METHODS OF MANUFACTURE AND CONTROL OF LEGUME INOCULANTS PREPARED IN NEW ZEALAND

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Prior to 1955 nearly all legume inoculants used in this country were supplied by the Plant Diseases Division of the Department of Scientific and Industrial Research. This service, which was mainly for lucerne, was introduced and controlled by Dr W. D. Reid and it was due to the retirement of Dr Reid and also because the large demand for cultures was proving an embarrassment to the Division that it was suggested that my company might undertake this service.

Although our laboratory was primarily a medical bacteriological one it was felt that our facilities and staff were suitable for the production of inoculants and on his retirement Dr Reid agreed to act as a technical advisor to the company and has been of great help over the subsequent years.

Initially only agar cultures were supplied, but as several merchants in New Zealand were importing powdered peat inoculants from Australia and America it was felt that this type of inoculant should also be produced. Considerable research on methods, types of peat, and suitable strains of rhizobia were undertaken before this product was finally marketed. At about the same time the Plant Diseases Division commenced a certification scheme whereby all inoculants sold in New Zealand were subjected to laboratory and field trials for effectiveness and keeping qualities.

Methods of Manufacture (Selection of Rhizobial Strains)

It is now intended to give a brief resume of the origin of the rhizobial strains used in the inoculants, with methods of fermentation and propagation of these strains, and a description of internal laboratory procedures that ensure the quality of the final product.

No strain work with rhizobial strains is carried out by ourselves and master cultures are obtained from either Professor J. M. Vincent, Sydney University, or the Plant Diseases Division, Auckland. These strains have been selected over the years as being the best for commercial use and it is essential that they be stable and do not give rise to avirulent mutants during fermentation and mixing

and storage. They must also be able to compete in the rhizosphere with other rhizobia.

For lucerne a mixture of three strains is used:

1. The standard Plant Diseases strain, which has been used for many years with most satisfactory results, known as P.D.D.
2. A strain originally isolated in Western Australia known as WA16/1.
3. A strain isolated by Sydney University known as SU277/1.

This combination of strains is able to live in harmony and gives most satisfactory results with all lucerne types.

In the selection of clover strains great care has been taken to select rhizobial strains which are effective for the three major clovers, subterranean, red, and white. The commonly used strains for this inoculant are obtained from cultures originally isolated in Australia. The current strains in use are designated NA30 and T.A.l. Several New Zealand strains have in the past been used, but at some time or other they have given rise to avirulent mutants. It should be emphasised at this point that all bacterial strains used in inoculants for this country have been fully tested in the field and have given very good results under New Zealand conditions. Individual strains are also available for peas, beans, cowpeas, lupins, soya beans, lotus, and peanuts, but the demand for these inoculants is very small.

To ensure that the bacteria remain stable and virulent they are grown on yeast mannitol agar covered with a layer of sterile paraffin and stored in the refrigerator until required. Under these conditions they remain in an excellent state of preservation for up to 12 months. A further method used is to grow the host plant in tubes of seedling agar, infect with the rhizobial strain, then deep freeze the plant and re-isolate from the nodules when required.

Methods of Manufacture (Propagation)

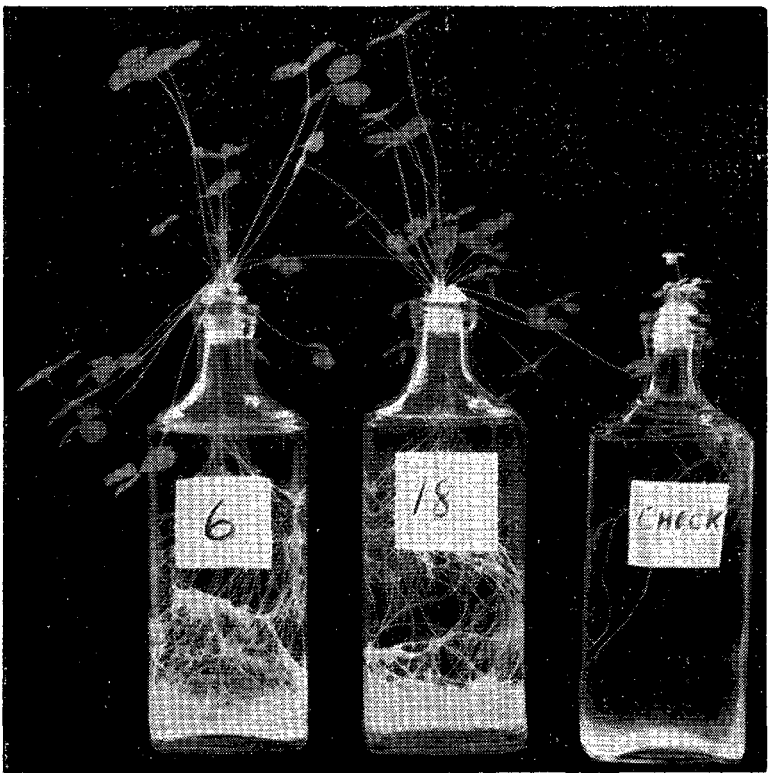
The fermentation and propagation of the rhizobial strains is carried out in fermentors under sterile conditions and the procedure is very similar to that used 'in the fermentation of antibiotics such as penicillin. The pure master culture is inoculated into the fermenting vessel containing the appropriate culture medium. This is composed of peptone mannitol and yeast extract at a pH of 6.8 and incubated for three days at a temperature of 27 degrees C. during which time the culture is continuously aerated and agitated. At the end of this time growths totalling up to 5,000,000,000 bacteria per c.c. are obtained and they are subjected to the following tests before addition to the peat carrier.

