LEGUME INOCULANT PRODUCTION

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The paper summarizes the methodology involved in Legume Inoculant production with particular reference to the conditions in Australia. The paper also describes a relatively simple fermentor recently employed by the author to grow rhizobia in mass culture. The factors affecting growth and survival of rhizobia in peat have been established together with enumeration of quality control methods usually employed to set standards for commercial manufacturers of legume inoculants.

INTRODUCTION

The practice of inoculating seed with artificial cultures of rhizobia dates from the 1890's, soon after Hellriegel's discovery of the ability of the inoculated legume to fix atmospheric nitrogen and Beijerinck's isolation of the rhizobia from the root nodule. However, prior to the use of artificial cultures, soil from around the roots of the desired legume was used either in large quantities to spread on the field to be sown or as a dust to coat moistened seed before sowing. The introduction of artificial cultures by Nobbe and Hiltner in 1896 represents the first attempt at large scale use (commercialization) of this technique. These early cultures were essentially the agar cultures of today but they soon changed to sterlized soil, then to peat (humus material) coated with agar and finally to peat (humus alone) during the period up to the early 1920's (Fred, Baldwin and McCoy 1932). This form of culture, either sterilized or non-sterile is stlll the accepted and best form of culture for modern day agriculture. The reason for its superiority lies mainly in the fact that in the peat form, better survival of the rhizobia on the inoculated seed is obtained than with agar, broth or reconstituted lyophilized cultures (Vincent 1970). Additional advantages are its longer shelf-life, ease of manufacture in sterilized or non-sterile carrier and ease of handling (distribution).

The use of peat by legume inoculant manufacturers brought with it problems of quality. It is interesting to note that even in the first years of artificial cultures the need for continuing control was recognized as an integral part of the system since "Cultures for inoculution of leguminous crops are essentially biological products and as such are subject to the natural laws of variation . . . ." (Fred, Baldwin and McCoy 1932). Despite this warning official control in U.S.A. was relaxed during the 1930's so that by the late 1950's many nodulation failures, attributed to poor quality inoculant, were reported by various investigators (pers. com.) In Australia the changeover from agar to peat cultures coincided with the handing over of production to commercial

interests. Again, in the absence of independent quality control, poor quality cultures were detected and field failures resulted (Date 1969). These two instances serve to highlight that even in situations of relatively advanced technological achievement, independent quality control procedures are necessary for maintenance of high quality legume inoculant cultures.

Production of cultures in carrier materials such as peat, peat-like materials or soil can be classed into two general methods. Firstly that commonly referred to as the Australian or U.S. system depends on multiplying the number of rhizobia in pure culture to a level that final mixture of broth culture plus carrier contains sufficient viable rhizobia to meet a minimum standard, i.e. no dependence on growth in the peat. Alternatively in the European system the carrier material is moistened with nutrient broth and sterilized before addition of a small volume of rhizobial broth. This method depends on considerable multiplication taking place in the carrier immediately after inoculation of the vessel. Various aspects of these systems will be discussed with main emphasis on the former system.

Preparation of Carrier

(*a*) *Selection of carrier material*

The most commonly used carrier is finely ground peat although soils high in organic matter and mixtures of soil plus peat or compost or lucerne meal have been used successfully (van Schreven, Otzen and Lindenberg 1954). More recently lignite (Kandasamy and Prasad 1971) and cellulose powder (Pugashetti, Gopalgowda and Patil 1971) have been tried but these seem less satisfactory than peat.

The essential characteristics of a suitable carrier are that it has a high organic matter content (>60 per cent), low soluble salt content (<1 per cent), a high moisture holding capacity (150 to 200 per cent, w/w. although materials of only 50 per cent have been used) (Roughley 1970*a*; Burton 1967), provides a nutritive medium for the growth of rhizobia and prolongs their survival both in culture and on the inoculated seed, where desiccation and seed coat toxicities can cause a rapid decline in the number of rhizobia. These characteristics should be used as a guide only in selection of a carrier material. It has been our experience that any potentially useful carrier material must be tested for suitability by conducting appropriate trials in which growth and survival of rhizobia are measured at intervals over a 12 to 26 weeks storage period under known conditions.

Many peat deposits are too acid to use as a carrier without prior neutralization to *p*H 6.5 to 7.0. The finely ground lime ($CaCO_3$) is a satisfactory neutralizing agent and should be added before milling so as to obtain an even distribution through the peat.

(*b*) *Drying and milling peat*

In most cases peats will be wet when harvested. They should be allowed to drain in the field and air dry before breaking into small pieces.

If desired peat can be dried in a force-air oven but temperatures must not exceed 100°C as higher temperatures cause degradation of peat with release of toxic substances which restrict the growth and survival of rhizobia (Roughley and Vincent 1967). High temperatures also cause excessive rises in temperature of peat when dry peat is mixed with rhizobial suspensions. Increases of up to 28°C can be incurred (Roughley and Vincent 1967). These effects may not be evident in all sources of peat since Burton (1967) is able to flash-dry (and partly sterilize) peat by momentary exposure to 650°C.

After drying peat materials are usually ground to a fine powder in a hammer mill and should pass finally a 250 to 300 mesh sieve (about 75μ pore size).

(*e*) *Sterilization of carrier*

The choice of method of sterilization depends mainly on the type of container in which the carrier is packed but also on the facilities available. The methods employed include flash-drying, autoclaving, gamma irradiation and chemical sterilants.

