# Production and Quality Control of Carrier-based Legume Inoculants

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## Abstract

Thompson, J.A. 1983. Production and quality control of carrier-based legume inoculants. Information Bulletin No. 17. Patancheru, A.P., India: International Crops Research Institute for the Semi-Arid Tropics,

Commercial production of legume inoculants began in 1895 in the USA and UK. In the 1980s they are produced in all continents. Most are used with powdered organic carriers such as peat, although other carriers have shown promise. The bulletin outlines the use of small fermentation vessels for production of *Rhizobium* inoculant Inoculant production is explained, with emphasis on quality control measures. Types and suitabilities of various worldwide inoculant carriers are discussed along with treatment and packaging techniques to ensure maximum effectiveness and sterility. Tips on contaminant-free handling in the workplace are included, stressing the need for qualified personnel and suitable basic facilities. Sources and selection of inoculant strains, and transportation of inoculants, are also considered. Examples of national and private bodies governing the manufacture of inoculants are reviewed, and standards for production are suggested.



## Résumé

Thompson, J.A. 1983. Production and quality control of carrier-based legume inoculants (*Production et contrôle de la qualité d'inoculants de légumineuses avec supports*). Information Bulletin No. 17. Patancheru, A.P., India: International Crops Research Institute for the Semi-Arid Tropics.

La production commerciale d'inoculants de légumineuses a débuté, en 1895, aux Etats-Unis et au Royaume-Uni. Aujourd'hui, ils sont produits sur tous les continents. La plupart de ces inoculants sont utilisés avec des supports organiques en poudre, telle la tourbe; mais, d'autres supports se sont aussi révélés prometteurs. Ce bulletin porte sur l'utilisation de petits vases de fermentation pour la production d'inoculants de *Rhizobium*. Les techniques de production sont expliquées, en soulignant l'importance des mesures de contrôle de la qualité. Les types et l'adaptabilité des divers supports disponibles dans le monde sont discutés, ainsi que les techniques d'emballage et d'inoculation, afin d'avoir une efficience et une stérilité maximales. L'on donne des conseils, afin que la manutention soit effectueé sans contamination et l'on insiste sur la nécessité d'un personnel qualifié et d'installations appropriées. L'on aborde également les sources et la sélection de souches d'inoculants, ainsi que le transport. A titre d'exemple, des organismes nationaux et privés régissant la production d'inoculants sont cités et des normes de production sont suggérées.



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## Acknowledgment


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I also thank D. Pulsford of Agricultural Laboratories Pty., NSW, Australia for supplying the metal fermentor drum which appears as Fig. 1 in this publication.

**J.A. Thompson**

# Historical Background

Commercial production of legume inoculants began with the application for patents in the UK and the USA by Nobbe and Hiltner in 1895 (Fred et al. 1932). In the USA, production expanded rapidly between 1929 and 1940 (Burton 1967). The product name Nitragin®, used by the company of the same name in the USA, was in fact the trade name for all preparations put out under the Nobbe and Hiltner process (Fred et al. 1932). The USA is probably still the largest producer of legume inoculants in the world, though commercial production is today carried out in all continents. The development of grain crops in both the developed and developing countries has provided a further impetus to production.

The initial preeminence of the USA is reflected in the descriptions by Fred et al. (1932) and Burton (1967). Many of the developed countries commenced production of inocula in official institutions early this century, e.g., Canada in 1905 (Newbould 1951); Sweden in 1914 (H. Ljunggren, Agricultural College of Sweden, Uppsala, personal communication), and Australia in 1914 (Roughley 1962). In 1932 Fred et al. listed 10 commercial manufacturers (9 in Europe and 1 in New Zealand).

Periodically, particular local requirements have precipitated considerable activity in inoculant production and associated research, e.g., polder reclamation in the Netherlands for over 20 years from the 1930s (van Shreven et al. 1953, van Shreven 1958). Although Canada does not currently support any commercial inoculant production, there was considerable study of inoculant production up to 1954 (Hedlin and Newton 1948; Newbould 1951; Spencer and Newton 1953; Gunning and Jordan 1954).

Australian involvement in commercial production coincided with the post-World War II boom in pasture development in areas lacking suitable rhizobia and has been fully described, particularly by members of testing authorities (Vincent 1965, 1968, 1970, 1977; Date 1969, 1970; Roughley 1970, 1976; Date and Roughley 1977; Thompson 1980).

In Uruguay and Argentina, the development of commercial legume inoculants has also followed post-war emphasis on pastures. In Brazil, production has been particularly geared to an enormous increase in soybean production. The present situation regarding availability of inoculants in Latin America has been comprehensively described by Batthyany (1977).

Inoculant production and use in New Zealand has been recently described by MacKinnon et al. (1977); and in South Africa by van Rensburg and Strijdom (1974).

The problems of starting production of inoculants in developing countries are well described; in Guyana, by Persaud (1977), and in Papua New Guinea, by Shaw et al. (1972) and Elmes (1975).

The earliest documented production of inoculants in India was in 1934, and, when the state of the industry was described by Sahni in 1977, it probably had more manufacturers than any other country.

Inoculant production is also well developed in many European countries, although poorly documented. Irradiated peat inoculants are produced commercially in the USSR (R.J. Roughley, personal communication), and in France (M. Obaton, personal communication). Inoculants based on autoclaved carriers have been produced by the Swedish College of Agriculture at Uppsala since 1914 (H. Ljunggren, personal communication).

The desirability of control measures to test legume inoculants was recognized early in the history of the U.S. industry. The U.S. Federal Government was responsible for testing cultures and issuing licences in the 1930s, and individual states had legislation as early as 1912 to control quality (Fred et al. 1932). However, by the late 1940s regulatory control by Federal authorites was no longer considered necessary (Burton et al. 1972). In recent years, the only authority to publish results of independent tests of commercial inoculants is the State of Indiana (Schall et al. 1975).

In a number of countries, government and university institutions supply inocula for commercial purposes, although the fact that the source of an inoculant is a reputable institution is not per se a guarantee of its value.

The scale of individual private enterprises often does not permit employment of fully trained personnel. Quality control is therefore more difficult. This led to the formation of the Australian testing authority, U-DALS (University-Department of Agriculture Laboratory Service) in 1956, and its successor AIRCS (Australian Inoculants Research and Control Service) in 1971.

While it is probable that legislation is available in many countries to allow checking of the quality of the product on sale, only a few countries have established regulatory authorities which control the release of the product to the market. Canada has recently invoked legislative powers to set standards and control quality (Anon. 1979a and b). The Australian body developed as a result of cooperation between manufacturers, scientists and state departments of agriculture, and functions without legislative backing. The Uruguayan system was established on the Australian model, but has legislative powers. The control body of South Africa functions in a similar fashion to that of Australia (van Rensburg and Strijdom 1974). India has recently prepared a detailed set of standards (Anon. 1977), although the mechanics of control require clarification. In New Zealand the Inoculant and Coated Seed Testing Service (ICSTS) has commenced operations (Anon. 1979c).

The need for legume inoculants in most countries may be satisfied by a variety of options ranging from purchase of the prepared product from another country to a full local program of inoculant development, including selection of local strains and use of local materials. The degree of commitment is clearly governed by the size of the potential market, availability of funds, technical expertise, and suitable raw materials.

The majority of legume inoculants currently produced in the world utilize powdered organic carrier materials. In spite of the wide range of tested alternatives, peat remains unchallenged as a carrier (Strijdom and Deschodt 1976). It undoubtedly has the desirable attributes of high moisture-holding capacity and can usually be used without additives, except, occasionally $CaCO_3$.

It is relatively easy to devise a substrate from a variety of materials which support satisfactory growth and survival of rhizobia (Strijdom and Deschodt 1976). The search for new carriers has revealed suitable materials which are usually cheap and locally available. However, the greatest attribute of peat, i.e., its protective effect on rhizobia used as seed inocula, has rarely been used as a criterion for the evaluation of alternative substances.

Alternative carriers which have shown some promise are coal (Strijdom and Deschodt 1976; Roughley 1976; Halliday and Graham 1978; Paczkowski and Berryhill 1979); charcoal alone (Newbould 1951), *or* with composted straw (Wu and Kuo 1969, quoted by Date and Roughley 1977); mixtures of soil and compost; and/or ground plant material such as those formulations popular in the Netherlands (van Schreven et al. 1954). In Sweden, the formulation containing soil, peat, composted bark, and wheat husks—in use since 1925 (H. Ljunggren, personal communication)—has changed little except for relative quantities. Where peats are scarce, the emphasis in tropical areas has naturally shifted to ground, fibrous plant material, e.g., cellulose powder (Pugashetti et al. 1971); bagasse; coir dust (John 1966); and composted corn cobs (Corby 1976). Filter mud, a waste product of sugarcane mills, has shown promise (Philpotts 1976), but in its raw state it can carry a very large fungal population which necessitates sterilization. The majority of Indian inoculant manufacturers use lignite (Sahni 1977), which Tilak and Subba Rao (1978) included in their comprehensive study of Indian carrier materials. Inorganic materials such as bentonite and talc have also been studied (Date and Roughley 1977). In the USA, commercial products based on vermiculite (Schall et al. 1975) are available. Dommergues et al.

(1979) have successfully prepared a polyacrylamide carrier.

# Production Techniques

## Fermentation vessels

### Scale of operations

Any fermentation vessel must be readily sterilized, allow access for inoculation and provide aeration of the culture. Access is also necessary for easy cleaning. The simplest fermentation vessel is a glass bottle or flask, aerated either by air bubbles or shaking. In the latter case, introduction of air is not necessary, but some degree of air exchange is preferable. Cotton wool plugs can be used provided they are not wetted by the culture. It is common practice for Indian manufacturers of inocula to use 1 -2 liter glass bottles or flasks on shakers (Sahni 1977).

The use of large shakers and glass flasks for commercial production can be defended on the following grounds:

1. the quantity of broth can easily be varied;
2. the volume of the autoclave used need not be large; and
3. the contamination of one flask does not ruin the whole batch.

However, the disadvantages are numerous, and include:

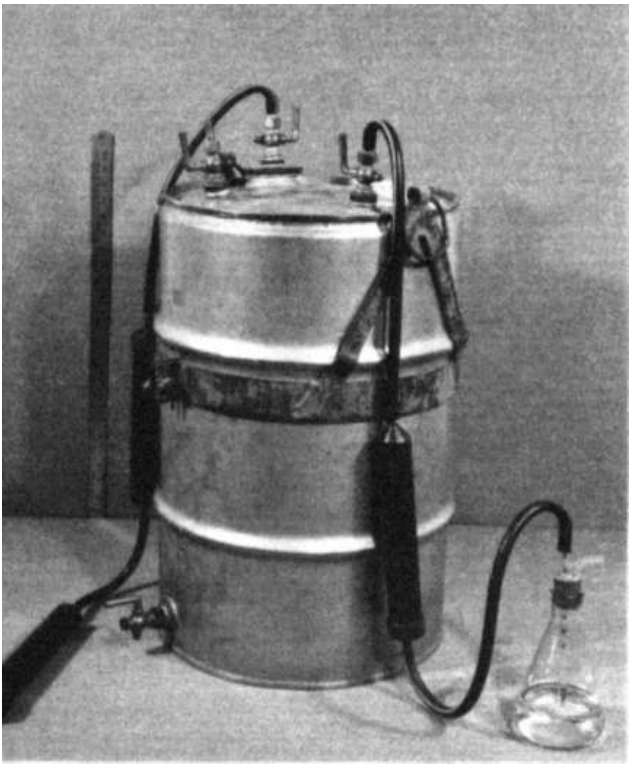
1. the high cost of shakers;
2. the high cost of glass flasks and the risk of breakage;
3. the inefficient use of space;
4. the noise of the operation;
5. the risk of contamination directly related to the number of flasks; and
6. the high labor requirement, especially for adequate quality control.

