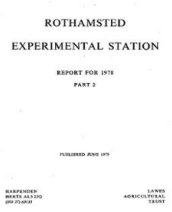
ROTHAMSTED RESEARCH

Rothamsted Experimental Station Report for 1978 Part 2



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M. Dye (1979) *The Rothamsted Rhizobium Culture Collection and Inoculant Use in the Uk ;* Rothamsted Experimental Station Report For 1978 Part 2, pp 119 - 130 **- DOI: https://doi.org/10.23637/ERADOC-1-34345**

The Rothamsted *Rhizobium* Culture Collection and Inoculant Use in the UK

M. DYE

Introduction

Rhizobia are soil bacteria capable of producing nodules on roots of leguminous plants and, as bacteroids, fixing atmospheric nitrogen within the root nodules. In this symbiosis the *Rhizobium* supplies the plant with combined nitrogen whilst the plant provides the necessary environment and energy required for nitrogen fixation. Species of *Rhizobium* are designated according to the range of host plants they will nodulate (Table 1). The group referred to as *Rhizobium* spp. is usually of tropical origin and contains rhizobia which nodulate a wide range of hosts, the 'cowpea miscellany', as well as those which nodulate only the species of plant from which they were isolated.

TABLE 1

The species of Rhizobium*

Species	Hosts nodulated
R. leguminosarum	*Pisum, Vicia, Lens*
R. phaseoli	Temperate *Phaseolus* spp.
R. trifolii	*Trifolium* spp.
R. meliloti	*Melilotus, Medicago, Trigonella*
R. japonicum	*Glycine* spp.
R. lupini	*Lupinus, Ornithopus*
Rhizobium spp.	Miscellaneous species, mainly of tropical origin

* After Buchanan and Gibbons (1974)

There is now a case for abandoning this classification of *Rhizobium* but until it is formally altered by an international committee on nomenclature, collections must retain it despite its faults. Accordingly, all strains in the Rothamsted *Rhizobium* Collection (RCR) are named according to the original host plant. The term 'strain' is used to designate a culture not known to have the same origin as another culture (Vincent, 1956).

Rothamsted has maintained a collection of *Rhizobium* since about 1925. Initially it consisted of strains used in the then Department of Bacteriology for research purposes and maintained by periodic subculture on agar media. As the collection grew maintenance became more and more time consuming so that in 1963 a part-time curator was appointed to reorganise and vacuum-dry the strains. It was at this time that the collection took on its present structure and 'service' role.

The function and organisation of service collections have been discussed by, for example, van Beverwijk (1963), Shewan (1963), Martin (1964), Clark and Loegering (1967), Lapage, Shelton, Mitchell and MacKenzie (1970) and Skerman (1976). The most important duty of a collection is maintenance of strains in a viable and genetically stable state; to this end all strains in the RCR have been vacuum-dried. Allied to this is the need to acquire cultures and to ensure, by appropriate tests, they meet the requirements for inclusion in the collection.

Another major role is to provide cultures on demand for teaching, research and industrial purposes and it is helpful for the customer to refer to a catalogue listing the strains available and their characteristics. The first edition of the RCR catalogue was

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published in 1965, with a revision in 1972. It is hoped to complete the 3rd edition within the next few months.

The curator of the collection also provides information and advice on the selection, culture, maintenance and use of strains and keeps detailed records of all data concerning strains received, their treatment in the collection and their dispatch to customers. Technical operation of the RCR has recently been reviewed in detail (Dye, 1979).

In this paper the development and usage of the RCR will be reviewed and the allied question of *Rhizobium* inoculants discussed.

Maintenance history of the RCR

In the early years of the collection, maintenance of strains at room temperature on yeast-mannitol agar (Fred, Baldwin and McCoy, 1932) in cotton-plugged tubes, was the best method available. It was necessary to subculture at 6-monthly intervals to prevent loss of viability due to drying of the medium. This practice was continued until 1963–64 when the strains were vacuum-dried. Although many of the organisms were maintained for extended periods by subculture until 1964 (Fig. 1), all acquisitions since 1964 have been dried on receipt.

