate (1 percent lactose, 0.1 percent yeast extract, and 2 percent agar). Several isolates can be applied to one plate. The plate is incubated for one to two days at 25°C and afterwards flooded with a shallow layer of Benedict's reagent and left at 25°C. If Ketolactose is present a yellow ring of Cu_2O becomes visible around the cell mass; after one hour it is maximal in diameter (about 2-3 cm). A positive reaction indicates a contaminant is present.

There are tests which utilize the cultural characteristics to distinguish rhizobia from contaminants.

Growth on yeast mannitol agar: Growth on YMA is slow for Rhizobium. Fast growers show moderate to excellent growth after three days while the slow growers show moderate growth after seven days.

Growth on YMA with congo red: Rhizobium absorb very little of the red dye while some contaminants absorb the dye strongly.

Growth and change of pH on peptone glucose agar: Rhizobium grows poorly on this medium and causes little change in pH.

There are other tests which are extremely specific and are excellent in establishing the identity of the particular strain you are studying.

Phage typing: The interaction between bacteriophages and bacteria results in either the formation of clear or turbid plaques. The formation of clear plaques is termed complete lysis i.e. the bacterial cell is destroyed while phage progeny is produced. In the formation of turbid plaques, not all the bacteria are destroyed, and the phage DNA

is incorporated into the bacteria. Both these cases have been reported in Rhizobium and the interaction between the bacteriophage and bacterium are highly specific (Schwingamer and Reinhardt, 1963 and Kleczkowska, 1957). In phage-typing the formation of clear plaques determines phage specificity of the particular strain you are working with. For example, if you had isolated phage from the soil and determined which Rhizobium strain is lysed, you could then use that phage to determine the identity of an unknown strain. To do phage typing in a laboratory would require the maintenance of a phage collection and in that collection there should be phage for all strains worked with in the laboratory. Phage typing can be accomplished at little expense in a very modestly equipped laboratory.

Serology: Serological techniques like phage typing are excellent in determining the identity of a specific strain. On the surface of bacterial cells there are antigens which elicit an immune response in animals. Antibodies are produced by the animal and can be used to determine the presence or absence of specific antigens. The development of antibodies (antisera) can be accomplished by using experimental animals such as rabbits. Vincent (1970), gives a detailed account of methodology for development of antisera as well as the methodology for the serological reaction, either agglutination or precipitation. His methodology is listed in this text in Appendix IX.

Cross inoculation groups: Rhizobium can be grouped together depending on which legume host they nodulate. Fred, Baldwin and McCoy (1932) proposed cross-inoculation or bacteria-plant groups which divide the genus of Rhizobium into groups based on their nodulation specificity.

Table 1 illustrates the cross-inoculation groups of Rhizobium as proposed by Fred, et al. While this table does not represent the absolute specificities, it does illustrate the general specificities of Rhizobium and its host legume.

Table 1. Cross Inoculation Groups of Rhizobia

<u>Bacteria</u>	<u>Plant Host</u>
<u>Rhizobium meliloti</u>	Alfalfa
<u>R. trifolii</u>	Clover
<u>R. leguminosarum</u>	Pea, vetch
<u>R. phaseoli</u>	Bean
<u>R. lupini</u>	Lupine
<u>R. japonicum</u>	Soybean
Cowpea rhizobia	Cowpea, peanut

Nodulation tests: The only absolute identification of Rhizobium is through nodulation tests. If the unknown bacteria does not nodulate its host legume then it is not Rhizobium, however, it should be noted that in certain instances there are examples of extreme host - strain specificity such as in soybean. The use of a highly promiscuous test plant could alleviate this problem. In practice small-seeded tropical legumes are used to determine nodulation capabilities because of their great promiscuity and small size. Several test plants (Table 2) other than the actual host have been used in determining the nodulating capacity of a particular strain. Siratro (Macroptilium atropurpureum) serves as a suitable test host for soybean and the cowpea group of legumes such as peanut, cowpea, velvet bean, poona pea, lentil, horse gram,

TABLE 2. Examples of "wide-spectrum" or "guinea-pig" test hosts useful for testing the nodulating ability of isolates from the nodules of various tropical and subtropical forage legumes.

(Date and Halliday, Unpublished manuscript)

A. Macroptilium atropurpureum, M. lathyroides or Teramnus uncinatus for true cowpea-type genera

<u>Arachis</u>	<u>Cyamopsis</u>	<u>Macroptiloma</u>
<u>Cajanus</u>	<u>Desmanthus</u>	<u>Psophocarpus</u>
<u>Calopogonium</u>	<u>Desmodium</u>	<u>Pueraria</u>
<u>Canavalia</u>	<u>Galactia</u>	<u>Rhynchosia</u>
<u>Centrosema</u>	<u>Glycine</u> (including <u>G. max</u>)	<u>Stizolobium</u>
<u>Clitoria</u>	<u>Indigofera</u>	<u>Stylosanthes</u> (some)
<u>Crotalaria</u>	<u>Labiab</u>	<u>Teramnus</u>
	<u>Macroptilium</u>	<u>Vigna</u>
		<u>Zornia</u>

B. Stylosanthes guianensis cv. Endeavour and S. hamata, cv. Verano for species of Stylosanthes, Zornia and Arachis

<u>S. angustifolia</u>	<u>S. leiocarpa</u>	Reaction of isolates from <u>S. bracteata</u> , <u>S. calcicola</u> , <u>S. capitata</u> , <u>S. sympodialis</u> unknown at this stage
<u>S. erecta</u>	<u>S. moccrocarpa</u>	
<u>S. fruticosa</u>	<u>S. montevidensis</u>	
<u>S. guianensis</u>	<u>S. scabra</u>	
<u>S. hamata</u>	<u>S. subsericea</u>	
<u>S. humilis</u>	<u>S. viscosa</u>	

C. Trifolium semipilosum cv. Safari for a range of East African clover species.

<u>T. africanum</u>	<u>T. reupellianum</u>	<u>T. usambaranse</u>
<u>T. baccarini</u>	<u>T. semipilosum</u>	
<u>T. burchellianum</u>	<u>T. steudneri</u>	
<u>T. masaiensis</u>	<u>T. tembense</u>	

and pigeon pea. White clover or subterranean clover serve as suitable test hosts for Rhizobium trifolii. Many of the test hosts are tropical in origin and are considered primitive evolutionary. These are generally smaller seeded which provides for easier laboratory study.

B. Maintenance of Isolates

As with any bacterial culture collection, accurate records must be maintained. In a Rhizobium culture collection accurate as well as descriptive records are essential. It is extremely important that data concerning the origin of a strain be noted as well as its symbiotic capacities. Vincent (1970) describes information that should be included in a culture collection file as follows:

1. collection number
2. origin
 - a. host
 - b. locality (soil pH, temperature range, rainfall, other environmental conditions)
 - c. if applicable, name of supplier and original collection number
3. resume of important features such as symbiotic capacities and other relevant reports

It is important that these records be made and/or maintained so that the particular strain may be studied in reference to its true environmental niche, symbiotic capacity, or other aspect of that strain. The preservation of rhizobial isolates and the maintenance of Rhizobium cultures is necessary to every testing laboratory as basis for further study and comparison to new isolates.

The maintenance of rhizobial cultures can be accomplished through two basic methods: 1) agar cultures and 2) dried cultures.

Agar slant cultures are the easiest method for maintenance of rhizobial cultures. Rhizobial cultures may be maintained in these agar slants for several years. These cultures remain stable under refrigeration and freezing conditions. Small screw-cap test tubes are adequate and also reduce desiccation.

Dried cultures provide the best method for maintaining rhizobia. Under these conditions the rhizobia are genetically stable, which is an important aspect to be considered. There are three types of dried cultures which can be used to maintain Rhizobia (Vincent, 1970): 1) Standard lyophilization or freeze drying, see Appendix X, 2) Porcelain bead (Norris, 1963), and 3) Dried soil (Vincent, 1970). Table 3 presents information concerning methods of preservation for strains of Rhizobium.

The maintenance of rhizobial cultures is of extreme importance in keeping an active culture collection. One should maintain their collection in duplicate to prevent losses of cultures due to unfortunate circumstances such as power failures or contamination. Ideally the duplicate collection should be lyophilized while the working collection could remain on agar slants for easy access. Cultures maintained on agar slants should be periodically (every six months) transferred, streaked for pure culture and tested for nodulation capability.