One critical study of the relative merits of this system vs two simple fermentors has been published by Gulati (1978). Biologically, the fermentors were superior on two important counts:

1. the lower proportion of contaminated batches; and
2. the superior economy of carbohydrate usage.

The range of fermentation equipment available makes valid economic assessment difficult, but some observations are obvious. The space and power requirement for a rotary shaker carrying 81 x 11 flasks is likely to be much greater than for four portable fermentors capable of growing the same amount of broth with air agitation. Power for agitation in most fermentors is 1 -3 watts/l. A stronger argument for economy, however, is that a proper control procedure requires checking of each flask or fermentor before use of the product—a 20-fold difference in numbers between flasks and fermentors in this example.

The following discussion of production technology is concerned with relatively small, portable, fermentors which can be manufactured in most countries including India (Gulati and Seth 1973). However, inoculant production is not restricted to this scale: many commercial inoculant operations, the best known being Nitrigin Co., Milwaukee, are based on large (approx. 1000 l) fermentors. Such fermentors are also manufactured in India, particularly in Pune, Maharashtra. They clearly need a source of steam for sterilization in situ, and it would be unusual for such fermentors to be agitated only by air. The size of such units justifies the cost of adequate seals to house agitation drives, although imported magnetic stirrers have been used in units designed at Central Food Technological Research Institute, Mysore (V. Srinivasa Murthy, Central Food Technological Research Institute, Mysore, Karnataka, India, personal communication).



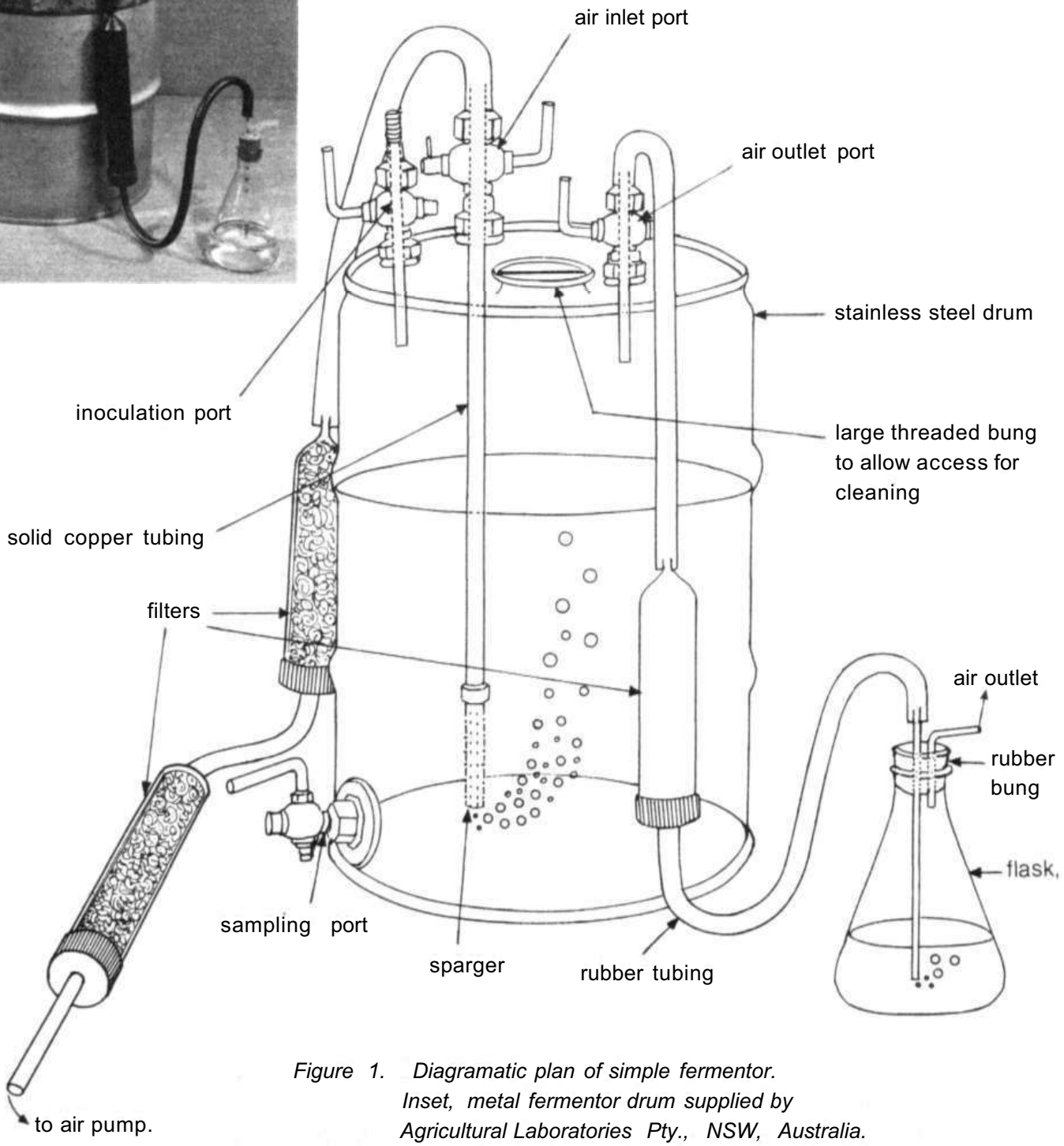



*Figure 1. Diagramatic plan of simple fermentor. Inset, metal fermentor drum supplied by Agricultural Laboratories Pty., NSW, Australia.*

Few inoculant manufacturers could justify the purchase of large industrial fermentors for inoculant production alone, although they are clearly a suitable means of growing rhizobia and may well be available from large companies already involved in preparation of microbial products.

The size of small fermentors can vary from a few liter to a working maximum of approximately 80 l, capable of containing 501 of broth. With an adequate autoclave, these can be sterilized complete with medium and all accessories. The essentials of a small fermentor unit for inoculant production have been illustrated by van Schreven (1958) and Date (1974) and are shown in Figure 1. In contrast to many commercially-available units which have mechanical stirrers, the unit illustrated is dependent on a source of air for aeration and mixing. Preferably, the unit should be made entirely of stainless steel. Mild steel is satisfactory, especially when coated with epoxy finishes, but rusting will result in short life. Cocks should be of brass or steel that can be flamed to high temperatures. A suitable fermentor should have at the top:

1. an air inlet port, a pipe to the base of the unit, and a metal or glass sparger for generating small bubbles;
2. an air outlet port;
3. an inoculation port; and
4. a large threaded bung to allow access for cleaning.

In addition, it is convenient to include a sampling cock at the base.

For autoclaving, the medium should not be filled to more than 80% of its total volume. When the fermentor contents approach 30-40% of the total volume of the autoclave, temperature gradients are such that the contents may not reach steam temperatures for a long period. For a 501 fermentor, it is necessary to autoclave for at least an hour. Care should be taken to prevent carbohydrate breakdown through excessive heating, which is most simply evaluated by the growth rate of the broth. The air supply should pass through filters packed with cotton wool or glass wool. Unless an oil-free compressor is used, an oil trap should be fitted in the line before the two large essential filters. At ICRISAT the filters conneced to the unit are routinely autoclaved. The air outlet should preferably be already vented through a filter to ensure sterility during cooling and to allow for the possibility of suck-back if the air supply ceases. During autoclaving, the air outlet should be left open to allow equalization of pressure, but the air inlet which reaches to the bottom of the fermentor must be closed to prevent loss of medium.

## Media for broth culture

The essential components for culture media for *Rhizobium* spp are generally available on the market. The standard medium (Fred et al. 1932) is based on yeast extract as the nitrogen and/or growth factor source with a suitable carbon source, and minerals. Considerable latitude is permissible in formulating a medium (Burton 1967, Vincent 1970), and the operator must experiment with the available materials. The components of the medium of Fred et al. (1932) were as follows:

| | g/l |
|---|---|
| $K2HPO_4$ | 0.5 |
| $MgSO_4.7H_2O$ | 0.2 |
| NaCl | 0.1 |
| $CaCO_3$ | 3.0 |
| Mannitol | 10.0 |
| Yeast water* | 100 ml |

*Supernatent obtained from 10% bakers' yeast standing 1 -2 hours in water.

In various media, the concentrations of inorganic salts have been varied at least two-fold (Vincent 1970), and at best are somewhat arbitrary, especially as the yeast source may contain at least some of the mineral requirement.

Ertola et al. (1969) found that the addition of potassium nitrate increased growth and maintained the pH near neutrality. Data by Balatti (1982) support this result. $CaCO_3$ is only required for long-term storage of fast growers on agar and can generally be deleted from both media (see Vincent 1970). Balatti (1982) includes $MnSO_4$ without comment.

In practice, concentrated yeast extract is commonly used, but care must be taken in view of the deleterious effect of amino acid supplementation (Strijdom and Allen 1966). A safe level is 3 g/l, although some media contain up to 10 g/l (Vincent 1970). A number of workers have demonstrated the deleterious effects of concentrations between 3.5 and 10 g/l on viable numbers, nitrogen fixation, nodulating ability or cell morphology (Staphorst and Strijdom 1972; Date 1972; Skinner et al. 1977). While bakers' yeast may not be readily available in India, a local retail supply in Hyderabad was found to cost half that of concentrated yeast extract.

For reasons of cost, mannitol as a carbon source has commonly been replaced by sucrose or glucose. Most rhizobia utilize both mono- and disaccharides, although the fast growers use a wider range than the slow growers (Graham and Parker 1964). Glycerol has been used commercially for *R. japonicum:* gelatin or arabinose is preferred by slow growers. It is probable that most sugars are not fully utilized, and slow-growing rhizobia do not utilize sucrose (Burton 1982). Not only will the excess be wasted, but it may contribute to unnecessary multiplication of contaminating organisms already present in unsterilized carriers, following impregnation with broth. Molasses, malt extract, and soybean extracts have been used successfully in commercial production units in India (Nandi and Sinha 1974); and Kumar Rao et al. (1980) found that sucrose—and in some cases commercial sugar—served quite satisfactorily for shaker cultures. Gulati (1978) found that the advantages of a fermentor over a shake culture included a more efficient use of mannitol as a carbohydrate source.

## Starter cultures for fermentors

A starter culture should be suspended in liquid, be in the log phase of growth to minimize the lag period in the new medium, and be sufficiently well grown to provide an inoculum of up to 1 % of the total broth to be prepared. Higher inoculum levels reduce time to maximum growth but may pose practical difficulties in preparation.

The volume requirements of any commercial production unit are such that the mother culture in a test tube must be multiplied to a larger volume. This may be done in liquid or on solid media and may even involve a multiple-stage process if a large fermentor is used. However, one starter culture should be available for each fermentation vessel because, not only is inoculation of the fermentor with the starter the point at which risk of contamination is the greatest, but attempts to inoculate more than one fermentation vessel with one starter greatly increase the hazards.

The hazards of contamination cannot be overstressed—not only is *Rhizobium* a relatively slow-growing organism, but the medium is not selective, and the strains commonly used in tropical countries are, medium to slow growers.