Flash-drying is used in the U.S.A. and depends on quickly reducing the number of contaminants to a very low level and on these survivors not interfering with the growth and survival of rhizobia. This method was found to be as satisfactory as autoclaving peat for four hours at 121° (Burton 1967). The advantage of autoclaving is that it is the only method which allows absolutely pure cultures to be prepared. It requires though the use of either glass containers or bags of autoclavable film. The former increases the costs of distribution and the latter are subject to bursting during autoclaving, and may restrict gas transfer into the inoculant (Roughley 1968). Some of the problems associated with bags may be overcome by venting the bags with a tube stoppered with cotton wool which allows pressure changes to occur in the bag during processing, serves as an inoculation port and allows gas exchange for rhizobial growth after inoculation. This port should be sealed off after numbers of rhizobia have reached their upper limit. Bottles two-thirds filled with moist peat may be sterilized by autoclaving for 3 hr at 125°C (van Schreven 1958), longer sterilizing times up to 5 hr had no long-term deleterious effects (van Schreven 1970).

Ethylene oxide has been used commercially in Australia to sterilize peat. Problems associated with adequately penetrating all the peat with gas and its subsequent removal necessitated treating the peat in bulk before packaging in an air-tight container which would withstand vacuum. The numbers of rhizobia developed in gas-treated peat varied from in excess of 10^9/g to undetectable levels. This variation was attributed to the problem in removing all traces of ethylene oxide and the ability of this gas to form toxic complexes with organic matter (Roberts *et al.* 1943). Because the peat was treated in bulk it proved difficult to package and inoculate without introducing contaminants which often multiplied to numbers in excess of 10^9/g peat.

In Australia we find the most convenient system is to package dry (10-15 per cent moisture), milled peat into polythene (0.002″=.05 mm thickness) bags and sterilize by gamma irradiation at 5 Krad. Peat sterilized in this way is easily handled and can be stored for long periods before impregnation with rhizobial broth cultures.

Preparation of Broth Cultures

Media requirements

Rhizobia are not highly demanding in their nutrient requirements. They are classified as heterotrophs and require nitrogen and carbon in readily available forms. Because of their unique association with plants many investigators have used plant extracts as supplements in nutrient media, however most researchers now use a yeast extract medium based on that described by Fred, Baldwin and McCoy (1932). Most rhizobia utilize mono-and disaccharides readily although some slow-growing strains, particularly those for lupin and soybean prefer pentoses (arabinose) to sucrose or mannitol (Graham and Parker 1964). An interesting case was reported by Roughley (1970*a*) where filter sterilization of galactose or arabinose was preferable to heat sterilization for the red strain of *Rhizobium* for *Lotononis bainesii*.

For the production of inoculants, a high count broth is required to ensure a high quality inoculant. For practical reasons, then, a short (rapid) growth period is desirable to reduce the risk of contamination and to reduce running costs of equipment. Rhizobia because of their comparatively slow growth rate can be readily outgrown by a chance contaminant during the fermentation phase. Higher viable cell yields can be obtained by varying the source and concentration of the yeast extract according to the individual strain requirements (Date 1972). Improved growth was attributed to stimulation by the increasing amino acid concentration of the yeast used, however, caution must be exercised to prevent loss of effectiveness (Hamdi 1969).

The inclusion of antibiotics in large scale fermentations has proved useful in Czechoslovakia where penicillin (100 I.U./ml) has been used to reduce contamination (Vintika and Vintikova 1958). This has not been found necessary in Australia but conceivably is an advantage where conditions for fermentations are less than satisfactory.

Fermentors

The fermentors or growth vessels for multiplication of rhizobia in submerged liquid culture have relatively simple requirements. A container such as a drum of 10 to 100 litre capacity fitted with an air-inlet port, an air-outlet port and an inoculation port makes a satisfactory fermentor. For convenience

a sampling tap can be included (see Fig. 1). The most convenient system is a drum of about 60-80 litre capacity to which 40-50 litres of broth are added. An air filter is attached to the air-inlet port and the whole unit placed in an autoclave for sterilization (Note that one of the air ports must be left open to allow for pressure changes during autoclaving). After cooling, the vessel is transferred to a suitable location, and attached to an air supply. This should be supplied at about 0.7 kg/cm^2 pressure (a reducing diaphragm may be necessary) and have a primary filter to remove particulate matter and be located in a "clean" environment. More sophisticated fermentation units such as those used in industry for antibiotic and enzyme production are satisfactory but unnecessary. Our experience in Australia suggests that the wholly autoclavable fermentation unit is more efficient than the larger units which require special coils and jacketing for sterilization (by live-steam) and cooling purposes, a special air filter and a number of additional pipes and taps all of which must be sterilized with live-steam from a separate steam generator.