V. EVALUATION OF SUPERIOR STRAINS OF RHIZOBIUM

The evaluation of rhizobial strains involves the assessment of the relative effectiveness of the particular strain. There is a criteria

TABLE 3. Information concerning methods of preservation for strains of Rhizobium. (Date and Halliday, Unpublished)

Method	Expertise and facilities	Length of useful storage period	Advantages	Disadvantages	Desirability
Agar slopes in screwcap tubes or agar covered with paraffin-oil	Basic bacteriological knowledge and facilities for pure culture transfer	1-2 years without transfer but can be longer if held at 50C	Simplicity, low cost minimum facilities and expertise	Short storage time increased contamination and variation. Higher risk of loss due to unsatisfactory storage conditions	Least desirable for long-term storage
Porcelain Beads	Ditto plus availability of beads, suitable airtight container and dry sterilizing facility for the silica gel desiccant	3-4 years. Experience suggests longer but small % strains lost (not viable) after 4 years	Low cost and longer storage times; less time before re-beading. Facility for number of subcultures	Not as long term. Good as lyophilization and risk of contamination and variation when rebeading. Time for re-beading.	
Lyophilized or freeze-dried	Basic bacteriological facilities, lyophilizing equipment	Minimum 15-20 yrs. Experience suggests much longer	Once ampouled no risk of variation or contamination. Virtually permanent storage at room temperature	Expensive for equipment and materials	Most desirable
Liquid N Storage	Ditto plus cryostat--source liquid	Years, but not much data available	Rapid	Very expensive; special precautions during freezing and thawing	Good

for the evaluation of rhizobial strains which depending on the sophistication of the laboratory, can be employed (in order of importance):

- (1) Effectiveness in nitrogen fixation;
- (2) Competitive ability (saprophytic competence and the ability to nodulate and form an efficient symbiosis in the presence of a less effective native rhizobial population).
- (3) Secondary characteristics (pH tolerance; survive in different soil types; grow in the presence of combined nitrogen, pesticide resistance; and characteristics important for inoculant production such as ability to survive in part culture).

In our laboratory we have been involved in the preliminary evaluation of rhizobia which nodulate peanut. The methodology which we use in our laboratory could be applied to a tropical agricultural evaluation of a rhizobial strain which nodulated peanut (Note should be made that these tests have been demonstrated in peanut, and our results may be a phenomenon only of peanut). Our methodology is listed below:

Experiments were conducted in the greenhouse to quantify the variation in nitrogen fixing ability of two sets of nine strains when they formed a symbiotic relationship with two diverse host genotypes. The strains were found to significantly influence plant color, plant dry weight, nodule number, nodule mass, the percent nitrogen, total nitrogen and nitrogenase activity (enzymatic measure of nitrogen fixation at a point in time) in both studies. Strains performed differentially on the two host genotypes for plant color, plant weight, total nitrogen and nitrogenase activity as was evidenced by a significant host \times strain interaction (Wynne and Elkan, unpublished). The strain-

host specificity found in this study indicates that the host genotype must be considered for screening rhizobia.

Total nitrogen was considered the best measure of the effectiveness of the rhizobial strains, however both plant color and plant dry weight were significantly correlated with total nitrogen. An arbitrary scale of plant color was established which allowed us to rapidly evaluate relative effectiveness of a strain in the greenhouse. Similar results have been obtained in the field which illustrate the validity of screening peanut rhizobia strains in the greenhouse.

Experiments were also conducted to evaluate the competitiveness of rhizobia strains in a field study in soil previously grown in peanuts and supporting a native rhizobial population. In this study one set of nine strains were evaluated in the previous greenhouse study to inoculate forty-two genotypes of peanut. Some strains were found to produce greater nodulation and nitrogenase activity than the native rhizobia control population when strain effects were averaged over all host genotypes. These studies illustrate the usefulness of greenhouse studies as a primary screening test for evaluating strains and also establish the validity of field tests as an evaluation of actual situations in the field. The results are analogous to results one might obtain in a tropical situation where one is evaluating a strain against a particular cultivar.

In these studies rhizobial strains have been evaluated on two parameters: (1) effectiveness in the greenhouse and field and (2) competitive ability in the field. These studies represent a preliminary assessment of infectiveness and effectiveness. Further studies can be

planned to test the rhizobia strains in the presence of combined nitrogen. Other secondary characteristics such as pH tolerance, pesticide tolerance, temperature tolerance, etc. could be substituted for combined nitrogen in a secondary evaluation of a strain.

In general terms the methodology we use in the evaluation of peanut-rhizobia exemplify a possible experimental method which involves a two-fold approach (1) preliminary testing in greenhouses and (2) final determinations in the field. There are certain fundamental guidelines connected with each of these tests.

In our greenhouse work plant growth is accomplished in Leonard jars or modifications of Leonard jars. The Leonard jar has been the standard device used for the aseptic growth of legumes during the study of *Rhizobium* strains. The assembly consists of three parts: a) a bottomless one liter Boston round flint glass bottle, b) a one-half liter cream jar, and c) a number 5 one-hole stopper equipped with a heat-resistant glass tube (8mm O.D. and 6.5 cm long) containing a nylon or polyethylene wick (6 mm in diameter and 17.8 cm long). The rubber stopper is fitted into the neck of the bottle. The bottle is inverted, and the neck is placed into the cream jar. The bottle is then filled to 2.5 cm of the top with growth substrate (each bottle takes about 1 kg sar'). The cream jar is then filled with 400 ml of nutrient solution (composition of nutrient solution--see Appendix III). The assemblies are covered with aluminum foil and autoclaved at 15 psi (250°F) for two hours. Some of the guidelines for Leonard jar experiments are listed below (Norris and Date, 1976):

- (1) Avoid Contamination. Care is needed to avoid contamination. A source of contamination can be the splashing of water during watering. The most common source of contamination is physical transmission from jar to jar. Inexperienced laboratory personnel may handle the jars causing contamination. A precaution to avoid contamination would be to cover the growth substrate (sand, etc.) with polyvinyl, polypropylene or polyethylene bends - all these plastic covers should be autoclaved before using to insure that no contamination can be attributed to the plastics.
- (2) Planting. The planting of seed is important in the preparation of plants for Leonard jar tests. The seed must be surface sterilized (see Appendix III). The seed must be germinated before planting in Leonard jar assembly. Germination can be accomplished in a separate container with the same growth substrate as in the Leonard assembly covered with foil or paper. The seed embryos are implanted aseptically (use sterile forceps) in small holes in the substrate of the Leonard jar. The radicals of the seedlings should be approximately 1 cm long.
- (3) Inoculation. Rhizobial cultures should be prepared such that the rhizobia will represent an active infectious population; in fast growing rhizobia 3-5 days growth on yeast extract mannitol broth and in slow growing rhizobia 5-7 days growth on yeast extract mannitol broth represent population of rhizobia which would provide adequate potential for infection. The rhizobia culture suspension is added to the Leonard jar plant assembly by pipetting 1 ml. At the same time uninoculated nitrogen controls receive 1 ml sterile water and 10 ml of a 1 mg Nitrogen/ml solution of NH_4NO_3 (100 kg/na ammonium sulfate). The nitrogen solution is added to the substrate not the nutrient solution. Norris and Date (1976) recommend nitrogen solution applications at 0, 2, 4 and 8 weeks or 20-, 10- and 10- ml at 0, 4 and 8 weeks depending on the early growth rate of the plant.
- (4) Care in growing plants. (See Appendix IV).
- (5) Watering. The nutrient solution during the course of the experiment is not replaced, but instead sterile water is added. This requires the preparation of an adequate supply of sterile water for the experiment.
- (6) Harvesting. Eight to twelve weeks is the general length of a particular experiment, however, a shorter experiment (four to six weeks) may yield information concerning ability to nodulate or form an efficient symbiosis. Short-term experiments cannot give information concerning the effect of a particular strain on the yield of the plant. The plants are removed from the jar gently, and the excess substrate is washed from the roots. The approximate number, size and distribution of the nodules are noted, then the dry-weight yield of the whole plant is obtained after drying at 60°-80°C for 24 hours.

A high correlation between dry-weight yield and total nitrogen eliminates the need for total nitrogen analysis. Nitrate controls are not to be included in statistical analysis with Rhizobium strain treatments, since they represent two totally different physiological processes. Nitrate controls indicate whether a strain is worthy of further testing. Norris and Date (1976) recommend that a strain worthy of continued study should increase dry weight yield by 70% over nitrate controls.

Testing in the greenhouse with Leonard jars at best only approximates actual field conditions. The primary use of this testing procedure is as an initial screening determination. An additional point should be made that growth chambers may substitute greenhouse facilities where available. However, growth chambers are smaller than greenhouses, and the testing may be modified such that shorter experiments would be more feasible in the confined areas. Plant growth systems such as agar-slope tubes (see Appendix II) may be more feasible in growth chambers in that they consume less space than the Leonard jar assembly. Methodology is listed in Appendix V for control of the growth environment in greenhouses and growth chambers.