The vacuum-drying technique used is based on that of Annear (1962) and has been

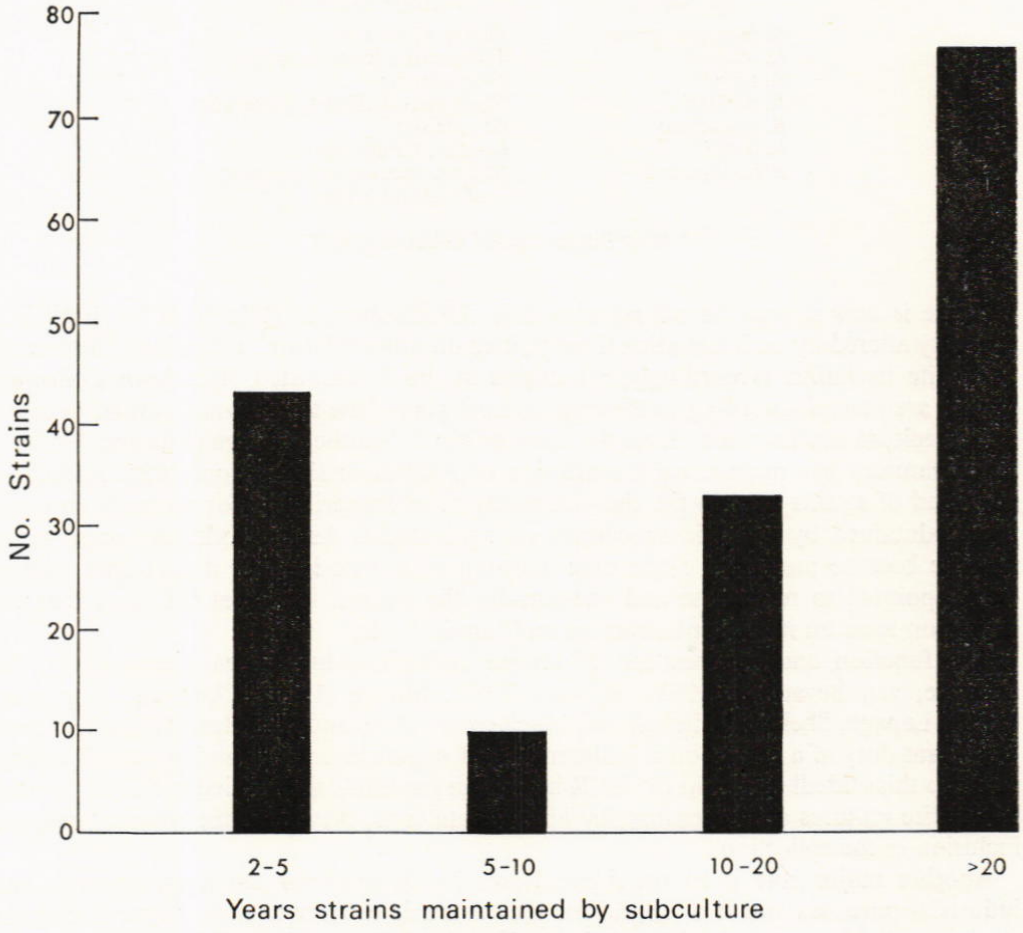

FIG. 1. Maintenance history of the collection.

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described by Dye (1979). Rhizobia are suspended in a protective fluid medium and absorbed on to cellulose fibres in an ampoule which is then evacuated via a chamber containing phosphorus pentoxide. Moisture from the culture is absorbed by the phosphorus pentoxide to leave a dry preparation and the ampoule is sealed under vacuum. The dried cultures are stored in a refrigerator. This process differs from conventional freeze-drying in that freezing is not an essential part of it. According to Annear (1957) '. . . freezing, if it does occur at all, must be extremely transient'.

In general, about 40–50% of the cells survive the drying process and preliminary results using the 'accelerated storage' test of Damjanovic and Radulovic (1968) indicate that a ten-fold reduction in viable bacteria may be expected after 10–20 years storage at 4°C.

Development of the RCR

Information is uncertain concerning the strains maintained prior to 1964, although the department's research papers indicate the holding of some scores of strains in the late 1920s and 1930s and of very many more over the next two decades. However, details after 1964 are accessible and show an erratic increase in the number of strains held (Table 2). This probably reflects changing world interest in *Rhizobium*–legume research, the department's involvement in various Ministry of Overseas Development projects, as well as the different policies of curators with regard to new acquisitions.

