LEGUME INOCULANT PRODUCTION MANUAL

Joe C. Burton, Ph.D.
Inoculant Production Specialist

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NifTAL Center - MIRCEN
University of Hawaii
Department of Agronomy
and Soil Science
College of Tropical Agriculture
and Human Resources
1000 Holomua Road
Paia, Maui, Hawaii, 96779, USA

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I. INTRODUCTION

Leguminous plants have the remarkable ability to work with special bacteria (*Rhizobium* spp.) in nodules on their roots and gather or fix atmospheric nitrogen. Through this phenomenal process, inert nitrogen gas is taken from the inexhaustible supply in the air and used by plants to build amino acids and proteins so essential to all life. This process is called "Biological Nitrogen Fixation" (BNF). Since nitrogen fertilizer is the most expensive input for food production, the BNF approach becomes a very attractive alternative to expensive nitrogen chemical fertilizers.

Rhizobia are not universally present in soils and often those present gather little nitrogen. In order to take advantage of this miraculous association of bacteria and leguminous plants, it is often necessary to provide dependable legume inoculants to assure effective nodulation of leguminous crops. The application of these bacteria to seed or soil is called "inoculation".

This manual is concerned with methods of growing rhizobia, making effective legume inoculants, and using them properly to maximize food, forage and fiber production. It describes culture media; systems of screening and culturing Rhizobium spp.; selection and processing of carrier materials; preparation, processing, and packaging of legume inoculants; quality control; regulatory standards; and proper application of inoculants to leguminous seeds.

Throughout this manual, the term rhizobia is used generically to apply to all root nodule bacteria including species of *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium*.

II. RHIZOBIA

A. Nature of Bacteria

The rhizobia were first characterized by their ability to induce formation of nodules on the roots of certain leguminous plants. These nodules often resulted in enhanced growth of the legume host as a result of the nitrogen fixed by the bacteria in the nodules. This ability, to induce nodule formation, was the criterion for the classification of *Rhizobium* without regard for N^2 fixation. Rhizobia are aerobic to microaerophilic gram negative, rod-shaped bacteria whose usual habitat is the soil.

Our knowledge of rhizobia has greatly expanded since 1886 when these bacteria were first discovered. Concepts of differentiation of *Rhizobium* species have changed. Nitrogen fixation is the function in which the inoculant producer is interested. Thus, effectiveness grouping of leguminous plants is of far more practical significance than cross-inoculation groups or nodulation *per se*.

B. *Rhizobium* Strains and Species

Legumes often require specific strains of rhizobia for maximum nitrogen fixation, but certain leguminous plants are nodulated by the same kind of rhizobia. Leguminous plants nodulated by a common *Rhizobium* comprise a "cross-inoculation group. For many years, bacteria isolated from the nodules on any of the plants in one of these groups were considered a single species of *Rhizobium*. Up to 1940, six *Rhizobium* species were named. The six *Rhizobium* species and their more common hosts were as follows:

1. *Rhizobium meliloti*	alfalfa and bur clover, *Medicago* spp.sweet clover, *Melilotus* spp. fenugreek, *Trigonella* spp.
2. *Rhizobium trifolii*	clovers, *Trifolium* spp.
3. *Rhizobium leguminosarum*	peas, *Pisum* spp., *Lathyrus* spp. vetch and faba bean, *Vicia* spp. lentils, *Lens* spp.
4. *Rhizobium phaseoli*	common, snap, scarlet runner, and field bean, *Phaseolus vulgaris* and *P. coccineus*
5. *Rhizobium japonicum*	soybean, *Glycine max*
6. *Rhizobium lupini*	lupine, *Lupinus* spp. serradella, *Ornithopus* spp.

The concept of using nodulation as a basis for designating *Rhizobium* species was questioned after studies of many leguminous species revealed numerous nonreciprocal crosses and incongruent reactions. A new system based on bacterial characteristics and numerical taxonomy is presented in Bergey's Manual of Systematic Bacteriology, Vol. 1.

Fast-Growers

R. meliloti	*Medicago* spp., *Melilotus* spp., *Trigonella* spp.
R. leguminosarum	
biovars: trifolii	*Trifolium* spp.
phaseoli	*Phaseolus vulgaris, P. coccineus*
viceae	*Pisum, Lathyrus, Lens, Vicia*
R. loti	*Lotus* spp. (fast growers), *Lupinus* spp.
R. japonicum	*Glycine max* (certain Chinese and Asian isolates)

Slow Growers

Bradyrhizobium japonicum	
biovars: glycinae	*Glycine max*
vignaea	*Vigna* - etc.
lupinea	*Lupinus, Lotus pedunculatus*

The rhizobia isolated from cowpeas and numerous other legumes were not given species names until recently. These were simply identified by the name of their parent host. This system is still being used with many leguminous species. Up to now, only about 16% of the 18,000 leguminous species have been examined for nodulation (Allen and Allen, 1981) and many of the *Rhizobium* strains isolated have not been adequately characterized for growth characteristics or the range of hosts with which they are able to symbiose.

C. Fast and Slow Growers

It is generally conceded that *Rhizobium* spp. fall into two distinct categories based on growth characteristics. *Rhizobium meliloti, R. trifolii, R. phaseoli, R. leguminosarum* and rhizobia isolated from nodules on sesbania, birdsfoot, chickpea, and others are considered "Fast Growers." When incubated at 28°C on a solid medium such as yeast extract mannitol (YMA), visible colonies develop in 4 to 5 days. In contrast, the "Slow Growers", *Bradyrhizobium japonicum* and others, require 6 to 8 days to produce visible colonies when incubated under the same conditions. However, within these two general groups, strains of rhizobia vary in their growth rates in culture; the division into slow- and fast-growers on the basis of time required to develop visible colonies is not absolute.

D. Cultural Requirements

Rhizobia are heterotrophic, aerobic to microaerophilic, gram negative, non-sporulating rods with a generation time of 3 to 8 hours. They will grow on the surface of solid media and also in static liquid media providing a large surface area is provided. Growth in submerged culture in fermentors with aeration is recommended for maximum viable cell production. The oxygen or aeration demands of rhizobia are not great. Five to ten liters of air per liter of medium per hour is adequate. Optimum growth occurs at 29° to 30°C.

Rhizobia must be supplied with a source of energy, nitrogen, minerals and growth factors. Sucrose is the most commonly used carbon source for fast-growers because it is universally available, satisfactory and inexpensive. Mannitol and glycerol are preferred by some manufacturers. The slow-growing rhizobia are reported to prefer pentoses (and hexoses), such as arabinose or xylose.

In submerged culture, growth obtained with sucrose is equal to that obtained with other sugars or glycerol. In culturing rhizobia, it should be remembered that the ability of these microorganisms to utilize particular carbohydrates depends to some extent upon the basal medium, the nitrogen source, the oxidation reduction potential, the method of sterilization, size of the inoculum used, and other factors.

Most rhizobia are able to use ammonium or nitrate ions as a source of nitrogen, but growth is usually better in media which supply an adequate amount of low molecular weight amino acids such as occur in plant extracts, yeast, alfalfa, cabbage, wheat straw, corn steep liquor, hydrolyzed casein, and other substances.

III. RHIZOBIUM STRAIN SELECTION

A. Strain Variation and Host Specificity

One of the first and most important steps in legume inoculant production is the selection of *Rhizobium* strains to be used in inoculants for the various leguminous crops. In some countries, government agencies specify and provide the approved *Rhizobium* strains for use by inoculant manufacturers. In other countries such as the United States, strain selection is the responsibility of each manufacturer. In any case, the prospective inoculant manufacturer should obtain all available *Rhizobium* cultures which have been tested and judged effective on the legumes for which he will prepare inoculants. Such cultures can serve as standards for evaluating new strains or isolates.

The criteria used in selecting strains of rhizobia for use in inoculant production are:

1. Ability to form effective N-fixing nodules on the legume plants for which the inoculum is recommended.

2. Ability to grow well in media, in the carrier and in the soil.

3. The ability to induce nodulation and enhance yield of leguminous crops under a wide range of soil and climatic conditions.

4. Good persistence in the soil.

The manual focuses on inoculant production and assumes that strains of *Rhizobium* have been selected by a thorough procedure, such as described by Halliday (1984) or Date (1976). The professional inoculant production microbiologist should be trained in methods of testing *Rhizobium* strains and should realize the importance of using only the most effective strains of rhizobia. Some strains of rhizobia may prove effective on many leguminous species; these broad-spectrum strains are highly desirable.

B. Effectiveness Groupings of Leguminous Plants

In the early development of the legume inoculant industry, it was common practice to produce a single inoculant for all leguminous species nodulated by a common kind or species of *Rhizobium*. One inoculant was produced for each cross-inoculation group of plants. Since the cross-inoculation grouping of plants was based entirely on nodulation with no relation to nitrogen fixation or growth enhancement (symbiotic effectiveness), these inoculants were worthless on many leguminous species.

As pointed out earlier, in some cases one strain of rhizobia may be an effective nitrogen fixer on several species and genera of leguminous plants. Further, it has been noted that certain leguminous genera and species tend to give effective response to the same strains of rhizobia. Leguminous species may thus be grouped for inoculation with a common inoculant (Table 1).

This grouping of leguminous species is very useful because it facilitates production of effective multiple host inocula when production of individual inocula for each genus or species of legume is not practical.

This grouping is particularly useful as a planning aid in searching and screening *Rhizobium* strains for legumes which have not been studied extensively. This grouping should not be used as a substitute for screening on strains of each legume on which the inoculant is to be used. Confirming tests with individual legumes should always be made.

EFFECTIVENESS GROUPING	LEGUMINOUS PLANTS

Rhizobium meliloti

1. *Medicago falcata, M. minima, M. sativa, M. tribuloides, Melilotus alba, M. denticulata, M. indica, M. officinalis*

2. *Medicago arabica, M. hispida, M. lupulina, M. orbicularis, M. polymorpha, M. praecox, M. rigidula, M. rotata, M. scutellata, M. truncatula, Trigonella foenum-graecum*

3. *Medicago laciniata*

4. *Medicago rugosa*

Rhizobium leguminosarum,* biovar *trifolii

5. *Trifolium alexandrinum, T. angustifolium, T. arvense, T. hirtum, T. incarnatum, T. subterraneum*

6. *Trifolium fragiferum, T. glomeratum, T. hybridum, T. nigrescens, T. pratense, T. procumbens, T. repens*

7. *Trifolium berytheum, T. bocconi, T. boissiere, T. compactum, T. vesiculosum*

8. *Trifolium africanum, T. burchellianum* var. *burchellianum*. *burchellianum* var. *johnstonii, T. pseudostriatum, T. rueppelianum, T. steudneri, T. tembense, T. usambarense*

9. *Trifolium cheranganiense, T. masaiense, T. rueppellianum* var. *lanceolatum, T. semipilosum* var. *kilimanjaricum*

10. *Trifolium alpestre, T. medium, T. sarosience*

11. *Trifolium ambiguum*

12. *Trifolium heldreichianum*

13. *Trifolium rubens*

14. *Trifolium reflexum*

15. *Trifolium rubens*

16. *Trifolium semipilosum*

EFFECTIVENESS GROUPING	LEGUMINOUS PLANTS

Rhizobium leguminosarum, biovar viceae

17. *Lathyrus aphaca, L. cicera, L. hirsutus, L. odoratus, L. sylvestris, Lens esculenta, Pisum sativum, Vicia hirsuta, V. tenuifolia, V. tetrasperma, V. villosa*

18. *Lathyrus ochrus, L. szenitzii, L. tuberosus*

19. *Lathyrus clymenum, L. sativus, L. tingitanus*

20. *Vicia faba, V. narbonensis*

21. *Vicia amphicarpa, V. sativa*

Rhizobium leguminosarum, biovar phaseoli

22. *Phaseolus angustifolia, P. coccineus, P. vulgaris*

23. *Lotus americanus, L. pedunculatus, L. strictus, L. strigosus, L. uliginosus, Lupinus albicaulis, L. albifrons, L. albus, L. angustifolius, L. arboreus, L. argenteus, L. benthami, L. formosus, L. luteus, L. micranthus, L. perennis, L. sericeus*

24. *Lupinus densiflourus, L. vallicola*

25. *Lupinus nanus*

26. *Lupinus polyphyllus*

27. *Lupinus subcarnosus*

28. *Lupinus succulentus*

29. *Anthyllis vulneraria, A. latoides, Dorycnium hirsutum, D. rectum, D. D. suffruiticosum, Lotus angustissimus, L. caucasicus, L. corniculatus, L. crassifolius, L. creticus, L. edulis, L. froindosus, L. subpinnatus, L. tenuis, L. tetragonologus, L. weilleri*

EFFECTIVENESS GROUPING **LEGUMINOUS PLANTS**

Rhizobium japonicum

30. *Glycine soja*

31. *Glycine max*, cv. Peking

***Bradyrhizobium* spp.**

32. *Alysicarpus vaginalis, Cajanus cajan, Crotalaria* sp. *Desmodium* sp., *Indigofera* sp., *Lespedeza stipulaceae, L. striata, Macroptilium lathyroides, M. atropurpureus, Psophocarpus* sp., *Vigna angularis, V. cylindrica, V. luteola, V. mungo, V. radiata, V. sesquipedalis, V. unguiculata*

33. *Canavalia ensiformis, C. lineata, Phaseolus aconitifolius, P. limensis, P. lunatus*

34. *Arachis glabrata, A. hypogaea, Cyamopsis tetragonoloba, Lespedeza bicolor, L. japonica, L. sericea*

35. *Centrosema pubescens, Galactia* sp.

36. *Lotononis bainesii*

37. *Lotononis angolensis*

Rhizobium loti

38. *Coronilla varia, Leucaena leucocephala, L. retusa, Onobrychis viciaefolia, Petalostemum candidum, P. microphyllum, P. multiflorum, P. purpureum, P. villosum*

39. *Dalea alopecuroides*

***Rhizobia* Unclassified**

40. *Strophostyles helvola*

41. *Robinia hispida, R. pseudoacacia*

42. *Amorpha canescens*

43. *Caragana arborescens, C. frutesceus*

44. *Oxytropis sericea*

45. *Astragalus canadensis, A. cicer, A. falcatus, A. mexicanus*, A. orbiculatus

C. Competitiveness

Competitiveness is a term used to describe a desirable quality in rhizobia. In essence, a *Rhizobium* strain with the ability to infect and dominate in the formation of nodules on a particular host when large numbers of other highly infective strains are in the rhizosphere is considered highly competitive. The factors contributing to this property are not understood. This quality is not related to the strain's ability to enhance growth of its host. It may be related in some way to the growth and survival of the rhizobia on the seed and in the rhizosphere, or to speed of infection. There is evidence that in some cases the host plant influences which strain infects its root hairs.

High competitiveness combined with good nitrogen-fixing ability are essential for yield enhancement in soils which harbor infective rhizobia. Serology is one method used in screening strains for competitiveness but it can be laborious and time-consuming. Further, interpretation of results be confounded by the lack of correlation between nodule numbers and nitrogen fixation. Field testing of individual high nitrogen-fixing strains as seed inoculants is perhaps the most useful nitrogen fixation. Field testing is the best integration of the *Rhizobium* strain's overall performance, including competitive ability, soil stress tolerance and persistence.

D. Stock Culture Maintenance

Rhizobium strain selection is a continual process. It is important to start with the most effective and competitive strains available and to strive continuously to find even better strains. Stocks of *Rhizobium* cultures important in the area should be maintained in the best way possible and checked frequently to make certain that they have not lost their desirable qualities. In order to avoid or reduce undesirable mutations, *Rhizobium* cultures should be maintained in the lyophilized or freeze-dried state in sealed glass ampoules. When lyophilization equipment is not available, stock cultures may be preserved satisfactorily by desiccation on porcelain beads. This method is described in Appendix C.

When bead materials are not available, cultures may be kept on yeast extract mannitol agar slants. Screw-capped tubes should be used to reduce the need for frequent subculturing. The cultures should be allowed to grow before tightening the caps, then stored at 4°C.

IV. INOCULANTS

Legume inoculants are preparations of live rhizobia designed for application to leguminous seeds or soils to insure effective nodulation of the legume seedlings and, therefore supply abundant nitrogen for crop production. Normally inoculants are designed for application to seeds because this is an easy and convenient way of putting the rhizobia in the root zone of the developing seedling where infection of the root hairs can occur and nodules develop.

A. Desirable Qualities of a Legume Inoculant

Strain selection and maintenance is a very important aspect of legume inoculant production. The qualities considered important are: 1) ability to form N-fixing nodules on the species and cultivars of legumes for which the rhizobia is recommended, (2) competitiveness in nodule formation and survival in the presence of other infective rhizobia in the soil, (3) prompt nodulation and good N_2-fixation over a wide range of soil types, (4) good growth ability in the carrier and in the soil, (5) persistence in the soil, (6) effectiveness on a wide range of host genotypes or cultivars, and (7) tolerance to soil stress factors including acidity, alkalinity, salinity, high concentration of aluminum and manganese.

Legume inoculants may consist of one strain selected for a particular legume host or they may comprise two or more strains effective on that particular host. The latter is often advantageous particularly in areas where several varieties or cultivars of the legume are grown or where the soils vary widely in characteristics. However, it is important to have compatible strains and the strains should be grown separately before combining to make the inoculant.

Multiple host inocula are often desirable. They are feasible. As shown in Table 1, certain genera and species of legumes tend to give a similar response to particular strains of rhizobia. For multiple host inoculants wide-spectrum strains are preferable. No strain should be included in a multiple host inoculant which could produce ineffective nodules on any of the leguminous hosts for which it is intended. Inoculants with only two or three strains are preferred over inoculants with many strains.

B. Soil Inoculants

While seed inoculants are the most common, there are times when it is not practical to apply inoculants directly to seeds. Chemicals applied to seeds to protect them from insects, pests, and diseases are often toxic to rhizobia. Rhizobia applied to seed planted in hot dry soils might be killed before the seeds germinate. Under these circumstances, it may be necessary to add the inoculant directly to the soil using large peat granules that are flowable. However, formulations of liquid broth or even powdered seed inoculant may also be used. Larger amounts of inoculum and special equipment are needed in most soil inoculation practices.

Legume seed inoculants are marketed in many forms: (1) peat or powdered solid base, (2) liquid or broth, (3) bottle or agar slants, (4) oil-dried, (5) lyophilized or freeze-dried powders, or (6) in polyacrylamides. The moist powder or peat-based inoculum is the most common seed inoculant and has generally been the most reliable. However, the true quality of a legume inoculant can be measured only by the results it produces in the soil and climatic conditions where it will be used. Inoculants which are

similar in appearance can vary greatly in the number of live effective rhizobia they provide and consequently, the ability to bring about effective nodulation. These aspects will be discussed in greater detail under Quality Control.

V. MASS CULTURE OF RHIZOBIA

A. Media for Fermentors

Rhizobia are moderately easy to culture and not particularly fastidious in their nutrient requirements. Nearly all rhizobia utilize monosaccharides and disaccharides readily and to a lesser extent trisaccharides, alcohols and acids. Starch is not utilized. The soybean and cowpea type rhizobia prefer pentoses such as arabinose or xylose. Sucrose and mannitol are probably the most commonly used energy sources. According to Graham and Parker (1964), *Rhizobium* strains within a species can differ in their ability to utilize carbohydrates. Consequently, it is important to make certain the *Rhizobium* strains selected can utilize the carbohydrate in the fermentor medium.

Yeast and plant extracts such as alfalfa, cabbage and peas, as well as casein and corn steep liquids are considered beneficial to growth of rhizobia. These extracts and hydrolysates can also provide carbohydrates for growth of rhizobia. This could mask the need for growth factors, micronutrients, and specific carbon sources, particularly when a generous amount of the extract is added. Yeast extract is the most commonly used growth factor supplement for rhizobia. Other suitable substitutes are listed in Appendix A.

The composition of some media which have been used successfully in culturing rhizobia is given in Table 3. Two of the media contain mannitol only, one contains sucrose only and another contains both sucrose and mannitol as a carbon source. The author has found that a small amount of lactic acid stimulates growth particularly of the slow-growing rhizobia. The lactic acid solution is used as a solvent for micronutrients, cobalt molybdenum, zinc, copper, iron and boron which are considered essential for N fixation by the legume but may not be required for growth of rhizobia (Table 2).