Field testing involves a slightly different methodology than what is used in greenhouse testing. There are two points where field testing differs from greenhouse testing: The first involves the method of measuring nitrogen fixation in the field, while the second involves methods for assessing the success of an applied strain in the field.

The recommended method of measuring nitrogen fixation is either total nitrogen analysis or the dry weight of plant tops (rather than whole plants as in the Leonard jar system) (Norris and Date, 1976). Acetylene reduction assays (Hardy, et al, 1968) have been used extensively in the measurement of nitrogen fixation, but this technique has

the disadvantage that it measures the rate of fixation at a particular time rather than over the duration of the experiment. In the past the equipment (gas chromatographs) needed for acetylene reduction assays was expensive; however, an inexpensive, simple and portable gas chromatograph has been developed by Mallard, et al (1977) which could be of value in modestly equipped laboratories where expensive gas chromatographs are prohibitive. Acetylene reduction assays should at most only supplement total nitrogen analysis or dry weight plant tops, as a method of evaluating the effectivity of a strain.

The second point of difference between Leonard jar - greenhouse experimentations and field determinations involves the need to assess the success of the applied strain in forming nodules in the presence or absence of a native population of rhizobia (presence or absence of nodules on uninoculated controls) (Norris and Date, 1976). Norris and Date (1976) present three situations in the field which one might encounter in evaluating a strain:

1. Uninoculated control plants without nodules; plants small and nitrogen deficient compared with inoculated plants which may be
 - (a) without nodules, thus indicating the unsuitability of the test strain, or
 - (b) nodulated but plants small and nitrogen deficient with no significant improvement in dry weight, indicating an ineffective test strain, or
 - (c) nodulated and showing substantial response in dry-weight yield.

Since no nodules were formed on the control in any of these situations it is safe to assume that those forming on the inoculated treatments are due to the test strain. This has been observed with such species as Lotononis bainesii and some of the African Trifolium species and in certain situations also with Leucaena leucocephala, Lablab purpureus, Desmodium uncinatum and D. intortum.

2. Uninoculated control plants with nodules that are judged as ineffective since inoculated treatments show healthy well-grown plants with significant improvement in dry-weight yield over uninoculated. Obviously in this instance only some of the nodules on the inoculated treatment will be due to the applied strain, but it is doubtful whether it is of practical value to determine the proportion of nodules due to the test strain, since only the test strain is contributing to nitrogen fixation.
3. Uninoculated control plants with active nitrogen fixing nodules and plant dry weight (or nitrogen) yields similar to those of inoculated plants. In such cases it is essential to determine how successful the applied strain has been forming nodules and to estimate their contribution to the total nitrogen fixed.

In this section we have elaborated on a series of procedures and recommendations for the evaluation of superior rhizobia strains in the greenhouse and in the field. In the greenhouse the conditions for the growth of the plant and the establishment and maintenance of the Rhizobium-legume symbiosis are maximized. Field tests, on the other hand, present special problems in one's attempts to maximize the symbiosis. These problems are as follows:

- (1) How does one select a site for field tests which would allow accurate determination of the particular parameter he is studying?
- (2) How does one determine nodulation capabilities in the field?

Site selection. The selection of a site for field tests involves a great deal of consideration depending on what is to be studied. In our field work in peanuts mentioned previously, we obtained results which indicate the applied strain was capable of increasing nitrogen fixation and nodulation. The results we obtained were fortunate in that in many cases the applied strain cannot compete with the native rhizobia population. In this case, it is necessary to select a site that has not been cultivated in the test legume in the past ten years. An alternative

to the above situation would be to test a strain and host legume in soil where no nodulating strain are present for that particular legume. The above recommendations involve selection of a site for the evaluation of nitrogen fixation. There are cases where a native rhizobia population is necessary. If one is testing the competitiveness and persistence of a test strain, a native population of rhizobia is needed. In this case special precautions should be taken to prevent cross-contamination between field plots such as installation of drainage ditches to prevent contamination from rainfall drainage.

Determination of nodulation. The prerequisite for determining the nodulating capabilities of a strain as well as evaluating other characteristics such as nitrogen fixation effectiveness is establishment of a plot size and row spacing to provide a maximal growth of the legume under test conditions. A design satisfactory for most species is one which each host - strain combination is represented by a row of uniformly spaced plants 25-30 cm apart within the rows and with a minimum of 0.5 m between the rows (Norris and Date, 1976). The general overall plot size would be 5m x 5m.

Nodulation is assessed at ten to twenty weeks depending on the the legume and the seasonal conditions, by sampling a number of plants in each treatment (Norris and Date, 1976). Nodule number is a valuable criterion in the first six to eight weeks of plant development, but it is of very little use thereafter. At that point the best criterion is nodule dry weight or nodule dry weight/unit dry weight of the whole plant or root system (Norris and Date, 1976). After determining the nodule mass then one should want to know whether these nodules are effective and what constitutes an effective nodule. For tropical

legumes it should be realized that effective nodules may be small (1-2 mm) or large (10-15 mm) depending on species and conditions. The Desmodium intortum and Macroptilium atropurpureum young (small) nodules are generally spherical but become lobed when older and larger (Norris and Date, 1976). As a general rule, effectiveness can be gauged by the degree of pink to red coloration of the nitrogen fixing bacteroid tissue inside the nodule; white or sometimes green tissue is inactive. The use of nodule number or nodule weight as a criterion of effectiveness can be misleading when many of the nodules contain a high proportion of inactive tissue. Nodule sampling. Nodule sampling involves the cautious removal of plant roots and the washing of excess soil from the nodules. With careful removal, an accurate determination can be made with respect to nodule mass and an accurate assessment of effectiveness can be made. It is important that all or most all nodules of the plant should be removed from soil or plant. A satisfactory method would be to take a large soil core sample (20-40 cm in diameter to a depth of 15 cm). This core sample should yield approximately 90% or more of the total root system of first year plants.

Supplemental information concerning field testing is in Appendix VI.

VI. SUMMARY

In this chapter we have attempted to present the current knowledge concerning the criteria for the selection of infective and effective strains of Rhizobium and the evaluation of those strains. Our current knowledge of legume bacteriology and strain selection differs little from the first comprehensive review by Fred, Baldwin and McCoy (1932). Effectivity still represents the fundamental criterion for selecting strains, however it is now considered only part of an overall selection program. Current thought establishes three criteria as basic to a strain selection program, and they are as follows:

- (1) Effectiveness in nitrogen fixation;
- (2) Competitive ability in the presence of a native rhizobial population
- (3) Ability to survive in various environmental conditions

The latter two points are extremely important for if a strain fails to compete successfully with the native rhizobial population for infection sites and does not survive environmental conditions where it is introduced, even a highly effective strain is useless in providing an efficient symbiosis.

Also in this chapter we have presented a methodology for evaluating superior strains of Rhizobium as to the criteria mentioned above. The evaluation of superior strains involves a two-fold approach: (1) preliminary testing in the greenhouse for effectivity and (2) final evaluation of a strain in the field, testing parameters such as effectivity, ability to compete in the presence of less effective native rhizobial population and the ability to survive under various environmental condi-

tions as previously mentioned in this text. The superior strains are evaluated for their ability to increase crop yield as measured as an increase in crop yield, itself or an increase in total nitrogen content.

Appendix I.

Routine medium for growth of Rhizobium
(Fred, Baldwin, and McCoy, 1932)

Yeast-extract mannitol broth.

K ₂ HPO ₄	0.5 g
MgSO ₄ . 7H ₂ O	0.2 g
NaCl	0.1 g
Mannitol	10 g
*Yeast water	100 ml
Distilled water	900 ml

*To prepare fresh yeast extract mix 100 g Bakers' compressed yeast with 1 liter of cold water and allow to stand for two hours at room temperature. Autoclave for one hour and allow to settle. The clear supernatant is adjusted to pH 6.8 and used as yeast water.

Solid medium contains 12-15 g/l agar.

With Modifications

(Date and Halliday, unpublished manuscript)

Bromothymol blue can be incorporated at 5 ml/l of a stock solution (0.5% w/vol of alcohol) as an indicator for acid or alkali production.

Actidione (cyclohexamide), when required for fungal suppression, is included at 0.002% w/v.

For isolation of acid-tolerant rhizobia mannitol 10 g should be replaced with 10 g arabinose.

Caution: actidione is highly toxic.

Appendix II.

Methods for Growth Chamber and Greenhouse
(Burton et al, 1972)

A. Glazed Jar (Fig. 2)

Jars of 1/2 gallon capacity without drainage holes are most commonly used. These jars should be thoroughly cleaned and free of any toxic chemicals. The bottom is layered with one inch coarse gravel and a heat-resistant glass tube 1 1/4-inch diameter is inserted and fitted at the side of the jar for watering purposes. The jar is then filled to about 3 inches from the top with horticultural grade vermiculite. After leveling the vermiculite in the jar, add a layer of mason's sand to about 1 1/2 inches of the top. Add 500 ml nutrient solution adjusted to bring the reaction of the total substrate to a pH of 6.5. Cover the top of the jar with a layer of tough wrapping paper and tie with a cotton string. Autoclave at 15-pound pressure (250°F.) for 4 hours.