TABLE 2

The number of strains in the RCR

Year	Number of strains
1964	211
1966	244
1970	345
1972	357
1974	496
1976	524
1978	536

More is known about the number of strains deposited in any given year (Table 3). The changes clearly mirror the research interests of the department. Early studies of the

TABLE 3

The number of strains deposited in the RCR over the past 50 years

	Number of strains deposited				
	1930–39	1940–49	1950–59	1960–69	1970–78
R. trifolii	15	63	11	65	7
R. leguminosarum	5	8	1	2	13
R. meliloti	0	0	1	1	16
R. japonicum	3	0	0	3	32
R. phaseoli	0	0	0	0	37
R. lupini	0	5	0	0	2
Rhizobium spp.	0	5	2	19	34

nodulation and cultivation of lucerne are not reflected in the table since several *R. meliloti* strains had been collected before 1930. However, the long-term interest in clover rhizobia and the increasing emphasis, over the past 10–15 years, on tropical and subtropical legumes (i.e. *R. japonicum* and *Rhizobium* spp.) is clearly shown. Attempts to cultivate Navy beans in the UK are also reflected in the increase in *R. phaseoli* strains held. It

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TABLE 4

Composition of the RCR

	Number of strains		
	Total in 1964	Total in 1978	Working collection
R. trifolii	156	178	38
R. leguminosarum	16	58	15
R. meliloti	14	43	11
R. lupini	4	11	6
R. japonicum	6	43	15
R. phaseoli	3	47	10
Rhizobium spp.	12	156	26
Total	211	536	121

should be noted that in 1964, when the collection was first dried, an effort was made to add strains from other collections so that rhizobia were available to nodulate effectively all the important legumes. This change in composition from a preponderance of temperate legume rhizobia to a more comprehensive collection is strikingly demonstrated in Table 4.

In 1964 all strains previously identified from their place of origin, experimental number, etc., were given a simple RCR number: 1–1000 for *R. trifolii*, 1001–2000 for *R. leguminosarum*, 2001–3000 for *R. meliloti*, 3001–3200 for '*lotus*' rhizobia, 3201–3400 for *R. lupini*, 3401–3600 for *R. japonicum*, 3601–3800 for *R. phaseoli* and 3801–5000 for *Rhizobium* spp.

Not all the RCR strains are able to form an effective symbiosis with appropriate host plants. Some produce an ineffective symbiosis (i.e. form nodules but do not fix nitrogen) whilst a few are non-infective and do not form nodules at all (Table 5). These strains are

TABLE 5

Distribution of ineffective and non-infective strains in the RCR

	Number of strains	
Species	Ineffective on all hosts tested	Non-infective on all hosts tested
R. trifolii	44	8
R. leguminosarum	3	2
R. meliloti	6	0
R. lupini	1	0
R. japonicum	1	1
R. phaseoli	23	6
Rhizobium spp.	0	3

useful in studying the physiology of the *Rhizobium*–legume symbiosis. Since rhizobia are identified principally by their ability to form nodules the taxonomic position of these non-infective strains is uncertain. However, the identity of some of the clover strains has been confirmed serologically and it is hoped, in due course, to test for internal group antigens (Vincent and Humphrey, 1970; Vincent, Humphrey and Skrdleta, 1973) in the remainder. Even amongst effective rhizobia there is a spectrum of strains with different nitrogen-fixing abilities, and the better ones are listed in the RCR catalogue as 'recommended strains'.

Details of the collection's holdings are made available to the scientific community primarily by means of the RCR catalogue. However, many of the strains isolated from sites in the UK are also listed in a world catalogue of *Rhizobium* strains (Allen, Hamatova and Skinner, 1973). The production of this catalogue was first suggested in 1966 at a meeting of the International Biological Programme's Primary Production subcommittee

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in Moscow. Data were collected in Prague, Czechoslovakia and the USA during 1968 and 1969 but difficulties arose in its collation. In 1972 Skinner undertook to edit the information and the catalogue was published the following year.

The RCR is also listed in the *World Directory of Collections of Cultures of Micro-organisms* (Martin and Skerman, 1972) and its holdings detailed in the computerised records of the World Data Center for Microorganisms at the University of Queensland, Brisbane, Australia. The Data Center periodically updates its information and makes it available on request; it was set up by the World Federation of Culture Collections, of which the RCR is an affiliated member.