Liquid starters are best prepared in Buchner flasks with sterile rubber tubing already connected to the sidearm for direct connection to the inoculation port of the fermentor. A starter can also be grown on solid medium. The advantage here is that the experienced operator can more readily see evidence of contamination. This agar-based culture can also be prepared in a Buchner flask connected to a second Buchner flask containing fluid for suspending the cultures (Figure 2). The whole unit can be autoclaved together and the solid medium sloped. After growth of the culture on the solid medium the suspending fluid is carefully decanted across, the culture is suspended after standing, and then introduced to the inoculation port via the rubber tubing removed from the flask containing solid medium.

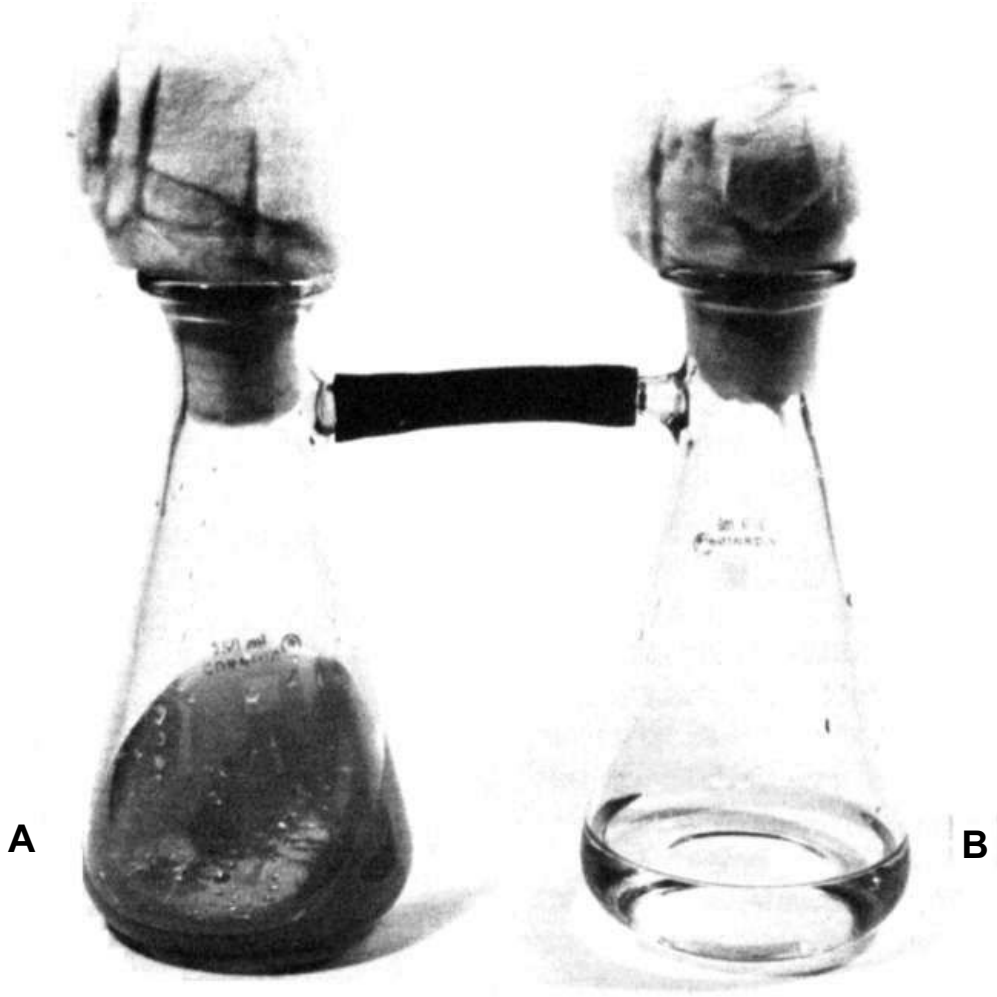



***Figure 2. Buchner flasks containing sloped solid agar-based culture medium (A) and suspending fluid (B)***

For inoculation with the starter, the inoculation port must be thoroughly sterilized by flaming, allowed to cool, briefly flamed again and the starter introduced. Risks of contamination may be further reduced by cutting the rubber tubing on the Buchner flask to provide a sterile end.

Commencement of aeration before inoculation ensures a positive air pressure at all outlets, further minimizing contamination.

## Aeration

Air flow rates as high as 120 l/l/hr are commonly recommended for some microorganisms, but 5 l/l/hr has been found satisfactory for small scale commercial units (van Shreven et al. 1953; Roughley 1970). The pressure necessary (0.7 kg/cm$^2$) may require a reducing diaphragm on the compressor outlet (Date 1974). Fine pore-size spargers (approx. 5µ m) ensure better solution of oxygen but impellers are not recommended because of the difficulty of maintaining a bacteriological seal on the shaft (Date 1974). Balatti (1982) favors 30 l/l/hr with a mechanically-stirred fermentor, claiming that the moving baffles produce turbulence, thereby increasing the interfacial area of the bubbles in the liquid.

Frothing is not unusual but is more common with fast-growing rhizobia. For this reason, fermentors may be filled with more medium for slow growers (approximately 80% full) than for fast growers (approximately 60% full). Frothing can be controlled to some extent by close monitoring and control of maximum numbers, but any commercial anti-frothing agent should be tested for compatibility with rhizobia before use.

It is also advisable to check components of the media, e.g., some dried yeast media induce frothing as soon as they are aerated.

## Incubation temperatures

Incubation temperatures for commercial production are usually 26-30° C for all strains, although Burton (1967) preferred 30-32°C to 35° C for *Rhizobium meliloti.*

## Inoculum level and incubation time

Inoculum levels are commonly of the order of 0.1-1.0%, providing $10^6$-$10^7$ rhizobia/ml of culture.

The mean generation time (MGT) will be affected by the stage of growth of the starter and the resultant lag phase, temperature of incubation, availability of nutrients, aeration and, of course, the size of the inoculum.

The following minimum batch times will be necessary for a starter containing $10^9$ rhizobia/ml, to provide a finished broth of 5 x $10^9$/ml:

| | Inoculum volume (%) | |
|---|---|---|
| | 0.1 | 1.0 |
| Fast growers (MGT 2-4 h) | 25- 52 h | 18- 36 h |
| Slow growers (MGT 6-12 h) | 78-156 h | 54-108 h |

Balatti (1982) favours 5-6% inoculum size which is reached by progressive scaling up through flasks and small fermentors. However, the organization of an inoculant production system may not benefit from the most rapid growth conditions. For example, it may be desirable to use a low-percentage inoculum to extend the period of growth, thus allowing for development of any contaminants (these commonly grow more rapidly than *Rhizobium*), and allowing the manufacturer to complete checks for their presence before the broth is ready for harvest. In all operations, however, it is essential to maximize the proportion of living bacteria available at the point of harvest. Manipulation of nutrient sources and aeration can also affect this during growth. Storage of finished broths should be kept to a minimum, but if it is unavoidable, cultures should be held at 4°C.

## Carriers

### Requirements

Apart from amenability to drying and grinding, there are no clear criteria for choice of suitable carriers. Many of the world's inocula are based on peat with a high percentage of organic matter. Australian inoculant peats have about 65% organic matter (Stephens 1943), and Wisconsin peat contains 86% (Burton 1967). However, India lignite can be 75% organic matter (Tilak and Subba Rao 1978).

In samples of peat from the Nilgiri Hills of India, organic matter ranged 13-31% because deposits are in restricted areas and subject to localized effects of erosion, admixture with soil, etc.

Carrier materials for inoculants should meet the following requirements:

1. the material should be finely ground to allow thorough mixing with other components and be compatable with its final use;
2. the pH should be readily adjustable to 6.5-7.0;
3. a good moisture-holding capacity is desirable, and is probably one of the major reasons for the popularity of peat;
4. the carrier should be sterilizable to favor survival of the inoculant; and
5. the carrier should be free of toxic materials.

It is necessary to thoroughly test a proposed carrier. Interactions between rhizobia, carrier,

method of treatment, and storage period are common, and even peats from the same area can vary in suitability (Roughley and Vincent 1967).

A great many carriers have been tested and used for inocula throughout the world. A wide variety of these have also been tested by Indian investigators.

Halliday and Graham (1978) tested coals in Colombia but only one of the nine deposits tested were found satisfactory for survival. Paczkowski and Berryhill (1979) also found various U.S. coals, including lignite, to be satisfactory carriers for *R. leguminosarum.*

Because it is difficult to obtain a suitable source of peat in India, experimenters and manufacturers have turned to lignite. In many Indian publications over the past 15 years, populations of rhizobia $>10^9$/g have been quoted, using lignite as the carrier (e.g., Kandasamy and Prasad 1971).

Filter mud or press mud has also attracted attention because of its ready availability in a number of countries (e.g. Godse et al. 1980; Philpotts 1976) although it is likely that considerable differences in its constitution result from different harvesting techniques (e.g., in many countries the leaf is burnt before the cane is cut) and different degrees of success in removal of sucrose from the final product. The presence of readily-available carbohydrate necessitates removal of the existing microflora by sterilization, and in ICRISAT experiments fungal populations have been difficult to remove by normal sterilization procedures. Another readily available carrier in India is charcoal, but its quality is dependent on its source.

There is voluminous literature in both India and abroad on the addition of organic materials to basic carriers. Unfortunately, the promising data obtained by workers in India is not followed up by publication of further data on the success or failure of these carriers when they are commercially produced. Short-term studies reported by Sharma and Verma (1979) show that the very good populations in sterilized lignite carrier can be further improved by further addition of lucerne meal.

A problem with Indian lignite is that some samples are difficult to wet, presumably due to the presence of waxes from the original vegetation. Surface-active materials may help overcome this problem.

It is very important that the data presented for experimental tests are critically evaluated. If a *Rhizobium* cell measures 1 micron x 0.5 micron, a cubic centimetre will hold $4 \times 10^{12}$ of packed rhizobia. It is difficult to visualize that broths or prepared carriers can contain the claimed $10^{11}$ cells/ml or per g since even frozen rhizobia in the form of a paste prepared by Northrup King and Co. of USA contains only $10^{11}$/g wet paste (Subba Rao 1982).

The ultimate test should be the suitability, for multiplication and survival, of the range of rhizobia to be grown. Strains of *R. meliloti* and *R. trifolii* differed in their tolerance of sodium and chloride ions when salt contamination affected the quality of Australian peats (Steinborn and Roughley 1974). The problem was only discovered by continued monitoring of the final product.

In examining organic carriers for suitability, the optimum moisture content must be defined. Moisture is commonly expressed as a percentage. The practice has developed in many countries of expressing moisture in terms of wet weight so that when the weight of water equals the weight of dry carrier, the moisture content is 50%. This contrasts with the method of expression in India where the normal method of expression is, as with soils, in terms of dry weight: equal quantities of carrier and water constitute 100% moisture. However, expression only in terms of percentage can be misleading when carriers of different moisture-holding capacities are compared. Water potential (i.e., suction or negative pressure) is a more precise criterion. This may be expressed in a number of units:

$$\begin{aligned}[1\ \text{bar} &= 0.987\ \text{atmosphere} \\ &= 1022\ \text{cm water} \\ &- 10^5\ \text{pascals} \\ \text{pF} &= 3 + \log(-\text{bars})].\end{aligned}$$

When moisture potential is related to percentage moisture content, the resultant graph provides the moisture characteristic curve of the material and allows realistic comparisons of materials with different moisture-holding capacities. Examples of pF value in an Australian peat illustrate the range of significant values. A pF value of 4.88 (moisture content of 30%) adversely affected growth in two of three strains, pF 4.15-3.42 was optimal tor the three strains in nonsterile peat (Roughley and Vincent 1967), while for sterile peat the optimum was 4.15-2.69 (Roughley 1968). Determination of moisture potential involves use of specialized equipment, but, provided that reference materials can be calibrated against this equipment, a simple procedure described by Fawcett and Collis-George (1967), can be applied. Calibrated filter paper is allowed to equilibrate with the moistened material and a moisture characteristic curve can then be derived. Different carrier materials can then be compared in terms of their moisture potential.