Table 2. Micronutrient - Stock Solution - Burton

Ingredient	Per liter
Boric Acid, H_3BO_3	2.780 g
Manganese sulphate, $MnSO_4.7H_2O$	1.540 g
Zinc sulphate, $ZnSO_4.7H_2O$	0.210 g
Sodium molybdate, Na_2MoO_4	4.360 g
Ferric chloride, $FeCl_3.6H_2O$	5.000 g
Cobalt sulphate	0.004 g
Lactic acid (88%)	580.0 ml
Distilled water	420.0 ml

* Addition of 1.0 ml per liter of medium gives: boron 0.5 microgram; manganese 0.5 microgram; zinc 0.05 microgram; molybdenum 1.0 microgram; iron 100 micrograms and cobalt 0.0005 microgram per liter or part per million (p.p.m.)

Table 3. Composition of Media for Growth of Rhizobia

Ingredient	Fred and Waksman 1928	VanSchreven 1963	Date 1976	Burton 1967
	----------grams per liter----------			
Mannitol	10.0		10.0	2.0
Sucrose		15.0		10.0
Dipotassium phosphate (K_2HPO_4)	0.5	0.5	0.5	
Tripotassium phosphate (K_3PO_4)				0.2
Monopotassium phosphate (KH_2PO_4)				0.4
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2	0.2	0.2	0.2
Sodium chloride (NaCl)	0.1		0.2	0.06
Calcium carbonate ($CaCO_3$)	3.0	2.0		0.2
Calcium sulphate ($CaSO_4.2H_2O$)				0.04
Iron chloride ($FeCl_3.6H_2O$)			0.1	
Yeast water	100.0 ml	100.0 ml	100.0 ml	
Yeast extract				0.5
Paraffin oil		0.5		
Ammonium phosphate $(NH_4)_2$ HPO				0.1
Water	900 ml	900 ml	900.0 ml	1,000 ml
Micronutrient Solution*				0.3

* See special nutrient solution - Burton, Table 2

B. Preparation of Yeast Water

Fresh starch-free cakes of yeast are preferred in making yeast water. Suspend 100 g of yeast in 1,000 ml of water and boil slowly or steam for 3 to 4 hours, replacing the water lost regularly. Allow the cooked suspension to stand until yeast cells have settled to the bottom, usually 10 to 12 hours. Siphon off the clear, straw-colored liquid; adjust the liquid to pH 6.6 to 6.8 with sodium hydroxide; bottle and autoclave for 30 to 40 minutes at 121°C. Following sterilization, the yeast water may be stored at room temperature.

Dried yeast may also be used in making yeast water. One pound of dry yeast is equal to about 2.5 lbs of wet yeast. Suspend 40 g of dry yeast in liter of water. Boil, decant, bottle and Sterilize in the same way as described for fresh yeast. One hundred milliliters of yeast water should contain about 75 mg of nitrogen.

Yeast extract powders prepared by spray-drying aqueous autolyzed yeast preparations are available in many countries. When these are available, about 0.5 g of the dried preparation per liter is used to replace yeast water. Dry preparations are convenient and usually satisfactory.

The media containing yeast described above may foam excessively when aerated vigorously in fermentor vessels. Foaming can be controlled by adding a small amount of sterile white mineral oil or silicone emulsion.

C. Culture Vessels

Rhizobia are relatively easy to grow and the medium is a simple one. Methods of culturing rhizobia vary with manufacturers, but aeration of the medium with sterile compressed air is the most common. Mechanical agitation may be used; its usefulness is primarily to expedite heat exchange during sterilization and cooling of the liquid medium. Mechanical agitation is not needed or recommended for small fermentors. Agitators are expensive and the shaft bearing can be a source of contamination.

Growth is not increased by violent aeration or agitation. An oxygen partial pressure of 0.15 atmosphere is optimum for respiration. The low oxygen requirement of rhizobia is undoubtedly associated with the organism's ability to grow in the interior of an active nodule where a low oxygen tension prevails. A temperature of 28 to 30°C is optimum for growth of rhizobia.

The fermentor for culture of rhizobia should have a simple design with the following specifications (Figure 1).

1. Ability to withstand internal pressure of 30 psi steam or greater.
2. Handy access port to facilitate adding medium and washing, and closure which provides a dependable seal during and following sterilization.
3. Metal such as stainless steel which is nontoxic to bacteria and easy to clean. Types 304 or 316 stainless steel may be used.
4. Ability to withstand direct heating with a gas or oil flame for easy sterilization.
5. Equipped to supply sterile air through a sparger to aerate the broth medium and provide oxygen for the rhizobia.
6. Inoculum port for adding the starter aseptically.
7. Sample port which is easy to sterilize to facilitate monitoring of the growth and purity of the culture.
8. Air exhaust tube with valve for regulation of aeration.
9. Fermentor should be equipped with an accurate, rugged thermometer of the bimetallic type, a pressure gauge, and a pop safety valve.
10. Strength and durability to withstand handling and use over a long period of time.
11. Permit aseptic removal of the broth culture and easy cleaning.

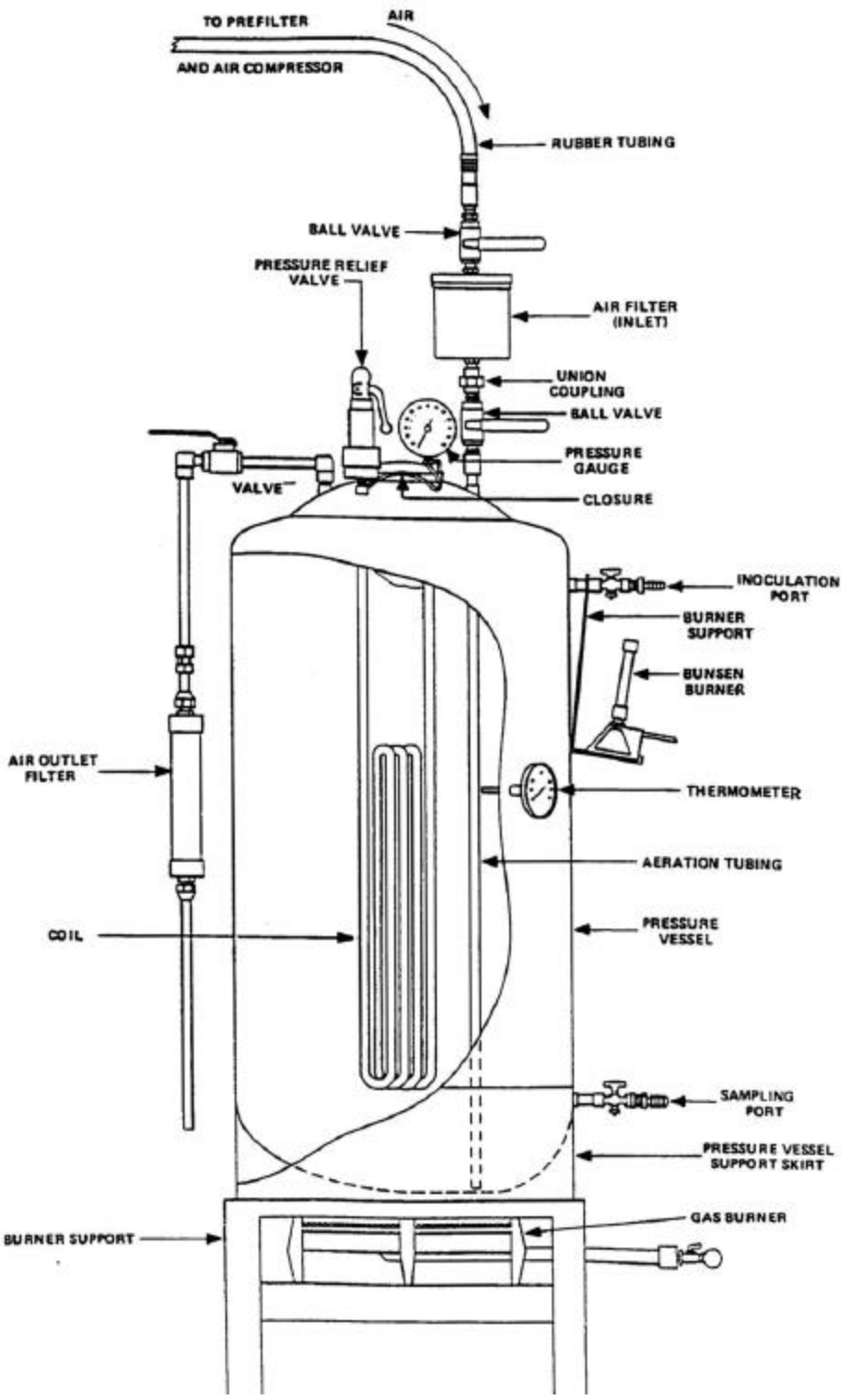

Figure 1. FERMENTOR

D. Fermentor Requirements for Various Production Goals

Production of legume inoculants begins with cultures grown in test tubes. The rhizobia grown in tubes are transferred to larger vessels, Roux bottles or Erlenmeyer flasks for shake culture. The rhizobia grown in Roux bottles or shake flasks are transferred to small or large fermentors based on needs. The various stages in inoculant production are shown in Figure 2. Each step in culturing rhizobia requires 48 to 72 hours and should bring about a 20 to 100-fold increase in volume of usable broth culture. A 3 to 5% larger starter is used with slow-growing rhizobia to offset their slower growth.

One of the first steps in preparing for legume inoculant production is to estimate as accurately as possible the kinds and amounts of various inoculants which will be needed each season of the year. In doing this, consideration must be given to the number of strains of Rhizobium which will be used in each inoculant and the leguminous crops to be inoculated. The equipment specified should allow flexibility and production of 130 to 150% of anticipated needs because for various reasons such as some unavoidable contamination the actual production will often be less than theoretical production. However, with well-designed equipment and good techniques, one can obtain 95 to 98% of theoretical production.

The data in Table 4 show the amounts of inoculant which can be produced and the area of land which can be planted to soybeans with various quantities of broth culture.

The data in Table 4 are based on the use of a pulverized sedge peat with an 8% moisture content as the carrier medium, the addition of 8 kg of $CaCO^3$ to 92 kg of powdered peat, a moisture content of 40% in the finished inoculant, and an application rate of 5 g of inoculant to 1 kg of soybean seed. The potential inoculant production with various fermentor capacities during a 9 month production season and conversion factors for acreages of various other legumes are listed at the bottom of the table.

The volume of broth culture grown should not exceed that which can be mixed with the carrier processed and packaged when it reaches the proper stage. The fermentors should not be used or considered as storage vessels. Rhizobia soon lose their ability to grow and multiply in the carrier medium after they reach the stationary growth phase. Broth culture should be mixed with the carrier while the rhizobia are in the logarithmic growth phase.

The weight of inoculant which can be made with a given volume of broth culture is similar for all legumes but the volume of seed this will inoculate or the hectarage of land covered will vary widely with leguminous species, seed size, and rate of sowing (Table 5). With small-seeded legumes, larger quantities of inoculant are needed to provide sufficient viable Rhizobium cells to assure effective nodulation.

Figure 2. Single Strain Inoculant Production

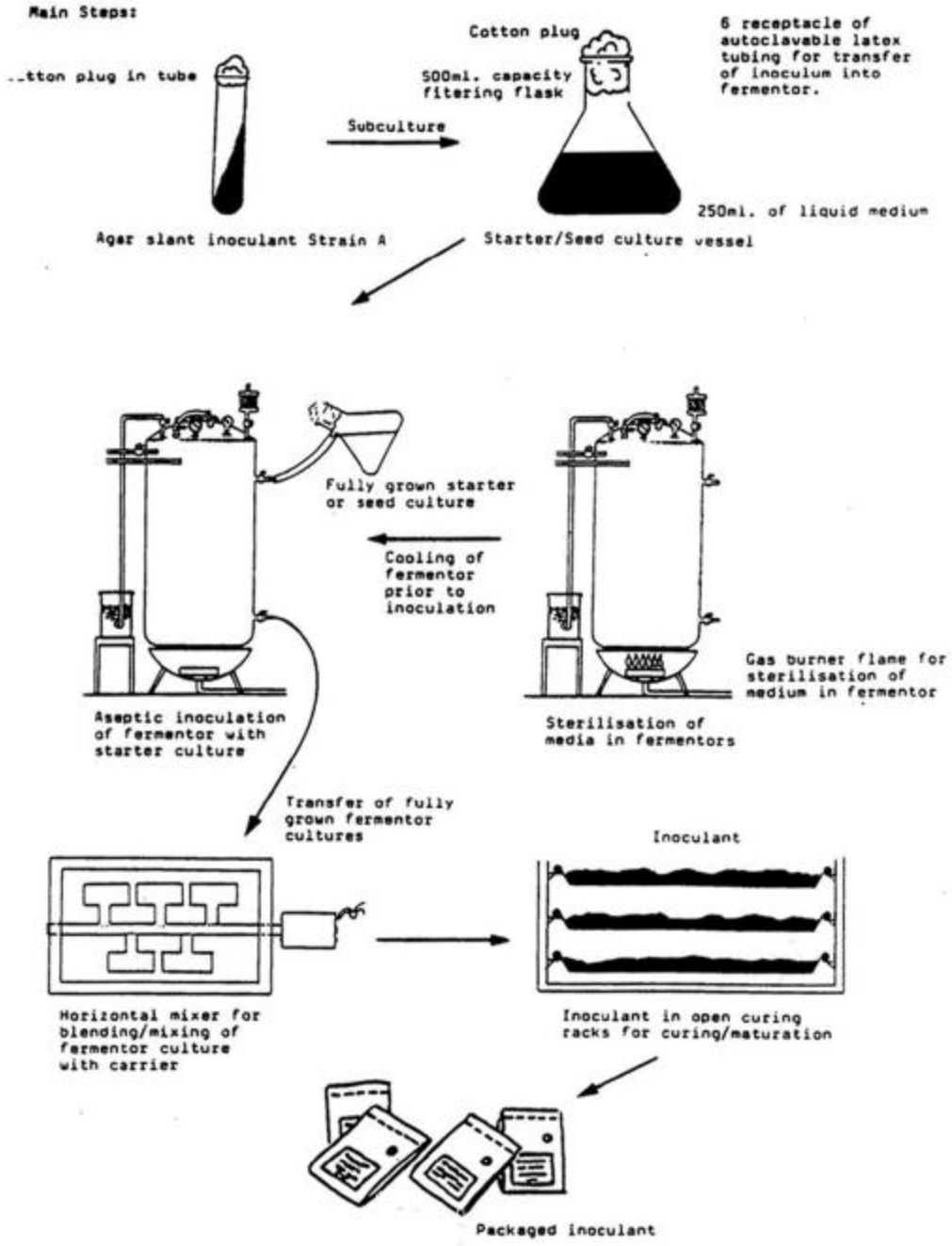

Table 4. Potential inoculant production with various sizes of fermentor

Fermentor Volume	Operating Capacity 75% volume	Weight of Inoculant/Batch Peat Base	Inoculant/9 months One batch per wk. 36 Batches	Enough for Soybeans hectares*
	Liters	Kilogram	Kilogram	
6 gal (22 L)	17	47	1,692	5,206
16 gal (60 L)	45	126	4.536	13,957
37 gal (140 L)	106	297	10,692	32,898
50 gal (189 L)	142	398	14,328	44,086
60 gal (227 L)	170	476	17,135	52,726
75 gal (284 L)	213	596	21,456	66,018
100 gal (378 L)	284	795	28,620	88,062

*soybeans planted 65 kg/hectare; inoculant applied at rate of 5 g/kg seed.

Conversion factors for other legumes are:

Legume	Planting Rate	Conversion Factor
	kg/ha	
Lotonois bainesii	2	X 32.5
Medicago sativa	16	X 4.0
Vigna radiata	76	X 0.85
Vigna unguiculata	80	X 0.81
Cicer arietinum	33	X 1.97
Vicia faba	87	X 0.75

Table 5. Relation of legume seed size and rate of planting to area which can be sown with inoculum prepared from one liter of broth culture (3 kg peat-base inoculum).

Leguminous plant	Seeds/kg	Rhizobia/Seed 5g Inoc/kg seed	Seed/ha kg	Hectares sown
Lotonois bainesii	3,700,000	135	2	300.0
Medicago sativa	500,000	1,000	16	37.5
Coronilla varia	250,000	2,000	20	30.0
Vigna radiata	25,000	20,000	76	7.9
Vigna unguiculata	10,000	50,000	80	7.5
Glycine max	5,000	100,000	65	9.2
Cicer arietinum	2,000	250,000	33	18.2
Vicia faba	1,250	400,000	87	6.9

In planning an inoculant production facility, it should be remembered that small culture vessels are always needed regardless of the production capacity required. The small fermentors are needed to grow seed or starter cultures for the larger fermentors as well as for inocula required in only small amounts. Further, with sufficient small fermentors, the production capacity can be greatly increased by acquiring one or more large fermentors for the final multiplication stage and by using a 5 to 10% starter.

Metal pressure vessels, which can be equipped for aeration with sterile air, are preferable to glass. Stainless steel is easier to keep clean than carbon steel. Vessels which can be heated directly by an open burner have advantages over those which have to be autoclaved.

VI. CARRIER MATERIALS

Peat is the most commonly used solid carrier in making legume inoculants. It is also the most dependable because rhizobia in a peat carrier remain viable longer both in the package and on the seed. However, good quality peat is not available in many countries.

Chemical and physical analyses of carrier materials are helpful but do not confirm the quality of a carrier. The quality can be determined only by placing viable rhizobia in the material and monitoring the growth and survival of rhizobia over a period of 6 months or longer.

The important qualities of a carrier material in legume inoculant production are:

1. Good absorption capacity,
2. Easy to dry and grind,
3. Nontoxic to rhizobia,
4. Free of abrasive minerals,
5. Low in content of soluble salts,
6. Easy to sterilize,
7. Available in adequate amounts at a reasonable cost.

Many other materials -- bagasse, sugar cane filter mud, coir dust, coal, lignite, charcoal, straws, various compost mixtures, clays, and minerals such as apatite and vermiculite, have all been tested and are acceptable (Date and Roughley, 1977; Burton, 1979; Paczkowski and Berryhill, 1979). Nonetheless, it is still desirable to carry out tests with each individual material because of the wide variation which occurs within each type of carrier. Some of the chemical and physical properties of materials used as carriers in inoculant production are given in Table 6.

It is difficult to make valid comparisons of organic materials used as carriers. In essence, the choice of a carrier material should be based on its ability to support large populations of rhizobia over a long period of time. This will depend upon the processing as well as the physical and chemical properties of the substance. Processing equipment can be far more expensive than the fermentor equipment used in growing rhizobia.

Table 6. Some physical and chemical characteristics of various carrier materials (Subba Rao, 1983).

Carrier	Organic Matter %	Total N %	Bulk Density g/cc	Porosity %	Water-holding Capacity %
Sedge Peat	76	0.95	0.82	45	200
Farm Yard Manure	79	0.93	0.79	55	153
Filter Press Mud	76	0.83	0.75	56	155
Compost	55	0.55	0.75	59	171
Vermiculite Clay	1	0.01	0.98	63	152
Lignite	75	0.31	1.08	35	198
Charcoal	22	0.01	0.43	73	200

Some of the properties of a U.S. processed peat (DEMILCO) are given in Table 7

Table 7. Sieve analysis of DEMILCO processed sedge peat carrier

Particle size	Powder Inoculant	Granular Inoculant
850 - 1200 ìm * (16 - 20 mesh)	0.00%	0 - 10%
600 - 850 ìm (20 - 30 mesh)	0.00%	30 - 40%
300 - 600 ìm (30 - 50 mesh)	1.00%	50 - 60%
150 - 300 ìm (50 - 100 mesh)	5.10%	4.0%
60 - 150 ìm (100 - 200 mesh)	5-10%	Trace
<60 ìm (through 200 mesh)	80-90%	Trace

*Micrometer - mesh - ASTM (Amer. Soc. Testing Methods)
The flash dried peat is sieved to separate particles in the range between 16 and 40 mesh (400 - 1200 micron diameter) to obtain carrier for the granular inoculant. the peat granules are thus natural and of various sizes. The fine powder carrier is obtained by passing the flash dried peat mix through a high speed hammermill and then screening to obtain a high percentage of particles of the 60 to 100 micron diameter size.

The chemical analysis of the DEMILCO peat is as follows: organic matter 80 - 85%; nitrogen 1.5 - 2.0%; crude ash 15 - 20%; and a pH of 4.5 - 5.0.

