STUDIES ON LEGUME NODULE BACTERIA.

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Introduction.

<u>Studies on Legume Nodule Bacteria</u> is offered as a thesis to the Faculty of Science of the University of Edinburgh in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

For convenience of presentation the thesis has been divided into three sections - I. Variation in Strains of Clover Nodule Bacteria; II. Effects of Calcium in Media on Clover Nodule Bacteria; and III. Nodule Bacteria of Various Legumes of Scotland. In the first is discussed certain aspects of clover inoculation and field and glasshouse trials of strains of legume organisms in both New Zealand and Scotland. The second covers work on the effects of culture media on characters and efficiency of strains of the clover organism. In the third section an outline is presented of the nodule organisms of Scottish legumes together with an analysis of the classification of nodule bacteria.

The outbreak of war unfortunately necessitated a return to New Zealand before these studies were completed; with the result that certain phases of the work had to be finalized in the Dominion. While at Edinburgh the work was carried out under the direction of Professor T.J. Mackie and Dr. T. Gibson of the University of Edinburgh.

Grateful thanks are offered to Miss B. Shearer, B.Sc. (Hons.), assistant to Dr. Gibson, for harvesting and forwarding clover material to New Zealand, and for arranging for photographs to be made of final glasshouse clover trials.

STUDIES ON LEGUME NODULE BACTERIA.

I. VARIATION IN STRAINS OF CLOVER NODULE BACTERIA.

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I. VARIATION IN STRAINS OF CLOVER NODULE BACTERIA.

PRELIMINARY INOCULATION TRIALS IN NEW ZEALAND.

In the years 1933, 1934 and 1935 the Department of Agriculture of New Zealand carried out in various parts of the Dominion some 160 experiments, under the direction of the author, to test the value of inoculating red and white clover seed with cultures of nodule bacteria. The seed was included in a grass-seed mixture, and a liming experiment was incorporated in the trials. Each trial was laid down according to the following plan:-

1 chain

	Limed	Not Limed
Seed plus inoculated clover		
Seed with uninoculated clover		

The culture used was obtained from nodules taken from a vigorous plant of certified white clover grown at the farm of the Grasslands Division, Palmerston North. The inoculum was added as a suspension in milk in the manner recommended for the inoculation of lucerne seed in New Zealand* (Reid, 1930).

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* 1. Into each bottle pour repeated small quantities (2-3 tablespoons) of skimmed milk, agitating bottle thoroughly each time. Pour into vessel until sufficient milk is used to moisten seed ($\frac{1}{4}$ pint sufficient for 30 lb. seed).
2. Strain through muslin to remove jelly, which should be thrown out.
3. Mix seed with filtered milk culture in clean basin.
4. Spread inoculated seed on clean cover and dry in cool place. Drying, with occasional stirring, will not take more than $\frac{1}{2}$ hour, when seed is ready for sowing.

Periodically plots were examined by Instructors in Agriculture stationed in the different districts, and on 1 August 1936, reports from the experiments were summarised in Table I.

TABLE I. Summary of Reports of Field Trials of Clover Seed Inoculated with a Strain of White Clover Nodule Bacteria.

Result of Inoculation	Reports from	
	South Island	North Island
a. Growth after inoculation consistently better.	3	8
b. No improvement in early stages, but fair to good results later.	8	11
c. Good results in early stages and moderate results later.	0	5
d. Slight improvement in early stages, but no response later.	15	8
e. Uninoculated better than inoculated.	0	3
f. Experiments failed through poor "strikes".	14	2
g. No effect apparent.	50	33

Liming generally improved the stand of clover both in inoculated and non-inoculated plots. The differences in response to inoculation in the two Islands are discussed on page 11.

Results showed that improvement in growth of clover occurred in only 33 per cent. of the trials. They suggested that the strain of organism employed was not suitable for all conditions. Accordingly further experiments were undertaken to determine if a more efficient strain of nodule organism could be selected. Early in 1936 bacteria were isolated from clover nodules obtained from many sources covering a wide range of New

Zealand soils. A strain from subterranean clover was also secured from the Waite Institute, Adelaide, South Australia. The cultures were used for inoculating white clover plants grown under semi-controlled conditions in a glasshouse, and variations in growth of the plants were recorded. Cultures selected from these strains were later used in field experiments.

GLASSHOUSE TRIALS IN NEW ZEALAND.

Isolation of Nodule Bacteria.

Small portions of roots bearing nodules were washed in running water, immersed for one minute in 95 per cent. ethyl alcohol, washed in three or four changes of sterile water, steeped for five minutes in 1/1000 acidulated mercuric chloride, and finally washed in four changes of sterile water. A small portion of the interior of a nodule was transferred to a Petri dish containing mannitol medium, crushed on the wall of the dish, and then spread over the surface of the medium with a bent glass rod. The medium was made up according to the following formula*, and is similar to that used by Ashby (1907).

Mannitol	1.00 per cent.
$MgSO_4,7H_2O$	0.02 " "
KH_2PO_4	0.02 " "
NaCl	0.02 " "
$CaSO_4.2H_2O$	0.01 " "
$CaCO_3$	0.50 " "
$FeCl_3$	2 drops of 1.0 per cent. solution.
Agar	1.50 per cent.

* Private communication (1928) from Dr. H.G. Thornton, Harpenden, England.

After inoculation plates were incubated for five or six days at 25° C. when single colonies were transplanted to slants of Thornton's (1931) lucerne root extract medium, prepared as follows:-

K_2HPO_4	1.0 g.
$MgSO_4.7H_2O$	0.2 g.
$CaCl_2$	0.1 g.
NaCl	0.1 g.
$FeCl_3$	0.02 g.
$CaCO_3$	5.0 g.
Sucrose	10.0 g.
Lucerne root extract	from approximately 100 g. roots.
Agar	15.0 g.
Water, made up to	1.0 litre.

Treatment of Material.

Seed from a single-plant selection of a certified strain of white clover was used for the tests. Seeds were disinfected in the manner used for roots as described on the preceding page. While still moist they were germinated in two four-inch pots of sand previously steamed for two hours in an autoclave at 30 lb. pressure, and each pot was placed in a cellophane cage to lessen the chance of contamination. Seedlings were then **transplanted** to the test pots, a practice which avoided inclusion of dead or hard seeds and possible effects on subsequent inoculation of mercuric chloride not removed in washing.

For the inoculation tests grey-wacke river sand, with particles ranging from 3 mm. to 0.25 mm., was used. The sand was washed in running tap water and placed in six-inch unglazed flower pots together with five grams of calcium carbonate. Each pot with its contents was then sterilised in the autoclave for four hours at 30 lb., transferred to the glasshouse and placed in separate shallow trays.

Six clover seedlings, in the first leaf stage, were planted in each pot, and six wheat seeds, sterilised in the same manner as the clover seed, were planted between the clover seedlings. Pots were then inoculated with the various cultures of nodule bacteria, by pouring around each clover seedling a few drops of a water suspension from a three day old slant. Treatments were prepared in duplicate and arranged in randomised form in the glasshouse. After inoculation pots were saturated with Bryan's (1922) nutrient solution, and each week thereafter were flooded with two applications of sterile tap water and one of nutrient solution. Any excess of nutrient solution collecting in the trays was discarded.

The pots were sown 20 October 1936, and on 21 April 1937 the clover and wheat plants were cut off at sand level, dried and weighed.

Results.

Results are given in Table II, together with the origin of those cultures used in the test.

TABLE II. The Effect of Strains of Clover Nodule Bacteria on the Dry-weight Yield of White Clover and Wheat Plants.

Sown Palmerston North, New Zealand, 20.10.36, and harvested 21.4.37.

Strain number	Origin of culture	Dry-weight yield in grams					
		Wheat			Clover		
		Per pot		Av'g[e]	Per pot		Av'g[e]
	Check, not inoculated	9.85	9.45	9.65	7.2	12.5	9.8
1	Red clover Christchurch, S.I*	2.95	3.40	3.1	23.1	30.9	27.0
2	White " Greymouth, S.I.	3.50	4.75	4.1	39.4	44.2	41.6
3	" " PioPio, N.I.	5.25	4.30	4.8	30.8	18.5	24.6
4	" " KeriKeri, N.I.	5.35	4.85	5.1	18.8	29.3	24.0
5	Red " Hororata, S.I.	7.35	4.20	5.6	18.1	31.3	24.7
6	White " " S.I.	5.10	6.35	5.7	21.7	46.7	34.7
7	" " Blenheim, S.I.	6.35	5.25	5.8	23.6	27.8	25.7
8	" " Christchurch, S.I.	5.30	7.65	6.5	41.2	39.3	40.2
9	Red " Otorohanga, N.I.	9.80	5.50	7.6	45.3	30.5	37.9
10	White " Tangimoana, N.I.	14.30	5.80	10.0	25.2	27.0	26.1
11	" " Winton, S.I.	12.20	7.60	9.9	17.2	34.5	25.8
12	" " Tiritea, N.I.	5.05	21.15	13.1	22.8	12.0	17.4
17	Subterranean clover Waite Institute	12.10	14.40	13.2	18.0	29.6	23.8

* S.I. = of South Island origin: N.I. = of North Island origin.

e Av'g = average dry-weight yield of duplicate pots.

Discussion of Results.

During the period of growth the wheat plants in all treatments were yellow and unthrifty, whereas the clover plants were dark green and generally vigorous. The uninoculated checks were stunted and pale in colour. In a number of treatments a decided variation in yield, both of wheat and clover, occurred in the duplicate pots. This was particularly noticeable in those which produced the higher average yields of wheat, namely in treatments 10, 11, 12, and 17. A high wheat yield in a pot was usually associated with a low yield of clover, suggesting that growth of the wheat was governed largely by the available growing space. Wide variations in yield of clover also occurred in duplicate pots of treatments where the wheat yield was low in both pots. The cause of these is not known. Average yields of the treatments suggested, however, that the strains of nodule bacteria varied in efficiency in promoting growth of white clover plants.

In April 1937, two additional trials were carried out. They differed in some respects from the earlier one in that the wheat seed was omitted, treatments were carried out in triplicate, and three additional strains were used. The latter were secured from the following sources:-

No.13 from white clover, Palmerston North, North Island, New Zealand.
No.14 " " " Otorohanga, " " " "
No.15 " subterranean clover, Christchurch, South Island," "

Trials were laid down on 7 and 21 April 1937, and terminated 12 November 1937. Growth of the clover plants was examined periodically. Plants were not weighed, visual differences in growth being sufficiently great to permit grouping of the treatments.

On this basis the strains were arranged in the following four groups, ranging from group 1 which comprised those with the most luxuriant growth - approximately six inches in height - to group 4 which included those with the least growth - approximately 1.5 in. in height.

Group 1. Cultures 5, 6, 9, 12.
Group 2. " 1, 3, 4, 13, 8.
Group 3. " 2, 7, 10, 11.
Group 4. " 15, 17.

Growth of plants of group 4 was similar to that of the plants of the uninoculated check pots. The grouping does not appear to bear any relation to yields obtained in the combined clover-wheat trials.

FIELD TRIALS IN NEW ZEALAND.

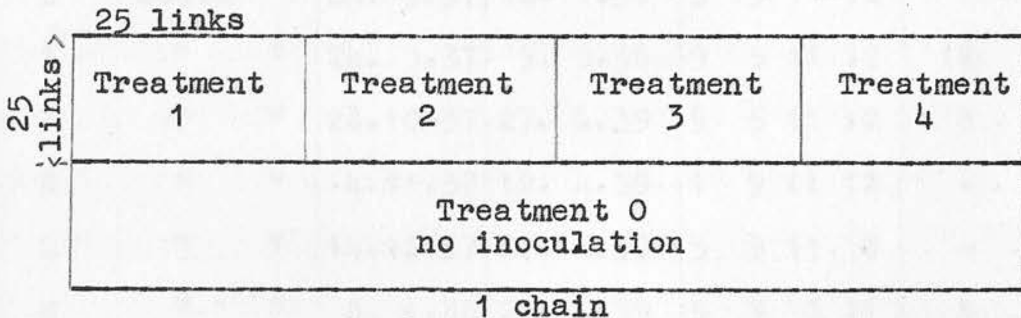
In 1937 field trials were laid down in many districts to find out if an efficient strain of nodule bacteria could be selected for general use in New Zealand, using for the purpose some of the cultures selected according to the above grouping.

Preparation and Lay-out of Experiments.

Strains 5, 9, 12 and 11 were employed in the majority of the trials, the first three because they produced excellent clover growth in the glasshouse, the last because it produced consistently poor clover growth. Trials were laid down on different types of farm lands - dairy, sheep and agricultural - distributed over both Islands. Each was arranged according to the following plan and was usually included as a central portion of a field being sown to a similar grass-clover seed mixture. The seed was weighed,

treated and mixed in the laboratory within two or three days of the time of sowing. Sufficient of each seed was obtained in advance for all trials, and the seed mixture was made up at the time of treating the clover seed. The red and white clover seeds were first mixed in amounts required for each treatment and disinfected by soaking in water for ten minutes, then in 95 per cent. ethyl alcohol for ten minutes and finally by washing in five or six changes of sterile water. Surplus water was drained off and a milk suspension of the selected culture mixed with the seed. The clover seed of each treatment was then dried and added to the untreated ryegrass. The disinfected clover seed for the control plot was treated with sterile milk.

Plan of Field Trials of Clovers Treated with Four Strains of Nodule Bacteria.



The grass-clover mixture contained certified perennial ryegrass, 15 lb. per acre, certified white clover, 4 lb. per acre, Montgomery red clover, 3 lb. per acre. Fertiliser composed of 16 lb. superphosphate and 56 lb. carbonate of lime was applied before seeding. The seed was distributed over well rolled ground and raked immediately, care being taken that the non-inoculated or adjoining areas were not touched in the process.

Trials were kept under observation for 9 to 24 months and differences in growth of clovers and ryegrass were periodically recorded.

Results.

Results are indicated in Table III.

TABLE III. Field Trials of Clovers Inoculated with Strains of Clover Nodule Bacteria.

Trial	Location	Sown	Completed	Cultures used	Efficient cultures
A	North I'd	10. 3.37	10.12.37	9 5 13 12	-
B	" "	17. 3.37	25. 8.38	9 13 11 12	12
C	" "	19. 3.37	16. 5.38	5 1 11 12	12* 5
D	" "	25. 3.37	27.9.38	9 13 11 12	13
E	" "	12. 4.37	1.11.39	5 9 4 12	12* 9
F	" "	28. 4.37	28. 6.39	5 9 4 13	13* 9
G	" "	14. 4.37	5. 8.38	9 13 11 12	-
H	South "	24. 3.37	12. 1.38	5 13 11 12	-
I	" "	24. 3.37	3. 3.38	9 5 11 12	12
J	" "	28.10.37	27. 4.39	9 5 11 12	5
K	" "	4.11.37	12. 4.39	1 9 11 12	-
L	" "	14.12.37	27. 4.39	5 9 11 8	-
M	" "	5. 1.38	27. 4.39	5 9 3 11	5
N	" "	26. 1.38	27. 4.39	5 9 15 11	15
O	" "	1.12.37	27. 4.39	5 9 11 8	5 11
P	" "	14. 1.38	27. 4.39	9 11 1 10	10

* Where two cultures showed to advantage the better one is denoted by *. The - sign indicates that no difference between treatments, including uninoculated plot, was observed. Apparently the soil of the areas was already infected with nodule bacteria and good nodulation was obtained on all plots.