## Milling and drying

As more critical studies have been made on peat than on any other carrier, its problems need to be borne in mind. Although peat can often be successfully air-dried sufficiently to allow milling, heat may be used to assist the drying process. Roughley and Vincent (1967) found that, with Australian peats, heating to 135° and 160°C caused changes which were lethal to subsequently-added rhizobia while maxima of 80-100°C were not harmful. They ascribed the harmful effect to production of inhibitory substances in the peat. In contrast, Burton (1967) found that flash-drying peat in a rolling drum with an air inlet temperature of 650°C produced a satisfactory peat. It is evident that a distinction must be made between drying temperature and product temperature (which could be expected to approximate the wet bulb temperature until drying is complete). The apparent absence of toxic materials makes it .probable that the temperature of Burton's peat did not exceed 100° C. However, it is clear that particular care should be taken with drying procedures and that oven-drying should be avoided.

Air-drying should be used where practicable. Drying with heat should be effected at the lowest possible temperatures and certainly below 100°C.

Australian and South African peats are commonly ground following air-drying (Roughley and Vincent 1967; Strijdom and Deschodt 1976).

Although the required particle size is dependent on final use, peat is commonly milled to pass through at least a 250 micron sieve (Burton 1967); some require 50% to pass through a 75 micron sieve (Strijdom and Deschodt 1976). Australian peats generally pass this standard. Not only does grinding to 75 microns improve adhesion of peat to dry seed, but Australian peat was found to be more subject to caking when moistened, if ground to only 150 microns (R.J. Roughley, personal communication). Care must be exercised with very fine grinding of a number of materials because of the risk of spontaneous combustion. A criterion of acceptability could be the bulk density of the final material. A very 'fluffy' product may require an unacceptably large volume per unit weight. Milling to a fine particle size can also be difficult if the material is very fibrous.

Lignite from Neyveli collieries, near Madras, India, has been favored as a carrier in India probably because of its ready availability. Charcoal is also used and filter mud (press mud) from the sugar industry has some useful attributes. Samples of Indian carrier materials commercially ground in a hammer mill at Hyderabad had the following particle size distributions and can be compared with an Australian peat. Thus these materials, though "pulverized" in a mill which did not contain fine sieves, reach the standards commoly quoted.

**Table 1. Particle size distribution of carriers.**

| Carrier | Percentage of sample retained by sieves | | | |
|---|---|---|---|---|
| Mesh size* of sieves | Lignite | Charcoal | Filter mud | Australian peat (ground in Australia) |
| 60 | 4 | 1 | 4 | 1 |
| 100 | 9 | 7 | 12 | 2 |
| 150 | 6 | 3 | 9 | 2 |
| 200 | 5 | 11 | 10 | 8 |
| >200 | 76 | 78 | 65 | 87 |

*Particle size has traditionally been defined in terms of the mesh number of the sieve through which the material will pass. Although this classification has generally been deleted from standard specifications, it remains in common usage in India British standard mesh number = the number of wires/linear/inch in a wire mesh sieve. Thus aperture sizes, which approximate particle sizes, are related to mesh numbers as given below:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| British standard mesh number | 8 | 30 | 60 | 100 | 150 | 200 | 250 |
| Aperture size (microns) | 2000 | 500 | 250 | 150 | 106 | 75 | 62 |

## Sterilization

Sterilization means killing of all organisms. A sterilized carrier can only be expected to remain sterile if it is retained in the same enclosed container in which it was sterilized. It is common practice to sterilize carriers at least once in an autoclave and then to inoculate in open trays. Without extreme precautions this procedure is likely to produce contaminated cultures, and worse, it is possible that the fewer contaminating organisms may be more of a problem than the very diverse populations of organisms in the original carrier. Most Indian inoculants tested at ICRISAT contained nonrhizobial contaminants. The inoculant producers in India with whom we have checked do not use the procedure of adding sterile broth to the carrier, to test for contamination.

The following discussion, therefore, is concerned with two clear-cut situations:

a) a nonsterile carrier and its impregnation; and

b) a sterile carrier and its impregnation while still known to be sterile.

Any operation involving the use of a sterilized unpackaged carrier must be considered as an example of a nonsterilized carrier. The discussion, however, covers techniques and materials not necessarily available in India.

## Inoculation of nonsterile carriers

Inoculants of good quality can be produced with nonsterile carriers. The published studies on nonsterile carriers have mainly been concerned with peat.

Nonsterilized carriers are normally held in the dried form after grinding, so that their natural populations of organisms have no opportunity to multiply before the *Rhizobium* broth is added.

Mixing with inoculum may be achieved by spraying or pouring the broth onto the pow-

dered carrier while it is being agitated by hand or in a batch mixer such as concrete mixer. The proportions of broth and carrier are governed by the nature and moisture-holding capacity of the carrier, but, with peat, it is generally desirable to add broth to the point where the carrier remains friable without forming balls (Date 1974). It has generally been considered essential to use broths of the highest quality, so that minimal multiplication is necessary for the rhizobia to dominate the other organisms. However, a recent study of data from 277 commercial batches of nonsterilized peat inoculant produced in Australia (prior to the change to sterile peat) revealed that when broth counts exceeded 5 x $10^8$/ml, the final number of rhizobia in peat cultures bore no relationship to inoculum size (Roughley and Thompson 1978). No data were available for broth counts less than 5 x tOVml, but Somasegaran and Halliday (1982) found similar effects with *R. japonicum* and *R. phaseoli* diluted to $10^6$/ml.

After mixing, the carriers should be covered to prevent desiccation and held during a 'curing' period—usually 1 week for peat. During this period growth of the inoculum can occur; moisture levels equilibrate, and any 'heat of wetting' is dissipated. Heat of wetting results from water additions to particles whose heavily-bound water has been removed by high-energy inputs. There is a positive relationship between temperature of drying and heat of wetting (Roughley and Vincent 1967). If peat is dried at 135°C, temperature rises of 20°C can be measured, but with recommended drying temperatures ( <100°C), the rise is of the order of only 5°C (R.J. Roughley, personal communication). For any carrier held in trays during curing, it is desirable to restrict the depth to 7-8 cm so as to minimize the temperature increase.

Following the 'curing' period, during which some multiplication will generally occur, the inoculant should be passed through a coarse sieve or hammer mill to remove lumps. If the final inoculant is to contain more than one strain, the separate batches of inoculated carrier should be mixed at this stage. Ideally, the finished inoculant should then be packaged, ready for testing and sale. Storage in bulk may only be a practical alternative if suitable storage conditions can be provided (i.e., low temperature without loss of moisture).

Cultures based on nonsterilized peat and subjected to a 'maturation' period of 28 days after packaging have better survival rates on seed than peats 7 days old (Burton 1976). Similar benefits to survival on seed were found with sterilized peat held for 14 days.

## Inoculation of sterile carriers

The decision to use a sterile carrier necessitates the placement of the carrier in its final package and its sterilization before inoculation. Sterilization methods for packaged carriers usually involve either autoclaving or gamma irradiation. The only alternative—-fumigation-requires access of gases to the carrier, long exposure to ensure adequate diffusion, freedom from residues of the fumigant, and measures to prevent recontamination. Fumigation of South African peats with ethylene oxide and methyl bromide (Deschodt and Strijdom 1974) gave poorer subsequent *Rhizobium* survival than autoclaving, although the harmful effect was not due to residual fumigant. Some organisms also survived the fumigation treatment.

Although Roughley and Vincent (1967) claim that the temperature of peat needs to be kept below 100°C during drying, there is no published evidence that prior autoclaving at 121 °C is harmful to the *Rhizobium* subsequently added to these peat products. However, it is particularly desirable that tests for suitability of carrier materials should include confirmation of compatibility with the sterilization procedure, and with additives. Sahni (1977) found that the amount of CaCO3 required by lignite for pH adjustment was dependent on whether or not the material was sterilized.

Gamma irradiation is usually expensive but may well be justified if suitable containers for autoclaving are not readily available. Packaged

inocula (200 g/packet) in India costs Rs.0.50 (US$0.05), plus freight.

Particular care must be taken with suitable packaging materials for sterilization. Polyethylene, though normally used for gamma irradiation, does not withstand autoclaving. In contrast, polypropylene, which can be used for autoclaving, breaks down with gamma irradiation. The polypropylene tested in India is only satisfactory when subjected to autoclave pressures of a maximum of 10 $lb/in^2$ Even at such high pressure, it is essential to expel all air before sealing the package. Alternatively the package can be unsealed, but closed with a clip to allow equilibration during sterilization, and subsequently sealed.

Dosages of gamma irradiation used in Australia are of the order of 5 Mrad. This does not necessarily produce a sterile product but ensures that surviving contaminants are very few in number; such contaminants generally remain in a minority even after storage of the inoculant for 12 months.

Absolute sterilization by irradiation may not be economically feasible; hence, it is impracticable for treatment of enriched, high moisture-content carriers such as those favored in many european systems (van Schreven 1970). The method, typified by van Schreven is to add a small inoculum of rhizobia to a sterilized carrier already containing growth-promoting constituents, and held essentially at its final moisture content, in its final distribution pack. There may be a 100- to 1000-fold increase in the number of rhizobia within the carrier before distribution. Clearly, there is the grave risk of any contaminant multiplying during this period.

Experimental development of inoculants frequently leads to the use of techniques closely following normal laboratory procedures, van Schreven et al. (1954) used a sterile needle inserted between the cotton wool plug and the neck of the glass container of a sterile carrier, and aseptically siphoned a small volume of culture from a flask. It was, therefore not necesary to remove the cotton wool plug, and an important source of contamination was eliminated. Subsequent mixing was achieved by periodical turning of the container, which was only half-filled with carrier.

The alternative technique of preparation, more common in the USA and Australia, is for a large inoculum of well-grown broth (approximating 30-50% of the total final weight) to be added to the dry carrier powder (approx. 10% moisture). Since this method is less dependent on multiplication, it is even suitable for batch mixing with a nonsterile carrier. It is certainly successful for pre-packaged sterile carriers which are inoculated with a needle directly through the wall of the package. Electrically-operated automatic medium dispensers are most suitable for this operation, and the equipment available commercially allows syringes, attached tubes and needles to be removed for sterilization. Such units are also amenable to dispensing mixed, measured quantities of broths from separate containers (e.g., containing different strains). In Australia, for sterile peat prepacked in polyethylene, such equipment is used to inject broth directly through the wall of the polyethylene bag. The surface of the bag is sterilized round the point of injection, and the small hole is immediately covered with a self-adhesive label. The inoculum and carrier are mixed by manipulation immediately after sealing.

# Packaging

## Materials

### Glass

Bottles, traditionally used for agar cultures, can also be used for carrier-based inocula. Glass containers are fragile but have the advantage of being readily autoclavable. Although gas exchange is prevented, screw-caps are the most suitable method of sealing to facilitate handling, van Schreven (1958) used bottles for

inocula made with peat-soil mixtures, stoppered with cotton wool and a cellophane cover to prevent moisture loss. For many years, Czechoslovakian peat-humus cultures were packaged in glass bottles and, in Israel, all inoculants are produced and marketed in bottles.