A. Sterilization
Sterilization or partial sterilization of peat and other organic carrier materials can greatly improve the suitability of the material as a carrier. Generally, inoculants prepared from air-dried, unsterilized peat have a short shelf life. Heat-treated peat, even without aseptic handling afterward, is usually far superior to air-dried or untreated peat. Sterilization may be even more important when nutrients or undecomposed organic substances are added to the carrier because of the increased competition between the rhizobia and other microorganisms for the nutrients provided.

Roughley (1967) reports that dry heating peat above 100°C causes degradation with toxic effects on rhizobia. In the United States (DEMILCO), shredded peat is passed through a revolving drum with an inlet air temperature of 650°C and an outlet temperature of 121°C. The flash-drying, similar to that used in dehydrating high quality alfalfa, produces no adverse effects. The moisture content is reduced from approximately 50% to 8% in a matter of seconds.

In South africa, Strijdom (1981) reports great success in steam-sterilizing peat at 121°C for 3.5 hours. Inoculants prepared with the steam-sterilized peat reach populations of viable rhizobia in excess of 1 X 10^9 and maintain this level for 6 months and longer. Steam sterilization of peat for 3.5 hours at 121°C

was just as effective as gamma irradiation at the 50 kGr (5 X 10^6 rad) dosage. Complete sterilization of the peat does not appear to be necessary. Growth of cowpea rhizobia was just as good in the peat receiving 25 kGr (2.5 X 10^6 rad) irradiation as it was in the peat receiving the higher 50 kGr (5 X 10^6) treatment. Strijdom (1981) points out that optimal sterilization treatments may vary with *Rhizobium* strain and species. Gaseous sterilization of peat with either ethylene oxide or methyl bromide caused development of unfavorable side effects and is not considered satisfactory.

The amount of time needed to sterilize peat or other solid carriers will vary with the moisture content of the substance. Dry peat is more difficult to sterilize by autoclaving than is wet peat. A moisture content in the range of 8 to 12% (wet basis) in the carrier is preferred in making inoculants. Wetting peat with a moisture content of 6% or lower liberates energy as heat. This heat of wetting may result in a high temperature and killing of rhizobia, particularly when the freshly made inoculant is placed in layers thicker than 25 to 30 cm.

A high moisture content in the carrier favors sterilization by heat, but high moisture reduces the amount of broth culture which can be added. This can be overcome, however, by using a more concentrated broth culture of rhizobia.

B. Pure Culture *Rhizobium* Inoculants

The production of pure culture *Rhizobium* inoculants requires that the carrier material, calcium carbonate and any other amendments be placed in the package and sterilized as a unit. The polyethylene flexible film package is satisfactory when gamma irradiation is the sterilizing agent. The packages containing the carrier are sealed, stacked in cardboard boxes and irradiated.

With steam sterilization, a polypropylene flexible film is required. The packages can be filled with the carrier and the open end folded over during sterilization to prevent bursting while being autoclaved. The packages should be sealed quickly after sterilization. An alternate method is to fill the package with carrier and press to remove air from inside before sealing. The sealed packages can then be safely autoclaved.

In order to maintain a pure culture of rhizobia, the package containing the sterile carrier must be inoculated aseptically using a hypodermic syringe. The broth culture is then worked into the carrier by manually kneading the package. One or two weeks are required for the rhizobia to attain the desired concentration in the package.

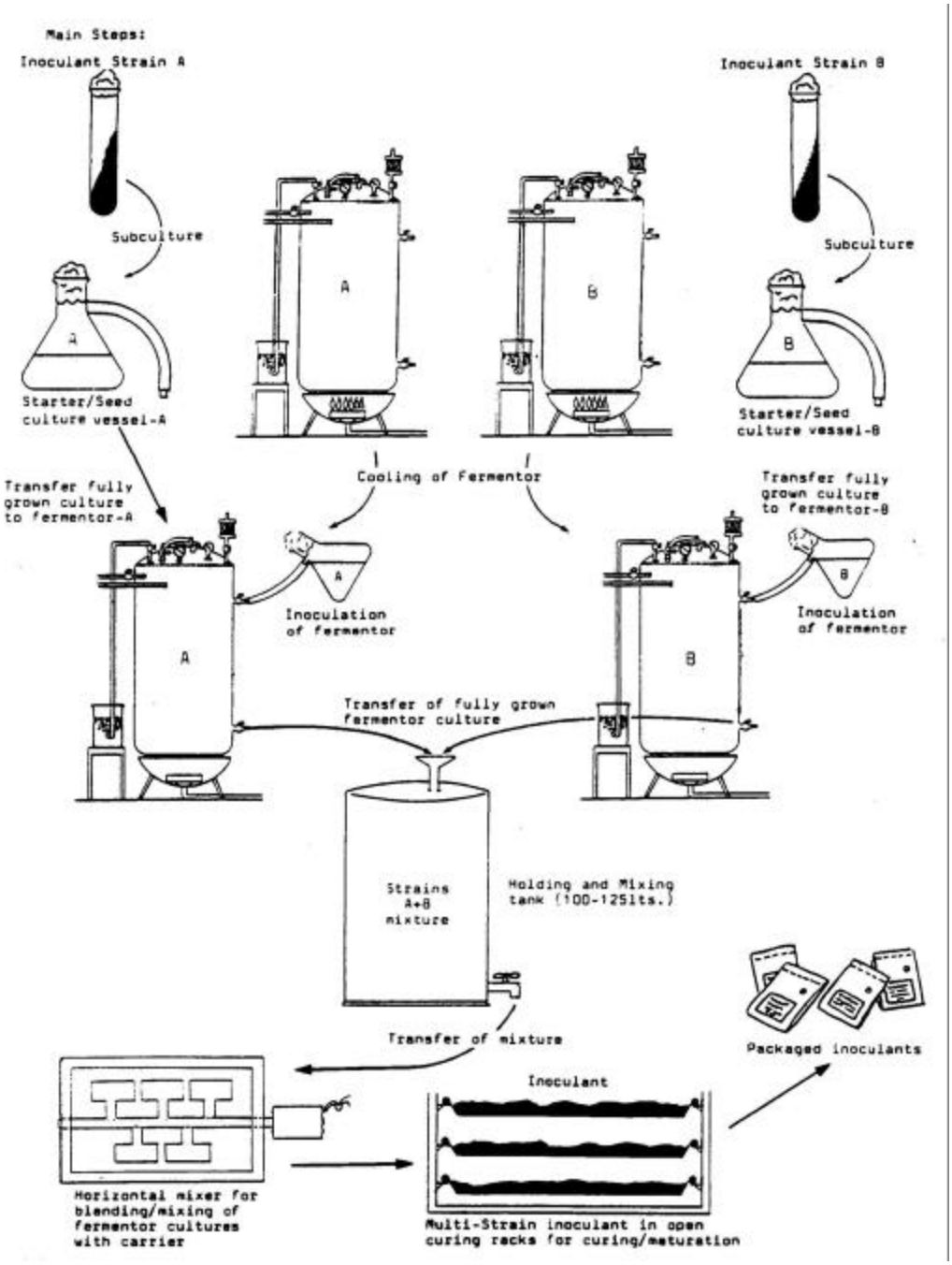

Figure 3. Multi-strain Inoculant Production

VII. MAKING INOCULANTS

Methods of selecting *Rhizobium* strains, culture media, systems of growing the nodule bacteria, and processing of suitable carrier materials have already been described. This section is concerned with the actual growing of the rhizobia culture and blending it with the carrier to make an inoculant.

Growth of the rhizobia is carried out in steps. Bacteria are multiplied by transferring cultures of the organism from test tubes, to bottles or flasks, to small fermentors and then to larger fermentors as required (Figure 3). The rhizobial growth should approach the peak of the logarithmic growth phase in each step before transferring to the next larger fermentor.

In a favorable medium at 28 to 30°C and a 1% starter, the population of viable rhizobia should attain a count of 5×10^8 to 1×10^9 per milliliter in 60 to 72 hours. With a 3 to 5% starter, the same concentration of viable cells should be reached in 48 hours or less. With an urgent need for inoculants, larger starter inocula can be used to speed production.

A. Medium Preparation

Medium preparation can be greatly facilitated by making a composite mixture of the nondeliquescent chemical phosphates, sulfates, sodium chloride, calcium compounds, etc., and by making one weighing of the composite for each fermentor. Reagent grade chemicals are not necessary; chemicals of the U.S. Pharmacopoeia (USP) grade are satisfactory and much cheaper.

A concentrate of the medium is prepared by dissolving the ingredients of the medium in warm water and straining before adding to the fermentor vessel. The water used should be of good quality (drinkable). The medium is sterilized in the fermentor vessel. The time required for sterilization will vary with fermentor design and size. Preliminary tests should be made to measure precisely the total capacity of the fermentor and just how much time is needed at 121°C to completely sterilize that medium. The operating capacity of a fermentor is approximately 75% of its total capacity.

Tests should also be made to determine the time needed to cool the medium to 28°C before inoculating with the seed culture. It is best to make a preliminary run with tap water to determine the cooling time required.

B. Monitoring Growth of Rhizobia

Growth of rhizobia should be monitored by drawing samples at least twice daily and testing for acidity, purity and growth. The pH can be tested colorimetrically by adding 5 or 6 drops of a 0.5% w/v alcohol solution of brom-thymol-blue indicator to 8 ml of broth culture in a tube. With either fast- or slow-growing rhizobia a yellow color indicates acid conditions and probable contamination. Stained smears on microscope slides will reveal stage of growth of the rhizobia and the presence of foreign microorganisms. Rhizobia have a characteristic banded staining with basic fuchsin dye. (See Appendix B for staining procedures).

In contrast to rhizobia, most contaminating microorganisms will stain uniformly dark red with the dye,

particularly the cell walls. Also, the size, shape, and arrangement of the cells are often quite different from rhizobia. The heat-resistant, spore-forming bacilli are the most common contaminants.

If there is any doubt that the bacteria are rhizobia a gram stain should be prepared (Appendix B). Rhizobia are gram-negative. Many of the contaminant microorganisms are gram-positive. They retain the crystal violet dye and will appear as blue or purple stained cells. Contaminated broth cultures should be discarded.

C. Multiple Strain Inoculants

When a multiple strain inoculum is desired, it is preferred that each strain of rhizobia be grown separately and then mixed in equal volumes at the time of making the inoculant (Figure 3). This method does not insure that there will be an equal number of rhizobia of each strain when the duct is used, but it does reduce the risk of one strain dominating the others at an early stage.

This type of production requires a greater number of culture vessels and more precise planning and scheduling. However, this should not be a major problem as long as the number of strains in any inoculum is limited to two or three. Use of multiple strain inocula does provide some protection against complete loss of effectiveness. Also, multiple strain inocula are usually effective on a wider spectrum (species and cultivars) of host plants.

D. Peat Inoculant In Bulk With Non-Sterile Carrier

The blending of a broth culture of rhizobia with the prepared carrier substance, e.g. peat, compost, bagasse, coal etc., is commonly referred to as "making the inoculant". The broth culture of undiluted and unconcentrated rhizobia is mixed with or sprayed onto the carrier in a horizontal mixer of the ribbon or paddle type. If the carrier is acid, $CaCO_3$ is added to neutralize acidity and raise the pH to 6.6 to 6.8. More uniform mixing and quicker action can be obtained by adding the $CaCO_3$ directly to the broth culture and mixing before spraying it onto the peat, but continuous agitation of the broth/$CaCO_3$ slurry is required to prevent settling. The moisture content will vary with different carriers but with peat, the moisture should be approximately 40% (wet basis) in the fresh inoculant.

If the carrier material is very dry, wetting it will bring about a release of energy and a rise in temperature referred to as the "heat of wetting". With continued use, heat from the mixer motor will build up and be transferred to the inoculant. Freshly prepared inoculant should be place in relatively thin layers, not over 25 to 30 cm deep in metal or fiberglass. Following mixing, the temperature should be monitored for 2 to 6 hours and should not exceed 38°C. During this period, the heat generated by the letting and mixing should be dissipated.

The curing process should take place in a clean, ventilated room maintained at a temperature of 25 to 28°C, and a relative humidity between 50 and 60% for 24 to 48 hours.

Following the curing, the inoculant should be milled or screened to break up any lumps which may have formed. It should be blended to give a homogeneous, moist powder which can be packaged easily. After curing and milling, the inoculant should either be packaged or placed in deep bins with tight covers

to protect its physical condition; the material dries out rapidly when stored in shallow or open containers.

The viable count of rhizobia should increase by at least tenfold in non-sterile peat during the first 2 to 4 weeks after preparation, but this can occur only when the inoculum is kept at a temperature favorable for growth. The rhizobia cannot grow when the inoculant is stored at freezing temperatures immediately after preparation. However, rhizobia will grow once the temperature of the inoculum reaches 26 to 28°C and should incubate at this temperature for one to two weeks.

E. Soil or Granular Inoculant With Non-Sterile Carrier

The soil or granular inoculant is prepared for application directly in the seed furrow. Its necessary physical characteristics may vary depending upon the method of application to be used. When mechanical granule applicators are to be used, the material must remain very flowable. With peat granules the moisture content must be in the range of 32 to 35% (wet basis). The carrier must support growth and survival of rhizobia and at the same time maintain flowability.

If the carrier is acid, it must be neutralized. This can be done with $CaCO_3$. The broth culture containing the suspended $CaCO_3$ is sprayed onto the peat in a horizontal paddle-type mixer. The mixing must be kept to a minimum to prevent breaking up of the peat granules. Many of the same precautions are also necessary with other carriers. When the soil inoculants are to be applied by hand or other methods, flowability may not be a major consideration.

F. Pure Culture Rhizobium Inoculant

The production of peat-base inoculants which contain only rhizobia requires a completely sterile carrier in a sterile container. A flexible film bag is most convenient, but glass bottles and high density polyethylene are also used. As described earlier, sterilization is by gamma irradiation or autoclaving at 121°C. The sterile carrier facilitates rapid growth of rhizobia in the package and a longer shelf life.

The sealed package containing the sterile carrier, usually 60 to 100 g, is inoculated with a pure broth culture of rhizobia using a syringe and needle such as the Becton-Dickenson disposable plastic syringe with an 18 G needle which is 1½ (3.8 cm) inches in length. The liquid culture is mixed with the carrier by kneading the package manually (Figure 4).

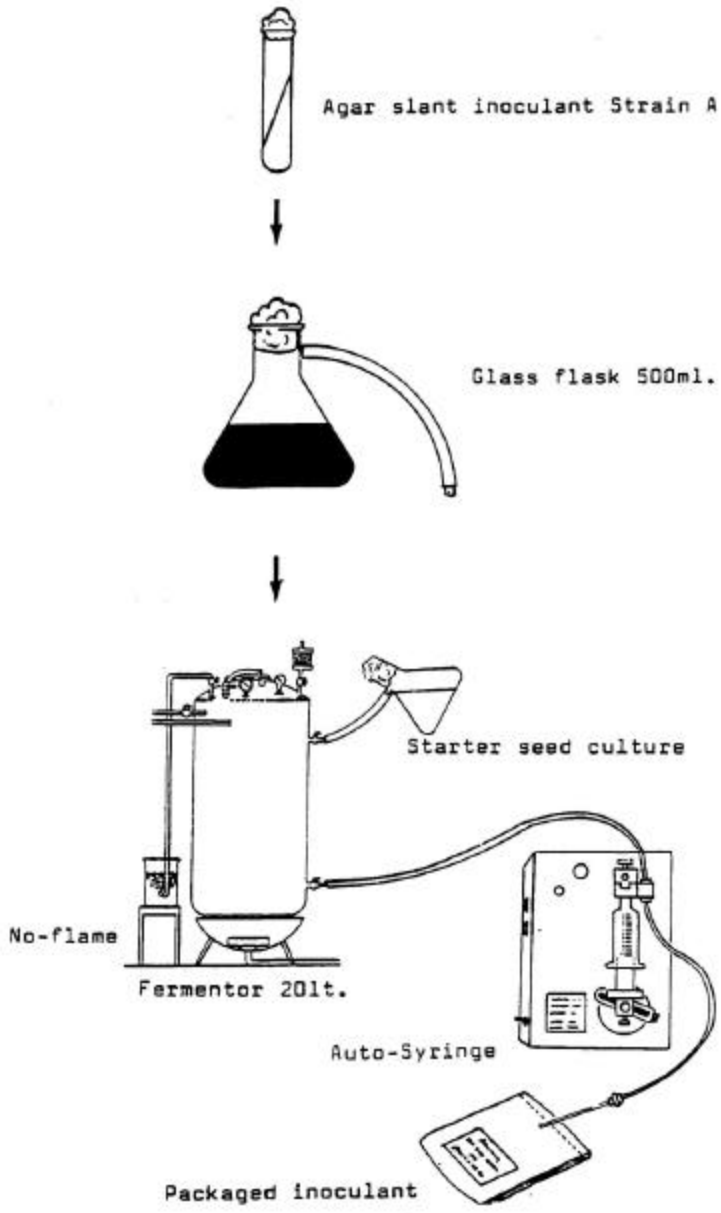

Figure 4. Pure Culture Inoculant System

This system of production is limited to small packages and is very labor intensive even when an automatic syringe is employed in dispensing the broth culture. Nonetheless, this system is being used successfully in Australia and other places where pure cultures of rhizobia are considered necessary and sufficiently advantageous to justify the additional cost.

VIII. PACKAGING

A. Evolution

Through the years, many types of containers have been used in marketing legume inoculants. The first inoculants (agar slants and liquid broths) were prepared in glass bottles because the bottles withstood the required autoclaving, could be sealed with stoppers or threaded caps and allowed visual inspection by the prospective buyer. With the advent of solid-base carriers, the bottles were replaced with metal cans using friction closures and at a later date with light-weight foil-lined fiber cans which could be sealed rapidly by machines. However, glass bottles are satisfactory and are still being used in many areas.

Today, the polyethylene flexible film is used in many countries because it is satisfactory and economical. It is light in weight and lends itself to rapid filling and sealing by automatic machines as well as to hand filling where the needs are smaller.

B. Requirements

An inoculant package must retain moisture for survival of the rhizobia and it must allow transmission of oxygen for respiration of the bacteria. The transmission of gases and water vapor by various flexible films (2 mil thickness) is given in Table 8.

The package must be strong and tough enough to endure the rigors of handling and transport. It must be easy to mold, fill, and seal by processing machinery. Also, it is very advantageous to have a film which accepts printer's ink so that cautions, directions for use, and other important information can be printed directly on the container. However, printers should be cautioned that printing can result in puncture of the film and make the containers unusable far inoculants. Polyethylene with a thickness of 2 or 3 mil (0.038 - 0.076 mm) apparently meets these requirements. However, polyethylene will not withstand autoclaving. If steam sterilization is to be employed polypropylene film should be used.

C. Sizes

Package sizes should be designed for the convenience of the farmer. The amount of legume inoculant needed will vary with the particular legume farm size, and method of planting. Also, there will be a variation in size of package with different forms of inoculant and different manufacturers. Actually, inoculants should be rated on the basis of numbers of effective rhizobia provided per seed rather than inoculant weight per seed.

Table 8. Transmission of gases and water vapor by various flexible films (2 mil, 0.038 mm).

Material	H_2O	CO_2	O_2	N_2
	cc's (STP)	cm^2/sec/cm	Hg at 25°C	X 10^{-8}
Polyethylene (density 0.922)	210	28	7.5	2.5

Material	H_2O	CO_2	O_2	N_2
Polyethylene (density 0.954)	30	6.5	1.6	0.5
Polypropylene	80	6	2.0	0.4
Polyester	280	0.25	0.05	0.008
Saran	2.7	0.06	0.005	0.001
Nylon 6	1,400	0.25	0.06	0.015
Pliofilm N1	202	0.5	0.09	--
Pliofilm N2	290	0.65	0.01	--
PVC	320	0.2	0.24	0.08
Polystyrene	2,400	18	2.0	0.5
Cellulose Acetate	15,000 - 24,000	14-39	1.5-2	0.4-0.5

SARAN is not satisfactory; water retention is good, but it is impermeable to oxygen. Cellophane (Cellutose acetate) does not retain water vapor and is unsatisfactory even though it is permeable to oxygen.

The dimensions of flexible film packages for various weights of peat-base inoculants and corresponding seed weights are given in Table 9.