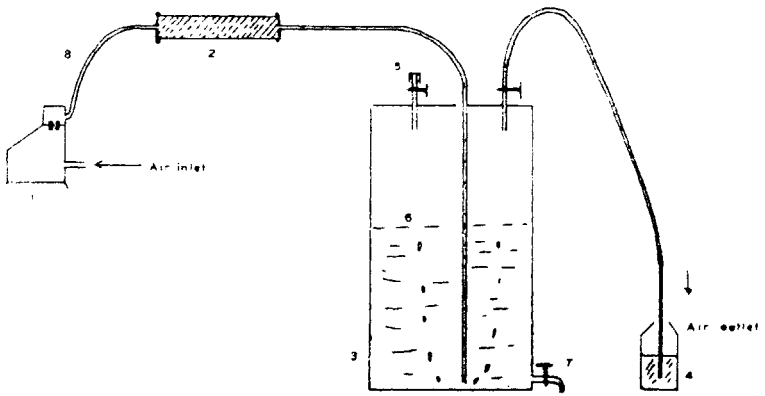

Fig. 1. Diagram of simple fermenter unit for production of rhizobial broth cultures.

Growth conditions

(*a*) *Aeration*—Although rhizobia grow satisfactorily for ordinary laboratory purposes in still culture, more rapid growth and higher cell yields can be obtained with vigorous aeration. Air-flow rates in the literature range from as little as 0.2 to 60 litre air/hour/litre of medium. Recent experience suggests that approximately 5L air/hr/L medium is satisfactory. In an experiment using clover strain TAl, increased viable cell numbers were obtained up to 5L air/hr/L of medium (100 to 3500 million rhizobia/ml for 1 to 5L/hr rates and 3300 million at the 10L/hr/L of medium rate (van Schreven 1958; Roughley 1970*a*). The use of a fine pore-size sparger and/or impeller will ensure better solution of oxygen into the medium in large fermentations, but impellers should not be used unless absolutely necessary as a bacteriological seal of the shaft is difficult to

maintain. Very adequate stirring is obtained in smaller units with the air-flow through the medium.

(*b*) *Inoculum level, incubation time and temperature*—Inoculum levels vary (0.1 to 1 per cent) but generally provide 10^6 to 10^7 rhizobia ml of culture medium at the beginning of the fermentation. "Fast-growing" strains (mean generation time 2-4 hr) reach maximum viable number in 2-3 days (36-72 hr) and may maintain this number for a further 24 to 36 hr depending on the growth conditions (but note that the proportion of viable cells decreases) whilst the "slow-growing" strains (m.g.t. 6-12 hr) require 5-8 days (108 to 192 hr) to reach maximum viable cell numbers. Aeration rate and time of harvest have a strong influence on the number of viable cells. Skinner and Roughley (1969) observed that clover *Rhizobium* strain TAl yielded a total of 5200 x 10^6 cells/ml after 24 hr growth with 65 per cent of cells viable but at 48 hr total numbers had increased to 140,00 x 10^6/ml with only 3 per cent viable cell, i.e., 3370 x 10^6/v 4200 x 10ml. Yeast extract source and quantity in the medium also have a very marked influence on the total to viable count ratio (Date 1972).

Temperature of incubation for rhizobia is not highly critical. General experience suggests that 26 to 28°C is a suitable temperature for all strains. Burton (1967) prefers 30-32°C as an optimum temperature for growth of all strains except those for *Medicago* species which prefer 35°C.

Storage of broths

It is inadvisable to store broths after the attainment of maximum viable numbers for more than 24 hr. Significant reduction in viable numbers has been recorded for clover rhizobia within 12 hr in unaerated cultures at 25°C. Cold room (4°C) temperatures are preferable if broth must be stored for any period.

Preparation of Peat Cultures

Mixing of broth and carrier

(i) *Non-sterile carrier*—Milled carrier may be impregnated with rhizobial broth either by spraying or adding a bulk suspension to the carrier rotating in a drum-type mixer. Usually about 1 part by weight of broth is required to wet 2 parts of dry carrier. Final moisture content varies from 35-40 per cent (Burton 1967) to 45-50 per cent (Roughley 1970*a*) and appears to depend on quality of the carrier. A good rule-of-thumb is to add broth suspension to the point where the wetted peat still remains friable without "balling-up".

When the broth is fully absorbed the moist peat is passed through a coarse sieve, to break up any lumps, and collected in flat trays about 10 cm deep of a convenient size. Each tray should be covered with a sheet of polythene to prevent drying out of the surface layer during a maturation period of 5 to 10 days, depending on the growth rate of the strain, at 25-28°C. After maturation peat is resieved to disperse and concentrated pockets of growth or unabsorbed moisture as well as to break any lumps, before packaing into polythene bags. It has been our experience that pinholes in the polythene bag are unnecessary and can leod to rapid drying of the culture.

(ii) *Sterile carrier*—In this case the milled carrier is already packeted and sterilized. In Australia this is by gamma irradiation of dry (15 per cent moisture) peat in sealed polythene bags or as in Europe where the carrier is moistened to approximately 40 to 50 per cent moisture with a nutritive broth and sterilized in a cotton wool plugged flask or bottle. In each case a high count broth suspension is introduced aseptically with a hollow needle, mixed with the peat by shaking or kneading between the fingers, and incubated as for the non-sterile carrier for 5-10 days depending on strain. In the case of the gamma sterilized peat the broth is added at the same rate as for non-sterile carrier (approximately 50 per cent moisture) but only a small amount is added to the wet carrier in the European bottle system. This system depends on each culture growing from the inoculum injected whereas the former system does not depend on further multiplication of rhizobia for the culture to reach the minimum standard i.e. sufficient rhizobia, are added to meet the standard, any increase in numbers due to multiplication in peat being bonus quality.