## Metal

Metal cans, used for many early preparations in the USA (Fred et al. 1932), normally contained soil or peat cultures. Inoculant production in Sweden has continued since 1914 to be based on a metal can of 150 ml capacity, containing sufficient inoculant for 0.5 ha (H. Ljunggren, personal communication). The junction of the cap is covered with a fixed label so that air exchange is minimal. Metal cans are not popular today, although they are autoclavable and more resistant to breakage than bottles.

## Plastic

The development of the plastics industry has changed the packaging of inocula and many other goods. The majority of the world's inoculant production is marketed in plastic pouches.

A choice of plastic material for pouches involves balancing the requirements for gas exchange, moisture retention, etc. with strength and resistance to temperature.

Polyethylene has commonly been used as a packaging material following impregnation of carriers. In Australia this low-density (0.038-0:051 mm thickness) material is used as a sealed container in which peat is $\gamma$-irradiated for sterilization. Polyethylene permits high gas exchange, allowing for CO2 losses and O2 uptake and an aceptable—though not negligible—moisture transmission. Thus, over a 6-month period of storage at 26° C, it was found that the moisture content of the peat declined from 50% to 45%. It is strong enough for normal handling and, though not normally autoclavable, can be heat sealed. Inoculation is effected by injection through the wall and the hole is then covered by an adhesive label.

Polypropylene is also available in India, and at a thickness of .040 mm, it has proved suitable as a sealed container for production of carrier by heating. Minimal stretching and damage occurs if sterilization of a dry carrier is done by autoclaving at 10 lbs/in$^2$ instead of the normal pressure. Sterilization is best conducted on two consecutive days. Care should be taken to prevent rapid loss of pressure after autoclaving. The major problem of an "autoclavable bag" is that the temperature exchange between the bag and the atmosphere is too slow during cooling. Also the higher temperature in the bag can cause swelling and damage to the bag. In ICRISAT experience, the swelling which causes stretching of parts of the bag is most easily minimized by expelling all air from the package before it is sealed. However, the safest procedure is to allow air exchange by folding the top of the packet with a clip or ventilating it with a removable tube so that the packet is then sealed after cooling in the autoclave.

For steam sterilization, polyethylene of a higher density (higher melting point) can be used; alternatively, lighter polyethylene or polypropylene can be strengthened by lamination with polyester or nylon. However, while the latter materials are stronger, they provide poor gas exchange. Foil laminates with polyethylene are, of course, completely moistureproof.

Polyethylene is unaffected by $\gamma$-irradiation, but polypropylene breaks down and therefore cannot be used for this purpose.

Commercially-available plastic bags are normally sealed on at least one side—at the base. But this seal is often too narrow and close to the edge of the bag to withstand the pressure associated with autoclaving. It may be necessary to reseal the edges to provide a wider band of sealing (approx. 1.5 mm). It is desirable to insert this seal a few millimeters from the edge. Hence, it may be best to purchase the plastic as tubing, so that only the base need be sealed before filling, and length can be varied. Since hand-operated sealers are not designed for continuous use as in a commercial operation, the more robust foot-operated models are preferable.

The plastic packs available around the world commonly contain 200-250 g of inoculant that may be used to treat up to 100 kg of soybeans or 50 kg of lucerne.

## Effects on survival of rhizobia

Packaging techniques reflect a range of influences: the form of the product and the method of its production; the need for resistance of the package to sterilization or distribution stresses; the availability of suitable containers; and the overall scale of the inoculant production process. However, all these techniques must finally be judged in terms of the survival of the rhizobia in the prepared inoculant—in this regard the available data are frequently not in agreement, and compromises may be necessary until a material is finally tested and selected.

### Moisture

With any carrier-based inoculum there is an optimum moisture content which ideally should be maintained for the life of the culture. If aeration is not considered necessary (see below), it is practicable to seal any container and prevent moisture loss. All of the early commercially-prepared inocula in the USA (Fred et al. 1932) were packaged in either tin cans with lids or screw-caps, or bottles with stoppers or screw-caps.

The change to pliable bags—normally polyethylene—is a relatively recent development, reflecting the need for ease of handling, and resulting from the availability of a wide range of synthetic materials. The result has frequently been loss of moisture. There is considerable evidence to show how this moisture loss adversely affects survival. For example, Vincent (1958) found that a moisture loss of 24% per week at 5°C gave a weekly logarithmic death rate of 0.085, which was reduced to 0.001 when the moisture loss was 0.7% per week. There can also be an interaction between rhizobia and contaminants at particular moisture levels; in Australian nonsterilized peats the optimum moisture content range was found to be 40-50% (pF 4.15-3.12), while in sterile peat the optimum was 40-60% (pF 4.15-2.69) (Roughley 1968).

Moisture loss may also be confounded with increased concentration of harmful soluble salts (Steinborn and Roughley 1974).

### Aeration

The conflicting data on the need for aeration seem in part to be a reflection of the wide range of materials used in the studies (van Schreven et al. 1954; Hedlin and Newton 1948; Roughley 1968). While Roughley's data support the need for aeration with Australian peats, and have greatly influenced the choice of package material, they illustrate the need for comparative studies of package types with any carrier. There are certainly no generally-accepted principles regarding aeration.

However, with sterilized carriers it is essential to ensure that any aeration is achieved without contamination. Thus the use of pin holes to simplify gas exchange in polyethylene is not recommended, and it is also important to take care when doing any additional packaging involving stapling, that could damage the bag. Iswaran's (1971) results clearly show reduced survival because of packet perforation.

### Temperature

With peats after maturation at 26-30°C, low-temperature (4°C) storage is generally more favorable for survival than higher temperatures, including those at which growth would normally occur e.g., 26°C. Temperature effects are frequently confounded with effects of moisture loss. By preventing moisture loss, Roughley (1968) found that there was little or no death during 26 weeks with sterile Australian peats stored at 4° and 26° C. More recent studies, with normal moisture loss, have been less predictable. While the survival of fast-growing strains of

*Rhizobium* was favored by storage at 4°C, with little decline over 12 months, the numbers at 26°C commonly declined, although generally within acceptable limits. Conversely, the survival of slow-growing strains was superior at 26°C with marked reduction occurring at 4°C.

Iswaran's (1971) data, however, make the point that, where cool storage reduced survival of *R. leguminosarum* in peat culture, exposure to 26° C for one week appeared to have resulted in recovery of numbers (indirectly measured by plant nodulation response).

A more worrying aspect of storage temperature is illustrated by Wilson and Trang (1980). Not surprisingly, they found that storage at 45°C and 55°C reduced populations in peat inoculants in the U.S.A. However, they found a much greater decline in numbers of infective rhizobia compared with numbers measured by plate count. This was attributed to a decline in cell vigor, preventing nodule development. The effect of such temperatures is particularly relevant to India where pre-wet season distribution of inoculants must be made when ambient air temperatures are commonly above 40°C.

## Labelling

Ideally, each separate packet of inoculant should contain the following information:

1. legume hosts for which the contents are suitable;
2. quantities of seed to be treated;
3. expiry date;
4. instructions for storage and use;
5. any certification by a controlling body;
6. batch number of inoculant; and
7. extent of manufacturer's legal responsibility.

The flat plastic package used for impregnated peat lends itself readily to provision of all this information. The polyethylene package is however, often enclosed in a further polyethylene, cardboard, or even foil package. Under these circumstances, instructions and other information may be provided on a separate sheet packed within the cover.

## Distribution

The essential requirements for distribution are that the inoculant should not be subjected to excessive temperatures; ideally it should be held at 4°C throughout transport and storage. Although this may not generally be practicable during transport, precautions should be taken to minimize exposure by using rapid transport, preferably at night. Inocula in bulk should be transported in strong cartons or boxes. Indian distributors and users are forced to face the fact that distribution for rainy-season sowings is during the hottest part of the year, and inadequate protection from direct sunlight may negate all the advances possible in the improvement of carriers.

To discourage sale of inferior products, manufacturers may elect to replace stocks unsold at expiry date with current material or to refund cost.

# Inoculant Quality Control

## Organization

The quality of any product is the responsibility of the manufacturer. His product may, in addition, be subject to official regulations. The powers provided under legislation may be periodically, or only occasionally, invoked by government agencies, and the degree of inspection, evaluation, or penalty can vary widely. Many biological products, particularly food and drug items, may be subject to particularly stringent external controls of this nature. If failure to pass regulatory requirements involves

loss of the product, or restrictions on its use or distribution, it is clearly essential for the producer to adopt rigid quality control. In the absence of any official regulation, the manufacturer may adopt very low standards, that can be readily attained and require minimal implementation.

The powers of external control bodies vary widely between countries. The various control measures in the USA since the 1930s served to protect the farmer from worthless products. But they were not designed to be—nor did they function as—real measures of inoculant quality (Burton 1967). The Indiana State Chemist (Schall et al. 1975), the only authority publishing data on inoculant quality in the USA today, presents results of tests only in qualitative terms. However, in specifying brand names and manufacturing companies, they are more explicit than any other testing authority in the world.

India (Anon. 1977) has set standards of both production and quality testing. Attainment of the standards allows the subsequent use of the ISI Certification Mark. The manufacturer is free to choose his own strains of rhizobia.

Probably the most centralized quality control system is that of the Australian Inoculants Research and Control Service (AIRCS)—previously the University-Department of Agriculture Laboratory Service (U-DALS). It has no official regulatory powers. The history of the development of AIRCS is worth noting in relation to the possible development of industries producing inoculants in other countries. In 1954, government laboratories withdrew from commercial inoculant production in favor of private companies. The absence of suitable, naturally-occurring rhizobia from large areas being sown with legume-based pastures highlighted the many failures (Waters 1954), and Vincent (1954) initiated positive moves to secure improved quality. It is significant that, initially, Vincent advocated setting up a licensing authority, and the evolution of quantitative and qualitative standards which, he stated, 'should be the definite responsibility of the manufacturer'. When poor-quality inoculants continued to appear, manufacturers and interested scientists met informally and resolved to establish mutually-acceptable standards of quality, and to ensure that only mother cultures from a central collection were used. Manufacturers provided financial support to the control laboratory, for testing the final products. However, the quality of the final products continued to be poor. The same group of people met once again. As an outcome of this meeting, a system of progressive control was introduced in the mid 1950s—a system providing for testing of initial broth, freshly manufactured inoculant, and the product on sale. This marked the beginning of an effective control program in Australia (Date 1969). The principles of the organization are still retained, the standards have been progressively raised, and the procedures modified as, and when, the need has arisen.

The functions of U-DALS expressed by Date (1969) remain the basic functions of AIRCS today:

1. selection, testing, and maintenance of suitable rhizobial strains;
2. control of the quality of legume inoculants; and
3. advice to, and research for, manufacturers, distributors, and users of inoculants on the problems of production, handling, and application that affect the quality and efficiency of inoculant cultures.

The original U-DALS organization was partly funded by the manufacturers. A large proportion of the funds was raised by the University of Sydney. In 1971, AIRCS was formed with major financial assistance from the governments of all states of Australia. Contributions from a state are calculated on the basis of the proportion of the total inoculant used in that state. For a long period, financial support by manufacturers was discontinued, although they were still charged for particular services outside the normal framework of local inoculant production. The manufacturers have once again been called upon for support in recent years. However,

compliance with the standards set by AIRCS is still undertaken voluntarily by manufacturers.

In 1978, AIRCS was also required by its funding authority to examine standards for preinoculated seed, i.e., seed inoculants before sale (Thompson 1980).