1. Microscopic tests by phase contrast microscopy and by stained smear for the presence of contaminating bacteria other than rhizobia and for fungal spores.
2. An estimation of total number of viable rhizobia cells.
3. The identification or confirmation of the strains by means of antigen-antibody tests using specific anti-sera prepared in rabbits.

When more than one strain is being used in an inoculant these are then pooled, having due regard to total viable numbers in each fermentor, and added to the powered peat,

The peat itself is, strangely enough, the most expensive substance used, as it is necessary that the raw peat does not contain large numbers of contaminating micro-organisms, particularly actinomycetes, as these can produce antibiotics harmful to the rhizobia.

The peat is dried and ground to a fineness of less than 320 mesh and during the process the fibrous portion of the peat is



Routine checks for nodule formation on plants growing in a nitrogen-free nutrient solution.

separated from the silica. Only this fibrous material is used and this is then sterilised in revolving heated drums at 120 degrees C. for one hour. Temperatures above this produce charring and are considered to be unsuitable for the maintenance of the cultures.

The peat is usually acid and this has to be brought up to pH 6.8, at which stage the bacterial broth is added. *Moisture content* has been found to be of great importance in the maintenance of viable rhizobia and a moisture content of 45 per cent is aimed at. When packed in polythene no difficulty is experienced in maintaining this water content.

Methods of Packaging: Life of Cultures, Agar, and Peat

Peat cultures are issued with a life of six months during which time the total number of organisms must not fall below 1 00,000,000 per gram. Agar cultures which are normally dispatched direct to the farmer have a dated life of only one month, but in effect they do remain potent for three months providing they are stored in a cool, shady place. In South Australia and Queensland where large quantities of agar cultures are still used laboratories there guarantee a life of three months to agar cultures.

Methods of Testing

In addition to the Plant Diseases Certification scheme in which all cultures are fully tested, rigorous control measures are carried out by our own laboratories. These include total numbers of viable rhizobia, and a minimum figure of 500,000,000 living organisms per gram is aimed at. Dilution tests in seedling agar with the host plant are carried out and a satisfactory culture at time of issue must still be able to nodulate its host plant effectively even when diluted 100,000,000 times. Pot trials using soil in which it is known that rhizobia do not exist are also used, and these in conjunction with the field trials conducted by Plant Diseases Division give a good estimate of how the culture will perform under practical conditions. Regular monthly. checks incorporating all these procedures are carried out during the !ife of the inoculant. It should be pointed out that of all firms manufacturing inoculants in Australasia our laboratories are the only ones who conduct such exhaustive internal control procedures and this we feel could be the reason why of all manufacturers we have never yet failed to reach certification standards.

Conclusion

For many years much time and research has been devoted by laboratories throughout the world in an endeavour to develop a method whereby the seed can be treated by the merchant and sold as inoculated seed.

Routine laboratory testing of inoculants using northern gum soil.

This is really a very difficult problem, as it must be remembered that we are dealing with a living organism which has been taken from its natural environment, subjected to all sorts of artificial procedures, and then put back into the soil with the hope that it will perform its natural functions. It can be seen that after living on the fat of the land as it were and having nothing but the best of food and conditions during several life cycles, it is perhaps asking a bit much of the bacteria to compete in the rhizosphere for available food with the other naturally occurring soil organisms.