Rhizobial counts of 10^9 to 10^{10}/g peat can be expected from both these systems compared with 10^8-10^9 for non-sterile carriers. In many instances with good quality non-sterile peat more than 10^9 rhizobia/g can be expected at the time of manufacture (Date 1969), but will depend on strain of *Rhizobium* (Roughley and Vincent 1967).

(iii) *Multi-strain inoculants*—This term refers to either mixtures of strains for different host inoculation groups or mixtures of strains for the one host species. Neither approach can be recommended as domination of one strain over another in the culture is likely to occur (van Schreven, Otzen and Lindenberg 1954; Marshall 1956). This domination is not necessarily related to a strain's competitive ability in the field. If two or more strains are necessary in the one inoculant then these should be prepared as separate peat cultures uptil the end of the maturation phase. Two or more fully grown peats can be then mixed in equal proportions before packaging. For cultures produced in sterilized carrier equal volumes of high count broth can be injected simultaneously in the system where $> 10^9$ rhizobia/g are added at the beginning. However, this practice is not recommended even though there is some evidence that the host selects the most effective strain under controlled conditions (Robinson 1969*a*). It is likely that other factors such as ability to survive and multiply in a soil will modify this relaionship in the field.

(iv) *Packing of inoculants*—Inoculants have been distributed in a wide range of containers including sealed cans, glass bottles stoppered with cotton wool and waxed or covered with cellophane to prevent drying, bags of medium density polythene 0.089 mm gauge, or low density polythene 0.038-0.051 mm gauge. Glass bottles are commonly used for inoculants prepared with autoclaved peat and low density polythene for gamma irradiated peat.

Factors Affecting Growth and Survival in Peat

Growth and survival of rhizobia in peat or peat-like materials is influenced by freedom from the other organisms (sterility of carrier), moisture

content, temperature of storage, type of container, type of carrier, soluble salt content and interactions between any of these factors.

(i) *Sterility and storage temperature*

The effects of temperature of storage (5 and 25°C) on growth and survival of rhizobia in sterilized (autoclaved) and non-sterile peat, at constant (45 per cent) moisture content are illustrated in Fig. 2 for the number of medic rhizobia per g peat recovered at intervals during 26 weeks storage. The essential features are the marked difference between sterilized and non-sterile peat; the rapid early growth and decline at the higher temperature for sterilized peat compared with the rather slow increase at 5°C; the similarity in numbers of rhizobia in sterile peat at both temperatures after about 12 weeks storage; the rapid and continuous decline which followed the initial increase in numbers at 25°C for non-sterile peat; and finally the fact that this culture would have failed to meet the standard by 10-12 weeks. Average weekly log. death rates at 13 and 32 weeks for the four treatments are listed in Table I. Similar results have been observed by Roughley and Vincent (1967), Roughley (1968) and van Schreven

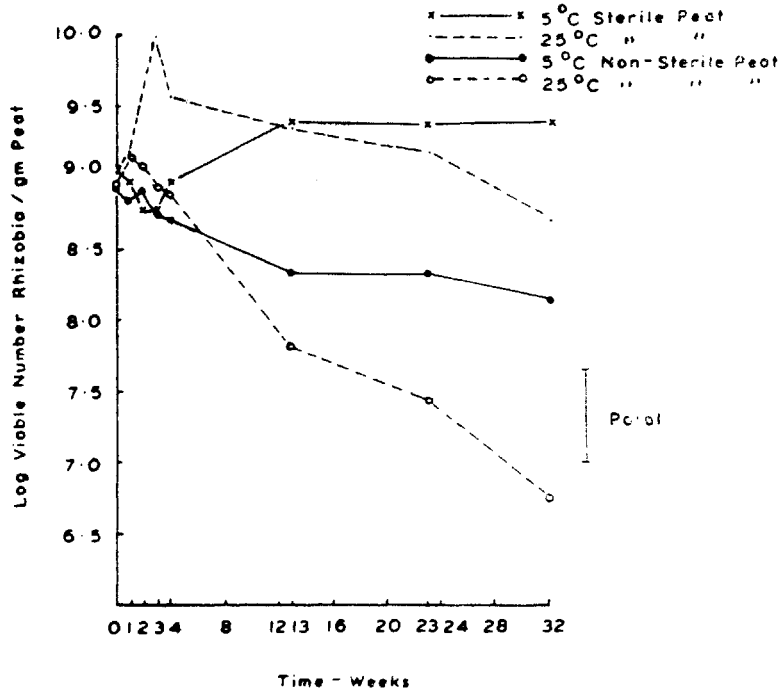

FIG. 2. Survival of lucerne rhizobia in sterilized and non-sterile peat at two temperatures of storage.

TABLE I

Average weekly log. death rate (k) at 13 and 32 weeks for lucerne rhizobia in sterilized and non-sterile peat culture at two temperatures of storage

	13 weeks storage		32 weeks storage	
	5°C	25°C	5°C	25°C
Sterilized	—0.028	—0.027	—0.012	—0.009
Non-sterile	0.048	0.125	0.026	0.068

(1958 and 1970). Experience in Australia has shown that while cultures prepared in sterilized peat (either autoclaved or gamma irradiated) provide better survival during storage and distribution, cultures in non-sterile peat have been prepared satisfactorily for medics and clovers provided they are used within the time which assures a minimum quality.