Table 9. Dimensions and capacities of various inoculant packages

Weight of Seed	Package Dimensions	Inoculant Net Weight
5 kg	8 x 16 cm	40 g
25 kg	12 x 18 cm	120 g
50 kg	16 x 24 cm	240 g
150 kg	18 x 27 cm	720 g
600 kg	30 x 40 cm	2,880 g

D. Label

The information required on the label for legume inoculant packages varies in different countries depending upon local requirements and regulations. Prospective inoculant producers should design their labels after consulting with the concerned governments or regulatory officials and then submit proofs of

their proposed labels for acceptance before actually having the packages printed. Regulations often change. Information printed on the label should include:

1. Name or names of legumes for which the inoculant is claimed to be effective (Latin and common names).
2. Name of the rhizobia (Latin or scientific).
3. Date beyond which the product should not be used.
4. Directions for use and rate of application.
5. Number of live rhizobia per gram of inoculum or per seed when used according to directions.
6. Net weight of inoculum.
7. Lot number of inoculum.
8. Caution on storage temperature.
9. Manufacturer's name and address.
10. Brand name if different from manufacturer.

IX. QUALITY CONTROL

Quality control is a highly important responsibility of the inoculant manufacturer. It is his duty to produce an inoculant which, when handled and used according to directions, will bring about effective nodulation for all the leguminous plants listed on the label during the effective shelf life stated on the package. The manufacturer's responsibility extends beyond production; it includes education of the handlers and users of the product regarding its limitations, the hazards of storage at high temperature, and other possible detrimental factors. Quality control is a continuous responsibility which begins with production and lasts until the product is used by the farmer.

A. *Rhizobium* Strain Selection

The selection and maintenance of highly effective strains of rhizobia for all leguminous crops for which inoculants will be prepared is one of the first and most important responsibilities of the manufacturer. All strains used in production should be tested each season to make certain they have retained their ability to effectively nodulate the leguminous plants on which they will be used.

B. Monitoring *Rhizobium* Growth in Fermentors

Samples of broth culture should be drawn from each fermentor daily and tested for purity and growth. The pH can be tested quickly with bromthymol-blue indicator. A grass green to blue color indicates a pH around 6.8, which is desirable. A yellow color of the broth culture indicates acid production and probable contamination.

Each sample should be examined under the microscope magnified 1,000 times or higher. A direct stain with carbol fuchsin dye is preferable because the banded staining characteristic of rhizobia is demonstrated. When contamination is suspected, a gram stain should be made for verification. Heat-resistant, spore-forming bacilli are the most common contaminants and these usually produce a strong acid reaction in the culture medium.

A *Rhizobium* cell concentration of 5×10^8 to 1×10^9 should be obtained in 60 to 72 hrs. when a 1% starter is used, or within 36 to 48 hrs. when a 3% or larger starter is added. A haemecytometer (Petroff-Hausser type) or Helber slide is very useful in quantifying growth. Broth culture with a *Rhizobium* cell concentration of 5×10^8 per ml or higher is considered good for inoculant production.

C. Mixing Broth Culture and Carrier

The suitability and required specifications of the carrier material should be established by research months in advance of inoculant manufacture. Further, when the supply of carrier is received, the necessary qualities should be confirmed. Uniformity in composition, particle size, pH and moisture content are very important in making a uniform good inoculant.

The operator should know exactly how much $CaCO_3$ is needed to neutralize the carrier and also the exact amount of the broth culture needed to attain the desired moisture content and concentration of rhizobia in the inoculant.

Moisture content is very important in obtaining good growth and survival of rhizobia in inoculants. A moisture balance for quick determination of water content, of carrier and of the freshly prepared inoculant, is essential. It is also very important to monitor the pH of the prepared inoculant.

D. Lot Numbers

In order to practice in-house quality control, it is necessary to have a manageable system of labeling different batches of inoculant. Lot numbers provide a convenient method of identifying inoculants made for a particular legume with a designated batch of broth culture on a specific date. The lot number is assigned to a particular batch of culture and all the pertinent information on that particular lot is entered in a permanent record book on production. The lot number should be put on each inoculant package filled with that inoculant.

E. Finished Inoculant

Samples of every lot of inoculant prepared should be collected and accurately labeled at time of

preparation. Lot samples should be tested for pH and moisture content 7 to 14 days after collecting and should be further tested for number of viable rhizobia. Samples of each lot should be tested on growing plants of the legume for which they were prepared. It is very important to verify that the rhizobia present are the proper kind to bring about effective nodulation on the target legumes and that there is a sufficient number of rhizobia to do the job. Methods of testing are described by Vincent (1970), Brockwell (1963), Weaver and Frederick (1982) and others.

It is possible to determine number of viable rhizobia by using the dilution plate method when the inoculants are prepared with a sterile carrier medium. Viable counts can be obtained in 5 to 6 days with fast-growing rhizobia and 7 to 10 days with slow-growing rhizobia. The dilution-plate method is fast and, of course, very useful in combination with the MPN grow-out test.

There is no completely reliable differential medium for rhizobia but those described in Appendix O have been very useful providing the carrier material does not harbor large numbers of foreign microorganisms.

F. Minimum Standards

The minimum standards for viable rhizobia vary in different countries, some of which are as follows:

AUSTRALIA: 1 X 10^9 viable rhizobia up to expiration date. An exception is made for *Lotononis* rhizobia, 3 X 10^7 is acceptable. (Date and Roughley, 1977).

CANADA: A minimum of 10^6 viable rhizobia per gram of inoculant is acceptable, but the inoculum must provide a minimum of 10^3 viable rhizobia for small seed (200,000 or more seed/kg) 10^4 viable rhizobia for intermediate size seed (30,000 to 200,000 seed/kg) and 10^5 for larger seeds such as soybeans or faba beans (less then 30,000 seed/kg), up to expiration date. This new concept of expressing a standard number of viable rhizobia per seed has considerable merit.

UNITED STATES: In the U.S., there is no federal regulation of quality in legume inoculants. State regulations vary and are generally based on so-called "Grow-out" tests. In the "Grow-out" tests, seeds are inoculated according to directions, planted in sterile sand or vermiculite, supplied with nutrients other than nitrogen, and plants are harvested after 5 to 6 weeks. The roots are then examined for nodulation and rated as satisfactory or unsatisfactory.

X. INOCULATION METHODS

The legume inoculant producer's responsibility does not end with the production of an effective product. He must tell the farmer or user how the product should be applied to attain the maximum benefit. It is a temptation for the producer to oversimplify application instructions just to encourage the farmer to use the product. Instructions on application of inoculants to seeds should be clear and simple but they should be honest and based on sound research under the prevailing field conditions.

A. Sprinkle or Slurry Method

The usual or conventional way of inoculating legume seed is to sprinkle them with water and then mix the inoculant with the moistened seed. An alternative method is to add the water to the inoculant and mix to form a slurry before adding it to the seed and mixing. These methods appear very similar but the latter or slurry method results in greater retention of the inoculant because of better adhesion.

Leguminous seeds vary widely in size, shape, nature of the seed coat as well as in surface area. All of these factors influence the amount of water which can be used on seeds without damaging them or adversely affecting their sowing qualities. The amounts of inoculant and water which can safely be added to various legume seeds is given in Table 10. This data can be used in testing and making recommendations for inoculating various legume seeds.

Gum arabic, vegetable and cellulosic gums are sometimes used as stickers for solid-base inoculants. When these are used, they should be dissolved in the water before sprinkling seeds or preparing the inoculant slurry. Gums are beneficial in adhering inoculant to seed and promoting survival of the rhizobia on the seed after planting.

B. Lime and Phosphate Coatings

In inoculating legume seeds for sowing under adverse conditions it may be advantageous to coat the seeds for protection. In highly acid soils, finely pulverized limestone is often used to coat seeds. The inoculant slurry is added first and then the extremely fine limestone is added and mixed quickly with the seeds in a bag or mechanical mixer. Seeds should be uniformly covered and remain separated for easy sowing. Excess powder should be removed by screening.

In tropical areas, limestone is reported to be detrimental to certain legumes. Finely pulverized rock phosphate may be substituted for the limestone.

Data on the amounts of water slurry and powders needed to coat various kinds of leguminous seeds are given in Table 11. When coating seed, it is often advisable to increase the amount of inoculum added. This can be done without fear of getting the seed too wet because the powder dries the seed quickly.

Table 10. Amounts of inoculant and water commonly used in inoculating leguminous seeds*

Inoculant	Water ml/kg	Rhizobia	Seeding Rate kg/ha	Inoculant**

Kind of seed	gm/kg seed+	seed	per seed	Rate kg/ha	gm/ha
White Clover (*T. repens*)	4.4	22	500	2.2	10
Birdsfoot trefoil (*L. corniculatus*)	7.4	25	1,800	3.4	25
Red clover (*T. pratense*)	4.4	22	1,500	10.0	44
Alfalfa (*M. sativa*)	4.4	22	2,000	17.0	75
Vetch (*V. atropurpurea*)	4.4	13	40,000	43.0	190
Soybean (*Glycine max*)	4.4	13	150,000	65.0	285
Lupine blue (*L. angustifolius*)	4.4	13	160,000	82.0	360
Peas (edible) (*P. sativum*)	4.4	6	200,000	260.0	1,150

* Data from the Nitragin Co. The amounts of inoculant per gram of seed vary slightly with manufacturers
\+ Based on rhizobia content of 200 X 10^6/gm inoculant
** At specified seeding rate

Table 11. Proper amounts of water and slurry to use on ten kilogram of legume seeds of various sizes for regular slurry and for lime-pellet inoculation

		Slurry method with 44 g inoculum to 10 kg seed		Lime pellet with 100 g inoculum/10 kg seed		
Legume Seed	Number of seeds per kg	Water ml/10 kg	Slurry ml/10 kg	Water ml/10 kg	Slurry ml/10 kg	Limestone 10 kg
Trifolium repens	2,000,000	250	300	420	470	4.0 kg
Medicago sativa	500,000	220	260	420	470	4.0
Coronilla varia	150,000	220	260	420	470	4.0
Vigna radiata	25,000	200	220	380	400	3.5
Vigna unguiculata	10,000	150	175	170	200	2.0
Glycine max	5,000	100	115	170	200	2.0
Cicer arietinum	2,000	100	115	160	190	2.0
Vicia faba	1,250	70	80	150	170	2.0

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XII. APPENDICES

APPENDIX A - ORGANIC MEDIUM INGREDIENTS FOR RHIZOBIA

Part A. Mineral and Major Components in Supplements to Growth Medium

INGREDIENT	PROTEIN %	CARBO-HYDRATES %	FAT %	CALCIUM %	MAGNESIUM %	PHOSPHORUS %	AVAILABLE P %	POTASSIUM %	SULFUR %
Alfalfa Meal	14.5	40.3	2.7	1.30	0.29	0.23	0.18	2.49	0.22
Beet Pulp	8.9	59.1	0.6	0.56	0.15	0.10	0.03	0.20	0.22
Corn	9.9	69.2	4.4	0.02	0.11	0.28	0.10	0.31	0.08
Corn Steeps	48.0	0.0	0.4	0.06	1.50	3.30	1.10	4.50	0.58
Cotton Seed Meal	41.0	28.9	3.9	0.15	0.49	0.93	0.31	1.25	0.40
Molasses Beet	6.7	65.1	0.0	0.16	0.23	0.02	0.01	4.71	0.47
Oats	12.0	54.0	4.5	0.05	0.16	0.35	0.10	0.42	0.21
Cotton Seed Flour	59.2	24.1	4.0	0.25	0.74	1.31	0.31	1.72	0.60
Rice	8.0	65.0	2.0	0.04	0.06	0.23	0.00	0.00	0.00
Soybean Meal	42.0	29.9	4.0	0.25	0.25	0.60	0.16	1.75	0.32
Whey	12.0	68.0	1.0	0.90	0.13	0.75	0.75	1.20	1.04
Yeast Brewers	43.0	39.5	1.5	0.1	0.25	1.40	1.50	1.48	0.49

Part B. Growth Factors and Amino Acids in Supplements of Growth Medium

Carrier	Niacin	Pantothenic acid	Riboflavin	Thiamin	Arginine	Cystine	Histidine	Isoleucine	Leucine	Methionine	Tryptophane
	----------mg/kg----------				---------- % ----------						
Alfalfa meal	44	30	12	_._	0.51	0.25	0.22	1.15	0.62	0.19	0.22
Beet pulp	16	2	0.7	_._	0.3	_._	_._	_.__	_.__	0.04	0.09
Corn	22	6	1.1	_._	0.5	0.09	0.20	0.40	1.1	0.17	0.10
Corn steeps	0.2	0.03	0.01	0.01	3.3	1.90	2.80	3.60	11.3	1.90	_.__
Cotton-seed meal	38	8.0	4.2	_._	4.3	0.60	1.00	1.5	2.3	0.45	0.5
Molasses	40	4.6	2.2	_._	_._	_._	_.__	_.__	_.__	_.__	_.__
Oats	13	13.0	1.1	6.6	0.8	0.2	0.2	0.6	1.0	0.7	0.2
Rice	33	11.0	1.3	3.0	0.5	0.1	0.1	0.4	0.6	0.6	0.1
Soybean meal	30	14.0	3.0	_._	2.9	0.6	_._-	_.__	0.6	_.__	_.__
Whey	11	48.4	20	4.0	0.4	0.4	0.2	0.7	1.2	0.4	0.2
Yeast brewers	498	121	35	75	2.2	0.6	1.3	2.7	1.0	1.0	0.8

Part C. Amino Acid and Vitamin content of Commonly Used Commercial Growth Medium Supplements

	Difco	Yeast	Hydrolyzed protein			
Total N	9.5	8.0	13.5	9.2	9.0	7.3
Amino N(%)	73.0	50.0	30.0	20.0	50.0	37.0
AMINO ACID(%)						
Alanine	---	---	2.6	2.3	---	5.6
Arginine	1.0	3.7	3.7	4.6	7.1	5.6
Aspartic acid	5.0	---	5.7	5.3	7.5	0.2
Cystine	---	0.8	0.3	0.6	1.4	---
Glutamic acid	6.5	8.5	20.1	12.1	17.4	0.7
Glycine	2.5	5.0	1.0	2.8	3.7	1.6
Histidine	1.0	2.3	2.0	1.8	2.2	1.2
Isoleucine	3.0	4.2	4.8	0.7	2.9	1.3
Leucine	3.5	6.0	9.4	3.8	5.5	4.7
Lysine	4.0	6.0	6.8	3.7	3.0	3.3
Methionine	1.0	2.0	2.8	0.8	1.7	1.0
Phenylalanine	2.0	4.0	5.5	3.3	4.9	1.5
Threonine	3.5	3.2	4.3	1.9	2.9	1.2
Tyrosine	0.5	3.0	4.4	1.9	3.1	---
Valine	3.5	4.0	6.2	2.0	4.0	2.4
VITAMINS			Micrograms	per gram		
Biotin	1.0	2.0	---	---	0.6	---
Niacin	280.0	550.0	---	---	74.8	---
Pyridoxine	20.0	25.0	---	---	15.0	---
Riboflavin	20.0	35.0	---	---	13.6	---
Thiamin	3.0	50.0	---	---	20.0	---
Calcium pantothenate	---	100.0	---	---	58.0	---

*Analysis by Difco

**Analysis by Amber Laboratories, Milwaukee, Wisconsin
***Christianson, D.D., et al, 1965

APPENDIX B - STAINS USEFUL IN STUDYING RHIZOBIA

1. General Stains

PREPARATION OF STAINS

A. **Ziehl's carbol fuchsin**: dissolve 3.0 g basic fuchsin (C.C.) in 100 ml 95% ethyl alcohol; mix 10 ml with 100 ml 5% aqueous phenol (made by adding 6 ml 88% liquid phenol to 94 ml H_2O)

For staining rhizobia, dilute Ziehl's carbol fuchsin with distilled water -- 1 part dye to 6 parts water by volume. Dilute immediately before using.

B. **Loeffler's alkaline methylene blue**: Dissolve 0.3 g methylene blue (C.C.) in 30 ml 95% ethyl alcohol and mix with 100 ml 0.01% aqueous KOH.

C. **Methylene blue in dilute alcohol**: Prepare like the Loeffler formula above but use distilled water in place of the dilute KOH.

D. **Ammonium oxalate crystal violet**: dissolve 2.0 g crystal violet (C.C.) in 20 ml 95% ethyl alcohol and mix with 80 ml 1% aqueous ammonium oxalate.

E. **Crystal violet in dilute alcohol**: Prepare like solution "D" but use distilled water instead of 1% ammonium oxalate.

F. **Carbol rose bengal**: Dissolve 1.0 g rose bengal in 100 ml 5% aqueous phenol. It is well to add 0.01 to 0.03 g $CaCl_2$.

Notes on stains described above:

(1) Ziehl's carbol fuchsin (A) is the most intense. That is the reason for diluting with distilled water before use.

(2) Crystal violet (D and E) are intermediate between basic fuchsin and the methylene blue stains and are, therefore, very good solutions to use for routine staining of rhizobia.

Staining schedule:

(1) Make dilute infusion with distilled water if from solid medium and make smear of loopful on clear microscope slide.

(2) Fix by brief flaming or drying on a flat surface over boiling water.

(3) Stain 30 to 60 seconds with one of the dyes.

(4) Wash in tap water and allow to dry in an inclined position.

2. **Negative Staining** - to show unstained bacteria in a dark background.

PREPARATION OF SOLUTIONS

Nigrosin: Add 10 g Nicrosin (C.C.) to 100 ml distilled water and dissolve by placing in a boiling water bath for 30 minutes. Replace water lost by evaporation and add 0.5 ml of formalin. Filter twice through double filter paper.

Procedure: Mix a loopful of the bacterial suspension on the slide with an equal amount of the Nigrosin solution and spread evenly on a microscope slide. Allow to air dry and examine under the microscope.

3. Gram Staining - Differentiation between gram Positive and Gram Negative cells. Rhizobia are gram negative

PREPARATION OF DYES AND SOLUTIONS

A. **Gram's Iodine**: Dissolve 1.0 g iodine and 2.0 g KI in 300 ml distilled water.

B. **Safranin Solution**: Dissolve 0.25 g Safranin 0 (C.C.) in 95% ethyl alcohol and mix with 100 ml distilled water.

C. **Ammonium oxalate crystal violet**: Dissolve 2.0 g crystal violet (C.C.) in 20 ml 95% ethyl alcohol and mix with 80 ml 1% aqueous ammonium oxalate. (Same as solution "D" under general stain).

Staining Procedure:

(1) Prepare smears and fix in flame.
(2) Stain for 1 minute with ammonium oxalate crystal violet.
(3) Wash in tap water.
(4) Immerse in Gram's iodine for 1 minute.
(5) Wash in tap water and blot dry.
(6) Decolorize with 95% alcohol for 30 seconds.
(7) Counterstain for 5 minutes with Safranin (Sol'n."B" above).
(8) Wash in tap water.
(9) Dry and examine.

APPENDIX C - BEAD STORAGE FOR STOCK CULTURES

Equipment Needed:

1. Beads: Flex-Sox insulating Part No. 780021 The Carborundum Co., Electronics Div., P.O. Box 311, Latrobe, Pennsylvania

2. Test Tubes: Bacteriological with molded screw caps Kimax 100 ml x 16 mm

3. Test Tubes: Bacteriological Pyrex, 100 mm x 13 mm

4. Silica Gel: Indicating 6-16 mesh

5. Glass Wool

Description Method:

Cultures of rhizobia are grown in yeast mannitol broth (YMB). When the broth becomes turbid with *Rhizobium* cells, it is poured over sterile beads contained in the 100 x 13 mm tubes. The excess liquid is drained off and the beads containing the rhizobia are stored in sterile screw-cap bacteriological test tubes filled about 1/2 full of indicating silica gel. The beads are kept separated from the silica gel by a layer of glass wool. In the dry atmosphere of the tube, the rhizobia absorbed on the beads remain viable for a long time.

When sub cultures are desired, single beads are removed from the tube with a sterile nichrome wire hook and are planted in fresh, sterile YMB. The tube is recapped and kept for future use.

Step by Step Procedure:

1. Prepare yeast mannitol broth and add 2 ml broth to each tube (13 x 100 mm); plug with cotton and autoclave.

2. Using test tubes of the same size, add 14 beads to each tube, plug with cotton, and autoclave for 2 hours at 121°C.

3. Fill the screw-capped culture tubes (16 x 100 mm) about 1/2 full of indicating silica gel (6-16 mesh) and add a thin layer of glass wool over the gel to form a bed for the beads. Place tubes in basket and wrap basket top with heavy brown paper. Sterilize by heating in the oven at 185°C for 4 hours. The caps should be sterilized in the autoclave in large covered petri dishes; the caps will not withstand the oven temperatures. The sterile caps should be placed on tube immediately after the tubes are removed from the oven. The caps should be screwed tightly to prevent moisture absorption by the silica gel.