B. Leonard Assembly (Fig. 1)

The technique used deviates from the original Leonard system in two ways: a) nutrients are added as a solution rather than as salts mixed with the sand, and b) nutrient solution is adjusted to bring pH of substrate to 6.5.

The assembly consists of three parts: a) a bottomless 1 liter Boston round flint glass bottle, b) a 1/2 liter cream jar, and c) a Number 5 one-hole rubber stopper equipped with a heat-resistant glass tube 8mm. O.D. and 6 1/2 cm. long containing a nylon or polypropylene wick 6mm diameter and 17.8 cm. long.

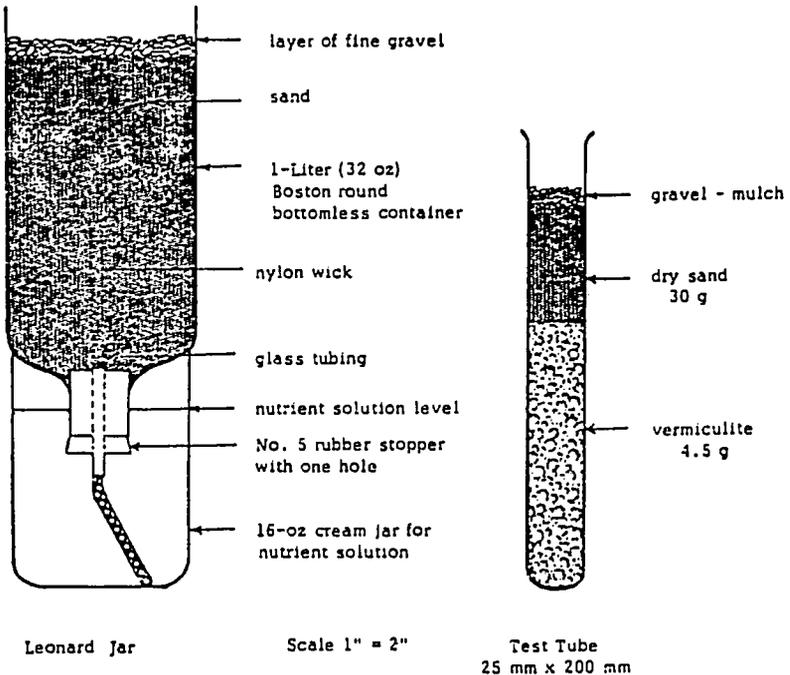
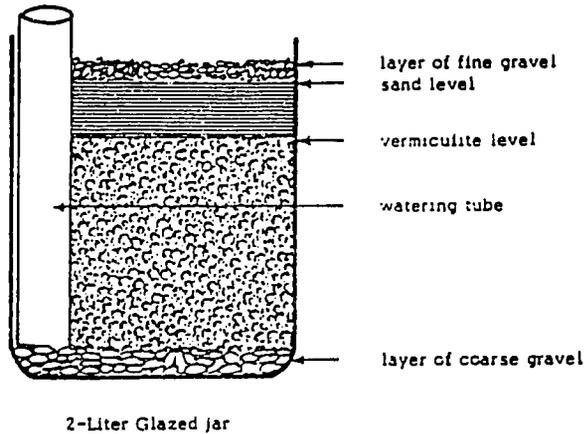


Fig. 1. Growing techniques for growth chamber and greenhouse (Burton, Martinez, and Curley, 1972).

After fitting the wetted stopper into the neck of the bottle, it is inverted and neck is placed into the cream jar. The bottle is then filled with 400 ml. nutrient solution. (Composition of the nutrient solution is given in the section which follows.)

The nutrient solution is added to the cream jar instead of to the sand so that functioning of the wick can be checked. The dry sand should be completely moistened by capillary action of the wick and sand in approximately 1 hour. The bottles are then covered with a petri-dish top or aluminum foil and autoclaved at 15-pound pressure (250°F) for a minimum of 2 hours.

C. Test Tube (Fig. 1)

Test tubes are less desirable than Leonard or glazed jars for growing plants, but they are often used. Tubes 2.5 by 15 cm. or larger are more desirable than smaller tubes. The growth obtained will approximate that obtained in the larger vessels providing the tubes are filled to within 4 to 5 cm. of the top. With 25 by 200 mm. tubes, fill about 2/3 full with horticultural grade vermiculite (4.5 g.).

D. Agar Tube Method (Vincent, 1970)

This method permits fairly good differentiation of symbiotic effectiveness with plants having seed up to a size approximately 10 mg. (such as subterranean clover).

Seedling agar -- Two commonly used media serve as examples:

<u>Jensen (1942)</u>		<u>Thornton (1930)</u>	
CaHPO ₄	1.0 g	Ca ₃ (PO ₄) ₂	2.0 g
K ₂ HPO ₄	0.2 g	K ₂ HPO ₄	0.5 g
MgSO ₄ · 7H ₂ O	0.2 g	MgSO ₄ · 7H ₂ O	0.2 g
NaCl	0.2 g	NaCl	0.1 g

<u>Jensen (1942)</u>		<u>Thornton (1930)</u>	
FeCl ₃	0.1 g	FePO ₄	1.0 g
Water	1.0 l	FeCl ₃	0.01 g
		Water	1.1 l

Agar (8-15 g/l), according to use as deep or slope.

Trace elements (Gibson, 1963) can be added as 1 ml/l of a stock containing:

BO	0.05%
Mn	0.05%
Zn	0.005%
Mo	0.005%
Cu	0.002%

pH adjusted to 6.5-7.0 (*Trifolium* - *Medicago*, respectively). Both media have undissolved constituents and therefore need to be kept agitated when being dispensed.

Taking 30 mg/plant as a reasonable dry weight yield by the enclosed method and 100 mg when only the roots are enclosed, a budget can be prepared with respect to the major elements supplied and required.

Ca and P are in considerable excess in both media; in fact, there is likely to be sufficient of the latter in the agar itself to meet the plant's needs and enough Ca at least for the enclosed plant. Mg would appear to be marginal in both media for the more extensive growth of the semi-enclosed plant. The same is true for K in the case of Jensen's medium. Trace element addition, if needed at all, is likely to be required only for the large plants in open assemblies.

Tubes

150 x 20 mm tubes are satisfactory for the smallest seeded species (e.g. white clover); 200-30 mm will permit a fair degree of differentiation in the larger (such as subterranean clover).

The approximate volumes of agar appropriate to tube size are set

out below. When tubes are sufficiently uniform, the medium can be dispensed to a constant depth.

Tube Size		Volume of agar (ml)	
Inches	mm	Deep	Slope
6 x 3/4	150 x 20	8	12
6 x 1	150 x 25	12	18
6 x 1 1/4	150 x 30	18	30
8 x 1 1/4	200 x 30	25	40

The tubes should be closed with cotton wool plugs of uniform depth (20 mm) and moderate compactness.

Enclosed methods may be modified so as to permit the shoot to grow without restriction whilst the roots are protected within the enclosed vessel. This method also permits different temperature to be used for shoot and root growth. The details of methods for small-seeded and large-seeded legumes are given below.

E. Tube Method (Fig. 2, Gibson, 1963):

1. Use sufficient seedling agar per tube to provide a long slope reaching to the top of the tube (13 ml for 150 x 20 mm tube).
2. Cap the tubes with thin circles of aluminum foil (0.03 mm thickness, 44 mm diameter, cut out with a punch after the foil has been interleaved with paper to prevent sticking). Secure the cap with a strong rubber or plastic ring.
3. Make a small (5 mm) watering and ventilation hole in the cap near the side of the tube, and plug it with cotton wool.
4. Autoclave the tubes and set as slopes so that the plugged hole is uppermost and the agar reaches the aluminum cap.
5. Prepare seeds by germinating them on water agar in inverted dishes so as to provide straight radicles 13-15 mm long.