Strains of rhizobia vary considerably in growth and survival in peat. For example Roughley and Vincent (1967) demonstrated that the cowpea-type strain CB756 would produce a satisfactory inoculant only in sterilized peat, whereas the medic strain SU47 gave good cultures in the same peat not sterilized. The clover strain TA1 was intermediate in response (Roughley and Vincen 1967).

(ii) *Moisture content*

The effect of moisture content on survival must be considered separately for non-sterile and pure culture inoculants as there is an obvious interaction between rhizobia and the contaminants at particular moisture levels (Roughley and Vincent 1967; Roughly 1968). For sterilized peat moisture levels of 40-60 per cent were satisfactory whilst 30 per cent gave fewer rhizobia after storage, suggesting that low moisture content alone was the reason for poor survival. In the case of non-sterile peats there appear to be several factors involved. At 30 per cent moisture the ratio of contaminants to rhizobia was higher than in the range of 40 to 60 per cent (Roughley and Vincent 1967) and at higher moisture levels soluble salts such as sodium chloride became more toxic, e.g. at 55 percent moisture there is 1.5 times as much salt in solution than at 45 per cent moisture (Steinborn and Roughley 1973). In addition to initial moisture content the rate of moisture loss is also important (Hedlin and Newton 1948; van Schreven, Otzen and Lindenberg 1954; Roughley and Vincent 1967), since numbers declined more rapidly with decreasing moisture content (Table II).

Because peats and peat-soil mixtures vary widely in their ability to absorb moisture the expression of moisture as either a percentage of the wet or dry

weight of the carrier is misleading. This inherent ability of different carriers to absorb different amounts of moisture may explain the different optima reported by van Schreven, Otzen and Lindenberg (1954) and Roughley and Vincent (1967). Comparisons may be made between different carriers if moisture is expressed on the basis of pF as determined by Fawcett and Collis-George (1967).

TABLE II

Influence of moisture level on the number of clover rhizobia, strain TA1, in peat culture after 12 weeks storage of 26° C and 100 per cent relative humidity (from Roughley 1970 a)

Moisture content	Log number viable cells	
	Sterilised peat	Non-sterilized peat
30%	6.057	4.562
40%	10.017	8.244
50%	10.114	8.376
60%	10.130	6.940
Difference for significance (P=0.05)	0.092	0.514

(iii) *Aeration*

There have been several conflicting reports on the requirements for gaseous exchange for growth and survival in peat cultures. Some of this confusion was possibly caused by the fact that the demand for gaseous exchange in peat cultures though definite is not high (Roughley 1968). Most reports compared only unrestricted gaseous exchange with sealed containers, however, these were not sealed under vacuum and the amount of air trapped may have allowed rhizobia to survive.

Rhizobia did die rapidly in peat when stored in tightly stoppered flasks but with access to air their numbers remained high until the carrier was desiccated (Hedlin and Newton 1948). This finding was later confirmed by van Schreven, Otzen and Lindenberg (1954) using mixtures of soil and peat. In contrast Newbold (1951), Gunning and Jordan (1954) and Spencer and Newton (1953) obtained satisfactory survival in either screw cap tubes or sealed cans. Roughley (1968) has measured growth and survival of clover, medic and cowpea-type rhizobia in peat packed in various polythene and mylar packaging films and compared these with sealed cans. The medium and low density polythene materials gave best results and mylar film and sealed cans the worst. Average weekly log death rate over 8-12 weeks storage was about 0.05 for polythene and 1.2 for sealed cans.

Quality Control and Culture Standards

Details of inoculant quality control have been reviewed on several occasions recently, e.g., Date (1969), and Vincent (1968,1970). Assessment of quality employs both qualitative and quantitative tests since it is important to have sufficient of the correct strain of *Rhizobium* present for successful seed inoculation. Inoculant quality therefore is a reflection of the relation between number of viable rhizobia on the seed and conditions affecting nodulation. This will vary from the one extreme under favourable conditions, e.g. pots in the glasshouse or seedlings in tubes, where a few rhizobia will be able to rapidly colonize the soil and rhizosphere resulting in prompt nodulation, to very adverse conditions, unfavourable for survival and multiplication, where a very heavy inoculum would be required for success in forming nodules. The level of inoculation can of course be influenced by treatments which ameliorate the adversities either as methods to improve conditions on or near the seed or to provide a totally more favourable environment by soil amendment(s).

Quality standards in Australia have evolved over the period since control began in 1957 and have been modified several times based on accumulated experience. The key to any standard for peat cultures depends on the number of rhizobia required per seed for successful nodulation. This will depend on (i) survival of the inoculum between application to the seed and seed germination, (ii) ability of the inoculum to colonize the seedling root, and (iii) the ability of the inoculum strain to compete with rhizobia already in the soil for infection sites on the seedling root. Early evaluation of these factors indicated that a minimum of 100 rhizobia/seed was sufficient, however, this was increased to 300 or preferably 1000 seed as evidence of establishment and nodulation difficulties were recorded where numbers were less. This was particularly so where seed was exposed to adverse conditions such as acidic soils, exposure on soil surface with oversown seed and as attempts at preinoculation were made. For a number of years this standard was acceptable and the quality of inoculants was judged accordingly (see Table III). However, more recent studies such as those of Ireland and Vincent (1968) suggest that even higher standards may be necessary. For example, these workers showed that where *Trifolium subterraneum* was sown, in an area occupied by rhizobia ineffective on that species, but fully effective on naturally occurring *T. repens*. with an inoculum level of 10,000 rhizobia/seed, only 30-40 per cent of plants were effectively nodulated in soil containing 280,000 rhizobia/g compared to 80 percent when the ineffective strain was only at 12,000/g soil. The same data illustrated strain differences, e.g. strain TA1 required 600,000 rhizobia/seed to obtain 90 per cent plants with effective nodules compared with more than 6,000,000/seed for strain UNZ29 for the same result. The minimal standard now accepted by AIRCS* is 100×10^6 rhizobia/g peat for cultures produced in sterilized peat. There are two exceptions: firstly cultures for *Lotononis* at 30×10^6 rhizobia/g and secondly those for medics at $50 \times 10/g^6$ peat. These exceptions are based on the