4. Prepare the *rhizobium* cultures by transferring from agar slants into yeast mannitol broth (2 ml) in the small tubes.

5. After sufficient growth has been attained, pour the broth culture over the sterile beads in the 13 x 100 mm test tubes. Replug the tubes and allow the beads to soak in the broth for about 2 hours.

6. Invert the tubes and allow the cotton plugs to absorb the excess broth. Transfer the beads to the sterile silica-gel tubes and cap immediately.

7. Label the bead culture accurately and boldly using a pencil on the frosted marking area.

8. Bead cultures will remain viable for a longer period when kept refrigerated at 4°C or lower.

9. Check for viability once each year.

APPENDIX D - LYOPHILIZING *RHIZOBIUM* CULTURES

Lyophilization or freeze-drying is a process for stabilizing biological materials. The material is quickly frozen and dehydrated immediately by subjecting to a high vacuum. Under vacuum, the water passes directly from the solid to a vapor state by sublimation.

Lyophilization is one of the best means of maintaining microorganisms in a genetically stable condition. When properly processed, lyophilized cultures will remain viable for several years. However, good equipment and technique are required.

The equipment and technique should be thoroughly tested on a few strains and viability confirmed before attempting to process a valuable stock of Rhizobium cultures. Viability and effectiveness should be tested at periodic intervals.

Procedure for Lyophilizing Rhizobia

1. The rhizobia strains to be lyophilized should be grown at room temperature in 50 ml of yeast mannitol broth (YMB) contained in a 250 ml Erlenmeyer flask on a rotary shaker set at 250 to 270 rpm.

2. When sufficient growth has been attained, 10 ml of the broth culture is pipetted aseptically into a sterile screw-capped tube and centrifuged for 15 minutes. Decant the supernatant aseptically.

3. Resuspend the cells with 0.5 ml of sterile water containing 10% sucrose and 5% peptone (Vincent, 1970). This gives a 2OX concentration.

4. Dispense a 0.1 ml aliquot into each of 6 to 8 sterile ampoules plugged with cotton. The tops should be flamed and the remainder of the cotton plugs then pushed down about halfway into the tubes. (MAKE SURE THE LYOPHIL TUBE IS LABELED WITH THE RHIZOBIUM STRAIN NUMBER.)

5. Turn refrigeration on and allow condenser to cool to at least -40°F. The drain line seal should be **CLOSED**. MAKE CERTAIN THAT CONDENSER IS CLEAN AND DRY.

6. Start the vacuum pump. The vac valves to each port should be **CLOSED**.

7. The culture in lyophil tubes is shell frozen by slowly rotating the ampoules in a low temperature bath. The material is frozen on the walls of the ampoules to expose as much surface to vacuum as possible. The thinner the shell, the faster the material will dry.

8. After freezing, immediately insert ampoules into the ports of the manifold.

9. Open vac valves to initiate drying.

10. The ampoules should be sealed with a gas-oxygen torch after the entire pellet is visibly dry. **Seal**

ampoules under vacuum. Make certain vacuum is not broken at any time.

Precaution:
Gas flame should be ignited before turning on the oxygen. Turn the oxygen off first and then the gas.

11. The vacuum in the system is broken by turning off the pump and opening the vac valve. Do not attempt to remove ampoule while the system is under vacuum.

A. **Viability Test**

1. To open an ampoule, make a file mark on the center of the tube.

2. Wipe tube with cotton moistened with alcohol before breaking.

3. After breaking, tube mouth is flamed and the dried cell pellet is transferred to a tube of broth (YMB).

4. Shake the tube until the cells are evenly suspended.

5. Streak a loopful of the suspension on yeast mannitol agar in a petri dish.

6. Incubate both the plate and the tube cultures at 26 to 28°C and then inspect carefully for colony characteristics and turbidity.

Viability tests should be made immediately following freeze drying and again after one, six and twelve month's storage.

B. **Infectiveness and Effectiveness**

The ability of a strain to form nodules and fix nitrogen on the host plant should be tested after 12 months' storage. The lyophilized culture should be compared with a culture maintained on agar slant.

It is recommended that lyophilized cultures be tested for infectiveness and nitrogen-fixing ability at least every 5 years.

APPENDIX E - SEEDS OF LEGUMINOUS PLANTS

Relationship between seed size, number of rhizobia per seed, and weight of inoculum per hectare when seed are inoculated with 4.4 g/inoculum per kilogram and the inoculum contains 5×10^8 rhizobia per gram.

Plant*	No. Seeds 1 kg	Rhizobia No./Seed	Kg Seed ha	Inoculum g/ha
Vicia faba	1,000	22×10^5	87	384
Arachis Hypogaea	2,000	11×10^5	44	194
Cicer arientum	2,000	11×10^5	33	145
Dolichos lablab	3,000	7.3×10^5	27	119
Lupinus albus	3,000	7.3×10^5	110	484
Pisum arvense	6,000	3.7×10^5	98	431
Glycine max	9,000	2.4×10^5	65	286
Cajanus cajan	17,000	1.3×10^5	11	48
Lens esculenta	19,000	1.2×10^5	15	66
Vigna radiata	22,000	1×10^5	76	334
Cyamopsis tetragonoloba	44,000	5×10^4	38	167
Vigna aconitifolius	44,000	5×10^4	40	176
Vicia hirsuta	44,000	5×10^4	49	215
Trigonella foenum-graecum	50,000	4×10^4	33	145
Onobrychis sativa	66,000	3×10^4	38	167
Coronilla cretica	242,000	9×10^3	20	88
Ornithopus sativus	352,000	6×10^3	22	97
Desmodium intortum	440,000	5×10^3	11	48
Indigofera hirsuta	440,000	5×10^3	10	44
Medicago sativa	440,000	5×10^3	16	70
Trifolium incarnatum	605,000	3.6×10^3	18	79
Lotus pedunculatus	1,940,000	1×10^3	4	18
Trifolium agrarium	2,200,000	1×10^3	4	18
Lotonois bainesii	3,840,000	6×10^2	2	9

*From largest to smallest seeds in descending order

APPENDIX F - SIEVES-SIZES AND DESIGNATIONS

U.S. Series Designation			
Alternate	Standard I.S.O.	Tyler screen equivalent	Sieve opening (inches)
No. 10	2.00 mm	9 mesh	.0787
No. 12	1.70 mm	10 mesh	.0661
No. 14	1.40 mm	12 mesh	.0555
No. 16	1.18 mm	14 mesh	.0469
No. 18	1.00 mm	16 mesh	.0394
No. 20	850 micron	20 mesh	.0331
No. 30	600 micron	28 mesh	.0234
No. 35	500 micron	32 mesh	.0197
No. 40	425 micron	35 mesh	.0165
No. 45	355 micron	42 mesh	.0139
No. 50	300 micron	48 mesh	.0117
No. 60	250 micron	60 mesh	.0098
No. 70	212 micron	65 mesh	.0083
No. 80	180 micron	80 mesh	.0070
No. 100	150 micron	100 mesh	.0059
No. 120	125 micron	115 mesh	.0049
No. 140	106 micron	150 mesh	.0041
No. 170	90 micron	170 mesh	.0035
No. 200	75 micron	200 mesh	.0029
No. 230	63 micron	250 mesh	.0025
No. 270	53 micron	270 mesh	.0021
No. 325	45 micron	325 mesh	.0017
No. 400	38 micron	400 mesh	.0015

APPENDIX G - ENUMERATING RHIZOBIA IN BROTH, INOCULANTS AND SOILS*

Routine enumeration techniques for determining the total and viable numbers of cells in a pure broth-culture of *Rhizobium* are described. The mean generation time of *Rhizobium* in broth culture is computed.

Key steps/objectives

1) Inoculate yeast-mannitol broth with *Rhizobium*

2) Calibrate Pasteur pipettes

3) Determine the total count

4) Measure the optical density of the broth cultures

5) Make a serial dilution and plate by the pour-plate, spread-plate, and drop-plate methods

6) Read and calculate the viable counts obtained by the three methods

7) Compare results of the counting methods

8) Inoculate flasks with diluted culture(s) for the generation time experiment

9) Determine viable counts periodically

10) Plot growth curve and determine the mean generation time.

(a) Preliminary culturing of fast- and slow-growing rhizobia (Key step 1).

Inoculate two flasks each containing 50 ml of YM-broth with a fast-growing *Rhizobium phaseoli* strain, TAL 182, and two other flasks with a slow-growing *Rhizobium japonicum* strain, TAL 379. Incubate the flasks at 26°C on a rotary shaker at 20 rpm. TAL 182 should be started 4-5 days in advance of the exercise; TAL 379, 7-9 days in advance. Take the culture of TAL 182 from the shaker after 4-5 days and remove a 20 ml subsample for the procedures (b) and (c).

(b) Determining the total count with a Petroff-Hausser chamber (Key step 3).

The Petroff-Hausser counting chamber (Figure 4.1) is a precision-machined glass plate that has a sunken platform at its center. The surface of this platform is exactly 0.02 mm below the surface of the plate and is etched with a grid system which consists of 25 larger squares, each of which is divided into 16 smaller squares. Because of the precisely machined gap between the grid surface and the overlying glass coverslip, it is possible to relate the number of cells observed in a field bounded by the sides of these squares to the volume of fluid in which they are suspended. Knowing the volume of each square, the concentration of rhizobia (total cells/ml) can be calculated.

The following data apply to the Petroff-Hausser counter as well as the Helber counter which has a grid system of an identical design.

Brief details of the Petroff-Hausser counting chamber

Squares	Corresponding Volume (ml)	Factor (1/volume)
Total grid area	2×10^{-5}	5.00×10^{4}
Large square	8×10^{-7}	1.25×10^{6}
Small square	5×10^{-8}	2.00×10^{7}

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

(c) Using the Petroff-Hausser counting chamber (Key step 3)
Chamber and coverslip should be soaked in a mild liquid detergent and thoroughly rinsed with distilled water and then air dried. This will assure an even flow of the liquid into the chamber and prevent the forming of air bubbles.

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

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

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

The *Rhizobium* suspension will quickly spread over the grid. Excess culture (if more than one drop was added) will overflow into the two channels at the edges of the etched platform. If these channels flood completely, the coverslip may not rest flush on the surface of the glass plate. If this happens, clean the counting chamber and start again.

Place the chamber under the 40 x objective of a phase-contrast microscope. Count cells in individual large squares. To avoid counting the same cells twice, omit bacteria on the upper and left borderlines of each square. Count at least ten fields (8-80 cells per large square) to obtain coefficients of variability of 10%.

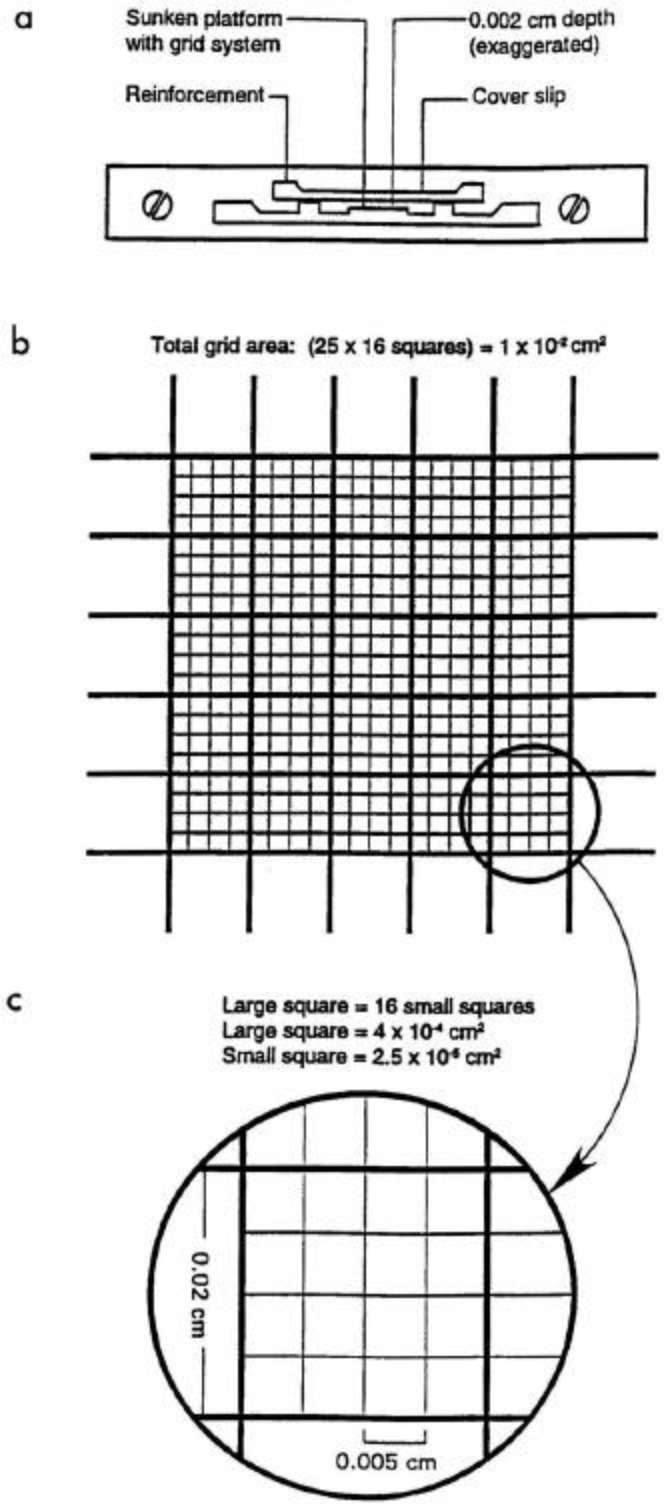
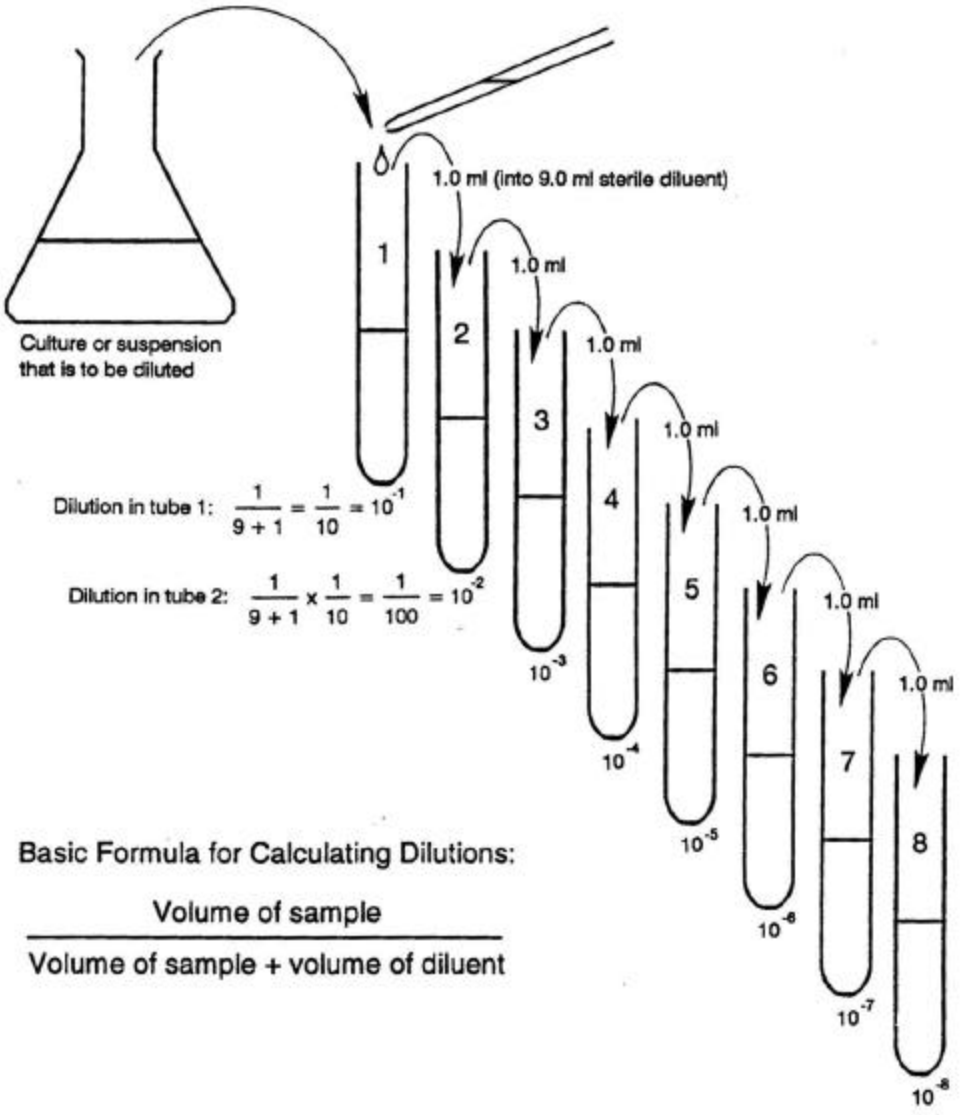


Figure 4.1 Petroff-Hausser counting chamber

Figure 4.2 Procedure for serial dilution



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

If 20 cells were counted in a large square and the original broth culture was diluted by a factor of 10^{-2}, and if a 20% suspension of this dilution was counted, the total number of rhizobia per ml of broth would be:

$$20 \times 100 \times \frac{100}{20} \times 1.25 \times 10^{6} = 1.87 \times 10^{9}$$

Remember that the count included dead as well as viable rhizobia and also the cells of contaminants, if present. Most direst counts are of variable reliability in that they overestimate the viable-count by a factor of more than two, i.e., 50% or more of the cells counted may be dead. This method is suitable only for counting cells in liquid media, and not in peat, soil, or other particulate materials.

(d) Estimating cell concentration by optical density (Key step 4).

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

Dilute 5-10 ml of the TAL 182 broth culture to 10, 20, 40, and 80% of its original concentration. Measure the light absorbed by each concentration with a spectrophotometer at a wavelength of 540 nm. relate the different concentrations to the actual cell count obtained with the Petroff-Hausser chamber by plotting the Optical Density (O.D.) against the total cell number. This method also has its limitations.

It is best suited for initially clear media. Dead cells and contaminants contribute to the O.D. of the culture, as well as gum produced by the rhizobia, undissolved salt or precipitate in the medium.

(e) Determining the number of viable cells in a culture by plating methods (Key steps 5, 6, and 7).

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

Use a fresh pipette for each strain and for each dilution in the series. Begin with the highest dilution in the series, With the aid of the suction bulb, fill and empty the pipette by sucking in and out 5 times with the diluted culture, then transfer 1 ml aseptically to a sterile Petri dish. Open the Petri dish only sufficiently to allow the pipette to enter and deliver the sample. Flame the pipette briefly (but do not overheat) by passing it through the bunsen burner flame each time prior to successive removal of aliquots for replication (2 per dilution) from the same tube. Similarly with the same pipette remove 1 ml aliquots in duplicate from the 10^{-7} and 10^{-6} dilutions into more Petri dishes.

Pour 15-20 ml YMA (kept melted and equilibrated at 50°C in a waterbath) aseptically onto each of the cell suspensions in the Petri dishes. To disperse the cells evenly, gently move each Petri dish clockwise

and counterclockwise allowing an equal number of swirls in each direction. To further ensure uniform dispersion of the cells, move the Petri dish three times forward and backward, then to the left and right. Allow the agar to set, invert the dishes and incubate at 26° - 28°C. Read the plates after 3-5 days. You have now completed the **pour-plate method**.

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

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

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

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

= 50 x 10^{7} cells per ml = 5.0 x 10^{8} cells per ml

A similar technique called the **spread-plate method** is also commonly used. Use the same serially diluted samples of TAL 379 prepared for the pour plate method above. Begin with the 10^{-7} dilution and deliver 0.1 ml of the sample into each of 4 plates of YMA previously dried at 37° for about 2 hours. Using the same pipette, dispense 0.1 ml samples from the 10^{-6} and 10^{-5} dilutions, in that order. Prepare a glass spreader by bending a 4 mm glass rod to the shape of a hockey stick, dip in alcohol, and flame; then cool the spreader by touching it to the surface of a separate YMA-plate. Lift the cover of each Petri-dish just enough to introduce the spreader and place it in position on the agar surface. Spread the sample evenly over the agar surface, sterilizing and cooling the spreader between samples. Incubate as before. Calculate the number of viable cells as outlined for the pour plate method, adjusting for the smaller volume that was plated (0.1 ml instead of 1.0 ml).