Discussion of Results.

Cultures 12 and 5 gave a growth response in four trials, cultures 13 and 9 in two, and cultures 15, 11 and 10 in one trial each. It is of interest that strain 15 was originally derived from nodules of subterranean clover and in trial N. was the most efficient strain in promoting growth of red and white clover. Strains 9, 12 and 13 were isolated from clovers collected in the North Island and strains 5, 11 and 15 from those obtained from the South Island. Strain 12 produced good results in three areas in the North Island and one area in the South Island, and strain 5 did likewise in three trials in the South Island and one trial in the North Island. Strains 9 and 13 were effective only in the North Island, and strain 11 only in the South Island, where its effects were limited to the district from which it was obtained. Strain 10, isolated from white clover grown in the North Island, was exceptional in that it produced the best growth in trial P in the southern part of the South Island. It was obtained from clover growing in a poorly drained area in sand dune country of the coastal district near Palmerston North. In Table I it was shown that most of the good responses to inoculation were obtained in the North Island. The strain of organism used for these first trials was obtained from the same plot of clover plants as strain 13, and within a few miles of the source of strain 12.

Results presented in Table III indicate that strains of nodule bacteria varied in their efficiency in promoting growth of clover. Most of the experiments showed that for efficient inoculation of clovers in the Dominion selected strains are required

for each Island. A similar inference has been drawn by Briscoe & Andrews (1938) with strains of <u>Rhizobium</u> of soybean for they stated that following collection of strains from various sources and testing them in one locality,-

> "There is an indication that strains of soybean bacteria isolated locally are more efficient than strains obtained from different climates".

This aspect of legume inoculation is of considerable economic importance; but more experimental work is required to ascertain if locality influences efficiency of strains under all conditions.

FIELD TRIALS IN SCOTLAND.

<u>Sources of Cultures.</u>

In the year 1938 work on the clover nodule organism was transferred to Scotland and numbers of field and glasshouse trials were carried out under the direction of the East of Scotland College of Agriculture, Edinburgh. The strains mentioned were used, together with additional ones from southern Scotland and the United States of America. Sources of the latter were:-

No.18. White clover nodules from old pasture, Baads Mill, Lothian, Scotland.
No.19. " " " " " " Bog Hall, East Lothian, Scotland.
No.20. Wisconsin Culture 186 from the collection of Dr. A. Cunningham, East of Scotland College of Agriculture, Edinburgh.
No.16. An atypical single colony isolation from culture No. 20.

The Wisconsin strain was being used successfully by the College for establishment of red and white clover on the peat reclamation areas of Southern Scotland. Strains 18 and 19 were isolated from nodules in the manner described on page 3, but were plated on yeastrel mannitol agar instead of on Ashby medium. Strains 1 to 15 and 17

had been maintained for approximately two years on either the modified medium of Ashby (1907) or the root extract medium of Thornton (1931), slants of the latter, in two ounce bottles, being used for storage and transport of the cultures.

For the field trials were used three of the strains tested in New Zealand, together with strains 17, 18 and 20 from South Australia, Scotland and the United States of America respectively.

Preparation and Lay-out of Experiments.

Field trials were carried out on a peat reclamation area at Carnwath, Lanarkshire, where a newly drained and cultivated field of 30 acres was being prepared for seeding to pasture. Three inches of the surface peat had been burned just previously. Further cultivation and rolling were given and lime at 1.5 tons per acre and phosphatic fertiliser at 4 cwt. per acre were broadcasted and harrowed in. No nitrogen was applied with the fertiliser. After being fenced, the area was subdivided to provide 49 plots, each 9 ft. 6 in. by 5 ft. 3 in., and separated by paths 5 ft. 3 in. in width. It was believed that the area was free from clover nodule organisms as no clover plants had been observed growing thereon. The only legume in the vicinity was Lotus corniculatus growing on the banks of a stream 50 yards away. Tractors and implements used in cultivation were not disinfected. The accompanying plan illustrates the lay-out of the plots.

Plan of Plots for Field Trials of White Clover Treated with Six Strains of Clover Nodule Bacteria.

Row	Plot 1	2	3	4	5	6	7
1	3	6	11	17	18	20	C
2	6	20	3	C	11	18	17
3	20	18	C	6	17	3	11
4	18	17	20	11	6	C	3
5	17	C	6	20	3	11	18
6	11	3	17	18	C	6	20
7	C	11	18	3	20	17	6

The figures in the plots indicate the culture treatment of each plot, checks being indicated by the letter C.

To avoid transference of nodule organisms from plot to plot implements and materials were disinfected after use on each. Commercial wild white clover seed was immersed in 80 per cent. ethyl alcohol for ten minutes, (Bushnell & Sarles, 1937), washed in five changes of sterile water, dipped for four minutes in water at 65°C. (Helz *et al.*, 1927), cooled by immersion in cold sterile water, placed in Petri dishes and dried for five hours over calcium

chloride in an evacuated dessicator. Samples spread on plates of yeastrel agar showed that the method of disinfection and the viability of the seed were satisfactory. Seed in 2.07 g. lots sufficient for each plot at a sowing of four pounds per acre, was distributed in test tubes.

To each slant of a four day old culture on yeastrel agar was added 5 ml. of sterile skimmed milk. Four drops of the prepared suspension were added to each lot of clover seed which, after being air dried in a Petri dish, was mixed with seven grams of timothy seed. The latter had previously been killed by heating in a hot-air oven at 143° to 146°C. for four hours, and was added to the clover seed to facilitate distribution at the time of sowing. Seven series of seven plots were sown with strains 3, 6, 11, 17, 18, 20 and a non-inoculated check. Seed for the check plots was disinfected, treated with sterile milk, and diluted with timothy seed in the same manner.

Plots were sown 24 and 25 May, rows one to four being sown one day and rows five to seven on the following. Seed of each plot was sown in nine drills seven inches apart, and replications randomized as is shown in the plan. Tools, boots and hands were disinfected between plot seedings, by immersion in a commercial disinfectant containing phenol. On 1 June perennial ryegrass, 30 lb. per acre, was sown on the paths separating the plots. Cold weather retarded germination and subsequent growth, but by 28 June clover seedlings were well developed on all plots.

Examination and Sampling of Clover Plants.

Except for one examination of nodule development on 5 July, plots were not disturbed until September 1938. No exceptional changes in weather conditions, or height of the soil water level were observed through the growing period. On the former date a few plants were taken from different parts of each plot. Nodules were present on practically all seedlings of the plots sown with inoculated seed, but in the check plots were fewer in number and present on only 30 per cent. of the plants. In plots sown with inoculated seed, nodules were situated chiefly in the vicinity of the crown, tap root and main lateral roots, whereas in the check plots they were chiefly confined to the smaller laterals. At the time of examination marked differences in growth in the various plots were apparent, differences which persisted for at least 15 months. (Figs. 1 & 2).

The source of the organisms forming nodules on the check plotsis not known. Although clover plants had not been observed on the area nodule bacteria could be carried thereto by soil attached to tractors and cultural implements, or by birds, insects or even wind. A possible source was plants of _Lotus corniculatus_ growing near the plots, though this is unlikely since in work discussed below it is shown that no evidence has been secured indicating that strains from species of _Trifolium_ and _Lotus_ are interchangeable.

On 12 September, approximately five months after seeding, work on sampling was commenced. Turves of clover, both tops and roots were removed from the first row of plots, and as time allowed samples were taken from the others. Delay was occasioned by the

Strain 17.

Strain 11.

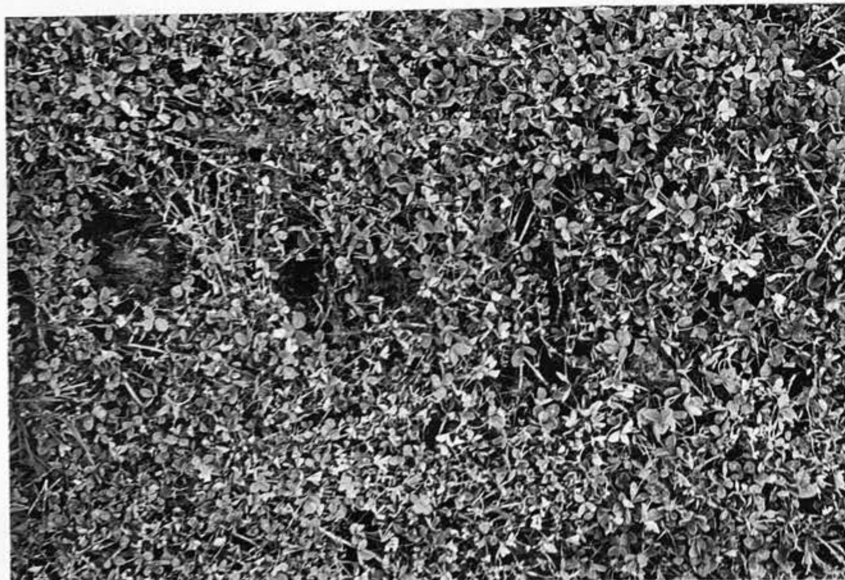


Fig. 1. Field Trials. Comparison of growth of white clover inoculated with strains 11 and 17 four months after sowing.

Strain 18.

Strain 11.

Fig. 2. Field Trials. Comparison of growth of white clover inoculated with strains 11 and 18 one year after sowing.

time required to separate the peat from the roots, so that sampling was not completed until 3 October. Sampling consisted of removing four one-foot turves from each plot, samples being taken 18 inches from each end of both the second and eighth drills. Where the growth was poor or uneven, further one-foot samples were taken, either as extensions of the first samples or from the middle drill of the plot. The peat was separated from the roots by washing in water, and the plants, tops and roots, dried at 103°C. ground in a mill and weighed. During the process of washing nodule development was noted. Dry-weight yields of the plots, based on four one-foot samples, are given in Tables IV and V. In the former, yields are arranged according to the plan of the plots; in the latter they are tabulated according to the treatment.

<u>Nitrogen Analyses of Clover Plants.</u>

The nitrogen content of the dried samples was determined by the Kjeldahl method as set out in the Statutory Rules and Orders, 1932, of the Fertilisers and Feeding Stuffs Act, 1926. Preliminary determinations by the standard method proved to be unreliable, in that repeated tests of one sample gave different results, consequently modifications in the procedure were necessary to produce consistent nitrogen yields.

For the purpose, finely ground clover material was dried for four hours at 103°C., and from 0.6 g. to 0.8 g. was weighed into each digestion flask. Nitrogen free acid of 25 ml. to 30 ml., 7 g. of potassium sulphate, 0.25 g. copper sulphate, and a small piece of solid paraffin were added to each flask. Samples were in duplicate. After digestion the contents of the flasks

were washed into two-litre distillation flasks and granulated zinc, indicator, and the requisite excess of concentrated sodium hydroxide were added. Distillation was into N/14 sulphuric acid. A sample analysis is as follows:-

Period of digestion	1 hr.	2 hr.	3 hr.	4 hr.
Per cent. nitrogen	3.99	3.95	3.92	3.52

Reduction in volume of the acid in the flasks varied considerably, and digestion was stopped when the volume approached 10 ml. Appreciable reduction in volume occurred in the 1 hr. and 2 hr. periods in the above test.

In two further tests boiling was maintained at an even rate in the flasks of each set; and in one the sand baths were removed and the gas flames allowed contact with the digestion flasks. These tests provided the following results:-

Period of Digestion		1 hr.	2 hr.	3 hr.	4 hr.
Per cent. Nitrogen;	with sand bath	3.57	3.63	3.87	3.92
" " " :	without sand bath	3.90	3.90	3.96	3.99

A lengthy period of heating was required to obtain the best results. As the work of Smith & Patterson (1937) suggested that the period might be reduced by using 0.3 per cent. selenium as the catalyst, 3 g. were mixed with 97 g. of potassium sulphate and 10 g. of the mixture was introduced into each flask. Two tests, over naked flames, gave slightly improved results, as follows:-

Period of Digestion	0.5 hr.	1 hr.	1.5 hr.	2 hr.
Per cent. Nitrogen (a)	3.90	3.95		
" " " (b)	3.84	3.97	4.01	4.00

Difficulty arose through occasional solidification of the contents on cooling, so that the amount of potassium sulphate-selenium mixture was reduced to seven grams, and the selenium increased to four per cent. In later tests it was found that when the condensation ring in the neck of the flasks was maintained at approximately 85 mm. from the bulb of the flask, consistent and maximum results were obtained.

It became evident that the best results were secured when seven grams of the potassium sulphate-selenium mixture were used and the digestion flasks were heated for 1.5 hr. over naked flames. The period of digestion is longer than that recommended by Smith & Patterson for nitrogen determinations of potato, but is much shorter than is required when copper sulphate is used as the catalyst.

Results.

Dry-weight Yields of Clover Plants.

Dry-weight yields of the plots, based on four one-foot samples, are given in Tables IV and V.

TABLE IV. The Effect of Strains of Clover Nodule Bacteria on the Dry-weight Yield of Clover Plants. Field Trials.

		Plot						
		1	2	3	4	5	6	7
Row 1	Yield	53.26	27.68	64.02	38.80	40.31	49.80	27.50
	Culture	3	6	11	17	18	20	C
2	Yield	46.19	25.87	23.16	29.62	67.16	15.27	24.40
	Culture	6	20	3	C	11	18	17
3	Yield	36.48	41.70	32.26	25.80	11.28	14.28	43.26
	Culture	20	18	C	6	17	3	11
4	Yield	21.90	27.61	25.65	53.15	23.05	31.02	26.22
	Culture	18	17	20	11	6	C	3
5	Yield	38.22	39.87	32.30	47.30	38.44	64.42	53.45
	Culture	17	C	6	20	3	11	18
6	Yield	74.62	41.72	25.20	18.28	34.35	51.83	41.47
	Culture	11	3	17	18	C	6	20
7	Yield	40.18	60.58	20.25	50.55	35.79	46.47	44.32
	Culture	C	11	18	3	20	17	6

The dry-weight yields are of tops and roots in grams, of total of four one-foot portions of drills, arranged according to plan of plots.

TABLE V. The Effect of Strains of Clover Nodule Bacteria on the Dry-weight Yield of Clover Plants. Field Trials.

	Strain Treatment						
	3	6	11	17	18	20	Check
Row 1	53.26	27.68	64.02	38.80	40.31	49.80	27.50
2	23.16	46.19	67.16	24.40	15.27	25.87	29.62
3	14.28	25.80	43.26	11.28	41.70	36.48	32.26
4	26.22	23.05	53.15	27.61	21.90	25.65	31.02
5	38.44	32.30	64.42	38.22	53.45	47.30	39.87
6	41.72	51.83	74.62	25.20	18.28	41.47	34.35
7	50.55	44.32	60.58	46.47	20.25	35.79	40.18
Average	35.37	35.88	61.03	30.28	30.16	37.48	33.54

The dry-weight yields are of tops and roots in grams, of total of four one-foot portions of drills, arranged according to treatments.

Results given in Table V show that the average dry-weight yield of samples from treatment 11 is considerably higher than of any other treatment. Conversely the yields associated with the other treatments are not widely divergent so that it has been necessary to employ the Analysis of Variance (Fisher, 1938; Paterson, 1934) to ascertain if the differences are significant.

Statistical Analysis of Yields.

Analysis of Variance.