Use of the RCR

Since 1964 when detailed records were started the number of requests for cultures has increased by about four-fold (Fig. 2) to a maximum of 110 in 1976. With the proliferation of *Rhizobium* collections it might be expected that the number of requests will level off

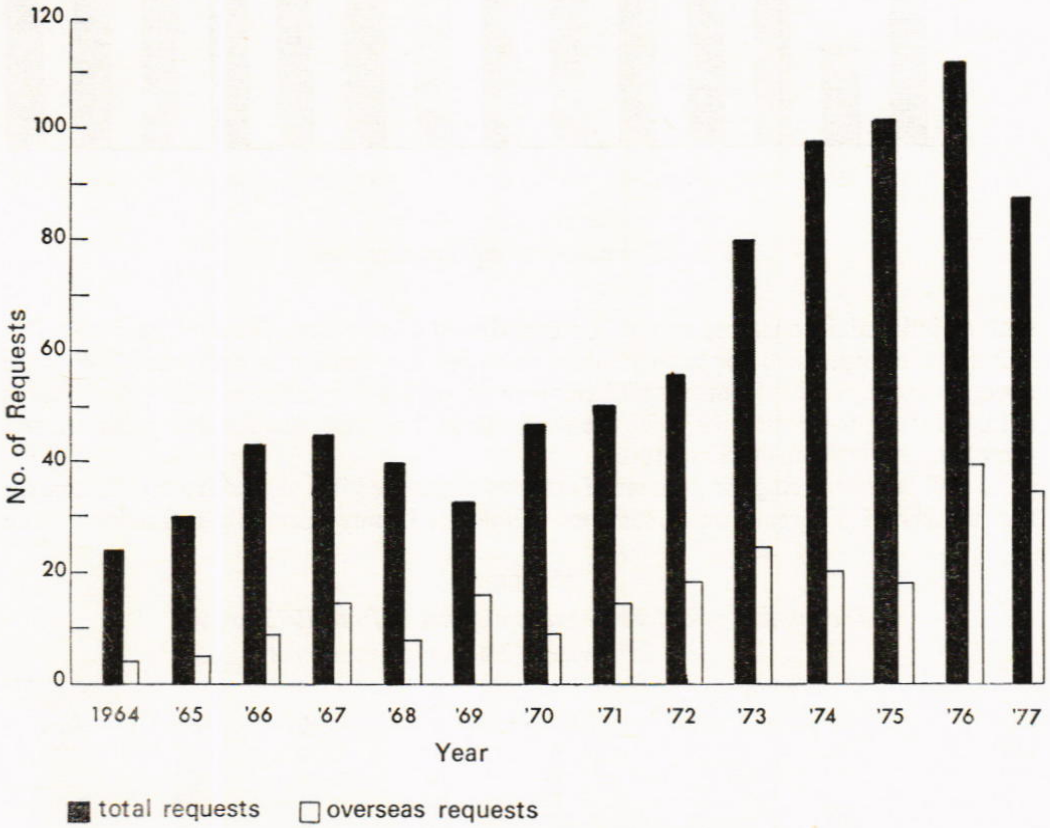

FIG. 2. The number of requests for cultures.

and may well decline. The beginning of this trend is suggested by the smaller number of requests in 1977 and reinforced by data for the first few months of 1978 which indicate a total of 80–90 may be expected over the whole year.

The number of cultures dispatched shows a similar pattern (Fig. 3), although the five-fold difference in scale should be noted. Included in the figures for 1964 are 273 cultures sent out, over the year, to a commercial seeding firm. Inoculants were not subsequently