6. Make a small hole in the aluminum cap opposite the plug and insert the radicle so that it lies along the surface of the slope. (In these operations the tips of the forceps and needle are kept in alcohol and flamed before use and cooled in sterile water before handling the seedling.)
7. Cover the planted tubes with specimen tubes containing a plug of moist cotton wool and resting on the rubber ring so as to prevent desiccation of the young seedling.
8. After three days most seedlings will have lost their seed coats, but in some cases this will need to be assisted with a sharp blade.
9. Add quarter-strength sterile seedling solution through the hole in the cap to within 12 mm of the top of each tube, preferably from a sterilizable automatic syringe, flaming the cannula every 4-5 tubes.
10. For N controls, use a small syringe to add nitrate to provide a total of not more than 5 mg N/ml. The first addition is made approximately 12 days after germination.
11. Add inoculum to the required tubes through the watering hole within a few days of setting up.
12. The tubes can be set up in enclosed rack, with roots protected from the light. The temperature for roots and shoot can be independently controlled by immersing the tubes in a controlled water bath. Illumination for this method should be overhead.
13. Maintenance - It is generally not necessary to add more nutrient solution until the plants are 18-21 days old; further additions

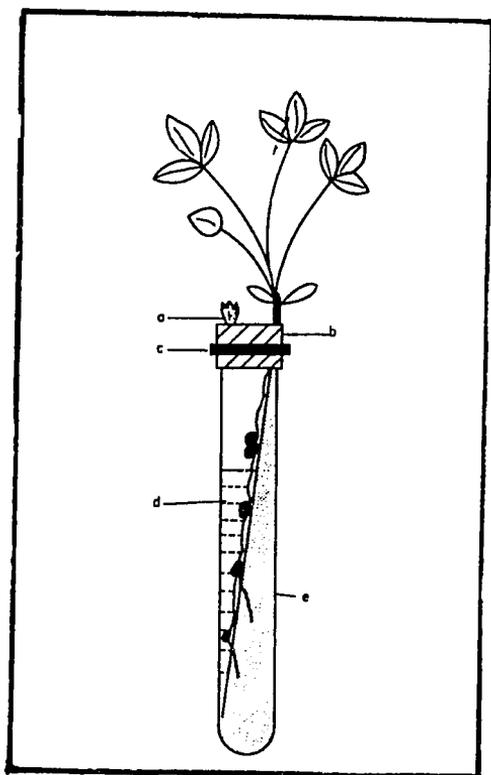


Fig. 2. Assembly for Gibson's partly enclosed seedling.

- a. cotton wool plug for watering
- b. aluminum cap
- c. strong rubber band
- d. seedling solution
- e. sloped seedling agar

are made after another 4-5 days and finally at 2-3 day intervals. The number of replicates required to establish significant values in the tube method are listed in Table 2.

The freedom from restriction secured by having the shoots growing outside the container permits rapid growth and very good differentiation. The method is, therefore, advantageous for the larger of the small-seeded legumes (e.g. 10 mg or more). Gibson has found that subterranean clover grew to four times the size (N-fixation: 5 x 8 x) in the partly enclosed method compared with the wholly enclosed plants. Moreover, growth and N-fixation were exponential for the growth period (between 14 and 34 days) so that the logarithmic growth and fixation rates could be determined as more precise measures of plant response than total growth and total plant nitrogen:

$$R_w(\text{relative growth rate}) = \frac{\log W_2 - \log W_1}{t_2 - t_1}$$

$$R_n(\text{relative fixation rate}) = \frac{\log N_2 - \log N_1}{t_2 - t_1} \text{ where } W_2, W_1 = \text{dry weight at times } t_2 \text{ and } t_1, \\ N_2, N_1 = \text{total plant N at } t_2 \text{ and } t_1$$

The chief disadvantage of this method is the additional labor and space involved. There is also a small loss of bacteriological control. The weight attached to these considerations will be largely determined by seed size, but also by the purpose of the investigation and the available facilities to hand.

TABLE 2. Number of Replicate Plants Required to Establish as Significant, Treatments with Values:

Clover	$\frac{\log N - \log C}{4}$	$\frac{\log N - \log C}{2}$
	(Quarter range)	(Half range)
White	12	3
Red	10	2
Subterranean	20	4
Crimson	14	4

Before the installation of any plant the plant seed must be surface-sterilized and the growth media should be completely sterilized. Outlined below is a method for surface-sterilization of legume seed.

Appendix III.

Surface Sterilization of Leguminous Seed (Allen, 1951)

No fool-proof method can be recommended for ridding leguminous seed of microorganisms. Certain seeds are satisfactorily cleansed after washing them in soapy water, others may not be completely freed of microorganisms after immersion for several hours in mercuric chloride.

Small seeds are more difficult to free of contaminants than are large seeds; rough coated seeds, more difficult than smooth coated ones, etc. Any disinfectant method now in general use may be used with some degree of satisfaction; the crux of the situation lies in obtaining evidence of its efficiency. The following method is an example of the conventional techniques:

1. Immerse the seeds in 95% alcohol for the purpose of removing the air bubbles from the region of the hilum scar and lowering the surface tension. Agitate for 3-5 minutes and pour off the alcohol.
2. Agitate the seed vigorously for 3-5 minutes in 1:1,000 mercuric chloride for 2-3 minutes; large, thick-coated seeds will withstand a longer time. The use of vacuum during the mercuric chloride treatment is advantageous. Wash the seeds various times in sterile distilled water. Washing under vacuum aids greatly in removing the residual traces of the disinfectant from the hilum region.
3. Immerse the seed with agitation for several minutes in water heated to 60-64°C. This not only has certain disinfectant action, but when used properly, the seed coat is softened and germination is enhanced.

4. Rinse the seed several times in sterile distilled water. Remove the seeds from the original container (vial, flask, or cheesecloth) to a sterile petri dish. A thin, or single, layer of seed over the bottom of the dish is preferred. Dry the seed in a 37°C incubator.
5. Tests for efficiency of the treatment:
 - a) Optimum conditions are obtained with small seeds (alfalfa, clover, Lotus spp., etc.) by spreading aseptically an adequate number (35-50 seeds) of seeds over the surface of an agar (any ordinary medium) plate. Incubate the plates at room temperature over a period of 72 hours, or until germination. Examine the germinating seeds periodically with a hand lens (10x). In such instances, it becomes necessary to select only those seedlings for greenhouse test purposes which are free of microbial growth.
 - b) Larger seeds (peas, soybean, cowpeas, beans, etc.) may be added to tubes of broth, followed by slanting the tubes so that only about one half portion of each seed is immersed. Occasional rotation or turning of the tube is advised. Turbidity of the broth is evidence of contaminating microorganisms; proof may easily be obtained by any staining method.

Appendix IV.

Care of the Growing Plants (Allen, 1951)

1. Sterilize all water and nutrient solution in 5 liter quantities in 6 liter Erlenmeyer flasks. At the time of use insert a sterile syphon attachment provided with the required length of rubber tubing, thumbpinch clamp and glass nozzle.
2. Seedlings need frequent brief waterings since the sand is pushed away during the germination period. The sand should be washed back around the rootlets; otherwise, the young rootlets will dry up because of the lack of nutrients.

The pots must never be flooded. Several small additions of water are better than one heavy one.

The seedlings should be watered as often as necessary. It is good practice to add nutrient solution about once every four waterings. Either of the following solutions is acceptable

Bryan's Modified Crone's Nitrogen-Free Solution

Stock Salt Mixture:

Potassium chloride	10.0 gm
Calcium sulphate	2.5 gm
Magnesium sulphate	2.5 gm
Tricalcium phosphate	2.5 gm
Ferric phosphate	2.5 gm

To Make the Solution:

Water	1,000.0 ml
Stock Salt Mixture	1.5 gm

Sterilize 15 lbs for 1 hour.

Mix all salts and grind to a fine powder.

Bond's Modified Crone's Solution

Stock Salt Mixture:

Potassium chloride	31.7 gm
Tricalcium phosphate	18.0 gm
Calcium sulphate	13.7 gm
Magnesium sulphate	5.5 gm
Boric acid (H ₃ BO ₃)	0.5 gm
Dipotassium phosphate	26.8 gm
	<u>100.0 gm</u>

To Make the Solution:

Water	1,000.0 ml
Stock salts	1.0 gm

Sterilize 15 lbs for 1 hour

Mix all salts and grind to a fine powder.

Distilled water is preferred, since tap water often contains appreciable nitrogen.

3. The plants should never be touched. Tough hardened seed coats are not to be removed. Nature will take care of these. If plants push out of the sand, consider them discards, or reinsert them with sterile forceps.
4. As soon as the seedlings have reached a good stand, thin the number of plants, if necessary, to a constant number in each pot. Be sure the plants selected are hardy. Discards should be chosen so that the selected plants will be spaced with about the same distance between them.

Appendix V.

Control of the Growth Environment (Vincent, 1970)

In the greenhouse - The simple unheated greenhouse can provide excellent growing conditions for part of the year in most localities. Its usefulness can be extended by heating and supplementary lighting in the winter and, in some degree by the use of a cooling system and reduced light intensity in the warmer times of the year.