Source of Variation	Degrees of freedom	Sum of Squares	Mean Square
Rows	6	1884.9	
Columns	6	588.9	
Strains	6	4755.0	792.5
Plot Error	30	3041.5	101.4
Total	48	10270.3	

The above analysis is based on the data contained in Table IV.

Mean square plot error 101.4

Standard error of 7 plots $\sqrt{\frac{101.4}{7}}$ = 3.8

Average yield of all plots 37.7 g.

Average yield of treatment 11 61.0 g.

The deviation of the average of treatment 11 is 23.3 or more than six times the standard error. Eliminating treatment 11, which appreciably affects the average yield of all treatments, the average yield of the remainder, including the check treatment, is 33.8 g. Allowing twice the standard error as reasonable, there is a range from 26.2 to 41.4. All treatments lie well within this range, which indicates that in the trials the dry-weight yields associated with strains 3, 6, 17, 18, 20 and with the check treatment, cannot be differentiated from each other. On the other hand strain 11 has given a most notably high result.

In August 1939, fifteen months after sowing, field plots were again sampled by cutting the herbage on an area within each plot of four feet long by two feet wide. The portions of the plots

previously harvested were not included in the area. Plots had not been treated or stocked in the intervening period, and in most the plants formed a more or less continuous mat on the peat. The dry-weight yields from the samples, arranged according to treatments, are given in Table VI. For comparative purposes average yields from the four one-foot turves of the first harvest, 1938, are also given.

TABLE VI. The Effect of Strains of Clover Nodule Bacteria on the Dry-weight Yield of Tops of Clover.

	Treatment and Yield in Grams						
	3	6	11	17	18	20	Check
Row 1	194	206	234	183	234	232	210
2	224	230	290	197	160	267	192
3	157	205	297	164	213	113	194
4	210	196	265	171	154	263	227
5	198	217	292	254	212	222	305
6	275	219	307	226	188	239	208
7	244	211	294	204	167	244	268
Average	214	212	282	200	189	226	229
Average 1938 Samples	35.37	35.88	61.03	30.28	30.16	37.48	33.54

Samples 4 ft. by 2 ft. taken 15 months after seeding.

The Table shows that average yields produced by the treatments are, in respect to each other, relatively the same as in the first harvest. Strain 11 maintained the highest yield, though its superiority over the other treatment yields was not so marked. Similarly there was a general levelling of the yields of the plots within a treatment, though those which gave high or low yields in the first harvest gave, in most cases, similar results in the final sampling. In this respect a few discrepancies are apparent. Strain 3, row 1, and strain 20, row 3, have given lower yields, and strain 20, rows 2 and 4, and the check plot in row 5, have given higher yields than would be expected from results of the earlier harvest. The cause of these fluctuations is not known, though possibly induced by differences in plot levels and therefore differences in depth of peat above the water level.

Nitrogen Content of Clover Plants.

Determinations were made of samples of the first harvest of all field plots, results being given in Table VII.

TABLE VII. The Effect of Strains of Clover Nodule Bacteria on the Nitrogen Content of White Clover.

Results arranged according to strain treatment.

Nitrogen per cent.							
Strain	3	6	11	17	18	20	Check
Row 1.	3.59	3.76	4.06	3.81	3.96	3.94	3.52
2.	3.68	3.42	4.06	4.06	3.81	4.05	3.97
3.	3.76	3.78	4.04	4.10	4.06	3.94	4.17
4.	3.96	3.60	3.92	3.95	4.06	3.91	3.94
5.	3.83	3.77	3.99	3.97	3.91	3.91	3.72
6.	3.75	3.98	4.02	3.70	3.69	3.97	3.75
7.	3.50	3.66	3.82	3.85	3.74	3.88	3.69
Average	3.72	3.71	3.99	3.92	3.89	3.94	3.82

Slight differences are apparent in the average nitrogen content of the strain treatments, but greater differences exist in results of individual plots of any one treatment. The differences between the average nitrogen contents are therefore not significant. It is only when the total nitrogen content is considered that a significant difference appears, and then only in treatment 11 where the difference is caused almost entirely by the high yield.

In these analyses the roots and tops were digested together. It was later thought that bulking in this manner might not give a true representation of distribution of the nitrogen. Further samples were therefore taken to ascertain if the percentage of nitrogen content varied in the roots and tops in (a) individual treatments and (b) all treatments.

On 21 October from each plot of row 4 a one-foot length of turf, representing the average growth, was selected, and a few plants taken from those plots where uneven growth facilitated the removal of single plants. When cleaned, weighed and analysed, results set out in Table VIII were secured.

TABLE VIII. The Effect of Strains of Clover Nodule Bacteria on the Dry-weight Yield and Percentage of Nitrogen of Tops and Roots of White Clover.

Plot	Strain	Sample	Dry-weight in grams		Percentage Nitrogen Content			Ratio of Tops-Roots
			Tops	Roots	Tops	Roots	Average	
1	18	Few plants	3.100	0.995	4.02	3.08	3.81	3.10-1
		Turf	8.252	2.312	4.17	3.06	3.93	3.57-1
2	17	Few plants	3.535	1.180	3.79	3.21	3.64	2.99-1
		Turf	9.475	2.702	3.98	3.21	3.80	3.50-1
3	20	Few plants	3.540	0.912	3.75	3.29	3.65	3.88-1
		Turf	9.295	1.955	3.80	3.34	3.73	4.65-1
4	11	Turf	21.225	4.760	3.82	3.13	3.69	4.45-1
5	6	"	4.082	1.422	3.76	3.00	3.56	2.87-1
6	Check	"	15.700	3.655	3.74	3.44	3.68	4.29-1
7	3	"	5.495	1.660	4.15	3.17	3.92	3.31-1

Results show that the nitrogen content of the tops is appreciably higher than that of the roots, and that, in general, a low nitrogen content of roots is associated with a high nitrogen content of tops. An exception occurs in the result given with plot 5. There does not appear to be any correlation between the nitrogen content and yield though in Table VII, compiled from larger samples from all the plots, such a relationship is suggested. On the other hand, in plots 1, 2 and 3, Table VIII, the smaller

samples of a few plants show a slightly lower percentage of nitrogen. This is associated with a low top-root ratio.

A flush of growth occurred in the 25 day period between the two harvests as is shown by the higher yields in the second samples. In general, however, the total dry-weight yields and average nitrogen content of the different treatments parallel the corresponding results contained in Tables V and VII. The average nitrogen percentage of each sample was also comparable with, though slightly lower than that of the earlier larger samples, as is shown in Table IX.

TABLE IX. The Effect of 25 Day Interval in Sampling on Dry-weight Yield and Nitrogen Content of Clover.

Plot	Strain Treatment	Yield in grams		Nitrogen per cent.	
		First harvest	Second harvest	First harvest	Second harvest
1	18	5.47	10.56	4.06	3.93
2	17	6.90	12.17	3.95	3.80
3	20	6.41	11.25	3.91	3.73
4	11	13.29	25.89	3.92	3.69
5	6	5.76	5.50	3.60	3.56
6	Check	7.75	19.35	3.94	3.68
7	3	6.55	7.15	3.96	3.92

For the purpose of comparison yields of the first harvest have been reduced to the equivalent of one-foot samples.

In plots 1, 2, 3, 4 and 6 higher dry-weight is correlated with a decrease in percentage of nitrogen. In plots 5 and 7 differences in yield are negligible and differences in percentage

of nitrogen in the two harvests are also very small.

Results suggest that bulking of roots and tops in the first sampling gave figures sufficiently reliable for estimation of the relative efficiency of strain treatments. It is probable that results based on the tops alone would have given similar conclusions, though the wider ratio of tops to roots in the high yielding plots would have indicated greater differences in yield.

The approximate doubling of the yield together with reduction in the percentage of nitrogen, suggests that growing conditions unequally favoured an increase in carbohydrate content. Wilson <u>et al</u>. (1933), Fred & Wilson (1934) and Wilson (1935), showed that increases in carbon dioxide supply do bring about such changes in the balance of carbohydrates and nitrogen. Wilson <u>et al</u>. (1933) also showed that additions of peat to sand pots can produce the necessary increase in carbon dioxide concentration and Waksman & Stevens (1929), likewise showed an increase by additions of phosphates and lime to peat. Similar improvements in yield and total nitrogen were obtained by Hopkins (1935), by subjecting plants to a 'short day'; but Orcutt & Fred (1935), did not secure significant differences in comparable 'short day' experiments. It is unlikely that in the Scottish trials reduction in 'day-length' alone in the months of September and October would cause such a marked increase in growth. It is more probable that some other environmental factor was responsible.

Nodule Characters.

While collecting and washing samples from row 4 the size and distribution of nodules were noted. They varied from large fan shaped bodies 9 mm. in width to small spherical nodules 0.5 mm. or less in diameter. Usually compound and large single nodules were grouped within 3 cm. of the crown, on primary and larger secondary roots. With increase in distance from the crown they diminished in size. It was not possible to make an accurate determination of the number or mass, mainly on account of the very many minute nodules present. Counts, however, are given in Table X of the approximate numbers of compound, single and extremely small nodules on individual plants.

TABLE X. The Effect of Strains of Nodule Bacteria on the Numbers of Nodules on White Clover.

Plot	Strain Treatment	Nodule type		
		Compound 2-9 mm.	Single 0.5-2 mm.	Minute 0.5 mm. or less
1	18	4	10	40
2	17	2	15	20
3	20	4	12	30
4	11	2	20	80
5	6	3	10	50
6	Check	1	24	100
7	3	4	10	30

The examination indicated that the presence of many large and a few small nodules was associated with low yields of clover and the converse. This is contrary to the conclusion reached by other workers (Helz et al., 1927; Baldwin & Fred, 1929a; Loehnis, 1930; Allen & Baldwin, 1931; Dunham & Baldwin, 1931; Eckhardt

et al., 1931; Ruf & Sarles, 1937), who found that the occurrence of large nodules at or near the crown was correlated with high yields of the host plant*. There are two possible explanations. Firstly, unusual nodule distribution may have been caused by a high concentration of carbon dioxide in peat soils. Wilson _et al._ (1933), in experiments with additions of carbon dioxide, noted that in the higher concentrations,-

> "There were many nodules both large and small; the long type was clustered near the crown on the tap root with the small round variety scattered among the secondary roots. The nodule development was characteristic of plants inoculated with poor strains of _Rh. trifolii_, ... indicating that CO_2 had changed the usual distribution of the nodules".

They held that in 'open air' the nodules were predominately on the tap root with some scattered on the secondary roots.

The fact that the carbohydrate-nitrogen ratio of the plants had also been changed suggests that the same cause, possibly a higher carbon dioxide concentration in the peat, had been responsible for both conditions.

Secondly, nodule distribution may have been affected by the presence in the area of organisms other than those introduced on the seed. Loehnis (1930) and Dunham & Baldwin (1931) have shown that simultaneous infection with two strains of bacteria is readily obtained. Where a good strain is used for inoculating seed sown in soil already infected with a poor one, nodules are formed by both, although the good strain predominates in its effects on the host plant. Such might have been the case with

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* Later trials under glasshouse conditions with 20 strains of clover nodule bacteria, including those given in Table IX, showed that nodule distribution conformed to that recorded by most workers.

strain 11 where nodulation was changed but efficiency not impaired. The explanation does not account for nodule distribution on plants of some other plots.

GLASSHOUSE TRIALS IN SCOTLAND.

Preparation of Material.

Shortly after the field trials were sown a similar experiment was set out in an unheated glasshouse. All strains listed in Table II, and strains 15, 16, 18, 19 and 20, were used for inoculating white clover grown under semi-controlled conditions. The interior of the glasshouse was washed with 1.3 per cent. formalin. Precautions were taken to protect containers and sand from bacterial contamination, but it was not possible to exclude Collembola and wind blown dust from the glasshouse. A few check pots did eventually become contaminated late in the experiment, fortunately without adverse affect upon results.

Tay estuary sand was sieved to secure particles with a range between 0.25 - 3 mm. The sand was then washed thoroughly in running tap water, and to each kilogram was added four grams of precipitated calcium carbonate. The mixture was packed into numerous three pound glazed undrained stone pots, which were covered with two layers of water-proofed paper then sterilised in an autoclave at 28 pounds pressure for four hours. In the glass-house, pots were embedded in three inches of sawdust previously saturated with 3 per cent. copper sulphate, and kept damp during the period of the trial by occasional watering with a weak solution of a proprietary disinfectant containing phenol.

To facilitate watering a sterilised glass tube 150 mm. by 9 mm., loosely plugged at the base with glass wool, was inserted in the middle of each pot, a method similar to that adopted by Hofer (1937), Fig. 3.

Wild white clover seed, from the same source as was used for the field trials, was disinfected by the hot-water method previously described. Samples were plated on yeastrel medium and gave a germination of 90 per cent. No bacterial colonies developed indicating that disinfection was complete. On 23 and 24 June 1938, eight treated seeds were sown in each pot at a depth of approximately 3 mm. Pots were then inoculated, watered with 100 ml. of nutrient solution*, and covered with an 8 mm. layer of sterilised ground cork. The nodule bacteria were applied by pouring on the seed water suspensions from three day old cultures grown on asparagus-mannitol slants. The strain treatments were carried out in quadruplicate and the bacterial growth of a single slant in 9 ml. of water, was used for the four pots of each treatment. Sterile water was used for subsequent watering, 20 to 40 ml. being added each week to each pot; the quantity being governed by the amount necessary to raise the level in the glass tube to within

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* Modified Bryan's (1922) Nutrient Solution.

KCl	10.0 g.
$CaSO_4.2H_2O$	2.5 g.
$MgSO_4$.	2.5 g.
$Ca_3(PO_4)_2$	2.5 g.
K_2HPO_4	2.5 g.
$FePO_4$	2.5 g.

The salts were ground together in a mortar and 1.5 g. of the mixture was added to each litre of water. The solution was steam sterilised in bottles of two litre capacity by slowly raising the pressure to 22.5 lb. The precipitate was not removed and only the one application of 100 ml. of solution was given to each pot.

8 mm. of the sand surface. The water was gravity fed through a sterilised rubber tube from an inverted bottle, flow being controlled by a stop-cock.

The strains of bacteria were tested in quadruplicate and, together with 28 non-inoculated check pots, were arranged in randomised form in the glasshouse. As germination of the seed was uneven it was found advisable to reduce the seedlings to five in each pot. Subsequent growth of the plants was not vigorous and harvesting was consequently delayed. Wide differences in growth between the treatments were apparent within six weeks of sowing, and persisted throughout the growing period. The check plants, and those treated with cultures 8, 16, 17 and 18, were consistently stunted and yellowish in colour.

Harvesting of Plants.

Tops and roots were removed separately. Tops were clipped off at sand level and the pots then emptied into a flat dish containing approximately 300 ml. water, from which main roots were removed by hand and small and broken roots were 'floated off' by gentle agitation. Further washing did not remove all adhering sand, the greater portion being separated by gentle sifting when dry. The small amount which remained was later determined by weighing the residues in the Kjeldahl digestion flasks. The method of Wilson & Georgi (1932) of estimating the amount of sand in a digestion flask by comparing it with a weighed sample of the same sand was found unreliable. The presence in the sand of fine mica flakes probably accounted for failure of their method.

The roots and tops were dried over a room heater, ground in a small coffee mill, dried at 103°C., weighed and stored in stoppered bottles.

Nitrogen Analyses.