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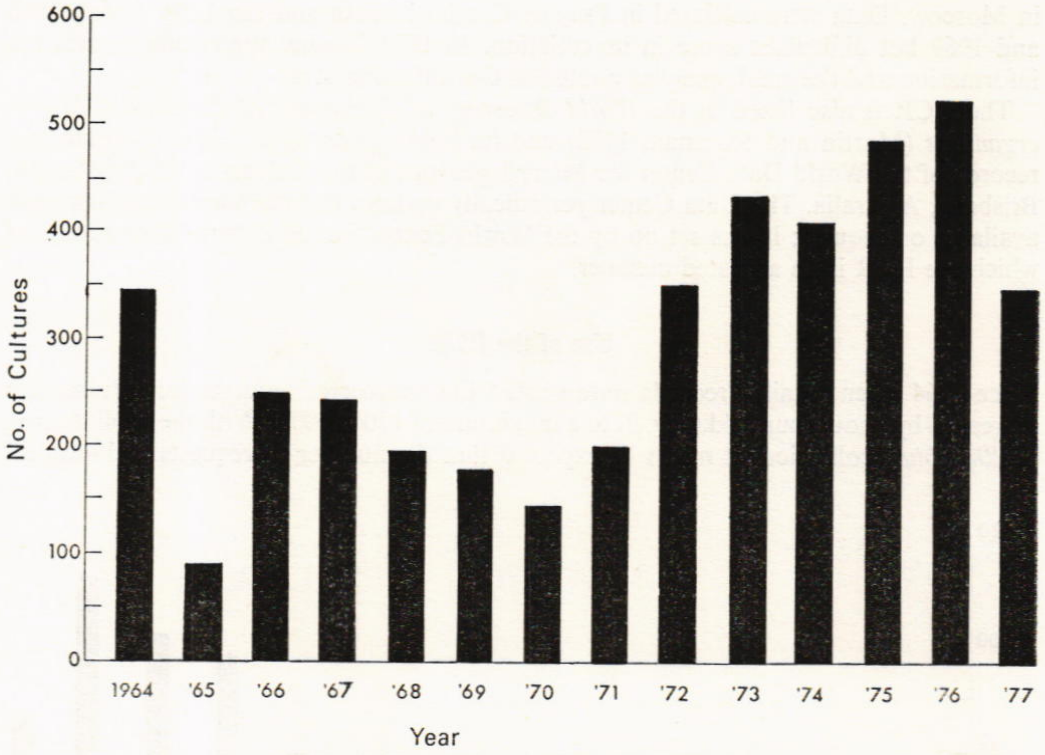

FIG. 3. The number of cultures dispatched.

sent to industrial concerns, except for experimental purposes. On average about five cultures are dispatched for each request, although the median is only two. This large average figure results from a small number of orders for large numbers of cultures (Fig. 4). Over the period 1973–77 the single largest request was for 104 cultures, and there were several for 40–50 cultures.

As well as considering the number of cultures dispatched it is also instructive to analyse the number of different strains sent out (Table 6). In any year only a fraction of the

TABLE 6

The number of different strains dispatched from 1973 to 1977

	Number of different strains sent out						
Year	*R. trifolii*	*R. legumino-sarum*	*R. meliloti*	*R. japonicum*	*R. lupini*	*R. phaseoli*	*Rhizobium* spp.
1973	63	18	5	10	4	7	27
1974	58	23	5	10	5	29	26
1975	29	50	7	30	7	47	16
1976	104	39	12	7	4	12	32
1977	22	13	12	14	5	15	35
1973–77 Overall	138	51	18	39	10	47	69
Total no. available	178	58	43	43	11	47	156

available strains are used, although there are notable exceptions. For example, in 1975 cultures of all the *R. phaseoli* strains were sent to one customer. Taking the last 5 years overall, a high proportion of the strains in the collection were actually sent out although

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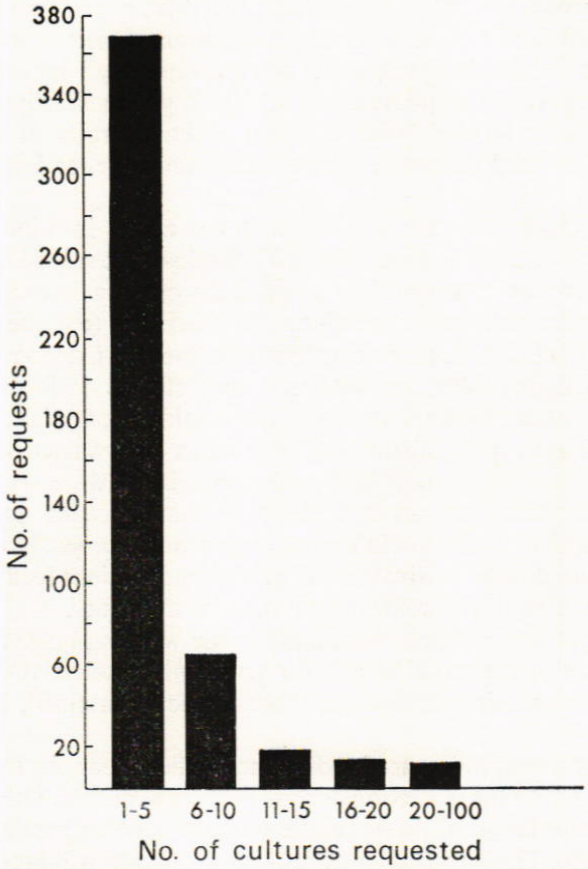

FIG. 4. The numbers of cultures dispatched per request over the period 1973–77.

few strains were dispatched more than twice in any year (Table 7) and only 12 of these were sent out, on average, more than four times a year over the period 1973–77. These latter strains, with the exception of 0403, are the 'recommended strains' mentioned earlier.