A system of adjustable louvres permits retention of a satisfactory level of illumination whilst reducing the input of heat (Hely, 1959). Evaporative coolers are efficient in relatively dry air and the same principle has been applied by surrounding the growth vessels with sand, itself within a porous container and kept wet (Dobereiner and Pimenta, 1964). In more detail this method involves the following assembly:

Large fully permeable clay pots (e.g. capacity 10 l) are filled to about one third with sand. Smaller impermeable pots (or tubes) are placed inside the large pots so that the root area will be surrounded by sand which is kept saturated with water.

In a growth chamber - Artificial illumination in growth chambers permits standard conditions to be maintained throughout the year. Relatively simple provision can also be made for the adjustment of light intensity, day-length and temperature to suit the major groups of host. The essential features of units used in Vincent's laboratory and capable of providing satisfactory and reproducible conditions for a regular testing program are described below.

Illumination - Satisfactory results have been obtained with close lateral illumination of enclosed tubes as well as with overhead lighting of plants growing in open containers or in enclosed tubes.

Warm-white fluorescent tubes (40 watts) have been satisfactory for clover and some species of Medicago, but need to be supplemented at the red end of the spectrum for some hosts, notably lucerne. Special tubes (Sylvania Grolux) are sufficient in themselves. These have high spectral peaks at 600-700 m μ (for photosynthesis) and 400-500 m μ (for synthesis of chlorophyll). They are produced in sizes ranging from 60 cm - 240 cm (20-215 watts) including some that provide for very high output (e.g. F48 T12/VHO/GRO; 110 watts for 120 cm length). Lower output lamps (40 watts, 120 cm) have the advantage of running with the standard 120 cm ballasts.

When overhead illumination is used the tubes are mounted below a white reflector in banks sufficient to cover the width of the bench. Intensity ranges from 500-1,000 lumens according to the height of the light bank (30-60 cm).

Hours of lighting are controlled by time switches, e.g. 12 hr per 24 for clovers, 16 hr per 24 for lucerne and summer legumes. Other variations can be made to suit particular legumes.

Temperature and humidity - Simple lighting assemblies can be used to increase day-length in the glasshouse or provide controlled illumination in a laboratory. These permit useful work to be done provided temperature extremes are avoided out of the normal growing season. The

temperature factor however, has to be watched very carefully particularly because the symbiotic association with host and bacterium is much more susceptible to it than is the plant receiving combined nitrogen.

When many lighting units are confined in the restricted space of a lightroom, there is sufficient heat produced to demand quite considerable cooling. Heating is also necessary to maintain a standardized temperature against cooling during the dark period. Thermostatically controlled cooling and heating systems can be linked to the time switches controlling the light and dark cycles.

A simple humidifier avoids excessive drying out by keeping relative humidity not less than 60%-70%.

Appendix VI.

Field Trials (Vincent, 1970)

Although growth chambers and greenhouse experiments are valuable for the primary assessment of the symbiotic capacity of particular Rhizobium/host combinations, the full evaluation depends on the field trial. This is because: (a) Greater differentiation may be secured in the field as a result of effective nodulation. This will depend, of course, on the supply of soil nitrogen available to the plant and the extent to which naturally occurring effective strains might confuse the result. (b) The association between bacterium and host is being tested in a natural and complex situation.

Examples of effects associated with the complex soil environment are (1) hydrogen ion concentration and soil moisture affecting survival and multiplication of rhizobia as well as the interaction among the host, Rhizobium and environment affecting competition between rhizobia, (2) the influence of calcium ions and temperature on nodulation, molybdenum deficiency affecting nodule function. Success in complex situations, and (3) particularly if they have been chosen to provide a cross-section of conditions that are likely to be encountered, is likely to have much more practical meaning than success under arbitrary, relatively favorable conditions.

The principles that apply to fertilizer and plant variety trials are equally applicable when dealing with rhizobial inocula. Special care is required to avoid accidental mixing or transfer of the rhizobia and some experience in order to recognize whether the applied inoculum has, in fact, established itself.

It is obviously not possible to cover every situation in detail but the following notes have been directed toward the more important features of rhizobial field experiments.

1. Selection of Site - The selection might be determined by the strictly practical reason that it is the soil or locality which needs to be investigated. On the other hand it might be chosen because it is specially suitable as an area for a particular investigation. Soils with low rhizobial count and low available nitrogen are likely to be most useful for strain trials, though often requiring some form of soil amelioration to remove a barrier to rhizobial survival and full expression of the symbiosis. On the other hand, if the competitive capacity of an applied strain is to be tested this can only be done when there are already many rhizobia in the chosen area. Ability to distinguish between nodulation due to experimental and that due to naturally occurring rhizobia will be an essential part of this kind of investigation.

If possible the site should be level enough to avoid surface wash after heavy rain; it also needs to be secured against animals and pests, and from human interference. The size of the area will be determined by the practical plot size, the number of treatments and the method of sowing in relation to available labor and machinery. The expected heterogeneity of an area (in its rhizobial as well as other characteristics) will have an influence on plot size and degree of replication. Machine sowing permits larger plots, and demands a larger total area with some wastage due to space needed to maneuver the machine. Hand-sowing is likely to limit the size of the plots

because of the labor involved, but also makes it easier to work in small plots.

2. Experimental Procedures - Decisions need to be made concerning the degree to which the experiment should simulate practical conditions or whether practical procedures need to be departed from in order to maximize the likelihood of establishing the inocula. When the purpose is to compare the nitrogen-fixing effectiveness of a collection of rhizobial strains it is fair to use fertilizer, adjust pH, and use a heavy inoculum to increase the likelihood that the strains being tested will have, in fact, produced nodules. Water might be necessary to overcome drought. On the other hand, if the purpose is to compare the strains' ability to survive and nodulate in the field, there is a case for including more exacting conditions as well as the more favorable.
3. Application of Fertilizer - The even spreading of fertilizer over the surface of the plot lessens the risk of an unfavorable effect on the inoculum, but will require heavier application to overcome a marked deficiency, or to increase pH. Alternative application of the fertilizer or lime in the drill can affect the seedlings' immediate environment favorably or unfavorably so far as the rhizobia are concerned. The use of calcium carbonate (CaCO_3) as a seed-pelleting material may cause a sufficient localized rise in pH to secure nodulation in a moderately acid soil. On the other hand, placement near the inoculated seed of an acid fertilizer, or one containing heavy trace elements, can reduce nodulation.
4. Inoculation - Strain performance can be affected in some measure by the form of inoculum and the number of bacteria contained in it. Unless

the field experiment is itself providing for such comparisons the inoculation procedure should conform with common practice in the region concerned (agar or peat culture, plain or pelleted seed: number related to standard required in commercial inoculant supply). An inoculum level equivalent to a good quality culture, giving 1,000-10,000 viable rhizobia per seed at the time of sowing, is generally suitable. Wherever possible, the size of the population of rhizobia in the applied inocula should be determined and recorded.

A regular seeding machine that sows a large number of rows simultaneously can be adapted by dividing its seed-box so as to sow seed inoculated with one of several different strains. Extra spacings between the different inoculum treatments can be secured by omitting one or two intermediate rows. There remains the problem of rhizobial cross-infection in the machine itself. Retention of the same drill rows for the same inoculum avoids the need for frequent cleaning and "sterilization" of the machine but imposes a nonrandom pattern on the plots that might become associated with bias in favor or against a particular treatment according to depth of planting, fertilizer flow, water movement on sloped plots.

Hand-seeding may be facilitated by using bottles as seed containers, closed with a stopper having a wedge-shaped incision of a size to suit the passage of the seed, or one can also use aluminum foil taped across the top of the tube; punch a hole in the foil to allow seed passage. This will also reduce risks of cross contamination during the planting operation.

Appendix VII

Isolation from a Nodule (Vincent, 1970)

Nodules will, according to their source, age and freshness, have other microorganisms besides rhizobia on their surface or within them. Undamaged nodules may be cleansed and surface-sterilized to remove the microorganisms external to the nodules, but there are occasions when this involves the risk of destroying the rhizobia within the nodule as well. Whenever possible healthy nodules should be used that have been shortly before removed from the root or stored at low temperature meanwhile. When old or desiccated nodules have to be used extra care will have to be taken to recover rhizobia from among many contaminants. Fungal contamination can be suppressed selectively by the incorporation of actidione in the medium.

When it is important to distinguish rhizobia that are within the nodule from any that might be on its outside, extra time might have to be used for sterilization and even dissection of nodule contents might be desirable. Medium to large nodules can be conveniently handled with forceps for dissection; small surface-sterilized nodules can be dropped into warm melted paraffin wax (m.p. 60°C) and cooled immediately to provide a more readily handled pellet.

For most purposes the simple procedure detailed below will enable rhizobia to be recovered.