Determination of the nitrogen content of the roots and tops was made by the Kjeldahl method. The majority of samples transferred to the digestion flasks weighed between 0.65 g. and 0.9 g. Where the yields were small, as in plants of the check pots and treatment 16, the tops, or roots, of two or more replications were bulked for sampling.

The sand of some of the pots was retained for determination of nitrogen content. Two methods were employed in its collection, the second proving more suitable as less broken roots were present. (1) The sand and water from which roots had been removed as previously described, was acidified and dried over a water bath. (2) Sand and roots were gently emptied on a 3 mm. wire grid placed over a shallow dish. When about half the contents of the pot had fallen into the dish the sand was collected and passed first through a 2 mm., then a 1 mm. sieve to remove any small broken roots, and stored in stoppered bottles. The main roots, and those collected on the sieves, were washed, dried and ground.

Determination of the nitrogen content of the sand was undertaken by the method recommended in The Statutory Rules and Orders 1932, Fertilisers and Feeding Stuffs Act, 1926. Sixty grams of air dried sand was weighed into the digestion flask,

followed by one gram of phenol in 35 ml. nitrogen free concentrated sulphuric acid. The flasks were kept cool for 20 minutes and occasionally agitated. Ten grams of a mixture of three per cent. selenium in potassium sulphate was added, and the flask heated vigorously for one and a half hours. After digestion the liquid was transferred by repeated washing to a distilling flask. It was then made alkaline with caustic soda and the ammonia was distilled into N/14 acid.

Results.

The plants were harvested 6 to 16 December 1938, six months after sowing. Dry-weight yields and nitrogen content of the roots and tops are given in Table XI. In Table XII are set out the average total yields and total nitrogen produced by each strain treatment.

TABLE XI. The Effect of Clover Nodule Bacteria on the Dry-weight-yield and Percentage Nitrogen Content of White Clover Plants grown in Sand Pots.

Strain	Row	Dry-weight-yield in grams				Percentage Nitrogen Content			
		Per pot		Average		Per pot		Average	
		Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
1	1	2.980	1.351			2.67	2.52		
	2	2.022	0.831			2.33	2.48		
	3	1.352	0.696			2.45	2.48		
	4	2.069	0.877	2.105	0.938	2.49	2.47	2.48	2.48
2	1	1.716	0.790			2.40	2.68		
	2	1.936	0.846			2.63	2.70		
	3	2.255	0.813			2.41	2.68		
	4	1.728	0.727	1.911	0.794	2.81	3.01	2.56	2.77
3	1	2.161	0.988			2.21	2.17		
	2	1.630	0.824			2.81	2.36		
	3	1.515	0.758			2.87	2.61		
	4	1.143	0.522	1.612	0.773	2.06	2.11	2.48	2.31
4	1	2.574	1.000			2.62	2.46		
	2	2.718	1.081			2.40	2.47		
	3	1.312	0.686			2.98	2.51		
	4	2.277	1.007	2.220	0.943	2.77	2.29	2.69	2.43
5	1	2.711	1.244			2.52	2.23		
	2	0.308	0.242			-	-		
	3	1.352	0.678			2.52	2.38		
	4	2.445	1.000	2.170	0.974*	2.58	2.36	2.54	2.32*
6	1	2.032	0.839			2.38	2.59		
	2	1.940	0.903			2.52	2.32		
	3	2.065	1.169			2.42	2.24		
	4	1.932	0.882	1.992	0.948	2.98	2.55	2.57	2.42
7	1	3.000	0.792			2.77	2.64		
	2	1.634	0.779			2.66	2.55		
	3	2.240	0.774			2.86	2.65		
	4	2.377	0.974	2.312	0.830	2.58	2.26	2.72	2.52

* Average weights and per cent. Nitrogen are of three pots.

TABLE XI Continued.

Strain	Row	Dry-weight-yield in grams				Percentage Nitrogen Content			
		Per pot		Average		Per pot		Average	
		Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
8	1	0.218	0.234			-	-		
	2	1.133	0.564			3.12	2.59		
	3	0.107	0.139			-	-		
	4	1.260	0.649	1.196	0.607*	2.72	2.88	2.92	2.73*
9	1	1.813	0.982			2.85	2.54		
	2	2.435	0.946			2.41	2.43		
	3	2.285	0.982			2.37	2.26		
	4	2.545	1.049	2.269	0.990	2.71	2.42	2.58	2.41
10	1	2.567	1.145			2.54	2.52		
	2	2.177	0.853			2.23	2.37		
	3	2.303	1.033			2.26	2.40		
	4	2.104	0.922	2.288	0.988	2.59	2.19	2.40	2.37
11	1	2.328	0.861			2.58	2.75		
	2	2.991	1.298			2.61	2.45		
	3	2.217	0.863			2.43	2.55		
	4	2.871	1.214	2.602	1.059	2.63	2.42	2.56	2.54
12	1	1.555	0.906			2.63	2.53		
	2	2.025	1.100			2.70	2.41		
	3	1.904	0.897			2.87	2.53		
	4	1.928	0.757	1.853	0.915	2.80	2.62	2.75	2.52
13	1	2.210	0.932			2.35	2.38		
	2	2.291	1.099			2.70	2.41		
	3	1.623	0.695			2.63	2.53		
	4	1.667	0.785	1.947	0.878	2.21	2.55	2.43	2.45
14	1	2.045	1.072			2.43	2.21		
	2	2.403	1.114			2.53	2.37		
	3	2.442	1.226			2.76	2.26		
	4	1.321	0.759	2.053	1.043	2.72	2.24	2.61	2.27

* Average weights and per cent. Nitrogen are of two pots.

TABLE XI Continued.

Strain	Row	Dry-weight yield in grams				Percentage Nitrogen Content			
		Per pot		Average		Per pot		Average	
		Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
15	1	1.727	0.738			2.35	2.33		
	2	1.542	0.990			2.61	2.52		
	3	1.096	0.594			2.60	2.70		
	4	1.179	0.589	1.386	0.728	2.80	2.40	2.59	2.42
16	1	0.075	0.205						
	2	0.233	0.220			1.43	1.63		
	3	0.345	0.219						
	4	0.221	0.241	0.218	0.221			1.43	1.63
17	1	1.143	0.468			2.37	2.60		
	2	1.660	0.849			1.79	1.83		
	3	0.563	0.308			1.48	2.20		
	4	1.196	0.527	1.140	0.538	2.49	2.42	2.03	2.26
18	1	1.462	0.718			2.45	2.84		
	2	1.185	0.501			2.61	2.95		
	3	0.826	0.465			2.61	3.09		
	4	1.312	0.701	1.196	0.596	2.22	2.53	2.47	2.85
19	1	2.575	0.948			2.19	2.50		
	2	0.801	0.535			2.49	2.41		
	3	1.793	0.701			2.48	2.84		
	4	1.336	0.644	1.626	0.707	2.29	2.71	2.36	2.61
20	1	2.341	0.959			2.54	2.32		
	2	1.737	0.780			2.27	2.43		
	3	2.114	0.819			2.70	2.69		
	4	2.174	0.933	2.091	0.872	2.37	2.25	2.47	2.42
Check	1	0.218	0.200			1.09	1.82		
	2	0.203	0.180			1.27	1.62		
	3	0.208	0.207			1.52	2.14		
	4	0.235	0.186	0.216	0.243	-	-	1.29	1.86

TABLE XII. The Effect of Strains of Clover-nodule Bacteria on the Dry-weight and Nitrogen content of White Clover grown in Sand Pots.

Strain	Average dry-weight gms.			Nitrogen content in m.g.		
	Tops	Roots	Tops+Roots	Tops	Roots	Tops+Roots
1	2.105	0.938	3.043	52.2	23.3	75.5
2	1.911	0.794	2.705	45.9	22.0	67.9
3	1.612	0.773	2.385	40.0	17.8	57.8
4	2.220	0.943	3.163	58.9	22.9	81.8
5	2.170	0.974	3.144	55.1	22.6	77.7
6	1.992	0.948	2.940	51.2	22.9	74.1
7	2.312	0.830	3.142	62.8	20.9	83.7
8	1.196	0.607	1.803	34.9	16.6	51.5
9	2.269	0.990	3.259	58.5	23.9	82.4
10	2.288	0.988	3.276	54.9	23.4	78.3
11	2.602	1.059	3.661	66.6	26.9	93.5
12	1.853	0.915	2.768	50.9	23.1	74.0
13	1.947	0.878	2.825	47.3	21.5	68.8
14	2.053	1.043	3.096	53.6	23.7	77.3
15	1.386	0.728	2.114	35.9	17.6	53.5
16	0.218	0.221	0.439	3.1	3.6	6.7
17	1.140	0.538	1.678	23.1	12.2	35.3
18	1.196	0.596	1.792	29.5	17.0	46.5
19	1.626	0.707	2.333	38.4	18.5	56.9
20	2.091	0.872	2.963	51.7	21.1	72.8
Check	0.216	0.243	0.459	2.8	4.5	8.3

Dry-weight Yields of Clover Plants.

The data recorded in Table XI and summarised in Table XII showed that wide differences in yield followed inoculation by different strains of clover nodule organisms. Yields ranged from 1.678 g. in treatment 17 to 3.66 g. in treatment 11. Similar differences occurred in the replications of many of the treatments, some of which could be attributed to the position occupied by the pots in the glasshouse. Some, however, particularly in replications of treatments 5, 8 and 17, were associated with poor or no nodule development. Only one plant possessed nodules in the low yielding pot treated with strain 5; and with strain 17 the low yield was associated with very small nodules. With strain 8 nodules were not present on plants of those pots shown with a low yield. A later trial with strain 8 gave similar erratic results for, in two of the three replications, only one or two nodules were present and then only on one plant in each pot. Strain 16 did not produce nodules on the roots and later tests under similar conditions with open pots and closed containers, have shown that this isolant from strain 20 (Wisconsin 186) is not capable of forming nodules on white clover roots.

Variations between replications have also been reported by Loehnis (1930), Wilson et al. (1937) and Hofer (1937). The cause of these irregularities is not known. Fred et al. (1932) suggested that variations in nodule formation and yield of plants could be caused by differences in moisture content of the sand, and in the temperature and shading of the plants.

Such variations in environment may have contributed to the irregularities shown in Table XI.

Average yields in glasshouse and field trials of strains used in both tests, are in close agreement. In both, strain 11 was responsible for the highest, and strains 17 and 18 for the lowest yields.

Statistical Analysis of Dry-weight Yields.

Considerable variations in yield resulted from the strain treatments. As they graduate by small amounts from lowest to highest it has been necessary to subject the data to statistical analysis to ascertain those differences which are significant.

The total yields of four replications of each treatment are as follows:-

Strain treatment	Total dry-weight	Strain treatment	Total dry-weight
1	12.17 g.	11	14.64 g.
2	10.80	12	11.06
3	9.53	13	11.30
4	12.67	14	12.36
5	*12.43	15	8.44
6	11.75	17	6.71
7	12.56	18	7.16
9	13.02	19	9.33
10	13.09	20	11.84

* Based on an estimated value of 3.01 g. for the plants of pot in row 2. Treatments 8 and 16 are not included in the analysis.

Analysis of Variance.

Source of Variation	Degrees of freedom	Sum of Squares	Mean Squares
Treatments	17	19.45	1.14
Rows	3	3.15	1.05
Error	50	15.64	0.30
Total	70	38.24	0.55

Variance ratio F = 1.14/0.30 = 3.78

Snedecor's (1937) table of 'F' gives a value of approximately 2.37 for 17 and 50 degrees of freedom for a probability of 1/100, which indicates that the effect of treatment is significant.

Standard error of treatment total $= \sqrt{4 \times 0.30}$

" " of difference of any two treatment totals $= \sqrt{4 \times 0.30 \times 2} = 1.549$

Therefore difference between two totals for significance $= 1.549 \times 2 = 3.10$ g.

General mean of treatment totals =11.21 g.

The total yield of treatment 5 is based in part on a calculated yield for the replication which showed poor nodulation. This replication is treated as a 'missing plot' and the method of Allan & Wishart (1930), has been used to calculate the yield.

K = the estimated yield of missing pot.

Then $K(n-1)(s-1) = (n-s-1)S - sS_t - nS_b$

where S = Sum of all known yields (not including unknown).

S_t= " " treatment totals, not including treatment 5.

S_b= " " row totals, not including row with treatment 5.

n = Number of replications of each treatment.

s = " " treatments.

$$K(3)(17) = 22(197.85) - 18(188.43) - 4(152.42)$$
$$= 4154.85 - 3391.74 - 609.68$$
$$= 153.43$$
$$K = 3.01 \text{ g.}$$

The analyses showed that the yield of treatment 11 was significantly greater, and the yields of treatments 17 and 18 significantly less, than the general mean of the yields. In a comparison of treatments 3, 6, 11, 17, 18 and 20, - also used in the field trials - the yield of 11 was significantly greater than the yields of 3, 17 and 18, but not greater than 6 and 20; and the yields of strains 6 and 20 were significantly greater than those of 17 and 18. In the field trials the yield of strain 11 was significantly greater than those of all other treatments, but no significant difference existed between the other yields. Results confirm the conclusion that there was close agreement between the field and glasshouse trials.

Nitrogen Content of Clover Plants.

Data contained in Table XI show that the percentage nitrogen content associated with the treatments varied considerably between the different treatments and within some of them. The average nitrogen content varied between 2.03 per cent. and 2.92 per cent. in the tops and between 2.26 per cent. and 2.85 per cent. in the roots. In individual treatments the range was equally wide; for example in treatment 17 varying from 1.48 to 2.49 per cent. in tops, and from 1.83 to 2.60 per cent. in roots of different pots. This irregularity in replications was also remarked upon by Loehnis (1930), Wilson *et al*. (1931, 1937) and Burton & Wilson (1939). All the extremes in percentage of nitrogen were associated with treatments giving low dry-weight yields. Conversely, treatments giving a high yield, namely 4, 5,

6, 7, 9, 10 and 11, had nitrogen contents which fell within a comparatively narrow range, 2.40 to 2.72 per cent. for tops and 2.27 to 2.54 per cent. for roots. In the majority of the results, tops showed a slightly higher nitrogen content than roots, and where the reverse was the case it occurred with treatments giving low yields. However, in all treatments except 5, 7 and 14, at least one replication showed a nitrogen content lower in the tops than in the roots. Other workers (Stevens, 1925, and Fred et. al. 1932), recorded similar results though according to Fred et al. (1932), it is more usual for the tops to contain a higher percentage than roots. In field trials the tops contained a higher nitrogen percentage than the roots, as is shown in Table VIII.

In the glasshouse experiments there did not appear to be any definite relation between nitrogen percentage and dry-weight yield, though a very high percentage (strain 8) and a very low percentage (strain 17) were both associated with low yields. Eckhardt et al. (1931) noted that a high percentage of nitrogen did not always indicate thrifty plant growth. On the other hand numerous workers (Stevens, 1925; Wright, 1925; Fred et al., 1926; Dunham & Baldwin, 1931; Allen & Baldwin, 1931; Wilson, 1935), held that high dry-weight yield was associated with a high percentage of nitrogen. In the field trials the nitrogen percentage was much higher in all treatments, but as in the glasshouse tests, there was no relation between nitrogen percentage and dry-weight yield. It is probable that the nitrogen percentage is a variable factor influenced by conditions of growth. In the trials, however, there was nothing to indicate that the factors of

moisture content of sand or peat, temperature, light, etc., were operating unequally in the treatments or their replications.