* AIRCS—Ausitralian Inoculant Research and Control Service

TABLE III

Peat standards and number of rhizobia per seed

(Old versus new standards)

Host species	No. seed per kg	Standard* inoculation rate (kg seed/70 g peat)	New standards: Minimum No. rhizobia per seed for cultures meeting current standards for purchased peat samples	New standards: Current standard for purchased samples ($\times 10^6$/g peat)	Old standards: Minimum peat quality to achieve 300 rhizobia/seed based on old standard for purchased samples ($\times 10^6$/g peat)
Trifolium subterraneum	154,000	10	4,540	100	9
Trifolium repens	750,000	5	1,870	100	50
Medicago sativa	430,000	10	820	50	30
Lotus pedunculatus	1,940,000	5	720	100	20
Pisum sativum	3,100	25	900,000	100	0.4
Phaseolus vulgaris	4,000	25	70,000	100	1
Lupinus angustifolius	7,500	25	37,400	100	1
Glycine max	11,000	25	25,400	100	2
Vigna sinensis	15,000	25	18,700	100	2
Macroptilium atropurpureum	88,000	10	8,000	100	5
Stylosanthes humilis	200,000	5	7,000	100	20
Lotononis bainesii	3,840,000	5	370	30	4

* This is a slightly higher rate than previously, e.g. for *T. subterraneum* 70/g peat suitable for 13.5 kg seed, *P. sativum* 27 kg, for *S. humilis* 7 kg.

fact that the higher standard is not possible with the particular strain for *Lotononis* and for medics on the fact that these rhizobia have lower death rates than other rhizobia in the same conditions (Roughley, pers. com). Some idea of the number of rhizobia per seed based on this standard which is only 10 per cent of the freshly manufactured minimal standard can be seen in Table III. Such compromises have been made in the past, e.g. see Vincent (1970), for ease of acceptance and enforcement of standards. It means in fact that the smallest seeded species in a host group receives at least the minimum number of rhizobia per seed whilst larger seeded species get proportionately more because of inoculation rates.

For many years we have adopted a double standard : one applying to freshly manufactured cultures and another to cultures purchased from retail centres. Standards for freshly manufactured cultures were obtained originally by determining the death rate of the rhizobia in peat culture at above average

temperature conditions and calculating so as to ensure that the minimum number would be achieved per seed throughout the recommended useful life of the inoculant. Present day standards are slightly higher than the minimum 500×10^6 rhizobia/g accepted by UDALS. Increased standards have been made possible by better technology, particularly the use of sterilized peat to the extent that all cultures now contain a minimum of 1000×10^6 rhizobia/g at manufacture compared with only 44 per cent with 1000×10^6 or more and 44 per cent with 100×10^6 to 1000×10^6/g in 1966-67. Based on the lod standard 86 per cent of samples obtained from retail centres contained more than the minimum standard (38 per cent were $< \times 10$ standard and 48 per cent $> \times 10$ standard, 14 per cent failed) (Date 1969).

There are few reports of actual quality at manufacture in other countries. Czechoslovakian cultures contain a minimum of 300×10^6 rhizobia/g (in sterilized soil-peat mixtures (Hamatova and Vintikova 1963), and in Holland 4000×10^6 to 25000×10^6/g using a sterilized loam-peat-lucerne meal mixture in cotton wool stoppered bottles (van Schreven 1958).

The problem of who is responsible for quality control is a difficult one. Experience only can decide whether this should be under the control of an official (Government) testing authority or whether manufacturers should be made to exercise their own control. In Australia we have been guided primarily by the circumstances that existed and by the history of inoculant control in USA. During the early days of commercial manufacture in USA the USDA exercised control but relinquished this role during the 1930's to competitive pressures of the then large number of manufacturers. Inoculant quality, except in isolated cases, had shown a gradual decline over the period to the 1960's when some official groups re-instituted limited control procedures because of the very poor qulity of cultures available. In Australia during the early 1950's the same situation existed as commercial production based on peat cultures began. Many failures were recorded and attributed to poor quality inoculant (Vincent 1954; Brockwell 1954; Waters 1954; Vincent 1958 quoted by Date 1969). Circumstances existing at the time were such that individual inoculant manufacturers could not afford to exercise the degree of control required but with financial aid from primary industry sources, contributions from manufacturers and the resources of the University of Sydney and NSW Department of Agriculture, the UDALS (now AIRCS) group evolved based on three guiding principles : (i) selection, testing and maintenance of suitable rhizobial strains; (ii) control of quality of legume inoculants during manufacture and before distribution to prevent poor quality cultures being distributed to farmers. Samples from retail centres also checked to detect any major loss of viability during distribution; and (iii) advice to and research for manufactures, distributors and users of inoculants on the problems of production, handling and application that affect the quality and efficiency of inoculant cultuers.