TABLE 7

The strains sent out more than twice in any year between 1973 and 1977

R. trifolii	*R. legumino-sarum*	*R. meliloti*	*R. japonicum*	*R. lupini*	*R. phaseoli*	*Rhizobium* spp.
1 (16)†	*1001 (54)	*2001 (78)	3402 (6)	3210 (12)	3601 (15)	3001 (14)
2 (3)	1003 (8)	2006 (4)	*3407 (82)	*3211 (44)	3602 (4)	3002 (8)
*5 (106)	1004 (3)	2009 (3)	3408 (3)		*3605 (49)	3007 (3)
6 (17)	1007 (7)	2011 (10)	3409 (3)		3607 (3)	*3824 (50)
9 (5)	1012 (3)	*2012 (33)	3410 (4)		3609 (3)	3826 (3)
16 (3)	1013 (4)	2035 (3)	3412 (14)		3610 (15)	3827 (13)
32 (9)	1016 (3)		3425 (3)		3611 (3)	3828 (3)
33 (3)	1017 (3)				3621 (3)	3829 (3)
49 (3)	1019 (3)				3622 (3)	3830 (3)
213 (3)	1038 (19)				3637 (3)	3832 (3)
*221 (32)	1044 (5)				*3644 (48)	3833 (3)
0402 (3)	*1045 (38)					3877 (13)
*0403 (28)	1048 (6)					
0412 (3)	1049 (4)					
0462 (3)	1055 (3)					

* Strains sent out on average more than four times per year over the period 1973–77
† Figure in brackets is the total number of times the strain was dispatched

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Requests are received from all over the world and there are few countries that have not obtained strains from the RCR. It is difficult to draw any general conclusions concerning the use cultures are put to since customers often do not say why they need the strains. Nevertheless, the majority of requests are for rhizobia giving effectively nodulated plants and so can be met with a small number of highly effective strains. It is evident therefore, that a collection of several thousand strains, common in general service collections, is unnecessary.

The RCR has recently been reorganised with the above in mind, 121 strains being selected to form a 'working collection' (Table 4). Strains were selected using the following criteria: their effectiveness, measured by greenhouse or, in a few cases, field trials; the possession of particular cultural or symbiotic characteristics (e.g. the formation of black nodules, induction of chlorosis, unusual colour reactions on Congo red containing media etc.); their citation in published reports; the completeness of information concerning their origin, a minimum requirement being the original host plant, date of isolation, country of origin and cultural history, although this was waived if, for example, the strains were highly effective or had been used in published work. A few ineffective and non-infective strains were also selected although the collection primarily contains a range of strains effective on the world's most important legumes. No attempt was made to include strains suited to all cultivars or all environments since local or national collections should maintain strains of relevance to their own locality.

A larger number of strains than is available in the working collection may be needed to meet the demands of taxonomists and for screening to select strains with particular characteristics. The remaining strains will therefore be maintained, but without further testing.

There is obviously a continuing need to find more efficient strains than the present ones and these will be added to the collection as they become available. Other new acquisitions must have some bacteriological or symbiotic characteristic of particular importance to qualify for acceptance. These restrictions mean the collection will grow more slowly than before.

Rhizobium inoculant production and control

Some soils do not have a natural *Rhizobium* population so appropriate rhizobia have to be added before a legume will grow without applied nitrogen. This was recognised as early as 1886, even before the organism responsible was isolated in pure culture.

Early attempts at inoculation involved transferring soil from a field successfully cropped with a legume, and hence containing a natural *Rhizobium* population, to one in which legumes had failed. This worked well but the cost of transporting the large volumes of soil needed was prohibitive and there was a real danger of spreading weeds and diseases. *Rhizobium* was isolated in 1888. This was announced at a meeting in Berlin, chaired by Sir Henry Gilbert, and the first pot experiments on inoculation at Rothamsted were done the following year. The first pure culture inoculants were produced in Germany during 1895, selling under the trade name 'Nitragin'. The rhizobia were grown on an agar medium, suspended in a fluid such as skimmed milk and used to inoculate the seed prior to planting. Realization that different legumes require different rhizobia was vital to the success of the industry. The early history of inoculant use has been reviewed by Fred, Baldwin and McCoy (1932).