Materials and Equipment

Nodulated roots or stored nodules. A tube with muslin or nylon mesh at one end, or other suitable device for washing nodules. Sets of small

petri dishes for sterilization and subsequent washing, or a siphoning device permitting successive washing in the one unit. 0.1% acidified mercuric chloride (HgCl_2 , 1 g; Conc. HCl, 5 ml; water 1l). Sterile tap water. 'Nichrome' spatula, beaten from heavy gauge 'Nichrome' wire to provide flat blade and cutting edge. Poured plates of yeast extract mannitol agar (with or without 0.002% actidione): (2-4 per nodule). Small tubes containing 1 ml water and a little clean sterile sand (1 per nodule).

Procedure

1. Take nodules, selected or at random according to the purpose of the investigation, cutting them so as to leave a small amount of root attached for ease of handling.
2. Wash thoroughly (over muslin or nylon mesh, or in a suitable container) so as to remove gross surface contamination. (The inclusion of a trace of detergent will assist).
3. Expose momentarily to 95% ethanol and immerse in 0.1% acidified HgCl_2 for 1-30 min according to the size of the nodule and the required vigour of sterilization. A 3-4 min period is usually sufficient. (Alternatively use 3% - 5% H_2O_2 . In this case washing (step 4) is not required).
4. Wash thoroughly in at least 6 changes of sterile tap water.
5. Crush, or dissect, the nodule aseptically and spread the milky fluid from within the nodule over the surface of one or two plates of yeast extract mannitol agar (containing 0.002% actidione, if heavy fungal contamination is expected).

6. Incubate at 26°C and look for isolated colonies along the line of spreading that are conformable with the growth expected of Rhizobium. Note that although many species will give relatively large gummy colonies after 4-5 days, others will be much smaller even after 10 days. Again although most will be watery, translucent or whitely opaque (sometimes differentiated), pink colonies are also on record in the case of the Rhizobium from Lotononis bainesii (Norris, 1958).
7. Pick from a typical isolated colony, either directly to a slope of yeast extract mannitol agar or, for more critical purposes, for restreaking and repeated single-colony picking. In the latter case, better separation will be secured if the growth picked from the first colony is agitated in a tube containing a little sterile water and clean sand before the second streaking.
8. Look for uniformity of colony type in the restreak and pick on to a yeast extract mannitol agar from a well isolated typical colony.
9. Immediately label and record the isolation to relate the culture number to its source (locality, host, operator, date).
10. Identify the isolate as Rhizobium either directly by means of an infection test with a suitable host or, according to the nature of the material, indirectly by some other character: e.g. serological specificity, cultural characteristics or biochemical properties.

Appendix VIII

Gram Stain Technique (Vincent, 1970)

MATERIALS

Clean, flamed and cooled microscope slide.
Gram reagents:

A. Crystal violet solution

Crystal violet	10 g
Ammonium oxalate	4 g
Ethanol	100 ml
Water (dist.)	400 ml

B. Iodine solution

Iodine	1 g
Potassium iodide	2 g
Ethanol	25 ml
Water (dist.)	100 ml

C. Alcohol (Iodinated)

Iodine solution (B)	5 ml
Ethanol	95 ml

D. Counterstain

2.5% safranin in ethanol	10 ml
Water (dist.)	100 ml

PROCEDURE

1. Prepare and fix smear as for the simple stain.
2. Stain with crystal violet solution (A) for 1 min.
3. Rinse lightly with water and drain off excess water.
4. Flood with iodine solution (B), drain off, replace iodine and allow to act for 1 min.
5. Drain off iodine solution and decolourize with iodinated alcohol (C) for 5 min.
6. Wash with water and drain off excess.
7. Counterstain for 5 min. with safranin (D).
8. Wash with water, drain and dry.
9. Examine directly under oil immersion. Gram positive cells appear dark violet; Gram negative are clear red.

Note:

Very crowded smears are likely to give a patchy result due to uneven destaining.

Appendix IX

Development of Antisera (Vincent, 1970)

The principles of developing antibodies in an experimental animal are substantially the same whether one is interested in agglutination of whole cells or precipitation of soluble antigens. There are some particular requirements, however, and these are set out below.

Growth from the surface of agar slopes is harvested aseptically in sterile 0.85% NaCl, assisted by rolling a few glass beads over the surface. The suspension can be freed of large clumps by a short period of low-speed centrifugation (or by filtering through a small pad of sterile cotton wool). The more uniform suspension thus obtained can be concentrated by centrifuging, taking off a portion of the supernatant and re-suspending to provide a heavily turbid suspension ($1 - 10 \times 10^9$ bacterial/ml).

Bacteria grown in liquid medium can be freed of large clumps and concentrated in a similar fashion.

Laboratory rabbits are inoculated, either: (a) intravenously (for agglutination) or (b) intramuscularly, followed by an intravenous injection (for agglutination and precipitation). A sample of blood (5 - 10 ml), collected from the large marginal ear vein before inoculation should be retained as a check on freedom of 'antibodies' prior to injection.

Inoculating Antigen for Agglutination

If the operator is interested only in the agglutination reaction, a complex medium (e.g. yeast extract mannitol agar) can be used to provide a good crop of rhizobia.

When the flagellar as well as the somatic reaction is to be studied, a significant proportion of a young motile culture should be included in the inoculating suspension.

Inoculating Antigen for Precipitation

Because, unlike agglutination, this reaction will detect soluble as well as fixed antigens, the medium itself should be free of complicating antigenic components. Complex materials like yeast extract must, therefore, be avoided by growing the bacteria on a relatively simple defined medium (e.g. p.31) either solidified with good quality agar or as a broth.

If, as is usual, the operator wishes to retain the specificity associated with surface-located antigens, care will need to be taken to avoid autolysis (and consequent liberation of deep-seated components) by using relatively young (2-5 day old) fresh cultures grown on a nutritionally adequate medium (Humphrey and Vincent, 1965).

Intravenous Injections

1. Use a medium sized needle (22-24 gauge, about 0.5mm) to inoculate the large marginal vein of the ear of a well-grown rabbit with 1, 2, 3 and 3.5 ml of bacterial suspensions on successive days.
2. Ten days after the last injection, collect a sample from the ear vein, allow to coagulate and separate the serum (with centrifugation to remove residual red cells).
3. Dilute the twofold steps to provide final dilutions at least up to 3,000 and test with the inoculating (homologous) strain. Positive agglutination is likely to be secured at 1,6000 x dilution or better (especially in the case of somatic agglutination).

The larger quantity of blood can be collected after a further 4 days if the trial bleed is satisfactory. Otherwise a booster dose of 3 ml is given and the blood collected after a further week.

Intramuscular Injection

1. Immediately before use emulsify the suspension of inoculating antigen with an equal volume of adjuvant (Freund's (Difco), by progressive incorporation of the bacterial suspension in the adjuvant. To achieve a good stable emulsion the mixture is vigorously sucked up into and expelled directly from the barrel of the syringe (without needle).
2. Use the syringe with an 18 gauge needle (1-2 mm) to inject 1-2 ml of the emulsion directly into the large thigh of the rabbit's hind leg.
3. Collect 5-10 ml from the ear vein after 1 month to check titre (as above) and then inoculate 1-2 ml of the bacterial suspension (without adjuvant) intravenously.
4. Take the main sample after a further 7-10 days.

COLLECTION OF BLOOD AND PRESERVATION OF ANTISERA

1. Firstly, it is most convenient to sacrifice the animal and collect its blood directly from the jugular vein into a large beaker without aseptic precautions.

Other procedures, such as bleeding from the marginal ear vein or by heart puncture, can be adopted if it is desired to maintain the animal for successive yields or if aseptic collection is aimed at. 10-20 ml of blood can then be collected at several 2-3 day intervals and the animal may be kept for a longer period with intravenous boosting injections of the Rhizobium. However, the antiserum becomes less strain specific with time (as antibodies to minor common antigens develop) and it is often troublesome to maintain the animals for too long a period.

2. The blood is incubated for 1 hr at 37°C to facilitate clotting, the clot is cut and held at 4°C overnight to help extrusion of the serum and the serum is then drawn off and centrifuged to free it of residual cells.
3. The clear serum can be preservative (e.g. with a concentrated phenol solution so as to give 0.05% final concentration), and held at 2°C, preferably in 0.5 ml or 1 ml sub-samples to avoid repeated exposure to room temperature.

As a better alternative it can be subdivided in 0.5 or 1 ml lots in small ampoules, without preservative and deep-frozen or lyophilized. Either method provides safe storage for several years.

4. Satisfactory antisera from separate bleeds, or even different animals, can be pooled to provide a stock of uniform serum. Stocks

should be divided and stored in different refrigerators as insurance against complete loss from the breakdown of a particular unit.

PRINCIPLE OF ANTISERA ABSORPTION

Antisera can be made more specific by absorbing common antibodies, leaving those specific for the strain against which the antiserum was prepared (homologous strain).