Because of the chance of unexpected problems of variation of strains [e.g. loss of effectiveness (Schwinghamer 1964) or invasiveness (Yao and

Vincent 1969] and survival problems in peat (e.g. toxicity of high salt content peats (Steinborn and Roughley 1973), which require immediate investigation, our approach has been one of accepting the necessity of continuing control at a level which is believed to be beyond the resources of individual manufacturers. Other groups in other circumstances will need to determine their best approach. We believe that quality control of commercially produced cultures by an official body is preferable to quality maintenance by competition between manufacturers in a free enterprise system (Burton 1967) for the reason that *Rhizobium* strain selection, maintenance and recommendation are under supervision and that the consumer is protected by prevention of poor quality products reaching the retail market.

Test procedures as indicated previously involve both qualitative and quantitative tests on cultures at three stages; (i) the broth culture before impregnation of the carrier peat base; (ii) the final peat culture immediately after manufacture and before distribution; and (iii) on peat cultures obtained from retail centres. These tests are described in detail by Date (1969) and Vincent (1970). Recent modifications to tests procedures and standards have been outlined by Date and Roughley (1975).

Rhizobium Strain Selection

Isolation and maintenance of strains

The techniques of isolation of rhizobial strains from nodules, soil or peat culture using aseptically growing seedling host plants as a trap host has been described in several places, e.g., Norris (1964), Roughly (1970*a*) Vincent (1970).

Maintenance of strains in conditions which reduce chances of variation and contamination is essential. Freeze-drying is the most successful method and is basically that described by Annear (1964) using a 10 per cent sucrose plus 5 per cent peptone solution to suspend the culture for drying. Very satisfactory storage can be obtained by drying on porcelain beads as described by Norris (1963). Short-term storage up to 12 months can be obtained with screw cap culture tubes.

A combination of freeze-dried and screw-cap test tube cultures was used by UDALS (Date 1969) to provide manufacturers of inoculants with fresh mother cutlures on a regular basis. The system described allows for only two culture transfers between any two cultures received by manufacturers, thus reducing the chance of variation and contamination. It also provides for regular checking of cultures for trueness-to-type, i.e., effectiveness in N-fixation, invasiveness, competitive ability, serological characters.

Criteria used for strain selection

The selection of suitable strains of rhizobia is the first and most important step in the production of legume inoculant. In general terms, the basic criteria of satisfactory strain performance were stated by Vincent (1956) as (*a*) the ability to form effective nitrogen-fixing nodules with all the hosts for which

the culture is recommended, and (*b*) the capacity to do this under a wide range of field conditions. The ability to form effective nitrogen-fixing nodules is still the main criterion used in strain selection and forms the basis for host groupings and strain specificity. However, various characteristics covered by the second of Vincent's criteria are now given greater emphasis in strain selection programmes. These are competitiveness in nodule formation, particularly with native rhizobial populations, and ability to survive and multiply in the soil. These aspects have been given greater value as selection criteria because of the wider and more adverse range of conditions in which agronomists require legumes to establish. Brockwell *et al.* (1968) have proposed an integrated programme of selection which includes in addition to the above points such characters as effectiveness over a range of root temperatures, promptness in forming nodules, ability to persist in the soil in the absence of the host plant, ability to grow in broth and peat, and ability to survive in peat culture and on the seed.

(a) *Strain specificity and effectiveness groups*—The term strain specificity in this context refers to the effectiveness of symbiotic associations rather than to the ability to infect or form nodules on a particular host. The old concept of "cross-inoculation" groups was based on the ability of an isolate from a nodule on one host species to form nodules on another legume species. Plants nodulating with the same isolates were considered to belong to the same "cross-inoculation" group and the isolate was given a species name after the genus or predominant genus for which the root-nodule organism was mutually interchangeable. The concentration of work with agriculturally important legumes (i.e. clovers, medics, peas, beans, lupins and soybeans) in temperate regions has fostered retention of the species names despite the work of Wilson (1944) who was unable to establish clearly defined boundaries around any single legume or group of legumes based on their ability to nodulate. The host legume has a dominant role in determining which strain forms a nodule (Vincent and Waters 1953; Gibson 1964). The effectiveness of the association in N-fixation is also strongly influenced by the host legume, e.g. specific strains for cultivars of subterranean clover (Gibson 1964), lucerne (Gibson 1962; Leach 1968), lines of *Centrosoma pubescens* (Bown and Kennedy 1961) or *Lotononis bainesii* (Norris 1958), and by host × strain × environment interaction (e.g. Roughley 1970*b*). Effectiveness groupings in clover, medic, lotus species and some legumes belonging to the "Cowpea-miscellany" have been reviewed by Burton (1967, Table 1.2). Obviously a dilemma exists : inoculants for individual leguminous species and difficulties in manufacture or wide spectrum strains grouping hosts where a strain is good to excellent in a range of legumes? In Australia a middle coarse has been adopted. Where a "wide-spectrum" strain has been available this has been used either alone or in combination with one other strain as the inoculant (Date 1969). Multiple strain inoculants have been avoided because of the danger of competition within the culture (Marshall 1956) and likelihood of competitiveness in nodule formation from the weaker N-fixing strains in the inoculant. This problem poses a delicate situation for the microbiologist who

must decide between practical aspects of inoculant production and scientific excellence in providing the best available material and legume inoculant manufacture.