Table XII shows that the total nitrogen of tops and roots, given for each treatment, and calculated from the average dry-weight yield is chiefly dependent on the amount of yield. Variations in nitrogen percentage, being relatively small, have thus little effect on the total nitrogen content, so that it would appear as if the efficiency of strains can be gauged by consideration of the dry-weight yield alone.

Nitrogen Content of Sand of Test Pots.

At the time of harvesting the glasshouse plants it was thought that the nitrogen content of roots and tops might not adequately represent the nitrogen efficiency of the strains. Accordingly, as is shown in Table XIII, determinations were also made of the nitrogen content of the sand in a number of pots. With the exception of three checks, the pots taken were the first four of each row at one end of the glasshouse.

The first six samples in Table XIII were obtained by the method of separating roots and sand in water, and the remainder by separating with sieves.

TABLE XIII. The Effect of Strains of Nodule Bacteria on the Accumulation of Nitrogen in Sand-pots.

Strain	Row	Nitrogen	
		Percentage	Total in pot in m.g.
13	1	0.0056	72.3
17	2	0.0058	71.3
9	3	0.0056	69.8
14	4	0.0053	68.1
Check	2	0.0056	71.8
*(Check	3	0.0055	69.2)
("	"	0.0054	67.6)
6	1	0.0052	64.3
1	2	0.0053	65.1
Check	3	0.0053	67.3
19	4	0.0058	71.2
Check	1	0.0053	63.6
18	2	0.0056	68.8
15	3	0.0058	70.8
16	4	0.0054	68.2
11	1	0.0058	68.4
8	2	0.0052	64.1
Check	3	0.0054	66.7
12	4	0.0056	67.2

* Estimations of the same sand, but sand collected by different methods. The first two checks are from different parts of glasshouse.

Very small differences were found in nitrogen content of the sand of the various treatments, and those which did exist could be readily accounted for by uneven sampling or by the presence of small roots which were difficult to remove completely from the sand. It is evident that the differences in the nitrogen content of the sand were negligible and need not be considered in determining the nitrogen efficiency of the strains of nodule bacteria.

Some recent workers (Virtanen & Hausen, 1935; Virtanen, 1937; Wilson & Burton, 1938; Isakova & Andrejev, 1938), have shown that nitrogen was excreted from the nodules into the surrounding medium, and that strains of nodule bacteria varied in this respect. Others (Bond, 1936; Horst & Roberg, 1938) with similar experiments did not obtain nitrogen excretion. Wilson & Burton (1938) reported positive and negative results according to the environmental conditions of the experiments, and Wyss & Wilson (1938) stated that excretion was associated with cool long days, and especially with sunlight of relatively low intensity but long duration. These conditions appear to have been present in Edinburgh during the months June to December. Bond & Boyes (1939) failed to obtain nitrogen excretion from soybean and broad bean plants grown at Glasgow in the period April-August 1938; and suggested that, if light values are of importance, the intensity at Glasgow as compared with Helsinki - where Virtanen had obtained marked excretion - might have been too low for excretion to take place. If this is so, then the light intensity at Edinburgh would also be inadequate for excretion since sunlight records for Edinburgh are comparable with those of Glasgow. However, the trials were not arranged to study this phase of nitrogen fixation, so that it is possible, in the absence of a suitable absorptive medium, i.e. kaolin, the sand system might not have been suitable for nitrogen accumulation.

Nodule Characters.

At the time of harvesting, the size, type, approximate numbers and distribution of nodules were noted (Table XIV). The Table gives the average results on all plants in a treatment.

TABLE XIV. Effect of Strains of Nodule Bacteria on Nodulation of White Clover.

Strain	Nodule description						Nodule distribution			Yield of host in grams
	Type		Size							
	Compound	Single	3-4 mm.	2-3 mm.	1-2 mm.	less than 1 mm.	Tap-root	Main laterals	Scattered	
1		+		+	++	+	++	+		3.04
2		++		+	+++	+	++	++	+	2.70
3		++		+	+	+++	+	++	+++	2.38
4		+		++	++	+	++	++		3.16
5		+		++	+	+	+	++		3.14
6	+	++		+++	+	+	+++	++	+	2.97
7	+	+		++	++	+	++	+		3.14
8	++	++	++	++			+	+++		1.80
9		+		+	++	+	++	+		3.26
10		++		+++	++	+	+++	+++		3.27
11		++		+++	++		+++	+++		3.66
12		+		++	++	+	++	+		2.77
13	+	++	+	+	+	+	++	++	+	2.82
14		+	+	+	++	+	++	++	+	3.09
15		++		+	+++	+	+	++	++	2.11
17		+++		+	++	+++	+	++	+++	1.68
18		+++	+	++	++	++	+	++	++	1.79
19		+			+++	+	+	+	+	2.33
20		++		+	++	++	+	++	+++	2.96

\+ In columns 1 and 2, + = 1 to 15 nodules per plant; ++ = 15 to 30 nodules per plant; +++ = over 30 nodules per plant.

\+ In columns 3 to 8 the number of + signs gives the proportion of nodules in the various groupings.

Particulars indicate that the presence of nodules on the tap root and larger laterals is correlated with high yields, and that small nodules evenly distributed over the root system are associated with low yields. Although this is the conclusion of most workers it does not agree with evidence secured in field trials. Exceptions exist, for example, in results of strains 8 and 20; the former produced large nodules on the tap root yet gave a low yield, whereas the latter, gave a moderate yield yet produced many small and widely distributed nodules.

The table does not indicate variations in nodulation in replicates of some treatments and on plants in the same pot. For instance, in three pots of treatment 19 most plants possessed a few small nodules situated on the tap root and main laterals within 1½-2 in. of the crowns. In the fourth pot more than twice the number of nodules were present, few being in the vicinity of the crowns, but mostly distributed over an extensive zone. Similar irregularities occurred in other treatments, suggesting that conditions of growth influenced nodulation. In one pot of treatment 19 the largest plant had only five large nodules on the upper portion of the tap root, whereas the other four had many more smaller nodules scattered over the roots within 1½ in. of the crowns. Variation in nodulation of plants in the same pot suggested that the host plant was partly responsible. It is probable that environment, strain of the organism and plant are inter-related and instrumental in delimiting the size, type, number and distribution of nodules.

Comparison of Results of New Zealand and Scottish Trials.

The results of field and glasshouse trials in Scotland do not, at first glance, bear any relation to those obtained in New Zealand. The results contained in Tables III and XI show that, of the cultures successful in New Zealand, those of South Island origin also gave good results in Edinburgh. Of the promising strains in both countries, cultures 5, 10 and 11 were efficient in the South Island, and culture 9 to a lesser extent in the North Island, New Zealand. A satisfactory comparison cannot be based on such a few examples, although the evidence suggests that some factor common to southern Scotland and southern New Zealand, perhaps similarity of climatic conditions, had influenced the efficiency of the organisms.

There is a possibility that, during the period of eighteen months between the trials in the two countries, storage on media relatively rich in lime had altered the efficiency of the strains, since it was found subsequently that repeated transfers to media deficient in or amply supplied with available calcium, changed the efficiency of nodule bacteria.

CULTURAL AND MORPHOLOGICAL CHARACTERISTICS OF CLOVER NODULE BACTERIA.

A study of the possible variations in the cultural, physiological and morphological characters of the strains of clover nodule bacteria was undertaken.

Methods.

Cultures of strains 1 to 15, 17 and 20 were first plated to check their freedom from contaminants. A small loop of each was spread with a glass rod on the surface of yeastrel mannitol agar (Topping, 1937)*. A second plate was prepared from the first by transferring the glass rod to the second Petri dish and rubbing the surface of the medium. The plates were incubated at 30° C. for four or five days and single colonies were transferred with a small loop to slants of yeastrel mannitol agar and the asparagus medium of Carroll (1934)⊕. The yeastrel agar supported

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* Topping's Medium:-

Yeastrel	0.25 per cent.	(a commercial yeast extract)
'Bacto' peptone	0.25 " "	
Agar	1.50 " "	
Mannitol	0.50 " "	

⊕ Carroll's Asparagus Medium:-

Mannitol	10.0 g.
K_2HPO_4	0.5 g.
$MgSO_4 \cdot 7H_2O$	0.2 g.
NaCl	0.1 g.
$CaCO_3$	3.0 g.
Asparagus extract	50.0 ml.
Agar	15.0 g.
Water	950.0 ml.

To a ten-ounce tin of asparagus was added sufficient water to make up the volume to 400 ml. The asparagus was macerated and the suspension filtered through four layers of cheese cloth. The filtrate was made up to 1 litre and sterilised. The completed medium was adjusted to pH 7.0 and sterilised in the autoclave by slowly raising the pressure to 22.5 lb. The reaction after sterilisation was pH 6.6.

a more luxuriant growth than did the asparagus medium. For example, culture 19 in six days formed colonies of 4-5 mm. diameter on the former, and 3-4 mm. on the latter. On slants, growth was also more abundant on yeastrel agar. As different batches of yeastrel medium were more likely to be uniform in composition, and were easily prepared, it was subsequently used as the standard for cultivation of the nodule bacteria. In later work mannitol was satisfactorily replaced by 0.5 per cent. sucrose.

For the study of cultural characteristics strains were grown on the following media:- Yeastrel mannitol agar slants; Yeastrel mannitol broth; Veal agar; Veal broth; Litmus milk; Potato slices; Nutrient gelatine.

Yeastrel media were prepared according to Topping's formula. Veal bouillon was prepared in the manner suggested by Hofer (1933), and a solid medium was made by adding 1.5 per cent. agar. 'Bacto' nutrient gelatine was used in preparing the gelatine medium. The litmus milk was made from cow's milk and was sterilised by steaming on three successive days. The other media were sterilised in an autoclave by raising the pressure to 22.5 lb.

Tubes of the above media were inoculated by transferring to each a 2 mm. loop of bacterial growth from yeastrel agar slants. The nutrient gelatine tubes, after inoculation with a needle, were sealed with rubber stoppers and incubated at 21°C. The other media were incubated at 30°C. Cultures were examined regularly for a period of one month.

The morphology of the organisms was examined in hanging-drop and stained preparations made from three day old yeastrel agar slants. The latter were stained by the gram method of Conn (1937). To demonstrate the presence of flagella stained preparations were made from one day old yeastrel agar slants. A loop of condensation water from the base of the slant was transferred to a larger loop of sterile water on a slide inclined to induce flow. The preparations were dried at room temperature. Methods of staining recommended by Gray (1926) and Leifson (1930), were used for the majority of the preparations. The former proved more reliable though flagella were less deeply stained.

Colony Characters.

Marked differences in growth were exhibited by the strains on yeastrel mannitol agar. Cultures of strains 9, 10, 11, 13, 14 and 15 made rapid growth, forming 2.5 mm. colonies in three days, whereas strains 4, 5, 6 and 18 formed colonies only 0.25 to 0.75 mm. in the same period. Apart from the growth rate colonies did not vary greatly in appearance. The following description of a four day colony on yeastrel agar could therefore apply to any of the strains:- Colonies convex, moist, smooth, glistening, entire, translucent to clouded, finely granular and mucilaginous. Strains 7 and 17 were more viscid than the others.

Two types of colonies were produced by strain 20 (Wisconsin 186) on both yeastrel and asparagus media; one being translucent, the other opaque and white. Single colonies of each were spread on separate plates of the two media. After seven days incubation all four plates developed both colony forms.

Further single colony isolations showed that each form persisted and from them cultures 20* and 16* were developed. In the following year a type similar to culture 16 was again isolated from culture 20.

Cultural Characters.

On agar slants growth was uniform, raised to convex, spreading, smooth, moist, glistening, translucent to clouded and later chalky, mucilaginous, and in most cases flowed to the base of the slant. Culture 16 alone exhibited marked divergence from the normal type of growth. On yeastrel agar slants the growth was dry, white, raised without basal slime, sometimes roughened or raised in horizontal folds up to 0.3 mm. in height. On potato slice the growth was raised, appearing in marked contrast with the barely perceptible growth of other cultures. Apart from those noted, differences produced by the strains were only slight and often transient. On yeastrel slants a chalky appearance was recorded for some cultures in three days, but by the end of the analysis practically all exhibited this condition.

In litmus milk all produced an alkaline reaction, though the three strains 7, 9 and 17, produced first an alkaline, then a neutral and finally an alkaline reaction. Similarly the depth of the 'serum zone' varied with the period of incubation, but rarely exceeded 3 mm. in any culture.

In veal broth a slight clouding was observed in some cultures from the eighth to eighteenth day of incubation. In nutrient gelatine stabs, a small raised growth not exceeding 0.5 mm.

* These isolations were made prior to initiation of field and glasshouse trials.

diameter, formed at the point of inoculation but no liquefaction was apparent within two months.

A summary of the cultural characters, from records taken at ten day intervals, is presented in Table XV.

TABLE XV. Cultural Characters of Strains of Clover Nodule Bacteria.

Medium	Character	5 days	15 days	25 days
Yeastrel slant	Translucent	1,2,3,4,5,6,7,8, 12,14,15,17,18,20.	1,4,7,14,17,18, 3,20.	4,14,17.
	Chalky	9,10,11,13,19.	remainder	remainder
	White	16.	16.	16.
Yeastrel broth	Not clouded	-	-	All except 8
	Slightly "	all cultures	3,7,8,9,11,13, 16,17.	8
	Moderately clouded	-	remainder	-
	Ring	3,7,8,9,14,16,20.	16,17,19,20.	-
	Pellicle	-	7,8,9,13,16,17.	-
Veal agar		No growth	no growth	no growth
Veal broth	Not clouded	all cultures	1,2,3,5,6,7,8, 9,10,11,17.	all cultures
	Slightly "	-	remainder	-
Potato slice	Slight growth	all cultures	all except 16	all except 16
	Moderate growth	-	16.	16.
Litmus milk	Neutral	-	7,9,17.	-
	Alkaline	all cultures slight	remainder	all cultures
	Serum Zone			
	up to 1 mm.	1,3,4,6,7,8,16.	8,11,13,16.	remainder
	1 - 2 mm.	remainder	remainder	1,4,6,7.
	2 - 3 mm.	-	9.	3,12,14,17,9,10.
Nutrient gelatine	Liquefied	-	-	-

Although the above differences are slight, similar ones have been utilised by some workers (Loehnis & Hansen, 1921; Stevens, 1925; and Hofer, 1933), for separating groups of nodule bacteria. Stevens, working with numbers of organisms from different cross inoculation plant groups, divided the nodule bacteria into sections according to the presence or absence of a serum zone. His treatment is impracticable, however, since in this analysis it was found that development of the serum zone was influenced both by strain of the culture and the period of incubation. Fred et al. (1932) have also noted variability in production of a serum zone by strains of bacteria from pea nodules. Hofer stated that only the organism of the alfalfa plant-bacteria group was able to grow in veal medium, whereas in this analysis slight cloudiness was produced by a number of the clover strains. It is possible that some strains of clover nodule organisms vary in their cultural characteristics as much as those of organisms from different plant groups. This aspect is further considered in Section III.

Morphological Characters.

All cultures exhibited irregular growth forms containing rods, cocci, gonidangia, and branched forms comparable with those described by Gibson (1928), though rods and banded rods as described by Thornton and Gangulee (1926) predominated. Club-shaped rods similar to the 'racket' forms in type F of Loehnis & Smith (1916) were also common. A few Y shapes were noted in some preparations.