Detailed absorption procedures for use with agglutination and precipitation will be described separately.

THE LONG AGGLUTINATION TEST

MATERIALS

1. Antisera (as above).
2. Testing antigen: This will be developed along the same lines as for the inoculating antigen but will be harvested without aseptic precautions. Unheated suspension will provide both flagellar (H) and somatic (O) reaction antigens. Heating at 100°C for 30 minutes destroys the flagellar antigens. The use of formalin does not mask the somatic antigen of the rhizobia as it does with the salmonellae.
3. Other materials:
0.85% NaCl. 10 ml pipettes calibrated in 0.1 ml 1 ml pipettes, finetipped and calibrated in 0.1 ml. Agglutination tubes 1 ml capacity, Dreyer pattern. Water bath (52° or 37°C) and racks suitable for Dreyer tubes. Reading-box to permit tubes to be viewed with indirect illumination against flat black background with provision for 2x-3x magnification. Test-tubes and racks for preparing dilutions of antisera.

PROCEDURE FOR SETTING UP THE TEST

1. Set agglutination tubes out in the agglutination rack according to

a recorded plan so that each treatment can be identified by its position.

2. For each antiserum to be tested, set up the test-tubes for serial dilutions in desired steps and to cover the chosen range. Actual volumes will depend on the number of antigens under test. The following schedule would be suitable for two-fold dilution to 6,400 tested with 8 suspensions of antigen:

Tube:	1	2	3	4	5	6	7	8
ml saline	9.6	5	5	5	5	5	5	5
ml serum (or ml of serum from previous dilution)(a)	0.4	5(1)	5(2)	5(3)	5(4)	5(5)	5(6)	5(7)
Final dilution (b)	50	100	200	400	800	1,600	3,200	6,400

Notes:

- (a) Tube No. from which sample is taken, shown in brackets.
- (b) 0.5 diluted serum + 0.5 ml antigen suspension.

A 10 ml and a 1 ml pipette are reserved for each serum and every care taken to avoid mixing sera in the course of the test itself and, most important, in the stock supply. When the dilutions are prepared, 0.5 ml quantities are dispensed with the smaller pipette, working from the most dilute to the most concentrated, with thorough rinsing of the pipette by sucking the lower dilution up and down in the pipette several times before using it to dispense the 0.5 ml amounts.

3. Provide a saline control (0.5 ml, 0.85% NaCl).
4. Dispense each antigen from its own serum-free 1 ml pipette, in 0.5 ml quantities according to the plan of the test. Be careful to avoid touching the tip on the agglutination tube, and thus transferring a little serum with the pipette.
5. Inspect the rack of tubes for completeness of dispensing and agitate it, or tubes singly, to remove air bubbles that might

separate the reactants or confuse the result by rising to the surface during the reading.

6. Set the racks in the water bath at approximately 52°C for a quick result (otherwise not lower than 37°C).

Note:

The water level in the bath should be between 1/3 and 1/2 the level of the reactants to promote mixing by convection. Failure to observe this precaution can slow down the reaction considerably or necessitate separate mixing of the contents of each tube. Mixing may still be required if the bacterial suspension is very viscous.

READING THE TUBES

The flagellar reactions proceed rapidly at 52°C (1-2 hr) and somatic reaction will generally be complete after 4 hr*. At 37°C the time has to be extended to about 4 hr and overnight respectively. A 'Vi-like' antigen reacts better at 37°C (Vincent, 1953).

The two kinds of agglutination can be readily distinguished when viewed with indirect illumination against a non-reflecting black background with the help of a low power lens.

Flagellar reaction--Large flocculent slow-settling aggregates which, when accompanied by the somatic reaction, leave a distinctly turbid supernatant.

Somatic reaction--Commences with a finely granulated appearance and either leads to a very compact deposit and completely clear supernatant or, towards the limiting dilution, remains suspended but is scintilla-

*The end-point may, however, be more accurately determined after standing overnight at room temperature.

tingly granular: an appearance quite different from the uniform dull turbidity of a negative reaction.

Combined reaction--The first-formed flocculent, flagellar deposit is accompanied by slower granular agglutination of the remaining cells and complete clearing of the supernatant, or the brightly reflecting condition noted under 'somatic reaction'.

Saline control--This should remain uniformly turbid. Occasional trouble is encountered due to 'auto-agglutination' of unstable antigen. A true positive reaction can still generally be distinguished by careful comparison with the control. Otherwise the test should be repeated with saline reduced to 0-5%, or left undecided.

Cases of unstable antigen might merit the more tedious complement-fixation test.

THE SHORT AGGLUTINATION TEST

MATERIALS

1. Antisera (as shown) but diluted 1/10 as working stock.
2. Testing antigen: Suspend the growth from a sloped culture in about 5 ml of 0.85% NaCl or use a suspension grown in a shallow layer of yeast extract mannitol solution (4 ml in 18 mm diameter tube, without CaCO₃). Unheated suspension provides both flagellar (H) and somatic (O) reactions; heating at 100°C for 30 m destroys the flagellar antigens.

Note: It is not practicable to remove clumps in a test involving a large number of cultures but confusion can generally be avoided by comparison with the saline control, particularly by depending on the finely granular scintillating appearance of the supernatant in the case of a positive somatic agglutination.

3. Other materials:

0.85% NaCl. Dropping pipettes, calibrated to deliver 50 drops for 1 ml. Antigen pipettes, roughly calibrated to deliver the equivalent of 16 drops when expelled. Agglutination tubes, 1 ml capacity, Dreyer pattern. Water bath (52° or 37°C) and racks suitable for Dreyer tubes. Reading box to permit tubes to be viewed with indirect illumination against a dull black background with provision for 2x-3x magnification.

PROCEDURE

1. Prepare antigen suspensions arranged in a systematic fashion to minimize labeling.
2. Set out agglutination tubes in the agglutination racks according to a recorded plan so that each treatment can be identified by its position.
3. Set up one or several dilutions of antiserum (or antisera), according to the nature of the test. The following schedule provides for 3 dilutions (50x, 100x, 200x) plus a saline control:

Dispensing Schedule (Drops)	
Saline	Antiserum 1/10
0	4
2	2
3	1
4	0

Add 16 drops of antigen to each tube.

4. Inspect the rack of tubes, if necessary agitate to remove air bubbles.
5. Set up at 52° or 37°C (see 'long test' for more detail).

Reading the tubes:

See details under 'long test'. Note that with the short test careful comparison with the saline control is even more important.

TRAY AGGLUTINATIONS

Disposable transparent plastic cavity trays can be used as a convenient alternative to agglutination tubes and are especially suitable when there is only a small amount of bacterial suspension available for testing; single large colonies can be used in this way. Dilution procedures have to be adopted to suit the smaller volumes and the trays incubated (37°C, 2 hr) in a humid chamber to avoid drying out. Readings are made with indirect illumination largely from below the tray. Until experience is gained comparisons should be made with regular tubes tests. Distinction between flagellar and somatic reactions is not easy with this method which is more suited to occasions when either or both types of reaction is acceptable or when only one type of reaction can occur.

The most convenient method of preventing the less specific flagellar reaction is to add the antigen suspensions to the tray first, heat at 80°C for 30 min (the tray having its cavities covered with a plastic sheet to prevent evaporation) and then cool before adding antiserum.

Appendix X

Lyophilization (Vincent, 1970)

1. One loop of bacterial culture is suspended in water containing 10% and 5% peptone.
 2. Dispense 0.1 ml aliquots into ampoules plug lightly with cotton.
 3. Apply phosphorous pentoxide to culture under high vacuum. (This provides quick freezing conditions and removes the bulk of the water in 1-8 hrs).
 4. The cotton plug is pushed halfway down the tube and the ampoule is constricted. (as to facilitate the final sealing off under vacuum).
 5. Drying is completed by maintaining the vacuum and exposure to P_2O_5 overnight.
 6. The ampoules are then sealed carefully by means of a fine twin-jet burner (taking care not to char the cotton wool).
 7. Inspect for cracks.
- * Ampoules can be checked for absence of leaks by means of a high frequency probe (e.g. Edwards T2HF Ampoule tester) Blue discharge color in the ampoule is satisfactory; leaky tubes will spark or fail to show any discharge color.

Reconstitution (Vincent, 1970)

1. Open ampoule
 - a) Mark the ampoule at the middle of the cotton, plug with a glass knife or file
2. Air is allowed to enter slowly through the cracked tube before the end is broken off.
3. Remove the cotton plug.
4. The mouth of the ampoule is flamed.

5. Add 0.1 ml sterile yeast extract mannitol broth.
6. Mix
7. The liquid media is then transferred to a plate of YEM agar.
8. The bacterial suspension is spread with a sterile loop.
9. Incubate.

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