Inoculants in the UK. In the UK early experience with inoculation was disappointing. At Rothamsted, H. G. Thornton began working on *Rhizobium* and developed a more successful inoculant. This was achieved by improving the growth medium, careful

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selection of strains and the development of a suspending medium which, at the time, was thought to help the bacteria to migrate from the seed into the root zone. Thornton then supervised field trials of lucerne at 29 centres throughout Britain. This work commenced in 1924 (Thornton, 1929) and showed that in the south-west, west and north every centre showed a gain from inoculation and untreated lucerne often failed completely. In the midlands and south most centres benefited although after one or two seasons the untreated plots often improved to equal the inoculated ones. Nevertheless Thornton considered that this strengthening of the young plant would be important in a bad season and recommended inoculation as an insurance. In the south-eastern counties inoculation was generally found to be unnecessary, although at acid sites (where rhizobia cannot survive) lucerne could only be grown after liming and inoculation. These results were later largely substantiated by trials at 44 more centres.

Demonstration of the advantages of inoculation prompted a demand from growers for lucerne inoculants. Rothamsted undertook to supply these cultures, 900 being provided in 1925 rising to about 2000 by 1929. Because of the time and cost involved in making inoculants it was decided to invite commercial firms to produce them and Allen & Hanburys Ltd. (then based in London) were finally chosen. A gentleman's agreement, rather than a legal contract, was entered into which allowed Allen & Hanburys Ltd. to print on their packets, 'Prepared . . . according to the Rothamsted Process. Approved by the Royal Agricultural Society of England'. This was conditional on Rothamsted regularly testing the product to ensure it was of an acceptable standard. One tube for every 1000 manufactured, starting at the 500th, was checked bacteriologically and by plant testing. Each tube contained sufficient culture for one acre of lucerne and cost 1/– (5 p).

This arrangement worked well, growers had confidence in the inoculants they used and consequently the sale of lucerne cultures gradually increased to a peak of about 40 000 annually in the mid-1940s, but then declined to 9–10 000 in the late 1950s and by 1964 to only 1–2000. This fall reflected, at least in part, the decreasing acreage sown with lucerne.

Inoculants for other legumes were added during the 1930s and 40s but their sale was insignificant compared with that for lucerne, and were undoubtedly uneconomic to produce. The company proposed stopping the sale of these lines in 1959 but were persuaded to continue by P. S. Nutman, Head of the Soil Microbiology Department at Rothamsted. However, when Allen & Hanburys became associated with Glaxo in 1963 these products were finally discontinued and Rothamsted again undertook to supply cultures (other than for lucerne) to growers. About 300 cultures were provided annually until 1965. Now they are supplied for experimental purposes only.

In 1965 Allen & Hanburys entered into an agreement with Chemicovens Ltd., with a view to withdrawing altogether from the inoculant business. In this agreement Chemicovens acquired exclusive rights for the sale of lucerne cultures. This company replaced the agar inoculant with a peat/soil-based one from Belgium, called 'Nodosit', which sold at 23/– (£1.15) per unit (to treat 2 acres of lucerne). Peat-based inoculants were developed in America and are made by mixing rhizobia, grown in liquid culture, with sterile neutralised finely ground peat. Well-made inoculants of this type are better than agar-based ones although Nodosit proved to be less successful than the Allen & Hanbury product.

Seed merchants began to import inoculants from various countries, some of which turned out to be useless, and Rothamsted lost all control over the quality of the products used by growers. This unhappy and chaotic situation lasted for more than 10 years although it has now improved to some extent. Today there are three major inoculant importers selling two Australian products, which are required to pass independent

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quality control checks in Australia, and an American product which, although not similarly controlled, has a good reputation amongst its American users. These inoculants are sold at about £3.50 for a packet containing enough material to treat 50 kg of lucerne seed.

Inoculants in Australia. It is instructive to compare the UK situation with that in Australia. Roughley (1962) has reviewed the development of the Australian inoculant industry. The Biology Branch of the New South Wales Department of Agriculture started distribution of cultures to growers in 1914, although very few were used initially. As in Britain a clear demonstration of the advantages of inoculation was required to boost demand for cultures. In 1945 about 3000 cultures were used, mainly for lucerne, pea, bean and clover, and by 1949 the total had not risen although the increasing use of subterranean clover as a pasture legume was apparent. The use of inoculants at this time was thus much lower than in the UK, although the greater diversity of legumes inoculated is notable.