The main criterion of effectiveness is the amount of nitrogen fixed, which may be assayed directly, or indirectly by determining plant dry weight. The two parameters are highly correlated (e.g. Erdman and Means 1952) although some degree of differentiation may be lost because of greater concentration of N (protein) in plants under N-sufficiency conditions than under N-deficiency levels.

(b) *Ability to survive and multiply in the soil*—Rhizobia introduced into the soil by way of inoculated seed may multiply rapidly in the soil solution and rhizosphere in numbers sufficient to allow prompt nodulation of the seedling plant. Failure to do so increases the time delay between exhaustion of seed reserves of N and newly fixed symbiotic N and so increases the chance of establishment failure. Failure to multiply at this early stage will also decrease the chance of the inoculum accounting for a significant proportion of the nodules in situations where there is sufficient soil N or native rhizobia able to symbiose with the legume. Also important is a strain's ability to survive or multiply in the soil in the absence of specific host plants. Chatel, Greenwood and Parker (1968) described this character as "saprophytic competence".

(c) *Competitiveness in nodule formation*—The ability of rhizobial strains to compete successfully with other rhizobia for nodule (infection) sites is perhaps the most important character after N-fixing abili ty used in rhizobial strain selection programmes. Brockwell and Dudman (1968) have shown marked qualitative differences in competitive ability between known effective strains on *Trifolium subterraneum* when introduced in approximately equal numbers and where they had to compete with a native population of rhizobia able to form effective associations with the host plant. However, where effective and ineffective strains were used in equal numbers as the inoculum in a tube experiment the host plant (*T. subterraneum*) appeared to have the ability to select the effective strain (Robinson 1969*a*). Nodules obtained from uninoculated field sown *T. subterraneum* and *T. pratense* also contained a predominance of the more effective strains (Robinson 1969*b*). On the other hand, studies by Ireland and Vincent (1968) suggested that ineffective strains are strongly competitive for nodule-sites.

(d) *Influence of the environment*—Selection of suitable strains of rhizobia for particular situations reflects the increasing refinement now found inr hizobial strain selection programmes. Such characters as relation of effectiveness of strains to soil base status. (Holding and King 1963), plant ecotypes as observed by Lowe and Holding (1970) for a group of strains isolated from white clover of the acid hill soils of Scotland and of the low temperature response for N-fixation in *Trifolium repens* selections (Brockwell, Bryant and Gault 1972).

Methods used in strain selection studies

Readers are referred to the publications of Norris (1964), Vincent (1970), Roughley (1970*a*) and Date and Roughley (1975) for details of methods used in *Rhizobium* strain selection studies. These references should be used as a guide since each situation will be different depending on the number of strains to be tested, local facilities and available labour.

Summary flow diagram of legume inoculant production and control

This flow diagram attempts to integrate the three areas of inoculant production, quality control (laboratory) and use in agriculture.

A selected rhizobial mother strain (1) is sent from the control laboratory to the production centre where starter cultures (2) are prepared for inoculation of the fermentor (3). Note that the fermentor depicted here is the larger industrial type. Broth from the fermentor is sampled (8a) and sent to the central control laboratory for testing (9a). On receipt of a satisfactory preliminary result, broth is mixed with peat (4) and sieved (5) into trays to mature. If more than one strain is used in the final culture each strain is treated separately until maturation. Separate peats are then mixed, sieved and packeted (6) and samples (8b) taken for testing at the central control laboratory (9b) before being offered for sale. Samples from the retail market (8c) are also tested (9b); the standards applied however being different (see Table II).

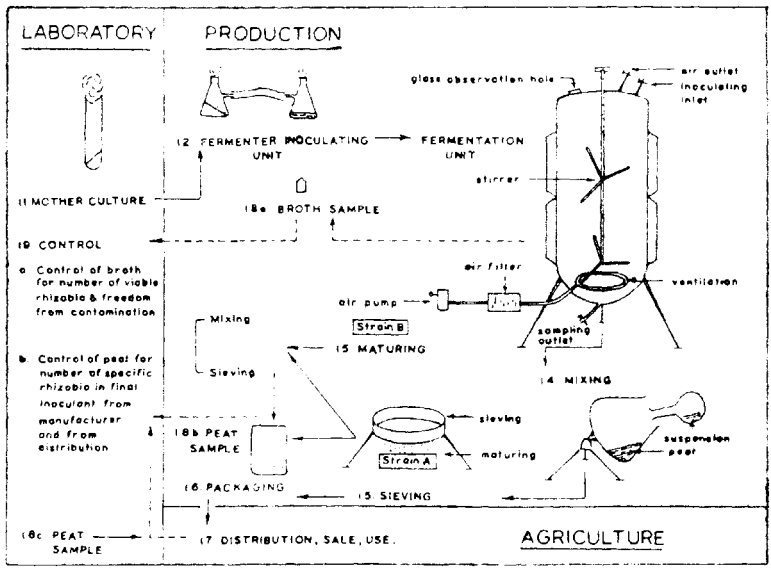

FIG. 3. Production of legume inoculants using a peat carrier-base.

For cultures prepared in sterilized carrier the same general principles apply except that mixing is done on an individual packet basis and sieving and blending omitted.

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