For this reason some form of protection must be given to ensure that it will survive until the seed germinates and the root hairs are suitable for infection. A further complication is that it has now been demonstrated that contained in the seed shell of clovers is a substance which is antagonistic to rhizobia bacteria. This undoubtedly is part of the seed's natural defence mechanism to disease.

The use of milk and sugar solutions has been found to be of great assistance in maintaining the life of the bacteria on the seed, and tests have shown that when the peat is used in conjunction with a molasses syrup, adequate numbers of bacteria remain alive for at least two weeks after treatment of the seed. Strangely enough the survival rate of rhizobia when dusted as a peat inoculant on the outside of lime-pelleted seed is very good. Lime pelleted seed treated in such a manner with a good quality peat inoculant gives satisfactory nodulation for up to one month from time of treatment. This, however, although substantiated by laboratory and pot trials, is now being carried a step further and is at present

being tested in the field. The obvious answer to the problem is an inoculant prepared as an ultra fine dry dust, but here we meet many technical problems, as for the survival of the rhizobia a moisture content of at least 25 per cent is considered necessary. How we reconcile this with the fact that rhizobia seem to exist quite satisfactorily in the soil in times of drought I do not know. It has been shown, however, that such dry dust can be made to support rhizobia, but the method is extremely expensive and at present is not a commercial proposition.

DISCUSSION

Q. (A. C. Hurst): Is there any advantage in inoculating seed where clovers have been grown for years?

A. This is a matter for field Officers. Inoculation is being done increasingly, even on good land.

Q. (Dr P. D. Sears): What are the principles involved in the use of carbon and lime pellets?

A. Pellets act as a home for the bacteria and protect them from toxic substances in the seed coat. If the inoculant can be separated from the seed coat this increases their chances of survival. This applies particularly to clovers.

Q. Dr K. F. O'Connor: How long is it safe to use peat culture once it has been opened?

A. This would depend on the extent to which the moisture content of the peat was affected. Peat has a great moisture-retaining capacity but if the percentage of moisture dropped below 25 per cent there would be very few viable rhizobia left. The safe period would depend mainly on how airtight the container was made after use. It is probable that the material should be used within a month.

Q. (Dr Fieldes): It has been stated that inoculants were ineffective on basalt soils in Queensland. Has this trouble been overcome, and are there soils in New Zealand where the same conditions apply?

A. I cannot say whether this trouble has been overcome in Queensland yet. 1 do know that in some parts of Australia, even where the inoculant rate has been increased up to ten times, no inoculation has resulted. In the Gumlands inoculation is sometimes not very effective. Soil antagonisms do exist in the rhizosphere while some soil organisms are antagonistic to rhizobia. Meat extract pellets in trial work have favoured the multiplication of rhizobia within the rhizosphere.

Q. (-. McLellan): Is it worthwhile inoculating pelleted seed to be sown by air because of the harmful effect of sunlight?

A. While this is not the ideal way it is better to inoculate. The whole of the seed is not exposed to direct sunlight and if a good quality inoculant is used there should be sufficient surviving bacteria to ensure satisfactory nodulation.

Q. (M. L. Smeatham): Why have lucerne failures occurred in paddocks which have previously grown lucerne? Is it possible that an antagonism occurs between the existing and introduced rhizobia?

A. The rhizobia present in the soil should be more than adequate for the inoculation of the new stand. Antagonism between strains has not been demonstrated. In fact it is unlikely to be an inoculation problem.

Q. (T. E. Ludecke): How long would the rhizobia remain viable if the seed were treated with molasses instead of pelleting and also if the seed were pelleted with molasses?

A. There is no specific data for survival times in the field. In the laboratory seed treated with syrup will survive for one month. Where syrup is used for pelleted seed other organisms attack the syrup in the ground. For this reason syrup is not considered satisfactory.

Q. (H. R. Scott): It is claimed that milk from cows treated with penicillin will kill rhizobia.

A. Tests have proved that all strains of rhizobia are completely resistant to penicillin. But the use of the other antibiotics will affect the culture.

Q. In the making of pellets it has been claimed that the glue should be heated until it feels warm to the finger. Would this kill the bacteria?

A. A fair test is to hold the jar of glue against the cheek. If it does not burn then it is quite safe to mix the inoculant with the glue.