## Facilities required

Suitably qualifed personnel are essential. Ideally, the laboratory should be in the charge of a professional microbiologist with at least one technically trained assistant. Premises need to cater for contaminant-free handling of cultures. If a suitable room is not available, it may be desirable to provide an suitable aseptic cabinet (e.g., a laminar flow cabinet). Normal aseptic procedures in a clean, closed room should be adequate for control procedures, provided air conditioning is not necessary.

Preparation of antisera requires suitable facilities for housing of small animals (preferably rabbits). The requirements for facilities and correct handling procedures are outlined by Kingham (1971). In certain countries the performance of many of the necessary procedures requires the possession of a valid animal licence.

Controlled-environment facilities are essential to grow plants for routine tests of infective ability and nitrogen fixation. These commonly need to provide conditions to suit both temperate (15°C night, 20°C day) and tropical or subtropical species (20-25°C night, 25-30°C day) although strict adherence to a temperature regime is less important than the avoidance of excessively high temperatures. Glasshouses or shade houses are suitable for growth of plants in pots or similar open units, but the particular specialized assemblies used for testing of infectiveness or effectiveness of *Rhizobium* subspecies (e.g., closed test tubes or the modified Leonard jar) may require special precautions. Temperatures within enclosed test tubes in a glasshouse can readily reach 35°C in an ambient temperature of 25°C. Shading the outside of the glasshouse with louvres (Hely 1959) or blinds (Norris and Date 1976) can allow good control in hot environments. Even a metal frame covered with a shading cloth protecting a small area within a glasshouse can effectively prevent heating of tubes above the ambient glasshouse temperature. Alternatively, tubes may also be housed in controlled-temperature water baths. Controlled-environment rooms should provide adequate light, air circulation, and temperature control, and the day-length should be controlled by time switches. Ballasts for fluorescent lights are sources of heat which must be housed outside the room. Warm-white or cool-white fluorescent tubes have a limited light spectrum, and this needs to be supplemented at the red end of the spectrum by either incandescent bulbs or Grolux® fluorescent tubes. The latter are not readily available in many countries and, even with the full expected life, replacements are frequent and expensive. Controlled environment cabinets have built-in refrigeration and are expensive in terms of cost of unit space compared with controlled-environment rooms. In countries with an unreliable electricity supply, a commitment to such equipment must include a standby generator.

It is useful to have field-testing facilities nearby, but any full-scale program will require access to a number of sites in different environments. The special requirements of strain evaluation and the need to avoid cross-contamination between treatments, render it essential for any test area to be under the full control of the testing body.

Equipment requirements are not elaborate, but an autoclave and dry sterilizer are essential, and a refrigerator is highly desirable.

A shaker or blender is necessary for suspension of inoculant or inoculated seeds in water for quantitative evaluation. While wrist-action shakers are commonly used, it has been found that the Stomacher Lab-Blender (available from A.J. Seward, Bury St. Edmunds, Suffolk, UK) is faster (15-20 sec per sample) and provides a superior suspension. The essential feature of the Stomacher is a pair of flat, vertical paddles alternately moving horizontally

towards a vertical surface on which is held a closed plastic bag containing the diluent and suspended material. The paddles approach within about 3 mm of the vertical surface, but the mixing is caused toy the pounding action of the paddles, producing a sponging and shearing action on the sample. When rhizobia are being suspended from seed, the chances of puncturing the bag are reduced by mounting it between thin layers of foam plastic.

A microscope is necessary for examination of stained cells and should preferably be fitted with phase-contrast equipment for total counts and a bacterial counting chamber (e.g., Petroff-Hausser).

For serological (agglutination) tests, suitable racks and a controlled-temperature water bath are necessary.

A freeze-drier provides the most satisfactory method for long-term storage of *Rhizobium* strains, and an ampoule tester and ampoule constrictor are valuable extras (all available from Edwards High Vacuum, Crawley, Sussex, UK).

# Methods

The outline given here is restricted to the particular requirements of inoculant quality tests. For more details refer to Vincent (1970) and Brockwell (1980).

## Counting of rhizobia

### Total cell count

A Petroff-Hausser or similar counting chamber is used because its shallow depth (0.002 cm) requires less microscope adjustment than a haemocytometer slide of depth 0.01 cm. A microscope with phase-contrast illumination and a 20-40x objective is necessary.

The slide is divided into clearly marked squares of measured area so that conversion factors, provided with the slide, allow calculation of numbers per unit volume. Populations up to $10^8$/ml can be counted without dilution and the lower limit for a reliable estimation is about $10^7$/ml. The estimate includes all cells, whether dead or alive, commonly doubling the plate count estimate.

### Plate count

The plate count is used for estimating numbers of rhizobia in broth cultures and inocula prepared from sterilized carriers. Dispersed suspensions are diluted serially in 10-fold steps to a level where 30-300 cells are expected to be in the sample aliquot. The materials required are:

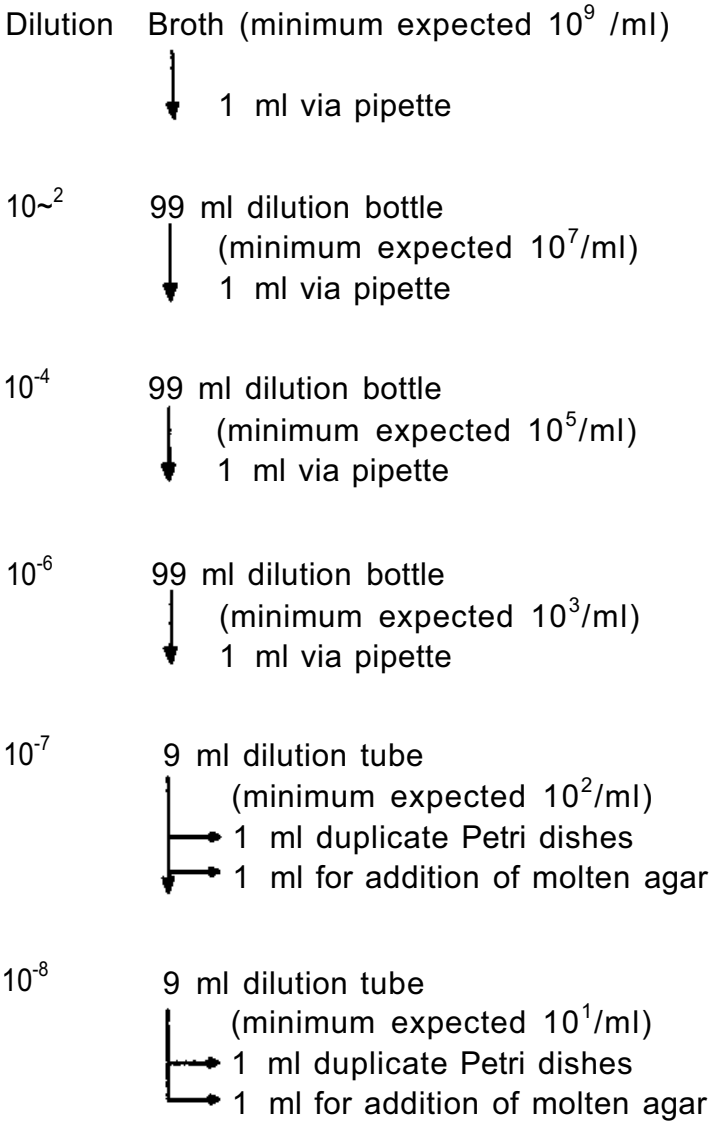
1. *Diluent.* The proposed water supply should be checked for suitability as a diluent. Protective salts may be necessary additions for good survival of rhizobia, or demineralization may be necessary. Sterile diluent is prepared in bottles (capacity 99 ml) or in stoppered, capped, or cotton wool-plugged tubes (capacity 9 ml).

2. *Pipettes.* Straight-sided 'blow-out' pipettes of 1 ml capacity are best, although a volume of 2 ml may be useful where additional 1 ml aliquots are necessary. A fresh pipette is used for each dilution step; 1 ml capacity rubber bulbs may be used to avoid sucking by mouth.

3. *Yeast-mannitol agar medium and Petri dishes (9 cm diameter).* Yeast-extract-mannitol medium is usually used, but $CaCO^3$ is omitted to avoid clouding the plates. Congo red (10 ml of a 0.25% aqueous solution per liter of medium) may be included to indicate possible contaminants. Rhizobia absorb less dye than most other bacteria, ɣ-irradiated carriers are not necessarily absolutely sterile as some organisms can withstand irradiation. It was also found at AIRCS that some of these resistant organisms are Congo red sensitive (Pulsford and Thompson, unpublished) so that they are only evident when Congo red is excluded from the medium. For poured plates, the medium is held at 50°C

in a water bath until 10-15 ml is added to the plate and mixed with a 1.0-ml aliquot of diluted sample. Alternatively, 0.2 ml of aliquot may be spread evenly over the surface of a pre-poured plate with a bent glass rod.

In quality control work, it is necessary only to prepare a dilution series for counting at predetermined levels. An example of the procedure to examine, with poured plates, a broth expected to contain at least $10^9$ rhizobia/ml is shown below. A fresh pipette is used for each transfer, avoiding contact between the pipette and the contents of the next dilution tube. Six pipettes would be used in the example. The contents of bottles and tubes are mixed by shaking or by sucking and expelling the suspension with the pipette. Duplicate plates are prepared for each dilution cultured.




The estimate of numbers from such a series is derived from a mean of the numbers of colonies on duplicate plates on which 30-300 colonies develop. Use of spread plates, which receive only 0.1-0.2 ml aliquots, reduces the series by one 10-fold dilution, as the minimum expected number at dilution $10^{-6}$ is 100-200.

## Plant infection count

There are no reliable culture tests for identification of legume root nodule bacteria. Thus, the plant infection dilution count must be used to estimate their numbers where other organisms are present in a suspension or culture. The technique is described by Brockwell (1980).

Under the particular conditions of quality control, where it is necessary only to determine whether a population reaches a certain minimum number, tests at four selected levels of a 10-fold dilution series are adequate, and five levels will provide a precise estimate considerably above the minimum. Thus, the table need only cover four levels and adjustment can be made for the primary dilution. Table 2 has been prepared on the basis of three plants per dilution, to illustrate the minor differences between two popular sets of tables, but alternative numbers can be readily calculated from Fisher and Yates (1963).

If the standard required of a peat is $10^9$/g and each plant receives a 1-ml aliquot, plants should be tested at dilutions $10^{-7}$, $10^{-8}$, $10^{-9}$, and $10^{-10}$. Thus in the following example:

| Dilutions tested | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ |
|---|---|---|---|---|
| No. of positive tubes | 3 | 3 | 3 | 1 |

the estimate is 424 x the primary dilution, in this case 10: i.e., 424 x $10^7$ = 4.24 x $10^9$. If all plants were nodulated, the peat would contain at least 1.726 (or 2.3) x $10^{10}$. Use of 0.2-ml aliquots

**Table 2. Estimates of *Rhizobium* numbers obtained by two methods of calculation from 10-fold serial dilutions with three tubes at each level.**

| No. of positive tubes | | | | Estimates of no. in aliquot of lowest dilution | | |
|---|---|---|---|---|---|---|
| Relative dilution | | | | MPN estimate from Brockwell et al. (1975) | Total no. of positives | Estimate from Fisher and Yates (1963) |
| 10 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | | | |
| 3 | 3 | 3 | 3 | ≥ 2300 | 12 | ≥ 1726 |
| 3 | 3 | 3 | 2 | 919 | 11 | 861 |
| 3 | 3 | 3 | 1 | 424 | 10 | 424 |
| 3 | 3 | 3 | 0 | 230 | 9 | 180 |
| 3 | 3 | 2 | 1 | 147 | 9 | 180 |
| 3 | 3 | 2 | 0 | 91.8 | 8 | 88 |
| 3 | 3 | 1 | 0 | 42.4 | 7 | 38 |
| 3 | 3 | 0 | 0 | 23.0 | 6 | 17 |
| 3 | 2 | 1 | 0 | 14.7 | 6 | 17 |
| 3 | 2 | 0 | 0 | 9.2 | 5 | 8.6 |
| 3 | 1 | 0 | 0 | 4.2 | 4 | 3.8 |
| 3 | 0 | 0 | 0 | 2.3 | 3 | 1.7 |
| 2 | 1 | 0 | 0 | 1.5 | 3 | 1.7 |
| 2 | 0 | 0 | 0 | 0.9 | 2 | 0.9 |
| 1 | 0 | 0 | 0 | 0.4 | 1 | 0.4 |
| Approximate range factor for 95% fiducial limits (x,÷) | | | | 4.1 | | 4.8 |

would make it preferable to test at lower dilution. In this example:

| Dilutions tested | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ |
|---|---|---|---|---|
| No. of positive tubes | 3 | 3 | 3 | 1 |

the estimate is 424 x $10^6$ x 5 (because only 0.2 ml used) = 2.12 x $10^9$ml.