Both of the above methods are lengthy and require a large number of Petri dishes. A variation known as the Miles and Misra **drop-plate method** is more rapid and consumes less materials. Use agar plates which are at least 3 days old or have been dried at 37°C for 2 hours. Mark off 8 equal sectors on the outside bottom of the Petri dish. Label 4 sectors for replications of one dilution and 4 for another, allowing two dilutions per plate.

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

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

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

Pipettes with the same tip diameter (e.g. external diameter of 1 mm) deliver drops of virtually the same volume. After the drop size of a calibrated pipette has been established, more pipettes of the same tip diameter may be selected using a wire-gauge. Alternatively, any Pasteur pipette may be cut to the same

tip diameter with a fine file after matching its tip with a wire gauge.

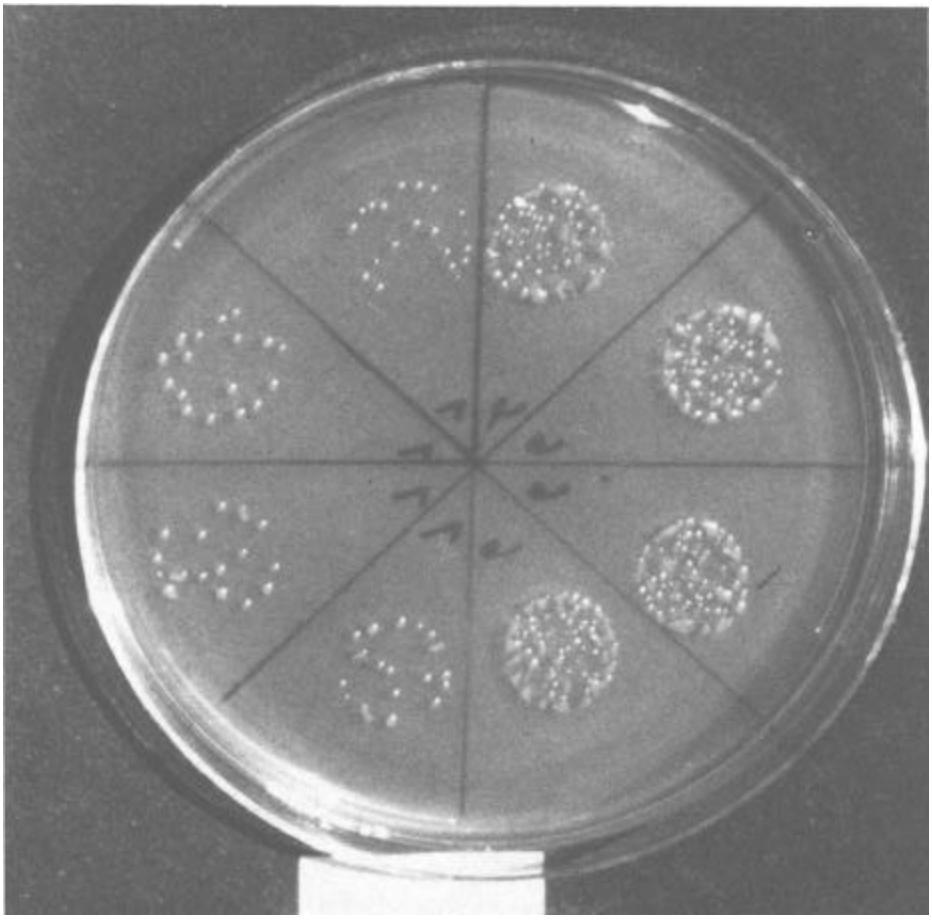


Figure 4.3 Growth of colonies of *Rhizobium* sp. from drops plated by the drop-plate method

Use the dilution series of TAL 379 which had been prepared earlier. Plate dilutions of 10^{-7}, 10^{-6}, and 10^{-5}. Begin with the highest dilution and deliver one drop to each of the appropriate 4 sectors of the plate. Use a calibrated Pasteur pipette equipped with a rubber bulb.

While holding the pipette vertically, about 2 cm above the agar surface, exert just enough pressure on the bulb to deliver one drop at a time. Use the remaining 4 sectors of the plate for the next dilution. Allow the drops to dry by absorption into the agar; then invert and incubate at 26°- 28°C.

The drop-plate method requires more skill than the other methods. Results may not match those of the pour-plate and spread-plate methods at the first attempts. It is advisable to practice drop-plating with plain water before using this method for the first time. Fewer colonies per drop require more drops to be counted to provide the same statistical precision.
After 3-5 days of incubation, with daily observations, count the colonies formed by TAL 182. Place an open Petri dish, inverted, on the illuminator of a colony counter. With a fine tipped felt pen, mark each colony-counted while simultaneously operating a tally counter. Record your counts.

If a pipette with a 14 gauge tip was used, one drop would be 0.03 ml. Divide 1 ml by 0.03 and multiply by the dilution factor and the average number of colonies per drop. Example, if the average number of colonies per drop is 60 at 10^{-5} dilution, the number of viable cells are:

$1/0.03 \times 60 \times 10^5 = 2000 \times 10^5 = 2 \times 10^8$/ml

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

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

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

(f) Determining the mean-generation (doubling) time of rhizobia (Key steps 8, 9, and 10).

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

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

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

A total of sixteen 250 ml Erlenmeyer flasks, each containing 100 ml of YM-broth into each flask and sterilize.

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

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

Inoculate each flask with 1 drop (0.03 ml $drop^{-1}$) of the diluted broth culture. Use a calibrated Pasteur-pipette for the inoculation.

Inoculate 16 flasks with TAL 182 and another 16 with TAL 369. Two flasks will be sampled each time for each strain.

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

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

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

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

Obtain the mean viable count for each day and transform the values to $\log_{10}$. Plot viable count (Y axis) versus time (X axis). Draw a smooth curve through the points.

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

$\log_a x = \log_b x/\log_b a$
if $a = 2$ and $b = 10$
$\log_2 x = \log_{10} x/\log_{10} 2$
$\log_{10} 2 = 0.3010$

Therefore, $\log_2 x = \log_{10} x/0.0310$

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

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

Requirements

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

Rotary shaker
Flasks (4) containing 50 ml YM broth each
Pipettes (10 ml sterile)
Slant cultures of TAL 182 and TAL 379

(b) Determining the total count with the Petroff-Hausser counting chamber

No requirements

(c) Using the Petroff-Hausser chamber

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

(d) Estimating cell concentration by optical density

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

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

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

(f) Determining the mean generation (doubling) time of rhizobia
Rotary shaker, colony counter, autoclave
Spreader, small beaker of alcohol, flame

Pipettes (1 ml, sterile)
Erlenmeyer flasks (32) with 100 ml YM broth each
Dilution tubes with 9 ml sterile ± strength broth each Plates of YMA

APPENDIX H - PLANT GROWTH SYSTEMS

A properly constructed glasshouse or plant-growth chamber can be very useful in assessing *Rhizobium* spp. (strains) for their nodule-forming and nitrogen-fixing abilities, competitiveness, and various other desirable and undesirable qualities. The usefulness of greenhouse techniques is greatly influenced by the system selected and also by the care which the researcher exercises in preparing the containers, surface-sterilizing the seed, planting the seeds or seedlings, and also by the daily care in watering, regulating temperature as required, and controlling pests.

In certain experiments with small-seeded legumes, the seedlings may be grown on agar slants in test tubes or in sand in tubes or small glass bottles. However, such tests often impose serious limitations and growth may be abnormal or limited. Differences attributable to treatments may be poorly defined. It is very important to have good growth of the test plant for manifestation of treatment responses in the host.

A. Containers

Three systems of growing plants are illustrated in Figures 1, 2, and 3. Each has its advantages and disadvantages. The 2-liter glazed jar (Figure 1) is excellent for large-seeded legumes. The bottom of the jar is layered with pea gravel. A glass watering tube 4 to 5 cm in diameter is footed on the gravel. The jar is then filled to about 5 cm of the top with sand or a layer of vermiculite and then a 5 to 6 cm layer of sand at the top to facilitate planting of the seed. The surface-sterilized seed or germinated seedlings are planted in the moist sand and inoculated. A thin layer, 1 cm, of fine gravel is then spread uniformly over the surface. It keeps the surface dry, reduces evaporation and serves as a mulch.

Vermiculite, "Terralite," absorbs much water and is a good substrate for the plants. However, it is very difficult to remove because the roots penetrate the vermiculite and become entangled in it. This is a big disadvantage in some instances.

The Leonard jar (shown in Figure 2) assembly consists of three parts: a) a bottomless l-liter Boston round flint glass bottle (a soft-glass bottle may be substituted); b) a ½ liter cream jar; and c) a No. 5 one-hole rubber stopper equipped with a glass tube (6 mm O.D. and 7 cm in length) inserted with a wick. The wick connects the sand with the water or nutrient reservoir and water is drawn up by capillary action. A braided olefin polypropylene 3/16" diameter wick is recommended because it withstands autoclaving and can be used repeatedly, but synthetics and natural ropes may be used. After filling the jar, the nutrient solution (400 ml) is added. The tops are covered with glass petri-dishes, aluminum foil, or brown paper, then autoclaved for 1 to 1½ hours at 121°C. After cooling, the surface-sterilized seeds or germinated seedlings are planted in the sand, inoculated, then covered with sand. A layer of sterile pea gravel is then added to the surface.

The advantages of the Leonard jar are: a) they take up much less space than the glazed jars, b) they require only 1½ to 2 hrs for sterilization, and c) a uniform moisture content is maintained in the sand and growth is more uniform with treatments.

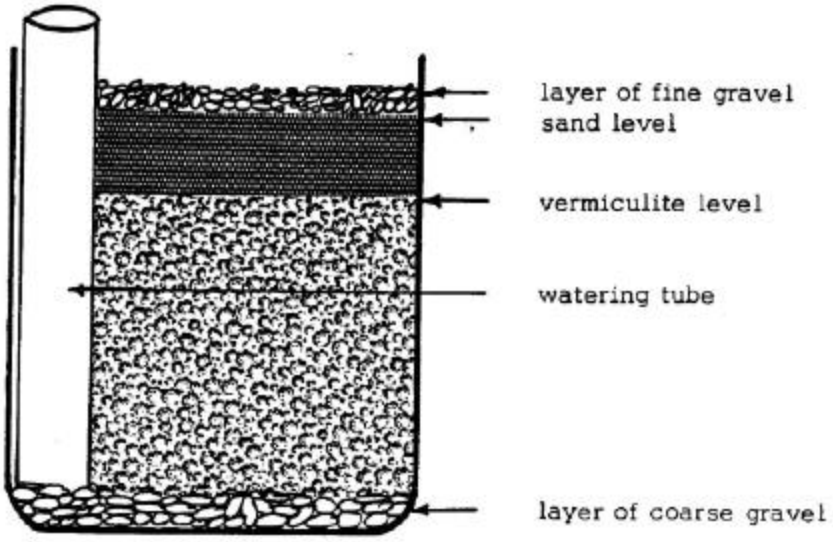


Figure 1
2-Liter Glazed jar

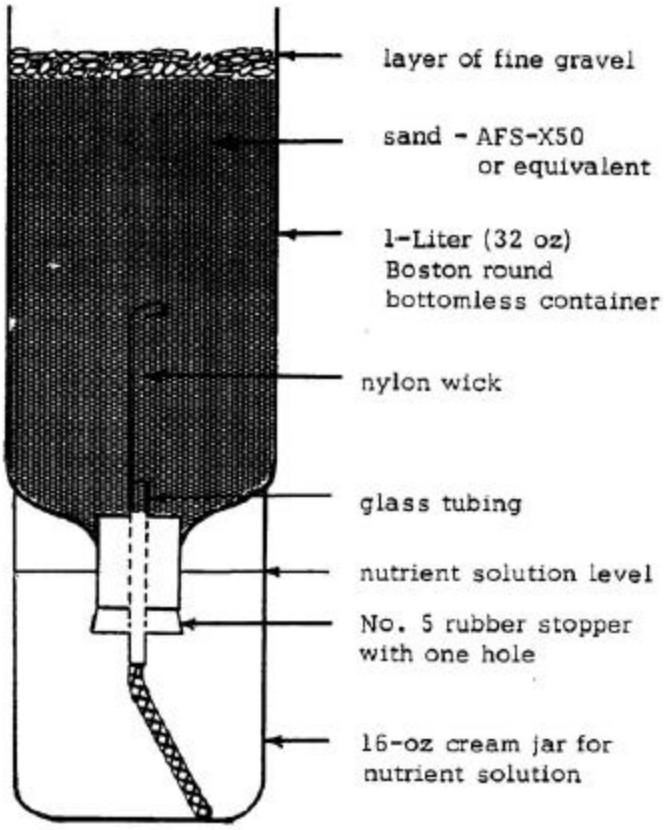


Figure 2
Leonard Jar

Scale 1" = 2"

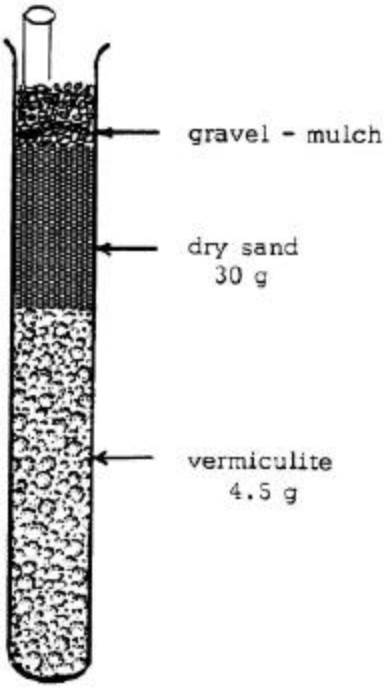


Figure 3
Test Tube
25 mm x 200 mm

The third system (Figure 3) is a **large test tube** 25 x 200 mm or larger. A glass watering tube (8 mm

O.D.) is inserted and the tube is filled with vermiculite. As in the glazed jar, a layer of sand is provided at the top to facilitate planting. A tube of this size will accommodate one or two seedlings. After planting, a layer of sterile gravel is added to the surface as a mulch. Watering is done through the tube using a sterile pipette. The vermiculite acts like a sponge and the water is drawn down quickly. Plant growth in these tubes is far better than that attained in a closed tube. The big advantage of this technique is that it is space saving and maximum use can be made of a scanty seed supply.

B. Support Medium - Sand and Vermiculite

In studying symbiotic nitrogen fixation by the *Rhizobium*-Legume association, it is important to grow the plants in a nitrogen-free or nitrogen-poor medium. If nitrogen is needed in some of the treatments, it can be added. Generally the growth medium should be neutral to slightly acid (pH 6.5 to 7.0). When the pH of the sand or vermiculite varies from this, it can be adjusted by adding an acid or base to the nutrient solution. This adjustment can be made also when the study involves tolerance of either of the symbionts to acidity or alkalinity.

The choice of sand is very important. Usually a sand of intermediate particle size is preferred over a very fine or very coarse one. Particle size of the sand is particularly important when the Leonard jar or wick system of feeding water is employed. The necessary capillary movement of water will not be possible if the sand is too coarse. With very fine sand, too much water will be drawn and the sand will become water-logged. Good growth cannot be obtained under these conditions. The sand preferred is one having an American Foundry Society (AFS) rating of 35 to 50. Seventy-five to ninety percent of the sand should be retained on a 50-mesh screen. About 25% of the sand should be larger than 30-mesh and around 55% should be smaller than 30-mesh, but larger than 50-mesh. The very fine particles (less than 50-mesh) can be easily removed by washing the sand. Washing can be done easily by adding a workable amount, 10 to 15 kg, in a strong woven cloth bag, immersing the bag in a tub of clear tap water, and agitating vigorously in several rinses of water. Sand should be spread out on a clean surface and dried before using.

Only a horticultural grade of exploded mica (vermiculite) should be used. Some of the vermiculites are toxic to plants. The chemical analysis of Western Minerals "Terra-Lite" vermiculite is as follows:

	"Exfoliated" sample %
Moisture	1.25
Silica (SiO_2)	39.00
Iron oxide (Fe_2O_3)	11.04
Aluminum oxide (Al_2O_3)	14.16
Titanium oxide (TiO_4)	1.40
Calcium oxide (CaO)	1.08
Magnesium oxide (MgO)	21.90
Sodium and potassium	
Oxides (Na_2O, K_2O)	4.88
Chlorine (Cl)	trace
Sulfur trioxide (So_3)	0.02
Loss on ignition	4.85

C. Nutrient Solutions

In studying the nitrogen-fixing ability of various *Rhizobium* strains in association with different leguminous hosts, it is desirable to grow the plants either in a nitrogen-free medium or one with a very low content of available nitrogen so that the maximum nitrogen fixation is obtained. All other nutrients should be present in adequate amounts and in balance. It should be remembered, however, that nutrients required by different plant species will vary among themselves and also to some extent with environmental factors.

While it is important to have adequate supplies of nutrients for plant growth, caution should be taken to avoid having an oversupply of any nutrient, particularly when the chemical ingredient is highly soluble. Adverse osmotic effects can develop and also harmful imbalances between nutrients may result. This latter effect can be avoided to some degree by using chemical ingredients which are only partially soluble and which can provide a reservoir without creating problems.

Some formulas for plant nutrition which have proven satisfactory are:

Bryan's Modification of Crones (Soil Science 13:279)

Potassium chloride (Cl)	10.0 g
Calcium sulphate $CaSO_4 \cdot 2H_2O$	2.5 g
Magnesium sulphate $MgSO_4 \cdot 7H_2O$	2.5 g
Tricalcium phosphate $Ca_3(PO_4)_2$	2.5 g
Ferric phosphate - Soluble	2.5 g
(Complete salt of sodium ferric citrophosphate containing 12-15% Fe, 15% P_2O_5 and 45% citric acid)	
	20.0 g

Mix all salts and grind to a fine homogeneous powder using a mortar and pestle. All 1.5 g of the Crone's salt mixture per liter of distilled or deionized water. The resulting solution should be supplemented with 5.0 ml of the Micronutrient Solution below:

Micronutrient Stock Solution

	g/L	ppm or mg/L (Final)
Boric acid H_3BO_4	0.57	0.50 BO
Manganese sulfate $MnSO_4 \cdot H_2O$	0.31	0.50 Mn
Zinc Sulfate $ZnSO_4 \cdot 7H_2O$	0.09	0.10 Zn
Copper sulfate $CuSO_4 \cdot 5H_2O$	0.08	0.10 Cu
Molybdic acid (85% MoO_3)	0.016	0.04 Mo
Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.0008	0.001 Co

The bond modification below of Crone's Byran Modification combines the macro and micronutrients in a single stock salt mixture. Special care must be taken to grind and mix all salts to obtain a completely homogeneous mixture.

Bond Modification of Crone's

Stock salt mixture	g
Potassium chloride	31.7
Tricalcium phosphate	18.0
Calcium sulphate	13.7
Magnesium sulphate	5.5
Soluble $Fe_2 (SO_4)_3$	2.7
Copper sulphate	0.5
Manganese sulphate	0.6
Boric acid	0.5
Dipotassium phosphate	26.8

Add 1 g of the salt mixture to each liter of water

The salt content of various other plant nutrient solutions is given below:

Chemical	Bryan Crone	Bond	Hoagland	Jensen	Thorton
		mg/Liter			
KC1	750	320			
$CaSO_4.2H_2O$	190	140	340		
$MgSO_4.7H_2O$	190	180	490	200	200
$Ca_3(PO_4)_2$	190	180			200
$FePO_4.2H_2O$	190				1,000
$Fe_2(SO_4)_3$		30			
$CuSO_4.5H_2O$		5			
$MnSO_4.H_2O$		6			
H_3BO_3		5			
K_2HPO_4		270		200	500
NaCl				200	100
$FeCl_3$				100	10
$CaHPO_4$				1,000	
K_2SO_4			435		
$Ca(H_2PO_4)_2.H_2O$			126		
		mg/L	or PPM		
Phosphorus	70	74	31	216	655
Potassium	393	289	195	90	224
Calcium	118	103	100	233	774
Magnesium	19	6	49	20	20
Iron	57	8		20	20
Sulfur	60	41	208	26	28

D. Surface Sterilization of Seeds

Legume seeds may harbor rhizobia and many other kinds of microorganisms. When studying the influence of rhizobia on growth of leguminous plants, it is important to remove all rhizobia which might nodulate the test plant and confuse responses obtained.