In most preparations staining was uneven and varied considerably according to the growth forms present. In cultures 5, 6, 7, 10, 11, 12, 13, 15 and 18, rod forms predominated and the cells were more uniformly stained. In the others staining was

uneven because of the presence of some or all of the various growth forms. There did not appear to be any connection between the rate of growth on yeastrel agar and the occurrence of any particular cell type.

Stained preparations from culture 16, when compared with slides made from culture 20, showed a similar range of growth forms. Short evenly stained rods and larger oval cells, stained around the periphery and at one or both poles, were the common forms present.

All the cultures showed one to four peritrichous flagella grouped near one pole.

The average size of the cells of different strains varied somewhat, but equally wide variation existed between the cells of any one culture. The dimensions in microns, as shown by gram stained preparations, were as follows:-

Culture	Dimensions	Average
1	1.2 to 2.7 x 0.4 to 0.45	1.87 x 0.43
2	0.9 3.6 x 0.45 1.0	1.67 x 0.5
3	0.8 4.5 x 0.4 1.3	1.7 x 0.45
4	0.9 2.7 x 0.45 0.7	1.7 x 0.5
5	1.2 3.0 x 0.35 0.45	1.7 x 0.4
6	0.9 3.6 x 0.45 0.55	1.8 x 0.47
7	1.0 3.0 x 0.4 0.45	2.1 x 0.4
8	1.0 2.0 x 0.4 0.5	1.5 x 0.45
9	1.1 3.6 x 0.4 0.6	1.9 x 0.45
10	1.2 3.6 x 0.4 0.55	1.77 x 0.45
11	0.8 3.0 x 0.4 0.5	1.3 x 0.45
12	0.8 3.4 x 0.55 0.8	1.7 x 0.7
13	1.2 3.2 x 0.45 0.65	1.8 x 0.55
14	0.7 3.5 x 0.4 0.7	1.8 x 0.5
15	0.6 2.5 x 0.4 0.8	1.7 x 0.6
16	0.7 2.2 x 0.45 0.8	1.4 x 0.66
17	0.8 3.6 x 0.35 0.45	1.33 x 0.4
18	0.8 2.0 x 0.4 0.7	1.2 x 0.5
19	0.6 2.2 x 0.4 0.6	1.3 x 0.45
20	0.8 2.0 x 0.4 0.7	1.3 x 0.5

SUMMARY OF SECTION I.

A study of 20 strains of clover nodule bacteria, collected from New Zealand, South Australia, the United States of America and Scotland, provided no evidence that strains can be differentiated by cultural or morphological characters.

Glasshouse trials in New Zealand, under semi-controlled conditions in sand pots, demonstrated wide differences in yield following inoculation with various New Zealand strains.

Field trials in North and South Islands provided some indication that cultures gave best results in the Island from which they were originally obtained.

In further trials on peat land in Lanarkshire, Scotland, with six cultures from New Zealand, South Australia, the United States of America and Scotland, a strain from the most southern district of New Zealand gave a dry-weight yield of clover almost double that of any other strain. No significant differences existed between the other yields. Nodulation on the peat land was unusual in that a few large and many scattered nodules were associated with high clover yields. The average nitrogen content was high in all treatments, ranging from 3.71 to 3.99 per cent. A second sampling taken nine months later than the first, showed a large increase in dry-weight yields, with a reduction in the percentage nitrogen content. The differences in yield associated with the treatments were still evident at the second harvest.

Glasshouse trials with 20 strains, under semi-controlled conditions in sand pots, gave equally wide differences in yield and total nitrogen content. The nitrogen percentage varied considerably in different treatments, and in replications of each treatment. It could not be correlated with plant growth, except that extremely high and extremely low percentages were associated with low yields. The strain which gave the highest yield and total nitrogen in the field trials, also gave the highest yield and total nitrogen in the glasshouse trials. Likewise the two strains which gave the lowest yields and nitrogen contents in the field gave the lowest yields and total nitrogen contents in the glasshouse trials.

Nodulation of glasshouse plants suggested that a few large nodules at or near the crown were associated with high yields, and many small nodules scattered throughout the root system were associated with low plant yields.

Nitrogen determinations of the sand in which the various treatments were carried out showed no differences in accumulation of nitrogen.

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II. EFFECTS OF CALCIUM IN MEDIA ON CLOVER NODULE BACTERIA.

INTRODUCTION.

The present investigation was undertaken to ascertain the effects on the nodule organism produced by cultivation on media deficient in and amply supplied with calcium. The media employed were simple modifications of one regularly used for the cultivation of nodule bacteria. Selected cultures were subjected to a series of transfers to the different media and then examined for variations in morphology and cultural characters, and efficiency in promoting growth of white clover plants.

It is generally maintained that, where acid soil conditions exist additions of lime in sufficient quantities to raise the hydrogen ion concentration to approximately pH 7.0, are beneficial to the host plant and to production of nodules. Additions of small amounts of lime to neutral soils may also be of advantage though large applications have been reported as harmful (Fred et al., 1932). Some workers (Scanlan, 1928; Albrecht & Davis, 1929a; Horner, 1936; Albrecht & McCalla, 1937a) have suggested that the improvement following liming is caused by the presence of the element calcium rather than to a modification of soil acidity. By additions of calcium chloride and calcium acetate, without materially affecting the pH, Scanlan increased the nodulation of soybean, and concluded:-

> "That possibly the influence of calcium towards increasing inoculation is one of keeping the bacteria alive during a long period of time".

Albrecht & Davis also improved nodulation in an acid soil with calcium chloride, and suggested that the beneficial effects of liming may be accounted for by the presence of the element calcium as well as by the change in acidity. In a second paper (1929b) they held that the lime exerted a physiological effect on the plants, and possibly on the organisms, bringing about greater nodule development.

Albrecht & McCalla (1937a) and McCalla (1937), using a colloidal clay medium for cultivating nodule bacteria, found that absence of calcium induced abnormal forms which did not produce nodules. When transferred to the same medium with addition of calcium, the abnormal forms became normal and were then capable of inducing nodulation. Albrecht & McCalla (1937b) also suggested that the ratio of exchangeable calcium to phosphorus in laboratory media, as compared with that in the soil, may account for the slow growths and variant forms of the bacteria and their dying in culture.

It is evident that the calcium element has a definite influence both on cultural characters of the nodule bacteria and inter-relation of organism and host. Conversely, Fred <u>et al</u>., (1932) held that cultivation on media - particularly those rich in nitrogen - did not adversely affect the efficiency of nodule bacteria.

EFFECTS OF MEDIA ON CULTURAL AND MORPHOLOGICAL CHARACTERS OF NODULE BACTERIA.

Methods.

The media employed in this investigation were prepared from a broth of the following formula:-

Yeastrel	0.25 per cent.
'Bacto' peptone	0.25 per cent.
Sucrose	0.50 per cent.

For a solid medium 1.5 per cent. agar was added. Broth and agar are referred to subsequently as 'standard broth' and 'standard agar'. Three forms, with and without agar, were employed, namely Standard broth; Standard broth + 0.15 per cent. calcium gluconate; Standard broth with all calcium removed.

The use of calcium gluconate as an available source of calcium, followed the recommendation of Albrecht & McCalla (1937b). The media containing calcium gluconate were adjusted to pH 7.0, with N/1 KOH, and distributed in tubes. After sterilisation by slowly raising the pressure in the autoclave to 22.5 lb., a considerable precipitate formed in the broth tubes. Additional broth was prepared by first sterilising the standard broth and then adding a sterile solution of calcium gluconate. The amount of precipitate was considerably less in the broth prepared by the latter method but as no difference was apparent in the growth of nodule bacteria in the two media, that prepared by the first method was used for the serial transfers.

The calcium-free media were prepared according to the method of Bordet (1930), by adding sodium oxalate to the standard broth and standard agar, and filtering three times though a pulp filter pad. The pad was treated before filtering the media, with an aqueous solution of sodium oxalate of the same concentration as in the medium to be filtered, and was then washed with distilled water. Sugar was added after filtration, and the media adjusted to pH 7.0 with sodium hydroxide. The media were distributed in tubes and sterilised in steam for 15 minutes at 15 lb. pressure. Bordet added 0.2 per cent. sodium oxalate, but tests showed that this concentration inhibited growth of nodule-bacteria in both broth and agar media. Concentrations of 0.05 per cent. and 0.033 per cent. also inhibited growth in broth but not in agar media. Media were prepared with the following concentrations of sodium oxalate:-

Standard broth + 0.006 % sodium oxalate
" " + 0.008 % " "
" " + 0.012 % " "
" " + 0.015 % " "
" " + 0.020 % " "
" agar + 0.054 % " "
" " + 0.066 % " "
" " + 0.076 % " "
" " + 0.087 % " "
" " + 0.096 % " "

Analysis of the broth prior to the addition of sodium oxalate showed a 0.0024 per cent. calcium content, requiring 0.008 per cent. disodium oxalate for precipitation.

A series of five transfers of three clover strains were made at two day intervals to the above media. Results showed that the two lowest concentrations, in both broth and agar, supported a luxuriant growth of all three cultures. The greatest concentrations, 0.02 per cent. in broth and 0.096 per cent. in agar, inhibited growth. Growth in media with concentrations of 0.012 per cent. and 0.015 per cent. in the broth, and 0.076 per cent. and 0.087 per cent. in the agar, was retarded, but by subculturing on every second day sufficient vigour was maintained to permit a continuous series of transfers. The experiment required media as free as possible from calcium and, as the media with the two lowest concentrations of sodium oxalate might have contained traces of calcium, the broth and agar with the higher concentrations were used for the series of transfers.

For the experiment strains 11, 18 and 20 were selected from those previously tested in field and glasshouse trials. Strain 11 was associated in both trials with the highest dry-weight yield and strain 18 with the lowest dry-weight yield. Strain 20 was associated with an intermediate clover yield and the source of the non-typical white colonies represented by strain 16.

As far as was possible daily transfers of cultures of the above strains were made to the standard and calcium gluconate agar slants. Growth in broth media and on the calcium deficient agar slants was not vigorous, and incubation for two days between transfers was necessary to ensure growth of the succeeding cultures.

The serial transfers on the standard and calcium gluconate media were continued for a period of 58 days, and on the calcium deficient media for 46 days. Transfers to the latter media, on account of the time required to determine suitable concentrations of sodium oxalate, were not initiated until 12 days after the commencement of the transfers to the standard and calcium gluconate media. A single 2 mm. loop of culture was used in each transfer. All cultures were incubated at 30°C. Details of the various transfers are summarised in Table XVI.

TABLE XVI. Summary of Culture Transfers and Media employed.

Medium	Transfers Number	Transfers Interval	Total period
Standard agar	46	1 day	58 days
" " + cal. gluconate 0.15 %	46	1 "	58 "
" " + sod. oxalate 0.076%	22	2 days	46 "
" " + " " 0.087%	22	2 "	46 "
" broth	29	2 "	58 "
" " + cal. gluconate 0.15 %	29	2 "	58 "
" " + sod. oxalate 0.012%	22	2 "	46 "
" " + " " 0.015%	22	2 "	46 "

Results.

Effects of Treatments on Cultural Characters.

Growth of the three strains on the standard and calcium gluconate agar slants* differed only slightly in appearance and

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* Allen & Allen (1939) obtained poor growth of nodule bacteria on media containing calcium gluconate, but in the present tests growth on media containing it was equal to that on the standard media.

amount. Cultures of strain 11 were raised, flat, smooth, shining, chalky, and with the margin entire. Cultures of strain 18 were similar, except that the surface was rough. Cultures of strain 20 were also similar to 11 but faintly clouded. The three strains formed a moderate amount of slime at the base of the slants, strain 18 forming the most and strain 20 the least.

On calcium deficient agar media all three strains, given sufficient time to develop, produced luxuriant growth. Cultures were convex, smooth, glistening, butyrous, with the margin thin and entire. Cultures of strains 11 and 20 were opaque white, and cultures of 18 faintly clouded. When touched with a needle all cultures could be drawn out into threads, up to 4 cm. in length. They did not flow down the slants nor exhibit the chalky condition observed on the other agar media.

In broth media the three strains formed a clouded growth, and usually a ring and pellicle were present. Clouding was more pronounced and the pellicle more definite in the calcium gluconate broth. Strain 18 grew slowly, and periodically a larger inoculum of two drops was required to maintain the continuity of transfers.

During the period of the serial transfers five platings of all the cultures were made at intervals of ten days. In the first each culture was transferred to Petri dishes containing the three agar media, standard, calcium gluconate and calcium deficient, but in subsequent ones only the standard medium was employed. Plates were inoculated by spreading over the agar surface a 2 mm. loop from the broth tubes and from the agar slants, a 2 mm. loop of a suspension prepared by adding a loop of inoculum to 6 ml.

sterile water. The plates were incubated at 30°C. and examined daily for ten days. Any colonies differing from the normal were transferred to standard agar slants.

The majority of the colonies on the plates were typical of the normal growth produced by each strain. At three days single colonies of strain 11 consistently averaged 2-3.5 mm., strain 20 2-2.5 mm., while those of 18 were much smaller, not exceeding 1.0 mm. The only unusual colonies were of two types - small opaque white ones frequent in the first and second platings and small glassy colonies which appeared in the later platings of strain 18. The former occurred in (a) the three strains on calcium gluconate agar, (b) strains 11 and 18 on the standard agar, and (c) strain 11 in the calcium gluconate broth. They were white, strongly convex, firm, and would slide freely on the surface of the medium when touched with a needle. Colonies transferred to standard agar slants formed chalky tenacious growth, with a minimum of mucilage. Three cultures labelled 11v, 18v, and 20v, from the ninth serial transfer of strains 11, 18 and 20 on calcium gluconate agar were used in subsequent tests described below. Another, 20-2, macroscopically similar to 20v, was isolated from a plate of strain 20 prepared at the commencement of the serial transfers. In the third, fourth and fifth platings the opaque condition became less distinct and on transfer to standard agar slants colonies produced normal chalky growth.

The small glassy colonies of strain 18 were most common on plates from standard broth, standard agar and calcium gluconate agar. Colonies were similar to those described save that they were translucent, and when transferred to slants of standard yeastrel agar also gave rise to normal growth.

Apart from these small differences the serial transfers did not affect the macroscopical appearance of the cultures or colonies.

The variant cultures 11v, 18v, 20v, 16 and 20-2, together with stock cultures which had not been subjected to the serial transfers, were inoculated on yeastrel mannitol agar slants, yeastrel sucrose broth, veal agar slants, veal broth, litmus milk, potato slices, and nutrient gelatine. The cultures were examined daily for 14 days and periodically thereafter for seven weeks. In the first few days all cultures on yeastrel agar were clouded or chalky, and as the period of incubation lengthened the chalky condition became more pronounced. In 14 days cultures of 11v, 20-2 and 16 were distinctly roughened. In the other media the reactions were similar to those of the stock cultures, and to those already described for the strains 1 to 20. A slight clouding was apparent in the first few days in yeastrel broth. No growth was apparent on veal slants, and only a faint clouding was visible in veal broth inoculated with strain 16, and the stock cultures. No definite growth was apparent on potato slices or in gelatine stabs, and no liquefaction of the latter medium occurred within three months. In litmus milk all cultures produced an alkaline reaction and a shallow serum zone within seven days.

Effects of Treatments on Morphological Characters.