The materials required are the same as for the plate count except for growing plants aseptically. For estimating numbers of rhizobia, it is essential to use a test host on which nodules can be formed by the *Rhizobium* of interest; it is not necessary that they fix nitrogen. It is desirable to use as small a seed as possible to minimize the need for growing plants in large assemblies. The following is the list of useful test hosts commonly employed (exceptions are tested only on the correct host):

1. *Rhizobium trifolii* - *Trifolium repens* (white clover) (exception T. *semipilosum).*
2. *Rhizobium meliloti* - *Medicago sativa* (lucerne) N.B. This is a particularly complex group (Brockwell and Hely 1966) so that exceptions are likely e.g., *M. rugosa.*

3. *Rhizobium leguminosarum* - *Vicia dasycarpa* (woolly pod vetch) or other small-seeded *Vicia* spp.
4. *Rhizobium lupini* - *Ornithopus sativus* (serradella).
5. *Rhizobium* spp (cowpea group) - *Macroptilium atropurpureus* (siratro).
6. *Rhizobium japonicum* (soybean group) - *Glycine ussuriensis* (wild soybean).

Van Rensburg and Strijdom (1974) found that siratro was adequate as a test host for the presence of *Rhizobium lupini, R. japonicum,* and *R. phaseoli* but the results with the latter two have been inconsistent in Australian tests (Vincent 1970).

*Cicer* (chickpea) rhizobia are diluted using chickpea plants grown from seeds with excised cotyledons and grown in coarse sand in tubes (O.P. Rupela, personal communication).

## Serological identification

Although a wide variety of methods are available, the simplest for control purposes is somatic agglutination.

### Materials

1. *Antiserum* is commonly stored in volumes of a few millilitres and held frozen without additives. The stock is normally diluted to 1:100 or 1:200 although the titre of rabbit antiserum is commonly at least 1:1600.
2. *Antigen* suspension must be cloudy (i.e., at least $10^7$ /ml) so that a positive reaction will be clearly visible.
3. *Saline:* 0.85% NaCl.
4. *Agglutination* tubes, capacity 1 ml. The Dreyer pattern is to be preferred, although Durham tubes are satisfactory alternatives.
5. *Water bath,* at 52° or 37° C.

### Procedure

1. Mix equal parts of antigen suspension and saline in a capped test tube and hold in boiling water bath for 30 min to inactivate flagellar reaction.
2. Using a Pasteur pipette, mix 18 drops of boiled antigen suspension with 2 drops of antiserum in one tube, and 18 drops of boiled antigen with 2 drops of saline in a second tube (control).
3. Place in a rack in the water bath with the water level below the level of the reactants to promote mixing by convection.
4. The somatic agglutination should be visible after 4 h at 52° C, or overnight at 37° C. The reaction commences with a granular appearance which should proceed to full settling out. Auto-agglutination of the saline control necessitates repetition of the test, and possibly reduction of the concentration of saline to 0.5%.

## Testing for contaminants

### Gram stain

This is the standard procedure for testing agar and broth cultures for presence of Gram + contaminants, spore formers, and cells with distinctly different morphology (the procedure is outlined by Vincent 1970). In broth cultures, allowances may need to be made for a few dead Gram + cells carried over from the autoclaved medium components; and some yeast cells are commonly visible in media based on yeast-water.

### Glucose-peptone test

Glucose-peptone does not favor growth of most rhizobia, but many contaminants readily grow and produce pH changes. The medium consists of glucose 5 g, peptone 10 g, agar 15 g, water 1 l. Bromocresol purple (1.0% in ethanol)

(10 ml) is added to the melted agar before dispensing into 28-ml McCartney bottles or 15 x 150 mm test tubes for sterilization and sloping. A loopful of culture streaked on a slope and incubated at 28-30° C should be examined after one and two days. Marked growth, especially if associated with change of pH, indicates gross contamination. Some strains of rhizobia show slight growth but generally without appreciable change in pH.

## Strains of *Rhizobium* for inoculants

### Single strain or multistrain?

Inoculants are generally used for more than one cultivar of a legume species (e.g., soybeans), or for more than one legume species (e.g., clovers), and even for a number of legume genera covering a number of legume families or subfamilies (e.g., cowpea cross-inoculation group). However, mixing species of *Rhizobium* (e.g., *R. meliloti* and *R. trifolii),* which do not normally crossinfect is not favored, largely because it reduces the number of organisms available to each host. It is essential that an inoculant should never contain a strain which will form ineffective nodules with any of the hosts for which it is recommended.

The advantages of single strain inoculants are:

1. that one doesn't face the problem of differential cell multiplication, which can result in dominance by one strain (Marshall 1956). Strains within a *Rhizobium* species can differ in ability to survive in peat. Use of mixtures of *Rhizobium* species is even more likely to lead to differential death rates because of differing resistance to adverse conditions (e.g., salt concentrations: Steinborn and Roughley 1974);
2. that any change in host requirements can be catered for by developing a separate inoculant which can be clearly and specifically labelled;
3. that any unfavorable variation showing up in a strain (e.g., loss of effectiveness or infectiveness) is not masked by other strains in the inoculum; and is readily evident if the host is grown in a low nitrogen environment with adequate controls.
4. that it is much simpler to check the identity of the single strain if quality control is applied only to the final product.

The disadvantages are:

1. that there is the need for a larger range of inoculant types. This can lead to organizational problems at both manufacturing and retailing levels; and
2. that loss of effectiveness or infectiveness will result in complete failure of inoculation, while multistrain inoculants will provide an infective alternative.

In view of our relative ignorance of factors governing rhizosphere colonization, infective processes and selection by the host plant, the proponents of mixed-strain inoculants can justifiably claim that the inevitable host x strain x site interactions will be best catered for by a mixture of effective strains, of which at least one may reasonably be expected to form an association. However, even if all added strains have survived (Marshall 1956) the published evidence is ambivalent on the ability of a host to select the most effective strain (Vincent and Waters 1953; Robinson 1969), and the competitive ability of strains in soil is even less understood. Thus, recently, separate research groups (Gibson et al. 1976; Roughley et al. 1976) found that *Rhizobium trifolii* strain WU95 was consistently competitive with other strains for nodulation of subterranean clover, but the other strains were ranked differently by the two groups of workers.

It is perhaps significant that the greatest emphasis on single-strain inoculants has been in Australia, which has thorough quality control,

thus reducing the risk of undetected strain failure.

## Sources of strains

A collection of strains is normally built up with a combination of field isolates and accessions from collections of other workers. Some of the principles and procedures have been discussed by Date (1976) and Norris and Date (1976).

### Other collections

Many strains in use for culture of legume inocula have originally been obtained directly from collections of other workers. The possible sources have been discussed by Dalton (1980) and Brockwell (1980). It is important that such accessions should retain their original collection number, even though a local renumbering system is normally necessary for storage. There is clearly an ethical requirement on the part of the recipient to advise the custodian of a collection if he proposes to use a strain for commercial inoculant production.

### Field isolates

Initiation of a program to produce legume inoculants commonly arises from the need to successfully inoculate species introduced into a new environment. If this need has been demonstrated by poor nodulation of test plants, it is unlikely that the natural population of rhizobia will yield a suitable isolate. However, good nodulation of isolated noninoculated plant hosts may indicate the presence of a small population of rhizobia that may be potentially useful as an inoculant. Guidelines have been set out by Brockwell (1980).

## Criteria for selection of inoculant strains

The number of criteria to be considered in the selection of strains for legume inocula has steadily increased with improved understanding of the legume symbiosis and of the ecology of *Rhizobium.* The most obvious criteria are:

1. the ability to nodulate the legumes for which the strain is recommended;
2. the effectiveness in nitrogen fixation in the nodules so formed;
3. the suitability for inoculant production; and
4. the usefulness under field conditions.

The first two criteria may be tested under controlled conditions; certain environmental factors—especially temperature—can modify the symbiotic response. There is little point in pursuing tests of strains which are poor performers over the normal temperature range in (1) and (2), when alternative good performers are available.

At the manufacturing level, it is sometimes found that strains differ in their reaction to normal growing procedures, and use of alternatives may be justified to avoid changing these procedures.

Usefulness under field conditions embraces a wide range of attributes (Brockwell 1980). These may not only be difficult to test for technical reasons such as availability of adequate test criteria, but also for logistical reasons, particularly as inoculants are us.ed over a wide range of environments. Thus, the essential test is to ensure that the strain can form nodules and fix nitrogen in normal field situations at least as well as alternative strains. It is particularly important that such ability is demonstrated with the full range of proposed hosts.

## Evaluation under controlled conditions

This involves use of a *Rhizobium-free* medium in a container sufficiently large to allow good differentiation of growth of nodulated and non-nodulated plants. Although the use of a nitrogen-free nutrient medium is common,

there is evidence that a more realistic evaluation of strain performance will be obtained in the presence of a small quantity of added nitrogen (Gibson 1976). Such conditions are more akin to the normal field situation so that the choice of strains favored by the presence of nitrogen should provide more generally useful inoculant strains. Optimal conditions for plant growth should be chosen. For nitrogen controls, combined nitrogen should be applied immediately after nodules are formed on the inoculated treatments.

Because of the need to prevent cross-contamination, the most commonly used assemblies have been sterilized, often enclosed, containers e.g., tubes or modified Leonard jars.

## Evaluation under field conditions

These tests take much more time, labor, and space because of soil variation within sites, the need to sow treatments sufficiently well-spaced to minimize cross-contamination, and the possible presence of soil nitrogen which may result in some growth of noninoculated controls, so that differentiation between treatments is delayed. The reduced differentiation between treatments can also result from the presence of naturally-occurring rhizobia and, in fact, the uninoculated controls may be as effectively nodulated as the treatments. In this case it may be necessary to carry out serological identification of nodules to determine whether the inoculant strain has formed the nodules. Inoculation rates should be normal, and nitrogen-free fertilizers added to ensure adequate nitrogen fixation and plant growth.

### Strains for grain legumes

The common cessation of fixation in grain legumes prior to pod-fill may result in interactions during the period between pod-fill and harvest e.g., plants well provided with nitrogen by an efficient strain may suffer more from a post-flowering moisture stress than smaller plants with less nitrogen. Further, such stress may be evident in grain quality which is not readily measured in simple terms. If labor and facilities permit, measurements of yield of dry matter at flowering and in grain are desirable, but, in the latter case, it is important to also measure fallen leaf.