In 1952 a method was developed for the large-scale production of peat-based inoculants. The Biology Branch sold about 20 000 of these in 1953 and because of greatly increasing demand handed over their production to commercial interests. This repeated the situation encountered by Rothamsted in 1929. Unfortunately most of the commercial products were of poor quality so in 1957 a voluntary quality control scheme was instigated and run by the University-Department of Agricultural Laboratory Service, later called the Australian Inoculant Research and Control Service (AIRCS). Originally this was financed by the manufacturers but is now wholly Government sponsored. The AIRCS control scheme undoubtedly 'weeded out' companies unable to produce good quality inoculants and today there are only two manufacturers active in this area.

In addition to its monitoring function, AIRCS also has an ongoing programme of trials to select better *Rhizobium* strains and provides mother cultures to the manufacturers.

Improvement of inoculants in the UK. The early introduction of peat-based inoculants in Australia and their independent quality control was a major advance, which ensured an excellent inoculant for use in that country. However, this implies no guarantee of their success in Britain for the following reasons: (1) adverse conditions during transport to this country may reduce the number of rhizobia in the inoculant; (2) incorrect storage at the distributors or retailers in Britain may have similar consequences; (3) *Rhizobium* strains suitable for conditions in Australia may not be so well adapted to conditions in Britain; (4) the UK farmer is less well informed on the proper application of inoculants than his Australian counterpart.

The first two points can be controlled by sampling the inoculants in Britain; this has hitherto not been possible. However, ADAS has now offered the British distributors a testing service and Rothamsted has undertaken the work in 1978 while ADAS sets up the necessary procedures ready for next season. All three major distributors have taken advantage of the scheme and all the samples tested so far have been of adequate quality. The testing procedures and standards are based on those given by Date (1969) and Date and Roughley (1977). If the scheme can be extended to include testing of samples from retail outlets it should eliminate crop failures caused by useless inoculants.

Seemingly there are no reports of attempts in Britain to select better lucerne strains since the early work of Thornton in the 1920s. To remedy this, 17 strains from the RCR have been compared, in a greenhouse trial at Rothamsted, with those used in the imported inoculants. Several RCR strains were as effective as the imported ones, but none was more effective. However, given enough effort, better strains can probably be

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found. In Australia cultures are supplied to AIRCS by regional government agencies who have tested them under local conditions. Regional ADAS laboratories could serve a similar function in this country, although a centre to do the final testing would need to be selected. It is important to note that strain selection is a two-stage process. Firstly, strains are screened in greenhouse trials and then the best of these are compared in the field, preferably at several sites.

If more efficient strains are isolated then it is likely that the Australian manufacturers would be willing to make inoculants using them. Alternatively a UK organisation could be set up for their production. Many British companies have recently approached Rothamsted to ascertain the possibilities in this field. It is unlikely any of them will pursue it further since the market is too small, at about 2000 packets (50 kg size) annually, for it to be profitable. However, there is room for expansion of the market particularly if improvement of hill pastures with white clover proves feasible, if a cold-tolerant Navy bean (*Phaseolus vulgaris*) cultivar suitable for the British climate is developed and if new fodder or grain legumes, such as lupins, are grown more widely. In addition, few of the EEC countries have local inoculant manufacturers. Another alternative to commercial inoculant production in the UK is for a government establishment to undertake their manufacture.

Summary

1. During the early years when the collection was used only for departmental purposes, strains of *Rhizobium* were maintained by subculture. In 1964 the strains were vacuum-dried and in the same year the collection took on a 'service' role.
2. The holdings of the collection have increased from 211 in 1964 to 536 at the present time and have become more diversified, reflecting the changing research interests of the department.
3. The number of requests for cultures has increased four-fold over the past 14 years, although the rise is now tailing off. This trend is matched by the number of cultures dispatched; two (median value) being sent for each request.
4. Over the period 1973–77 a large proportion of the available strains has been requested, although in any 1 year few strains are sent out more than twice. Only 12 strains were sent out, on average, more than four times over the same period; these are the 'recommended strains'.
5. Requests are received from all over the world and the majority can be met with a few highly effective strains.
6. The RCR has recently been reorganised; 121 strains being selected to form a 'working collection'. Other strains will be maintained but no more work carried out on them.
7. The history and present problems of *Rhizobium* inoculant use in the UK have been discussed, and suggestions made for improvement.

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https://doi.org/10.23637/ERADOC-1-17