Leguminous seeds vary widely in size, shape, seed coat, structure, color, and in other ways. Some seeds have very hard impermeable seed coats and have to be scarified either mechanically or by using concentrated sulfuric acid before they will germinate. There is no method of surface-sterilizing seed which can be considered universally dependable. High quality seeds with good germination are always preferred. It seeds are treated with fungicides or insecticides, these pesticides should be removed by careful washing in water and then drying the seed on paper towels.

Certain seeds are very easy to free of bacteria while others are very difficult. Small seeds are usually more difficult than large seeds; rough coated seed or seeds with hulls are more difficult to surface sterilize. With seeds which require scarification to allow imbibition of water, this be done physically using a metal file or highly abrasive sand paper. The scarification should be done before attempting to surface sterilize the seed. Another method of scarifying seed is to soak them in concentrated sulfuric acid (98%) for 10 to 12 minutes. When this method is used, the seed are scarified and surface-sterilized in one operation.

A number of different disinfectants are satisfactory for seeds which need no scarification and also those which have been mechanically scarified. It is always important to use four or five volumes of disinfectant for one volume of seed to avoid overdilution. Seeds should be rinsed five or six times with sterile water after the disinfectant is poured off. Several methods of surface-sterilizing leguminous seeds which need no further scarification are suggested below:

a) **Hydrogen peroxide** - Immerse the leguminous seed in 3% hydrogen peroxide for 30 minutes. Rinse the seed several times in sterile distilled water and dry.

b) **Calcium hypochlorite** - Agitate the seed for 15-20 minutes ln calcium hypochlorite. Rinse the seed several times in sterile distilled water.

c) **Mercuric chloride** (A reasonably good method but it should be used with care.

1) Immerse the seed in 95% alcohol to remove the waxy seed coating. Rinse two or three times in sterile water.
2) Place the seed in a 1:1,000 mercuric chloride solution and agitate for 2 - 4 minutes. Vacuum treatment may be employed. Wash the seed several times in sterile distilled water.

d) **Hot water treatment** - Immerse the seed with agitation for several minutes in water heated to 60-64°C. This not only has disinfectant action but serves to soften the seed coat.

Sulfuric Acid Scarification and Surface Sterilization

1. Place seeds in a sterile Erlenmeyer flask with at least 5 times the volume of seed and close with a rubber stopper.
2. Cover the seeds with acid. Allow sterilization and scarification to proceed for 10 minutes. DRAIN OFF THE ACID.
3. Add sterile water quickly in sufficient volume to dissipate the heat generated by the residual sulfuric acid and avoid injury to the seeds. Rinse and pour off the water. The first rinse should be done quickly to avoid damaging the seeds. Rinse the seeds five times with sterile distilled water.

Surface-sterilized seeds which are to be planted as seeds rather than seedlings should be spread out on absorbent paper towels immediately after treatment in a clean warm room and allowed to dry. As soon as they are thoroughly dry they should be placed in a clean sterile bottle or covered petri dish until ready for planting.

Producing Seedlings for Plantings

In glasshouse or growth chamber experiments, planting seedlings is often preferable to seeds because more uniform stands and placement of plants are obtained. This is particularly true when it is necessary to use seeds with poor germination. Surface-sterilized seeds may be germinated by placing them on a sterile moistened white blotter or filter paper in sterile petri dishes until young, plump seedlings develop. The seedlings can be easily planted in a layer of cultivated sand on the surface of the culture vessel.

Some workers prefer to germinate the seed in sterile petri dishes poured with agar water (1% w/v); large petri dishes are preferred. Plates should not be overcrowded with seeds. With seeds 3 mm or less in diameter, the plates should be incubated inverted, which will allow development of straight radicles from the seed; these are easier to plant.

E. Preparation and Sterilization of Plant Culture Vessels

The system of filling the plant culture vessels is illustrated in Figures 1, 2, and 3. It is advisable to determine the number of vessels or containers which will be needed (total treatments times number of replicates plus about 5% extra for breakage in handling and sterilizing.) Make certain all containers are clean and free of all toxic substances. Generally, the containers are filled with the supporting medium or media; the desired amount of nutrient solution is added to each container and the container is covered securely for sterilization in the autoclave. Thick kraft paper or aluminum foil works well on the glazed jars and the tubes; glass petri dishes, which fit snugly over the 32-oz. boston round bottles, work well for the Leonard jar assemblies. It is important that paper covers be tied firmly with twine over the glazed jars or tubes.

The table which follows contains information which may be useful in the planning of your experiment. The weights of sand and vermiculite should serve only as a guide. Generally, the volumetric proportions shown in Figures 1, 2, and 3 should be followed. The same is true for the nutrient solution. The substrate should be saturated with water before autoclaving to reduce the time required for sterilization to a minimum.

Guide for Preparation, Sterilization and Planting of Plant Culture Vessels

Ingredient or Specification	Glazed jar 2-Liter	Leonard jar 1-Liter Bottles	Test Tubes 25 x 200 mm
Sand	2,000 g	1,000 g	Not recommended
Vermiculite	500 g	Not recommended	4.5 g
Sand (Fig.1)	500 g		30.0 g
Nutrient Solution (Volume)	600 ml	400 ml	30 ml
Surface Area for Planting	130 cm^2	65 cm^2	5 cm^2
Seeds/SeedlingsSmall	15 to 20	10 to 15	2
per ContainerLarge	5 to 10	3 to 5	1
Autoclave times at 121°C	4 hrs	1 hr	1 hr

Pea gravel for mulching purposes should be sterilized along with the plant culture vessels. One liter Erlenmeyer flasks filled to the neck with washed pea gravel and plugged with cotton and secured with brown kraft paper for sterilization provide a very easy system for adding the mulch layer.

A sufficient quantity of water (either distilled or deionized) should also be sterilized in glass bottles or flasks and be ready for use during planting in case some of the sand or substrate becomes excessively dry.

F. Suggestions on Planting

The plant culture vessels have been filled, charged with nutrient solution and autoclaved. Seeds have been surface-sterilized and germinated when required. *Rhizobium* cultures have been prepared. It is planting time. Whenever possible, planting should be done in the glasshouse or growth chamber to avoid unnecessary handling and possible contamination in moving the jars or bottles from one place to another.

The table or bench where planting is to be done should be washed with disinfectant. Wind drafts should be avoided and all materials should be handled aseptically. Culture vessels should be arranged and labeled clearly for easy planting. It is usually best to plant the non-inoculated controls first.

Covers should be removed carefully from the culture vessels so as not to scatter sand or other extraneous matter which might contaminate jars.

Immediately before planting, the surface sand in each jar should be moistened with either nutrient solution or distilled water when needed and the sand levelled with a sterile spatula. The surface can be marked with a sterile tool to indicate spacing and planting depth of seeds. The germinated seedlings should be added aseptically to the indentations in such a manner that the small roots are projected downward in the dented sand areas. Large seeds should be properly inserted and fixed in the area with the hilum downward.

To each seed or seedling add one or two drops of the inoculum. Then cover the seeds with sterile sand using a sterile spoon. Small seeds should be covered with about 1 cm of sand; slightly more should be used for larger seed. The surface sand should be moistened with sterile water and this should be done very gently to avoid disturbance of the seed. Some investigators prefer to cover the planted surface with a layer of sterile gravel or coarse sand to prevent excess evaporation.

When any one strain or treatment has been planted, the replicate jars should be moved to a distant area on the table. The jars should be handled from the lower sides or bottom to avoid contamination. This procedure should be repeated with each treatment. Following planting, the individual jars should be randomized. Jars of the negative controls should be randomized among those of the inoculated treatments.

When planned, thinning of seedlings should be done 3 or 4 days following emergence before lateral roots become extensive. Thinning can be done easily using forceps and flame-sterilizing them between each jar or pot.

If the glasshouse or growth chamber has controls for temperature and day length, these should be checked and adjusted as required immediately after planting.

The jars, pots, or tubes should be monitored regularly and watered as needed with sterile water. Unless the plants are grown for longer than 6 weeks, no additional nutrient solution is needed. Excess nutrient addition can cause problems in imbalance or osmotic effects.

APPENDIX I - TO PRODUCE BROTH CULTURES IN SIMPLE GLASS FERMENTORS*

Glass fermentors are set up in the laboratory and used for the small scale production of liquid inoculum. The inocula are monitored for cell number and contamination during growth.

Key steps/objectives

1) Initiate starter broth cultures
2) Assemble small fermentor units
3) Sterilize fermentors
4) Become familiar with operation details
5) Inoculate the fermentors
6) Take broth samples periodically for cell count and check for contamination
7) Test for contamination
8) Perform total counts and optical density measurements
9) Perform viable counts by the spread plate method on the presumptive test media
10) Perform agglutination tests with the homologous antisera

(a) Inoculating starter cultures

(Key step 1)

Prepare four 50 ml flasks or tubes each containing 25 ml of YM broth. Obtain slant or bead preserved cultures of a slow-growing *Rhizobium* (e.g., *Rhizobium japonicum* TAL 379) and a fast-growing *Rhizobium* (e.g., *Rhizobium phaseoli* TAL 182). Inoculate two flasks with each *Rhizobium* and aerate at 26 - 28°C. These will serve as "starter" cultures for inoculating the YM-medium in the fermentors.

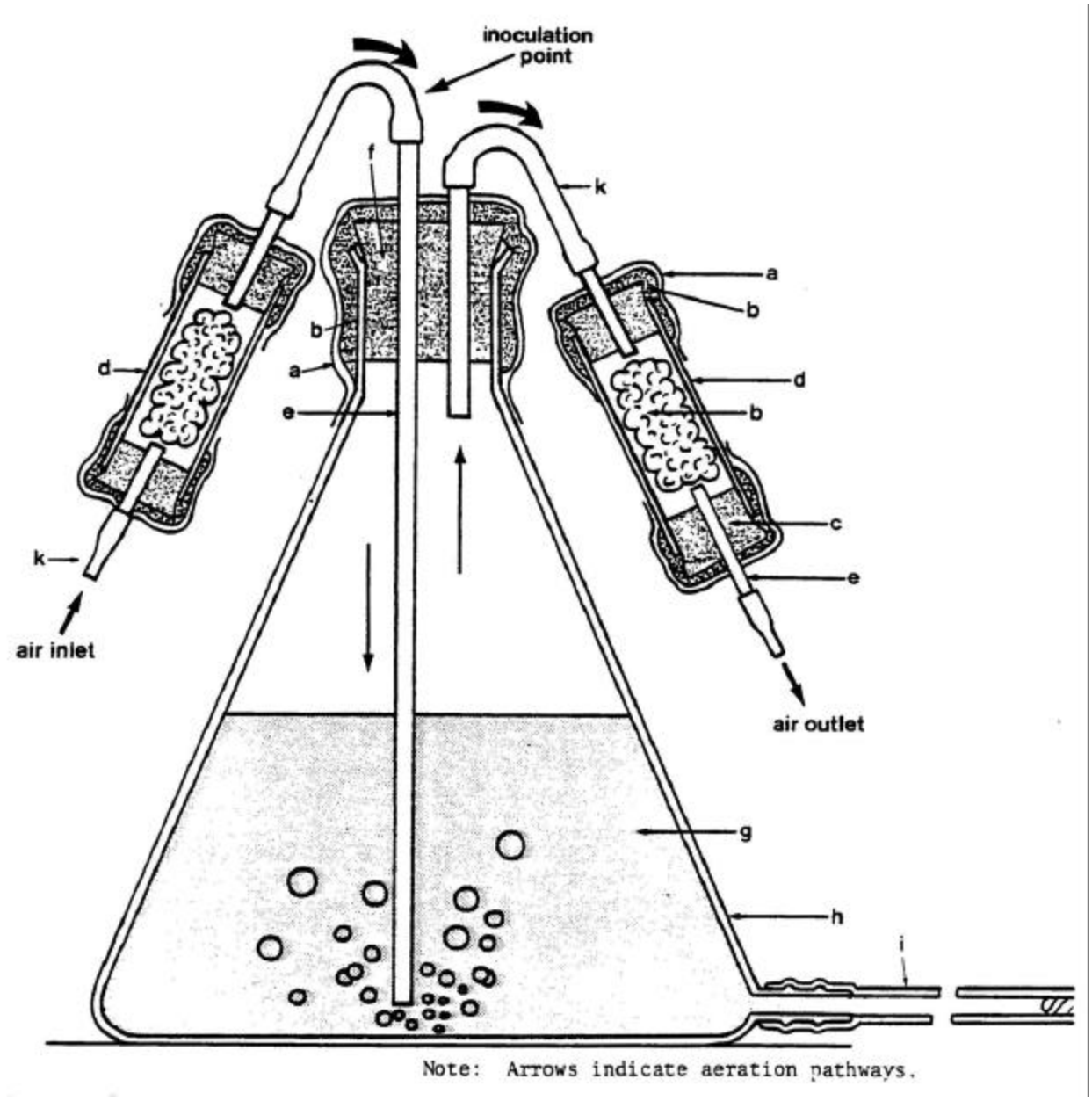
(b) Assembling simple fermentors

(Key steps 2 and 3)

Set up 2 fermentors (1 for each strain) as shown in Figure 19.1. The main fermentation vessel is a slightly modified 4 liter Erlenmeyer flask with a sampling port (glass tubing 4mm ID) fitted close to its base. (The assistance of a glass blower is needed for this modification). Fill each fermentor with 2-3 liters of YM broth. Connect the cotton packed filters to prevent the entry of contaminants via the air lines. All rubber stoppers and tubings must be autoclavable. Insert the large rubber stopper which holds the air inlet and outlet tubes with their respective filters, firmly into the neck of the flask.

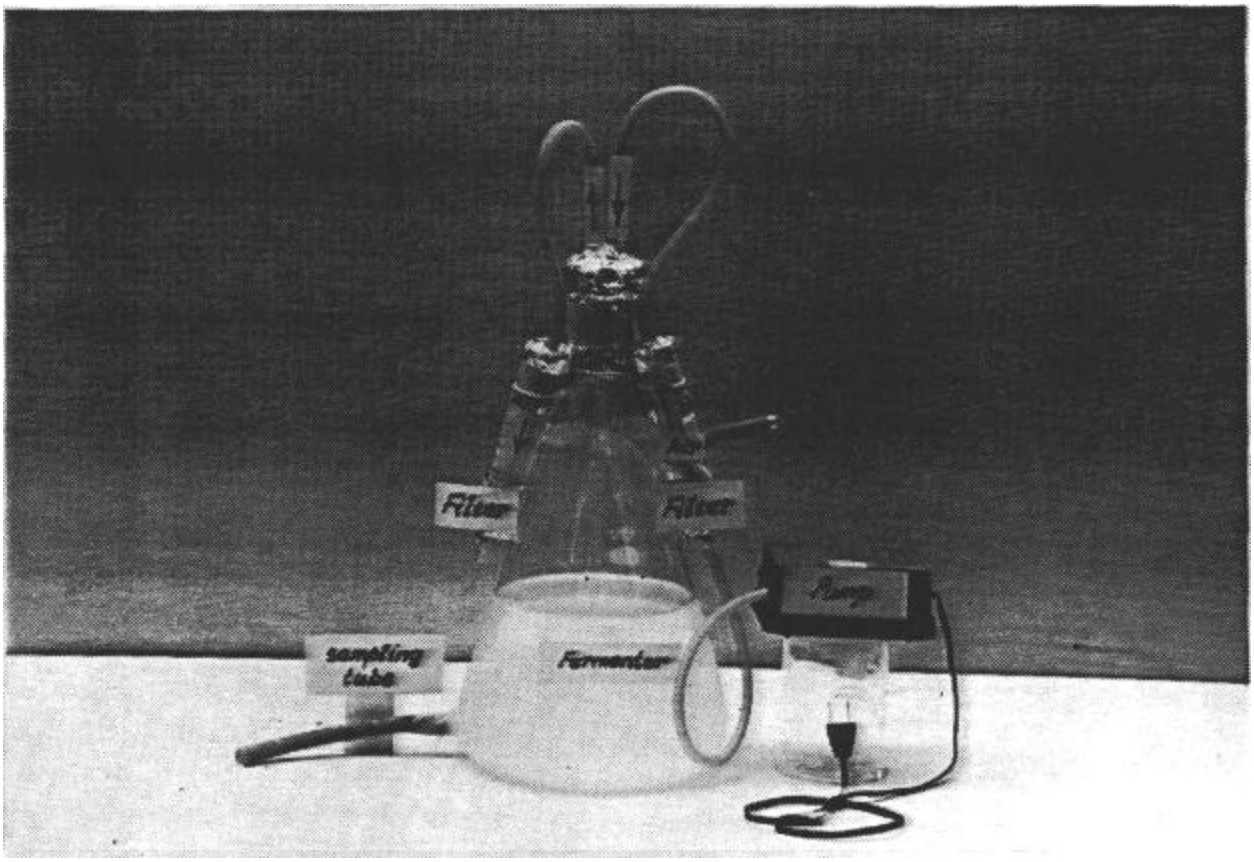
*Taken from exercise 19 of the NifTAL Manual for *Methods in Legume-Rhizobium Technology*, by P. Somasegaran and H. Hoben.

Figure 19.1. Scheme of simple fermentor unit



a - Aluminum foil: b - Nonabsorbent cotton: c - Autoclavable stopper: d - Filter unit:
e - Glass tubing: f - Autoclavable stopper: g - Growth medium: h - Flask: i - sampling tube: j - Plug: k - Rubber tubing

Figure 19.2 Simple Fermentor in ope ration



Connect the air inlet tube to an aquarium pump. Activate the pump and check the air inlet and outlet filters for air resistance. Air should flow freely through both filters while bubbling through the broth and aerating and agitating the medium. The cotton in the filters should be packed uniformly but loosely. Overpacking the air inlet filter can cause resistance to incoming air and lead to poor aeration. Overpacking of the outlet filter can lead to poor air escape and pressure build up in the fermentor.

Disconnect the fermentor from the pump and prepare it for autoclaving. Make sure that the stopper which holds the air tubes is still firmly seated. The air system must be well protected to prevent entry of contaminants. Wrap each flask with a wide band of non-absorbent cotton and secure with string. Add a protective wrapper of aluminum foil (Figure 19.2). Close the air inlet tube with a clamp at the spot indicated in Figure 19.1 to prevent the broth from leaving the flask due to pressure build up in the flask during autoclaving. Pressure relief during autoclaving occurs through the air outlet tube which must be left open. The filters should remain connected to the fermentor during autoclaving. To provide a convenient place for them, make an oversized wire ring around the neck of the fermentor vessel and twist it to an eyelet on each side. Each filter may then also be fitted with a piece of wire ending in a small hook. Hook the filters onto the eyelet (Figure of 19.2). Sterilize the assembly for one hour, if it contains approximately 2 liters of broth. Adjust the sterilization time according to the volume of liquid; increase time by 20 minutes for each additional liter.

After the fermentors have cooled, remove the clamp from the air inlet tubing. Connect the air supply to check for proper aeration once again and for leaks in the system.

Various types of air systems have been used to aerate small fermentors including compressors, compressed air in tanks, aspirators, and aquarium pumps. The latter have been very satisfactory air suppliers for small units. They are inexpensive, silent, and dependable. Although a pressure relief valve may be desirable, most aquarium pumps generate only low pressure sufficient however, for several fermentor units which may be connected to one aquarium pump using a manifold.

(C) Operating the glass fermentors

(Key step 4)

If, after autoclaving, each fermentor has been inspected and found to function properly, it is ready for inoculation with the starter culture. (If desired, the autoclaved fermentor may be left for 24 hours prior to inoculation for further check on sterility.) If an aquarium pump is used, and more than one fermentor is attached, adjust the air to achieve an equal flow to each fermentor. For other air supply systems, adjust the air flow on the bypass.

The glass fermentors are inoculated through the latex air inlet tubing with a sterilized syringe fitted with an 18 gauge needle. Care must be taken that no contaminants are introduced. Twenty ml of the starter culture are removed aseptically from its flask. The air inlet tubing is swabbed with 70% alcohol about one inch above its connection to the glass tube. The needle is inserted into the tubing pointing downwards toward the fermentor and the culture is injected. The airstream will facilitate speedy entry and incorporation of the inoculum into the YM medium. The culture is incubated at 26 - 28°C under continuous aeration.