With clovers sown in rows and swards, Thompson et al. (1974) found that the only reliable guide to the proportion of nodulated plants was obtained by using spaced plants with no possibility of plant-to-plant cross-infection. Further, yield was best measured on a unit area or row length basis rather than on individual plants.

## Maintenance of stock cultures

A stock culture collection is an essential part of legume inoculant control, whether for one small manufacturer or a number of users. While it is expensive, time-consuming and demands of careful manipulations and good records, it is, nevertheless, the basis of the whole operation.

The essential features of a good collection for servicing inoculant quality control are that it provides:

1. strains of proven ability for the legumes of interest;
2. 'back-up' strains also of proven ability; and
3. strains of current, anticipated, and potential usefulness.

### Agar cultures

The most convenient form of culture for storage is the slope of yeast-mannitol agar in a cotton wool-plugged test tube. However, cultures so stored are most likely to be genetically unstable (Vincent 1970). On this medium, organisms survive for several months, and even up to two years at low temperatures. The biggest problem is moisture loss. This can be reduced by using screw-capped tubes or McCartney bottles; or by covering the cotton wool plug with

quality testing service, even when inocula are controlled during production. The major problem is to obtain a representative sampling because of the large number of outlets. Testing at the retail outlet is commonly the first point of examination and can lead to more thorough initial testing if defective inoculants are detected.

## Broth culture stage

### Sampling

Broth samples, (10 ml is sufficient) should be drawn aseptically from the fermentor at the time maximum numbers of live cells are expected. Separate samples must be collected for each strain and forwarded as rapidly as possible to the control laboratory in screw-capped bottles protected by insulation and packed with ice, but not frozen. The samples should be examined immediately on receipt.

## Priority tests

1. Serological identity is tested by agglutination as above.
2. The Gram strain should be made on undiluted broth.
3. Glucose-peptone agar is also streaked with undiluted broth.
4. The total count of rhizobia is obtained with the Petroff-Hausser chamber, but gross contamination with morphologically distinct organisms may also be observed. Commonly, at least half the cells counted are dead at this stage of the broth development.

   The above tests can provide presumptive evidence of a pass or failure within 24 hours, so that a decision can be made on the use of the broth for the next stage of manufacture.

   The following tests are also initiated on receipt. (Final results of slow-growers may need up to 10 days.)
5. For detecting presence of contaminants, a sample of undiluted broth is placed on yeast-mannitol agar. This can reveal contaminants which have not been detected in the above tests.
6. A viable count of rhizobia is obtained by a normal plate count.

## Optional tests

1. Measurement of pH on receipt is used primarily as a guide to possible contamination, depending on the *Rhizobium* species. Commonly, *R. meliloti* strains produce a pH of 5.4 but cultures of strains for *Lotononis* can exceed pH 8.
2. The plant dilution count can be combined with the normal plate count. A delay of 3-4 weeks will be necessary before reading results, but a plant test made at this stage replaces the plant count which would otherwise be necessary on the final product.

## Inoculant at manufacture

### Sampling

Samples should be taken from each batch and forwarded to the control laboratory as soon as preparation, or maturation, is complete. Temperature control during transport is probably less critical than for broths, because the final product is probably more stable numerically, but refrigeration is preferred and certainly high temperatures must be avoided. In Australia, six packets are collected per batch of inoculant, but the variation between packets is small. Five of the six must reach the standard. Where greater variation exists, heavier sampling intensity may be necessary.

### Priority tests following full broth tests

1. For the viable count with sterilized carrier, the normal plate count is adequate and provides information on the presence of contaminants. Autoclaved carriers should be absolutely free of contaminants, but gamma irradiation may not provide full sterilization. The requirement is therefore that there are no contaminating organisms at the lowest dilution examined (normally $10^{-6}$ in the AIRCS Laboratory). With the nonsterilized carrier the plant dilution count is essential for estimates of *Rhizobium* population, although the plate count may provide useful information on the relative number of contaminants.
2. The serological identity of the rhizobia is tested on cells obtained by suspension of colonies from the plate count. When more than one strain is used, it will be necessary to grow test material from colony picks. This is simplified if colony characteristics allow clear selection.

### Priority tests without prior broth test: sterilized carrier

1. For the viable count, the normal plate count is all that is necessary, as presence of contaminants at the lowest dilution ($10^{-6}$) should result in rejection.
2. At least one packet should be subjected to a complete plant dilution count procedure as confirmation that an effective symbiosis is produced by the majority of the rhizobia. Provided some plants nodulate effectively, an inoculant should not be failed on the basis of one such test, but nodulation failure at higher dilutions alerts the operator to possible problems requiring investigation.
3. Determination of serological identity is relevant only if the testing authority provides the strains.
4. The Gram stain is made on a mass streak of colonies from a low dilution of the viable count.

### Priority tests without prior broth test: nonsterilized carrier

1. For a viable count, the plant dilution test is essential.
2. No separate test of infectivity is necessary because information is obtained from the viable count.
3. Serological identity is best tested from nodules obtained in the above count, but is only relevant if the testing authority provides the strains.

## Inoculant from retail outlets

### Priority tests on previously tested inoculants

1. A check on labelling should show that batch number, expiry date, and hosts must agree with previous records.
2. The viable count is made by plate count or plant count, depending on the sterility of the carrier.
3. Serological identity is especially important with sterile carriers where a plant count is not necessary for estimation. The serological check, therefore, becomes the only proof of strain suitability.

### Optional tests on previously tested inoculants

1. The moisture content is useful for record purposes.
2. The plant test is the only definitive test for nodulating ability.

### Priority tests on previously untested inoculants

If the testing authority has had no jurisdiction over the strains used by the manufacturer, there is no point in attempting to identify the strains available in the inoculum, even if a sterilized carrier is used.

1. For the viable count and test of infectivity, the plant infection dilution count is essential, although a plate count is a useful indicator of sterility.

2. An effectiveness test should be carried out, but it may be combined with the plant count and the effectiveness of the association measured by growth of the host, provided the assemblies allow differentiation of treatments from the noninoculated controls.

# Standards

## Broth culture stage

Irrespective of the carrier and the number of strains in the inoculant, any broth or surface-grown culture of rhizobia must be a single strain and be free of contaminating organisms. It should provide as high a population as possible. The Australian standard has been 5 x $10^8$/ml for some years for all except *Lotononis* (3 x $10^8$/ml).

## Inoculant at manufacture

### Nonsterilized carrier

The standards set for impregnated carrier arise largely from the levels which can be achieved by competent manufacturers. There is little point in setting unattainable standards, but low numbers of rhizobia may be offset in part by modifying the total quantity of seed or area to be inoculated. Thus, a working minimum of 100 rhizobia per seed of small legume was adopted early in the history of Australian inoculant control, and the rate of application of the inoculant to the seed was chosen accordingly. While nonsterilized peat was used in Australian inoculants, a standard of $10^7$ -$10^8$ rhizobia/g was considered adequate for 2 months' expiry and greater than $10^8$/g was allowed 6 months' expiry. South Africa also sets a standard of $10^8$/g (van Rensburg and Strijdom, 1974). The standard set by ISI in India requires $10^8$/g at manufacture and $10^7$ at expiry.

### Sterilized carrier

The introduction of sterilized peat in Australia allowed the standard for all inoculants (except *Lotononis,* 5 x $10^8$/g) to be raised to $10^9$/g. Such peats are also required to be free of contaminants at the lowest dilution tested (commonly $10^{-6}$). Standards set in New Zealand (Anon. 1979c) and Canada (Anon. 1979a) are similar.

## Inoculant from retail outlets

The minimum standard for inoculants on sale will ultimately be based on measured survival and will obviously be dependent on time to expiry. For some inoculants stored in the frozen state, or perhaps freeze-dried preparations, it is possible that minimal decline may occur. The carrier-based inocula in Australia have generally been allowed a 10-fold drop before expiry. Thus, on present standards, the fresh peat requirements of $10^9$/g allows for a count at expiry of $10^8$/g.

## Expiry periods

A balance must be struck between reasonable storage life, which can be ascertained only by measurement of survival, and commercial requirements for distribution and availability. A minimum period for the latter is probably 2 months if transport is adequate, but there seems to be little justification for a maximum exceeding 12 months.

In Australia, manufacturers are allowed a maximum expiry date which is 12 months after the date of commencement of the tests. The manufacturer may store the product at 4°C for any period up to expiry, but is restricted to a maximum of 6 months after release from store.

Thus, marked expiry dates can fall anywhere within 6-12 months from the testing date. Before the maximum expiry date, sample packets of inoculant can be resubmitted for testing, and are subjected to the same tests as at manufacture. If they pass, they are allowed a further 12 months' expiry, provided they also contain more than a specified moisture percentage (currently 40% compared with approximately 50% for fresh peat—expressed here in terms of wet weight).

### Calculation of realistic standards

If a standard considerably below these is set, the attainment of even 100/seed on alfalfa would require a fresh 250-g pack of inoculant to be applied to 5 kg of seed. With a decline to one-tenth of these numbers during its life to expiry, the 250-g pack could then only be applied to 0.5 kg of seed. It is doubtful whether such a quantity of peat inoculum could be successfully attached to the seed.

### Implementation of standards

It is essential that adequate records are kept by both a control body and manufacturers; the need is most obvious when more than one stage of manufacture is checked.

Broth samples normally need to be checked rapidly to determine whether the next step in manufacture should proceed. Manufactured inoculants should be accepted for testing only if the broth has passed the previous test. Within certain defined limits it may be practicable to retest a stored inoculant for extension of the expiry data. The results of all tests should be available in the records at the control laboratory and at the manufacturing plant.

## Regulatory powers

Procedures and standards such as those described above were developed in Australia during a long and close association between manufacturers, the control body, and its advisory committee. As a result, there is no dissent regarding the application of standards, and the control authority has had no need for regulatory powers or legislative action. It has clearly been in the interests of manufacturers to retain the right to quote official approval of the product. As the main funding authority for AIRCS, the State Departments of Agriculture are kept fully informed on the standard of the commercial products. The one state which has legislative authority to confiscate unsatisfactory material—Queensland—has not found it necessary to invoke its powers

The less the control authority is involved in tests of the process during preparation the more arbitrary its standards are likely to be, and, because the contact between the producer and the controlling authority is reduced, the implementation of standards may be more dependent on regulation.

## Practicality of inoculant quality control in India

The key to quality is a good quality control system. The future of Indian inoculants is wholly dependent on the implementation of a successful control system. Whatever the organization may ultimately be, the first moves must be made by the manufacturers on their own product.

At the time of writing, the industry is fragmented into three groups of manufacturers:

a) private companies;

b) state government laboratories including cooperatives; and

c) agricultural universities.

As seed supplies are distributed by state authorities, the inocula provided come preferentially from the state and university sources. Some states also purchase from private manufacturers; others have legislation preventing such collaboration.

The Indian Standards Institution (ISI) has outlined standards which appear to be reasonably attainable, at least by some research institutions—$10^8$ rhizobia/g at manufacture and $10^7$ at expiry. Few of the inoculants made available to the author from any of the manufacturers in 1980-82 reached the standards set by ISI.

Although ISI has appointed centers for granting of ISI registration, not all of these have adequate facilities for testing contaminated cultures.

In this bulletin it has been unequivocally pointed out that, in the presence of contaminating organisms, the plate count is inadequate and estimates of numbers must be made on the plant dilution series.

So far as is known, adequate quality control of the final product by the producer is not practiced and the farmer may receive a substandard product.

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