From two day old cultures on yeastrel slants slides were prepared of all the final cultures arising from the serial transfers of strains 11, 18 and 20, and from cultures of 16 and 20-2. Smears were stained by four methods; Gram (Conn, 1937), a weak solution of carbol-fuchsin (Fred et al., 1932), 5 per cent. erythrosin in phenol (Topping, 1937), and 3 per cent. crystal violet in ethyl alcohol. The most suitable were the carbol-fuchsin and crystal violet stains. Staining of the cells was improved by flooding slides with acetone and drying before adding the stain.

All organisms exhibited active motility, and those from cultures of 16 and 20-2, when stained by the technique of Gray (1926), exhibited peritrichous flagella.

Preparations showed the usual variety of cell forms associated with clover nodule bacteria, and described by Loehnis & Smith (1916), Gibson (1928), and Thornton (1931). The cell forms did not differ from those observed in the examination of strains 1 to 20, though the relative number of each type varied somewhat according to the medium on which the cultures had been grown in the course of the serial transfers. The usual forms present were commonly short and banded rods, with fewer coccoids and oval cells of varying sizes. These types were common to all the cultures and, except for a few gonidangia and Y shaped cells, comprised all the cells present in the cultures grown on standard yeastrel agar. Gonidangia were present in greater numbers in

cultures from calcium gluconate agar and, together with budding cells, were also prevalent in cultures from calcium free agar. A few Y shaped forms were also present in cultures from the calcium gluconate agar. No significant variation in the proportion of the different forms was observed in the cultures of the three strains from the same medium.

EFFECTS OF MEDIA ON EFFICIENCY OF NODULE BACTERIA.

Methods.

On completion of the serial transfers glasshouse trials were laid down to ascertain if the cultivation on different media had affected the efficiency of the bacteria. As stated previously since the microscopic and macroscopic characters of the strains had not been appreciably changed only cultures from the agar media were used for inoculation tests.

Commercial wild white clover seed was disinfected by two methods, one portion of seed being immersed for four and a half minutes in water at 65°C. (Helz <u>et al.</u>, 1927) and the other portion being treated by the corrosive sublimate process*. The treated portions of seed were sown in two separate jars of sterilised sand which were placed in sterilised glass vessels and covered with lids to prevent contamination during the period of germination and seedling growth. Both methods of disinfecting the seed proved effective in providing seedlings free from nodule bacteria.

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* The seed was immersed for seven minutes in 95 per cent. ethyl alcohol, washed in four changes of water, immersed for five minutes in 1/1000 corrosive sublimate, followed by a wash in sterile milk and finally washed in three changes of sterile water.

In the previous glasshouse 'strain' trials growth of the plants was not vigorous, and it was thought that the estuarine sand might contain a sufficiently high content of magnesium salts to produce a **detrimental** effect on plant and nodule growth (Horner, 1936). Sand was therefore washed in running water, then treated three times with one per cent. solutions of hydrochloric acid, and finally washed in six changes of tap water. Analysis had shown that the magnesium content of the sand, after the initial washing in water, was not excessive, being 0.8 m.eq. per 100 g. sand, which after the acid treatment was reduced to 0.44 m.eq. per 100 g. sand. After treatment the sand showed a lime requirement of 0.07 g. per 100 g. sand, consequently excess precipitated calcium carbonate was added at a rate of 2.0 g. per 1000 g. sand. Approximately 1200 g. sand was placed in each jar, 150 ml. nutrient solution added and the jars covered and sterilised. The nutrient solution was prepared by the same method as that used in the earlier glasshouse trials, and a trace of boric acid, 1 g. in 2.5 mill. (Brenchley & Thornton, 1925), and of manganese sulphate 6 g. in 1.0 mill. (Fred *et al.*, 1932) added. The presence of these trace elements did not noticeably improve growth of the clover plants.

On 17 July 1939, when the first true leaf of the seedlings unfolded, five plants were transferred to each jar of sand. Glass tubes, stoppered at the base with a loose wad of glass-wool, were placed in the middle of each. The sand was saturated with sterile water, and four pots for each treatment were inoculated with a water suspension of the appropriate culture.

After inoculation the surface of the sand was covered with a layer of sterile granulated cork 6 mm. in depth. As in the previous strain trials, jars were embedded in damp sawdust, and arranged on the benches in randomized form, (see Fig. 3). Subsequent watering of the jars was carried out by the method adopted in the earlier trials. The culture treatments were as follows:-

Checks . . . . . no inoculation.

11, 18, 20, derived from stock cultures of these strains.

11p, 18p, 20p, " " 46th. transfer on standard agar

11g, 18g, 20g, " " 46th. " " calcium gluconate agar.

11x, 18x, 20x, " " 22nd " " calcium deficient agar.

11v, 18v, 20v, " " plates of 9th. transfer on calcium gluconate agar.

16, 20-2 variant single colony isolations from strain 20.

Apart from periodical watering, in which the amount added was gauged by the height in the glass tubes, the jars were not disturbed until they were harvested 27 and 28 September 1939. When the plants were removed tops were cut off and roots washed free from sand, then dried at 100°C. and weighed. The dry-weight yields are given in Table XVII.

Because of the small amount of material available from single pots, tops and roots of two replications were combined to facilitate determinations of nitrogen content. The percentage nitrogen content of the combined replications, the average dry-weight yield, and the total nitrogen content for each treatment are given in Table XVIII.

TABLE XVII. The Influence of Serial Transfers and Calcium in Media on the Efficiency of Clover Nodule-bacteria as determined by the Effect on Dry-weight Yield of White Clover.

Medium	Cult -ure source	Row	Strain 11			Strain 18			Strain 20		
			Yield in grams			Yield in grams			Yield in grams		
			Tops	Roots	Total	Tops	Roots	Total	Tops	Roots	Total
Standard	Stock	1	0.649	0.297		0.042	0.023		0.371	0.174	
		2	0.658	0.228		0.239	0.122		0.360	0.137	
		3	0.413	0.138		0.105	0.040		0.483	0.216	
		4	0.941	0.363		0.195	0.084		0.477	0.177	
					3.687			0.850			2.395
Standard	46th trans -fer	1	0.483	0.234		0.128	0.049		0.421	0.161	
		2	1.096	0.294		0.207	0.121		0.261	0.145	
		3	0.674	0.221		0.170	0.083		0.249	0.124	
		4	0.664	0.255		0.224	0.111		0.565	0.169	
					3.921			1.093			2.095
Calcium	9th trans -fer	1	0.539	0.247		0.108	0.051		0.592	0.214	
		2	0.777	0.270		0.163	0.071		0.461	0.219	
		3	1.007	0.297		0.157	0.086		0.477	0.176	
		4	0.605	0.197		0.187	0.076		0.516	0.181	
					3.939			0.899			2.836
Calcium	46th trans -fer	1	0.520	0.156		0.117	0.062		0.392	0.194	
		2	0.913	0.343		0.210	0.098		0.788	0.291	
		3	0.697	0.306		0.170	0.071		0.958	0.331	
		4	0.749	0.238		0.228	0.096		0.550	0.217	
					3.922			1.052			3.721
No Calcium	22nd trans -fer	1	0.573	0.197		0.089	0.061		0.172	0.092	
		2	0.505	0.213		0.124	0.056		0.130	0.087	
		3	0.558	0.179		0.055	0.025		0.176	0.065	
		4	0.475	0.163		0.114	0.051		0.144	0.075	
					2.963			0.575			0.941
Checks not inoculated		1	0.087	0.071							
		2	0.099	0.082							
		3	0.076	0.051							
		4	0.106	0.065							
					0.645						

Results.

Dry-weight Yields of Clover Plants.

Results indicate that these cultural treatments altered the efficiency of some of the organisms. The yields associated with the cultures were, on account of the shorter growing period, lower than those obtained in the previous trials but the relative efficiency had not greatly altered in the intervening period of nine months. (Fig. 4).

The treatment involving 46 transfers on the standard agar showed a small increase in yield with strains 11 and 18, and a reduction in yield with strain 20, but these differences were not statistically significant.

The 46 transfers on calcium gluconate agar did not appreciably affect the efficiency of strains 11 and 18, but had a marked beneficial effect on strain 20. (Fig. 5). Repeated transfers of strain 20 on this medium increased the yield from 2.39 g. to 3.72 g., the latter being approximately the same as the yield of 3.93 g., associated with strain 11. Results obtained from the use of the variant culture 20v, selected from a plate of the ninth transfer of strain 20, showed a similar tendency. Yields of the plants inoculated with 11v and 18v were approximately the same as those associated with the stock cultures. The difference in yields associated with culture 20 v and the 46th transfer of strain 20 is not significant, but results suggest that the greater number of transfers to which the latter was subjected was, in part, responsible for the increase in efficiency.

Yields associated with the cultures grown on the calcium deficient agar show that efficiency of the three strains was considerably reduced, the yields of strains 11, 18 and 20 being lowered by 24, 47 and 55 per cent. respectively. (Figs. 6, 7, 8). The reductions in yields associated with strains 18 and 20 are statistically significant, but the effect of calcium deficient agar on strain 11 was not established.

Cultures of 16 and 20-2, isolations of abnormal white colonies in strain 20, did not induce nodule formation. Similar failure followed more recent inoculations of plants grown under controlled conditions in stoppered bottles.

Variations in yields of replications of some treatments were considerable and detract somewhat from the value of the results. In this experiment it was noted that the majority of replications giving high yields were situated at the south end of the glasshouse, where higher temperature, or better lighting, might have caused the increase in growth. Similar variations in replications, not necessarily from the same cause, were present in the earlier strain trials and appear to be frequent in this type of experiment (Loehnis, 1930; Wilson et al., 1937; Burton & Wilson, 1939). Because of these variations the data contained in Table XVII were subjected to statistical analysis. Differences in yields connected with media treatments of strain 11 were not significant so that an analysis of the data has not been included in the following 'Analysis of Variance'.

Fig. 3. Portion of glasshouse illustrating arrangement of pots.

Fig. 4. Growth of white clover inoculated with stock cultures of strains 20, 18, 11 and uninoculated check.

Fig. 5. Growth of white clover inoculated with cultures from calcium gluconate agar : reading left to right, strains 18, 11, 20, uninoculated check.

Fig. 6. Growth of white clover inoculated with cultures (left to right) of strain 11 from 46th transfer on cal. gluconate agar, 46th transfer on standard agar, 22nd transfer on calcium deficient agar, 9th transfer on cal. gluconate agar.

Fig. 7. Growth of white clover inoculated with cultures (left to right) of strain 18 from 46th transfer on cal. gluconate agar, 9th transfer on cal. gluconate agar, 22nd transfer on calcium deficient agar, 46th transfer on standard agar.

Fig. 8. Growth of white clover plants inoculated with cultures (left to right) of strain 20 from 46th transfer on cal. gluconate agar, 9th transfer on cal. gluconate agar, 22nd transfer on calcium deficient agar, 46th transfer on standard agar.

Statistical Analysis of Yields.

Strain 18.

Analysis of Variance.

Source of variation	Degrees of freedom	Sum of Squares	Mean Squares
Media	4	0.041	0.0102
Rows	3	0.064	-
Error	12	0.033	0.0027
Total	19	0.138	-

Variance ratio 0.0102/0.0027 = 3.7 (F test)

Judged by the 5 per cent. level of significance, the effect of the media is significant.

Standard error of treatment totals $= \sqrt{0.0027 \times 4}$

" " of 2 " " $= \sqrt{0.0027 \times 4 \times 2}$

$= 0.147$ g.

Therefore the difference between the totals for significance $= 0.294$ g.

Strain 20.

Analysis of Variance.

Source of variation	Degrees of freedom	Sum of Squares	Mean Squares
Media	4	1.064	0.266
Rows	3	0.04	-
Error	12	0.37	0.03
Total	19	1.474	-

Variance ratio 0.266/0.03 = 8.8 (F test)

The effect of the media, based on the one per cent. level of significance is therefore highly significant.

Standard error of treatment totals $= \sqrt{0.03 \times 4}$

" " of 2 " " $= \sqrt{0.03 \times 4 \times 2}$

$= 0.49$ g.

Therefore the difference between the totals for significance = 0.98 g.

Results show that both the calcium gluconate and the calcium deficient media caused significant differences in dry-weight yields. The former increased yields with strain 20 and the latter produced marked reduction in yields by strains 18 and 20.

It is thus possible to modify the effectiveness of nodule bacteria by serial transfers on media differing in available calcium content. Apparently all strains are not equally susceptible to variation under cultural conditions, since although strain 11 remained virtually stable, the effectiveness of strain 20 was modified by the presence or absence of calcium in the medium. The three strains had been cultivated and stored under similar conditions for one year prior to commencement of the serial transfers. In that time they had not developed any noticeable differences in cultural characters, and the conditions of storage on the standard medium evidently had not altered the relative efficiency of the strains.

Nitrogen Content of Clover Plants.

The amount of plant material from each jar was so small that two replications were combined to form samples for the nitrogen determinations. The figures given in Table XVIII are derived from the samples so formed.

TABLE XVIII. The Influence of Serial Transfers and Calcium in Media on the Efficiency of Clover Nodule-bacteria as determined by the Effect on the Nitrogen Content of White Clover.

Medium	Culture source	Strain 11			Strain 18			Strain 20		
		Yield in grams	Nitrogen %	Nitrogen total mg.	Yield in grams	Nitrogen %	Nitrogen total mg.	Yield in grams	Nitrogen %	Nitrogen total mg.
Standard	Stock	3.687	2.72) 2.58)	97.79	0.848	2.02) 1.81)	16.20	2.395	2.56) 2.53)	60.83
"	46th transfer	3.921	2.68) 2.82)	107.8	1.093	1.97) 2.00)	21.64	2.095	2.69) 2.66)	55.94
Calcium	9th transfer	3.939	2.62) 2.76)	106.0	0.999	1.81) 1.82)	17.93	2.836	2.50) 2.46)	70.33
"	46th transfer	3.922	2.66) 2.94)	109.7	1.052	1.93) 1.82)	19.66	3.721	2.57) 2.42)	93.00
No Calcium	22nd transfer	2.963	2.72) 2.71)	77.6	0.575	1.66) 1.65)	9.49	0.941	1.53) 1.60)	14.68
Checks Not inoculated		0.645	1.41	9.09						

A reduction in percentage nitrogen content is apparent in the results of strains 18 and 20 from the calcium deficient medium. Since the dry-weight yields were also small the total nitrogen associated with these two cultures is therefore extremely low and is similar to that of the uninoculated check plants. All the plants, other than the checks and those inoculated with cultures of 16 and 20-2, showed good nodule development.

Results of treatments with the stock cultures agree with those generally obtained by other workers who found that a high nitrogen percentage is associated with a high yield and a low nitrogen percentage with a low yield. This conclusion does not agree with that drawn in the earlier strain trials where no correlation existed between nitrogen percentage and dry-weight yield. At the time of arranging the 'effect of media' trials there was no intention of comparing the results with those of the earlier strain trials. Consequently no attempts were made to standardize operations. The sand was treated with hydrochloric acid before lime was added, the nutrient solution contained traces of boric acid and manganese sulphate, seed was of different origin, the date of sowing was different, and the period of growth was nine weeks instead of 24 weeks. The first two variations in treatment were introduced to overcome possible causes of the poor plant growth produced in the strain trials; the others were unavoidable. It is not known if the differences were responsible for the change in relative nitrogen content associated with the three strains.

Glasshouse Trials in New Zealand.