(d) Collecting culture broth from the glass fermentor

(Key step 4)

The broth medium is withdrawn from the glass fermentor aseptically by swabbing the outflow tubing with 70% alcohol and inserting a syringe needle attached to a sterile syringe. For quality control purposes, such a gram stain, pH measurements, optical density measurements, the total count, and plate counts, 5-10 ml are sufficient and may be withdrawn by using a 5 or 10 ml syringe equipped with a 22 gauge needle.

For injection of the broth culture into bags of sterile carrier (peat), to ml samples are usually withdrawn with a sterile 50 ml syringe and an 18 gauge needle. Alternatively, an automatic motorized syringe equipped with a 16 gauge needle may also be used if large numbers of bags are to be injected.

In another modified system, a 1 liter collection flask is connected to the fermentor as shown in Figure 19.3. This collection flask should be autoclaved together with the fermentor. It is connected to the fermentor via a tubing which is attached to a sampling tube which runs through the stopper on top of the unit into the broth culture. A sampling port at the bottom of the fermentor is not needed in this case. The broth culture is forced into the sampling flask by closing off the air outlet of the fermentor while the pump is running.

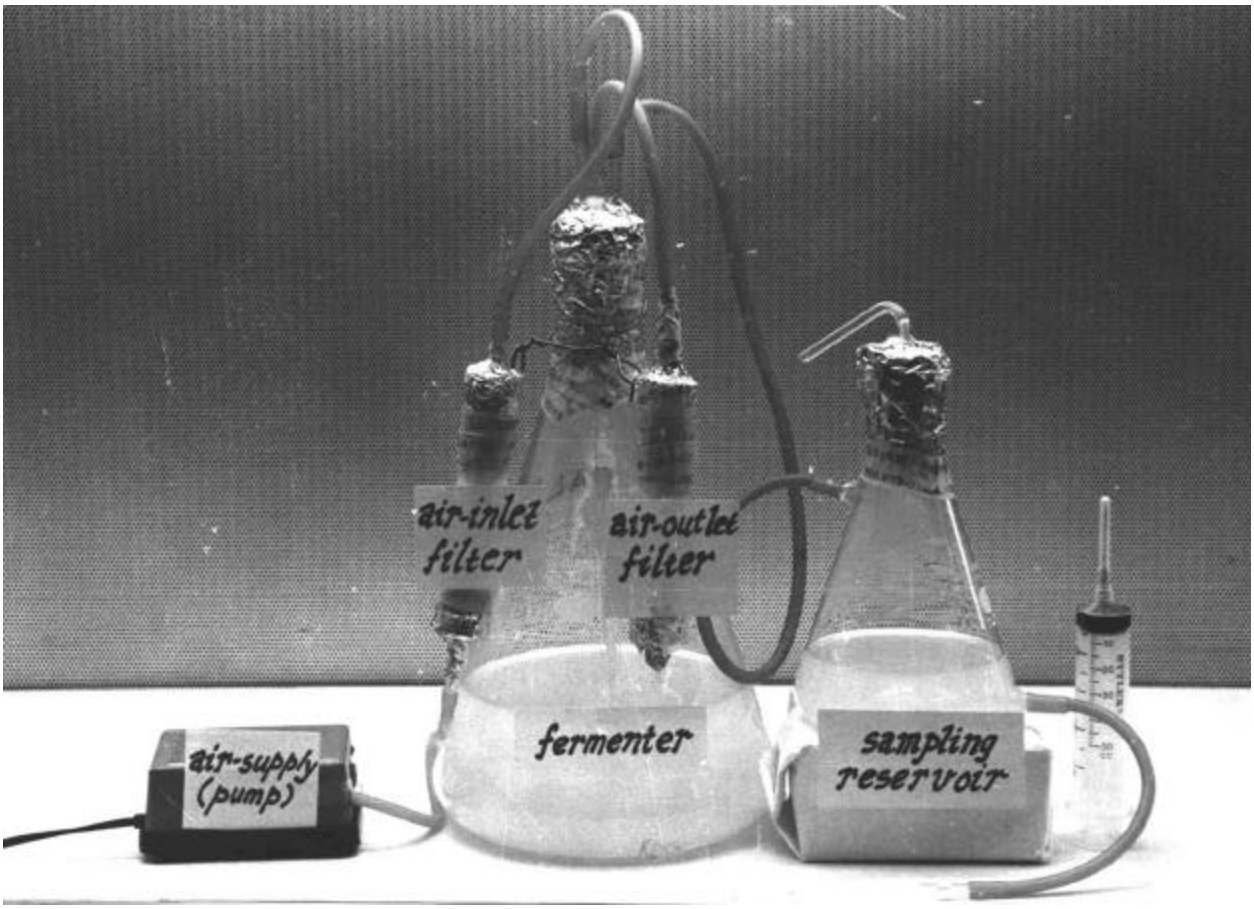


Figure 19.3. Modified fermentor

(e) Producing broth inoculum (Key steps 5, 6, 7, 8, 9, and 10).

When the starter cultures have reached the end of their log phase of growth (7 days for a slow-growing *Bradyrhizobium* and 5 days for fast-growing *Rhizobium*) they are ready to be used for inoculating the fermentor.

Inoculate one fermentor with the slow-growing *Bradyrhizobium* (e.g., *B. japonicum* TAL 379) and the other with the fast-growing *Rhizobium* (e.g., *Rhizobium phaseoli* TAL 182).
From both fermentors take a 10 ml sample at regular intervals once a day for 7 days and conduct the following tests:

1) pH measurement-a contamination problem is usually evident when the pH of the broth decreases toward acidity especially with slow-growing alkali producing rhizobia (e.g., *B. japonicum*). However, with fast-growing, acid producing rhizobia (e.g., *Rhizobium phaseoli*)

the pH test is less helpful since most contaminants are usually acid producers. Test the broth pH by adding two drops of bromthymol blue(0.5% in alcohol) in 1 ml broth. A yellow coloration indicates acidity and a blue coloration alkalinity.

2) Gram stain (Exercise 3)

3) Peptone glucose test (Exercise 3)

4) Total count with Helber or Petroff-Hausser counter (Exercise 4)

5) Optical density measurement (Exercise 4)

6) Spread plate count on YMA containing Congo Red and on YMA containing BTB (Exercise 4)

7) Agglutination with the homologous antiserum

This should be done just before harvesting when the culture has no less than 1 x 10*9* cells per ml. Dilute 2 ml of the cell suspension with 2 ml saline. Mix well and heat in boiling water for 30 minutes. After cooling, pipette 0.5 ml into an agglutination tube and add 0.5 ml of a 1:50 dilution of the homologous antiserum which should have a titre of at least 800.

Perform the agglutination test as described in Exercise 6.

The broth cultures may be incorporated into carrier material when the 1×10^9 cells per ml and purity of culture has been established.

Requirements

(a) Inoculating starter cultures

Transfer chamber
Platform shaker
Inoculation loop, flame
Erlenmeyer flasks or screw capped tubes of 50 ml capacity containing 25 ml YM broth each.

(b) Assembling simple fermentors

Large autoclave, aquarium pumps or compressor
Cork borer, small glass file, bunsen burner
For each fermentor:
Erlenmeyer flask, 4 liter, (This flask is modified by the addition of an outflow tube (ID 4 ml) at its base. These modified flasks are not available commercially, but any glass blower should be able to attach the short 3-5 ml glass tube.)
#12 autoclavable stopper
Glass tubing, inside diameter (ID) 4 mm, approximately 120 cm
Glass tubing, ID 30 mm, 2 pieces of 10 cm length for making air filters. Barrels of 50 ml syringes may be cut to size and used instead.
Rubber stoppers #4, autoclavable, 4 pieces
Hose clamps, 2, T-piece
Surgical rubber tubing, ID 4 mm, approximately 150 cm
Glass wool, Cotton wool, non-absorbent, Aluminum foil
Sampling tubes, ID 4 mm
YM broth (2-3 liters)

(c) Operating the glass fermentors

Syringes (30 ml), sterile with 18G needles
70% alcohol, cotton swabs or tissue paper
Broth culture of *Bradyrhizobium japonicum* TAL 379
Broth culture of *Rhizobium phaseoli* TAL 182

(d) Collecting the culture broth from the glass fermentor

Syringes, sterile (10 ml); 22G needles
Test tubes, sterile (for samples)

(e) Producing broth inoculant

Spectrophotometer; cuvettes, transfer chamber
Antisera homologous to the strains used (e.g. TAL and TAL 182)
Plates of peptone glucose agar
Plates of YMA containing BTB
Plates of YMA containing Congo Red
Syringes (20-30 ml); 22G needles
Test tubes, sterile (for samples)
Pipettes, sterile (1 ml); pipettes, sterile (10 ml).
Dilute tubes containing 9 ml sterile diluent, rack
Pasteur pipettes, sterile, calibrated
Solution of Bromthymol Blue (0.5% in ethanol)
Materials and supplies for Gram stain (Exercise 3)

APPENDIX J - CONVERSION FACTORS: LENGTH, AREA, MASS, VOLUME, TEMPERATURE

Approximate conversions to Metric Measures

Symbol	When you know	Multiply by	To find	Symbol
		LENGTH		
in	inches	2.5	centimeters	cm
ft	feet	30.0	centimeters	cm
yd	yards	0.9	meters	m
mi	miles	1.6	kilometers	km
		AREA		
in^2	square inches	6.5	square centimeters	cm^2
ft^2	square feet	0.09	square meters	m^2
yd^2	square yard	0.8	square meters	m^2
mi^2	square miles	2.6	square kilometers	km^2
	acres	0.4	hectares	ha
		MASS (weight)		
oz	ounces	28.0	grams	g
lb	pound	0.45	kilograms	kg
	short tons (2000 lbs)	0.9	tonnes	t
		VOLUME		
tsp	teaspoons	5.0	milliliters	ml
Tbsp	tablespoons	15.0	milliliters	ml
fl oz	fluid ounces	30.0	milliliters	ml
c	cups	0.24	liters	l
pt	pints	0.47	liters	l
qt	quarts	0.95	liters	l
gal	gallons	3.8	liters	l
ft^3	cubic feet	0.03	cubic meters	m^3
yd^3	cubic yards	0.76	cubic meters	m^3
		TEMPERATURE (exact)		
°F	Fahrenheit temperature	5/9 (after subtracting 32)	Celsius temperature	°C

Approximate conversions from Metric Measures

Symbol	When you know	Multiply by	To find	Symbol
		LENGTH		
mm	millimeters	0.04	inches	in
cm	centimeters	0.4	inches	in
m	meters	3.3	feet	ft
m	meters	1.1	yards	yd
km	kilometers	0.6	miles	mi
		AREA		
cm^2	square centimeters	0.16	square inches	in^2
m^2	square meters	1.2	square yards	yd^2
km^2	square kilometers	0.4	square miles	mi^2
ha	hectares (10,000 m^2)	2.5	acres	acres
		MASS (weight)		
g	grams	0.035	ounces	oz
kg	kilograms	2.2	pounds	lb
t	tonnes (1,000 kg)	1.1	short tons	
		VOLUME		
ml	milliliters	0.03	fluid ounces	fl oz
l	liters	2.1	pints	pt
l	liters	1.06	quarts	qt
l	liters	0.26	gallons	gal
m^3	cubic meters	35	cubic feet	ft^3
m^3	cubic meters	1.3	cubic yards	yd^3
		TEMPERATURE (exact)		
°C	Celsius temperature	9/5 (then add 32)	Fahrenheit temperature	°F

APPENDIX K - FORMS USEFUL IN KEEPING RECORDS IN LEGUME INOCULANT PRODUCTION AND QUALITY CONTROL

BROTH CULTURE PRODUCTION

FORM NO. _____ PAGE

RHIZOBIA FOR: _____

Name of Legume

FERMENTOR NO.	VOLUME LITERS	MEDIUM	SEEDING DATE	STARTER Kind/Amount	RHIZOBIA	DATE	RHIZOBIA	pH	LOT NO.

INOCULANT PRODUCTION

FORM NO. _____ Page

INOCULANT FOR: Name of Legume

DATE	LOT NO.	FERMENTOR	CARRIER Substance/Grind	WEIGHT kg	pH/DATE	MOISTURE % Wet Basis	VIABLE COUNT Date

ANALYSIS OF PACKAGED INOCULANTS

FORM NO. ——— Page

INOCULANT FOR:

DATE	LEGUME	BRAND	LOT NO.	EXP. DATE	SIZE	MOISTURE	pH	RHIZOBIA/g	SOURCE/HISTORY

PREPARATION OF INOCULANT

FORM NO. ——— Page

DATE	LOT NO.	LEGUME	CARRIER Substance and Grind	RATIO Broth/Carrier	ADDITIVES $CaCo_3$, etc.	CURING METHOD AND TIME	WHEN PACKAGED

INVENTORY OF CHEMICALS AND SUPPLIES FOR PRODUCTION OF BROTH CULTURE

FORM NO. ——— Page

ITEM	AMOUNT ON HAND	REORDER LEVEL	AMOUNT NEEDED	SUPPLIER	ORDER DATE	DATE RECEIVED

INVENTORY OF RAW MATERIALS

FORM NO. ——— Page

ITEM	AMOUNT ON HAND	REORDER LEVEL	AMOUNT NEEDED	SUPPLIER	ORDER DATE	DATE RECEIVED
Carrier						
Calcium carbonate						
Etc.						

BATCH TAB

Form Date

FERMENTOR NO.

VOLUME Liters

MEDIUM:

MINERAL SALTS Amount Added

K_2HPO_4

$MgSO_4 \cdot 7H_2O$

NaCl

$CaCl_2 \cdot 2H_2O$

NaOH

SUGAR

YEAST

STERILIZATION

Reached 121°C at

Turn-off Time

INOCULATED DATE

TIME

RHIZOBIA STRAIN

MONITORING PROGRESS

Date	pH	Microscope

DISPOSITION:

DATE
IF USED:

pH:

RHIZOBIA/CONTENT

LOT NUMBER ASSIGNED

APPENDIX L - PRESERVATION OF NODULATED LEGUMINOUS ROOTS FOR EXHIBITION PURPOSES

It is often helpful to have a real legume root with good nodules for display to show exactly what nodules look like. The preserved nodulated root makes this possible. The procedure described will preserve roots indefinitely as long as they are kept submerged in the solution.

Solution	**1 Liter**	**9 Liters**
Distilled H_2O	850 ml	7,650 ml
Formalin USP (37%)	100 ml	900 ml
30% Copper sulphate solution	50 ml	450 ml
Total	1,000 ml	9,000 ml

The copper sulphate solution is made by adding 30 g powdered copper sulphate (Mallinckrodt's N.F. grade of $CuSO_4 \cdot 5H_2O$) per 100 ml of distilled water and heating until all the salt is dissolved. The copper sulphate gives a bluish-green color and adds a live look to the specimen.

Nodulated roots which are to be preserved should be dug, washed thoroughly to remove all soil, and placed immediately in clean, cool water. Roots which have been allowed to dry do not make good pickled specimens. Roots should be placed in the formalin preservative solution as soon as possible.

Be certain to wash roots thoroughly, trim to fit the bottle, and make certain no dead leaves or trash is retained. These will spoil the preservative solution and give an undesirable cloudiness.

APPENDIX M - Recommended *Rhizobium* Strains

Leguminous spp.	*Rhizobium* Strain	Synonyms
Acacia albida	1B2	Nit. 176A22
	TAL 0169	
Acacia mearnsii	TAL 0309	CB 756
	TAL 0904	MAR 970
Acacia senegal	TAL 1145	CIAT 1967
	TAL 1428	ORS 901
Arachis hypogaea	TAL 0169	Nit. 176A22
	32H1	
	NC92 (Orbut)	
Cajanus cajan	P-241	
	32H1	
Calapogonium muconoides	TAL 0169	Nit. 176A22
	TAL 0196	Nit. 26Z4
Canavalia ensiformis	TAL 0201	Nit. 22A4
	Nit. 127E16	
Centrosema pubescens	TAL 0651	UMKL 44
	TAL 0655	UMKL 09
Cicer arietinum	TAL 0620	27A8
	TAL 1148	27A3
Desmodium uncinatum	TAL 0569	MAR 472
	TAL 1147	CIAT 299
Glycine max	TAL 0102	USDA 110
	142	USDA 3I1b142
	143	USDA 3I1b143
Lablab purpureus	TAL 0309	CB 756
	3302	
	32H1	
Lens culinaris	TAL 0634	Nit. 92A3
	TAL 0638	I2
Leucaena leucocephala	TAL 1145	CIAT 1967
	TAL 0582	CB 81
Lotus corniculatus	3071	3E0A6
Lupinus angustifolius	3053	3C2e1a

Leguminous spp.	*Rhizobium* Strain	Synonyms
	3061	3C2e1b
Lupinus luteus	3045	3C2d2
	3060	
Macroptyloma uniflorum	3301	3I6t2
Medicago falcata	1092	3D0n2
	1093	3D0n3
Medicago lupulina	1054	3D0b2
Medicago polymorpha	1066	3D0d10
Medicago sativa	TAL 1409	Nit. 102F77
	TAL 1372	POA 116
Mucuna pruriens	TAL 0309	CB 756
	TAL 0310	CB 1024
Pacyrhizus erosus	TAL 0655	UMKL 09
	Nit. 120B2	
Phaseolus coccineus	Nit. 127F1	
Phaseolus lunatus	TAL 0022	3I6d7
	Nit. 127E12	
	3258	
Phaseolus vulgaris	TAL 0182	
	Kim 5	
	CIAT 632	
	2668	3I6C15
Pisum arvense	2370	3H0Q18
	2391	3H0C1
Pisum sativum	Nit. 175G11	
	Nit. 128C53	
	TAL 1402	Nit. 128C75
Psophocarpus tetragonolobus	TAL 1021	Nit. 132B13
	C1204	
	3307	
Pueraria phaseoloides	Nit. 32H1	
	TAL 0768	CIAT 108
Sesbania grandiflora	TAL 1113	IC70
	TAL 1115	IC72

Leguminous spp.	*Rhizobium* Strain	Synonyms
Sphenostylis stenocarpa	TAL 0169	Nit. 176A22
	TAL 0173	Nit. 176A30
Stylosanthes guianensis	TAL 0309	CB 756
	TAL 0310	CB 1024
Vicia faba	Nit. 175F10	
	Nit. 175F12	
Vigna angularis	TAL 0169	Nit. 176A22
	TAL 0645	UMKL 12
Vigna radiata	TAL 0441	UPLB M6
	TAL 0169	Nit. 176A22
Vigna unguiculata	TAL 0209	
	TAL 0173	Nit. 176A30
	TAL 0169	Nit. 176A22
Voandzeia subterranea	TAL 0169	Nit. 176A22
	TAL 0556	

APPENDIX N - DIFFERENTIAL MEDIA FOR RHIZOBIA

1. **Basal: Yeast Extract Mannitol Agar (Medium 79)**

Ingredient	g/l
Dipotassium phosphate, K_2HPO	0.5
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	0.2
Sodium chloride, NaCl	0.1
Calcium chloride, $CaCl_2$	0.05
Yeast Extract	0.5
Mannitol	10.0
Agar (powdered)	15.0
Distilled water	1,000.0 ml

2. **Burton and Curley - unpublished**

Medium 79 plus:

50 ppm PCNB

30 ppm Oligomycin

7.5 ppm Rose Bengal

All additives are prepared as stock solutions and added to the molten agar immediately before pouring. For best results plates should be surface streaked.

Preparation of additives:

PCNB

Dissolve 0.1 g PCNB in 1 ml acetone. Add 4 ml of a Tween 85 solution (10 drops Tween 85 to 100 ml sterile water), shake well. Add 1 ml of the resulting suspension to 400 ml medium 79.

Oligomycin

Dissolve 1.2 g Oligomycin in 100 ml reagent alcohol. Add 1 ml to 400 ml agar.

Rose Bengal

Dissolve 1 g of Rose Bengal dye in 300 ml sterile water. Add 1 ml to 400 ml agar.

3. **Barber, Lynn** - Soil Science Soc. America J. 43:1145-1148.

Medium No. 1:	mg/l
Brilliant green	0.4
Sodium azide	0.2
Penta chloro nitro benzene (PCNB)	50.0
Medium No. 2:	
Brilliant green	1.5
Sodium azide	1.0
PCNB	500.0

The brilliant green and congo red solutions were autoclaved as 0.1% and 0.25% stock solutions respectively and added to the molten agar just before pouring plates. The PCNB was prepared as a 10% solution in acetone. The basic medium was yeast extract mannitol agar (YMA).