Glasshouse trials with the same cultures were repeated at Auckland, New Zealand, in December 1939. On 7 September the final cultures of the serial transfers were sub-cultured on yeastrel agar slants. The tubes were sealed with rubber stoppers and transported in a cool chamber to New Zealand, where they were transferred to standard agar slants. Growth of many was retarded and the culture of strain 20 from the 46th transfer on standard agar and culture 11v from the 9th transfer on calcium gluconate agar, failed to grow. Repeated transfers to various modifications of yeastrel peptone sucrose medium, and to the modified medium of Ashby (1907) did not improve the vigour of the cultures. No tests were made to ascertain if the media were deficient in any particular element, but since nodule bacteria had been previously grown in New Zealand on media rich in lime, 0.5 per cent. of calcium carbonate was added to the yeastrel agar. The cultures, other than the two referred to above, made normal growth on this medium. The period of storage after the completion of the serial transfers consisted of 16 weeks on the final slants of the serial transfers, ten weeks on standard yeastrel agar, and two weeks on yeastrel agar plus lime.

In these experiments the same procedure was adopted as that employed in the Scottish trials save that New Zealand certified white clover seed was used. Seedlings were transplanted and inoculated 2 December 1939, treatments being in triplicate. On 15 February 1940 the plants were harvested in the manner previously

described, nodules being present on roots of all those inoculated. The plants were dried and weighed, yields of the various treatments being given in Table XIX.

TABLE XIX. The Influence of Calcium in Media on the Efficiency of Strains of Clover Nodule-bacteria : the Effect of the Strains on Dry-weight Yield of Clover Plants.

Medium	Culture source	Row	Strain 11 yield in grams		Strain 18 yield in grams		Strain 20 yield in grams	
			Per pot	Total	Per pot	Total	Per pot	Total
Standard	Stock	1	1.240		1.016		1.141	
		2	1.857		0.483		1.783	
		3	2.326	5.423	0.542	2.041	2.470	5.394
Standard	46th transfer	1	1.951		0.712		--	
		2	2.047		0.536			
		3	2.564	6.562	0.792	2.040	--	--
Calcium	46th transfer	1	2.439		0.403		2.039	
		2	1.527		0.617		1.630	
		3	2.250	6.216	0.970	1.990	2.055	5.724
No Calcium	22nd transfer	1	1.691		0.543		0.425	
		2	1.590		0.647		0.536	
		3	1.797	5.078	0.675	1.865	0.326	1.287
Checks not inoculated		1	0.438					
		2	0.379					
		3	0.517	1.334				

Variations in yields of replications also occurred in this experiment, the majority with higher yields being situated in the northern portion of the house. At the time of the experiment, the roof of the glasshouse was covered with a lath blind to reduce the temperature, and because of this, the light intensity and possibly the temperature, would be greatest near the northern face of the glasshouse. (Fig. 9).

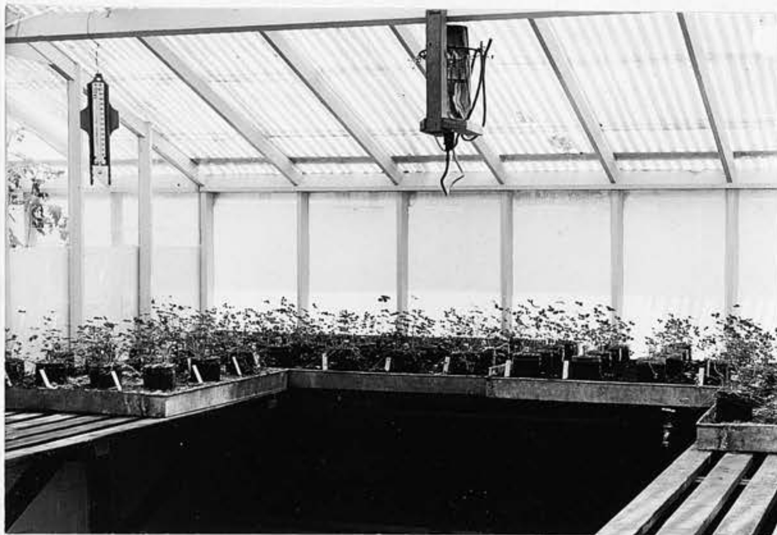

Fig. 9. Arrangement of pots and method of shading roof in New Zealand glasshouse trials.

Results, in the main, substantiate those obtained in the earlier trial (see Table XVII). A few discrepancies exist, especially in the improved yields given by the stock culture of strain 20, and the culture of strain 18 from the calcium deficient medium. Yields associated with cultures from the calcium deficient medium were all less than those of the other treatments, but only that of strain 20 was significantly lower. (Figs. 10 and 11). On the whole, storage of the cultures for approximately ten weeks on yeastrel sucrose agar and two weeks on the same medium plus lime, did not appreciably improve efficiency of the cultures previously grown on the calcium deficient medium.

DISCUSSION.

The effect of media on the nodule organisms has been investigated by numerous workers, and Fred _et al_. (1932) in discussing the various findings state,-

> "Contrary to the admitted possibility of variation resulting from cultural conditions, the fact remains that culture of the _Rhizobia_ on artificial media is not so degrading as formerly supposed".

Most of the work was concerned with the influence of prolonged growth on media rich or poor in nitrogen.

Burke & Hohl (1930) attempted to change the 'infective ability' of nodule bacteria by growing them on media containing plant juices of legumes belonging to other cross-inoculation groups, but they failed to extend the host-range of the organisms.

Fig. 10. Growth of white clover inoculated with stock cultures of strains 11, 20 and 18. (New Zealand trials).

Fig. 11. Growth of white clover inoculated with cultures of strains 11, 20 and 18 from calcium deficient agar. (New Zealand trials).

Almon & Baldwin (1932) stated that aberrant cultures, characterised by chromogenisis and inability to form nodules, were isolated from filtrates of normal cultures and from nodules. In an attempt to change the non-typical forms to typical nodule-producing ones by variations in media, oxygen tension, reaction, temperature, and frequency of transfer they secured a few normal cultures, but the effect on the host plant was not known. Albrecht & McCalla (1937a) and McCalla (1937) by using a colloidal clay medium, found that in the absence of calcium abnormal forms of nodule bacteria were produced which were either of 'low inoculating power' or incapable of forming nodules. With the addition of calcium abnormal forms became normal and produced good nodulation on plants supplied with calcium.

In 1930 Israilsky & Starygin found that the nodule organism from lupin dissociated into rough and smooth forms. Later Israilsky (1933) and Israilsky & Leonowitsch (1933) showed that the nodule bacteria from _Vicia_, _Medicago_, _Melilotus_ and _Onobrychis_ species also dissociated into R, O and S forms which, although capable of forming nodules, exhibited small differences in physiological reactions and morphological characters.

Most of the quoted experiments are concerned with the production of abnormal forms of nodule-bacteria, of which some S, O and R forms, are capable of producing nodules, and some, the chromogenic forms, produce either few or no nodules. There is no indication, apart from the failure of some abnormal forms to produce nodules, that efficiency of organisms was altered by cultivation on media.

In the present work abnormal forms, other than perhaps the chalky cultures from the 9th transfer on calcium gluconate, were not observed during the period of the serial transfers, nor did the cultures exhibit any unusual morphological features to suggest that the repeated transfers, or the media, had affected them in any way. On the other hand the definite effects on the host must have been caused by some physiological change in the organisms brought about by the media treatments. This is substantiated by the fact that the organisms previously grown on the calcium-free medium did not improve in efficiency after storage for some weeks on a medium - yeastrel sucrose agar - moderately rich in calcium.

The abnormal cultures, 16 and 20-2, isolated from one of the clover strains prior to the initiation of the serial transfers, were comparable with the rough colonies referred to by Israilsky (1933). Unlike the R forms described by him they did not differ greatly in their biochemical activities from normal cultures, and they did not produce nodules. These cultures were not subjected to the serial transfers on media deficient in and supplied with calcium.

SUMMARY OF SECTION II.

Three strains of nodule-bacteria were subjected to frequent transfers to yeastrel-peptone-sucrose broth and agar, and to the same media deficient in and with an excess supply of available calcium. Plates were periodically prepared from the cultures of each treatment, and on completion of the transfers, cultures from the agar series were used for inoculating white clover plants.

The strains were not affected morphologically or culturally by repeated transfers to the different media, but two treatments were associated with large differences in yields of the inoculated plants. The calcium rich medium caused a significant increase in yield of the plants inoculated with one strain, and the calcium deficient medium caused a significant reduction in yield, and in nitrogen percentage with two of the strains.

On completion of the transfers, each culture was held for 19 weeks on the final slant, and stored for a further 12 weeks on yeastrel-sucrose agar moderately rich in calcium. Inoculation tests showed that storage on the latter medium did not materially improve the efficiency of the cultures previously grown on the calcium deficient medium.

The three strains were not affected to the same extent by the treatments, one strain remaining comparatively stable.

The results showed that cultivation of nodule bacteria on media with either high or low calcium content altered the effect of the organisms on the host.

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III. NODULE BACTERIA OF VARIOUS LEGUMES OF SCOTLAND.

INTRODUCTION.

The work set out in this section was undertaken to ascertain the position of strains of nodule bacteria associated with various Scottish legumes in regard to their cross-inoculation grouping and to their place in Bergey's (1939) systematic key. The nodule bacteria from legumes of Scotland had not been previously studied, and many of the species and some genera of the host plants are not included in the recognised cross-inoculation groups.

Fred et al. (1932) brought together the extensive data on root nodule bacteria, and showed that organisms from some legumes will produce nodules on a limited number of others. On the basis of cross-inoculation they conveniently arranged the host plants and the organisms in cross-inoculation or plant-bacteria groups. Members of these may not belong to the same plant genus but are related in that organisms forming nodules on any one are also capable of forming nodules on other members of the same group. This classification assumes that crossing between bacteria and hosts of different groups does not take place. They listed 16 cross-inoculation groups, and Wilson & Sarles (1939) extended this list to include 21 groups, comprising some 266 species of leguminous plants.

Baldwin & Fred (1929b) proposed that five species of root nodule bacteria be recognised on the bases of morphological,

cultural and physiological characters. Eckhardt _et al_. (1931) added a sixth species. Both groups of workers regarded as the principal feature for separating species ability of the organism to form nodules on certain groups of legumes. Bergey _et al._ (1939), with minor modifications, followed the classification suggested by the above authors.

Recently J.K. Wilson (1939a) showed that nodule bacteria produced nodules on plants of cross-inoculation groups different from those from which organisms were obtained. He also showed that strains of nodule organisms from the same group varied considerably in their ability to form nodules, and that different species of hosts varied in susceptibility. He summarized the position (page 47) in the following sentence:-

> "The principal conclusion from the data is that neither a sufficient number of plant species nor sufficient number of diverse strains of the organisms have been employed by previous investigators, in a comparative study of the symbiotic relations, to justify the establishment of definite plant-bacteria groups or to encourage the use of those that have been arranged".

The greater part of the present work was completed when Wilson's paper was received and it was not then possible to arrange the experiments in the light of his results. Use of additional strains of organisms would have provided further data from which to draw conclusions, but it is doubtful if these would have been greatly changed thereby.

SOURCE OF CULTURES.

During the summer of 1938 cultures were prepared from nodules taken from the following legumes found growing in East Lothian, Scotland.

Culture No.	Host origin.		Host location.
18	Trifolium repens	White clover	Pasture
22	Ulex europaeus	Whin	Waste land
23	Cytisus scoparius	Broom	Waste land
27	Vicia cracca	Bush vetch	Roadside
29	Lotus corniculatus	Birdsfoot trefoil	Pasture
32	Medicago lupulina	Black medick	Waste land
33	Melilotus arvensis	Field melilot	Waste land
34	Ononis repens	Rest harrow	Sand dune
36	Astragalus danicus	Milk vetch	Sand dune

An additional culture, No. 25, from Cytisus laburnum was obtained from the collection of Dr. T. Gibson, East of Scotland College of Agriculture, Edinburgh.

METHODS.

Isolation of Nodule Bacteria.

Cultures were prepared from nodules by the same method as that used in work on nodule organisms of white clover. After sterilisation, small portions of nodules were crushed and spread on two media, yeastrel sucrose agar and asparagus mannitol agar. Following incubation for five or six days at 30°C. typical single colonies were transferred to yeastrel sucrose slants.

Cultural and Morphological Characters.

For the study of cultural characters cultures were transferred to the different media used in the examination of strains of

clover organisms, e.g., yeastrel sucrose broth and agar, yeastrel mannitol agar, veal broth and agar, litmus milk, potato slice, and nutrient gelatine. These media were prepared in the same manner as previously described. Nutrient gelatine stabs were sealed with a rubber stopper and incubated for three months at 21°C. Other tubes were incubated at 30°C. for three weeks, during which time they were regularly examined.

Smears for examination of morphological characters were stained with (a) a weak solution of carbol fuchsin and (b) three per cent. crystal violet in ethyl alcohol. In the preparation of smears for demonstrating the presence of flagella, cultures from yeastrel sucrose slants were transferred to slants of modified Hitchner's medium suggested by Stern & Sarles (1938). This medium formed a large amount of clear liquid at the base of the slant and, though a moderate growth was obtained on the slant surface, mucilage formation was considerably reduced. Two to three days after inoculation motility was at a maximum and a small loop from the expressed liquid was transferred to a drop of distilled water on a slide, allowed to flow down it, dried at room temperature, then stained by the method of Leifson (1930). A duplicate preparation was stained by the method of Gray (1926).

Cross-inoculation Trials.

The plants were grown in sand and in agar contained in stoppered milk bottles. The sand was from the same source as that used for the clover-strain trials, and was similarly treated with acid and washed with water. In each bottle was placed 250 g.

sand, containing 0.63 g. calcium carbonate, 25 ml. nutrient solution and sufficient distilled water to bring to saturation. The nutrient solution was prepared according to the formula previously used and given on page 32. The bottles were plugged with cotton-wool and sterilised for two hours in steam at 20 lb. pressure. The agar medium was prepared according to the formula of Cunningham (1924)*, and 250 ml. was poured into each bottle. After being plugged bottles were sterilised in the autoclave by slowly raising the pressure to 30 lb.

Preliminary experiments showed that the time required for germination by the different legume seeds varied greatly and that some seeds required treatment to accelerate germination. A number were tried including immersion in acid, abrading with a file, singeing in a gas flame, and cutting the testa with a knife. The last proved the most satisfactory and was applied to the 'hard seed' species of Cytisus, Ulex, Ononis and Astragalus. Seed was then disinfected by the alcohol-corrosive sublimate method used in the treatment of white clover seed. Seeds of Lathyrus, Lupinus and Phaseolus species being large and of high

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* Agar medium for plant growth (Cunningham, 1924).

KCl	10.0 g.
$CaSO_4.2H_2O$	2.5 g.
$Ca_3(PO_4)_2$	2.5 g.
$MgSO_4.7H_2O$	2.5 g.
$FePO_4$	2.5 g.
Agar	7.5 g.
Water	1 litre.

germinating capacity were sown directly into the test bottles, immediately after disinfection. The slower germinating species of Cytisus, Ulex, Ononis and Astragalus were germinated in bottles of sterilised sand. The small seeds which germinated rapidly, Trifolium, Lotus, Anthyllis, Melilotus and Medicago species, were grown on agar in Petri dishes. The method of germinating small and hard seeds in separate containers permitted selection of vigorous seedlings for transplanting to the test bottles. This was of particular advantage in the bottles containing agar medium in which germination is most erratic.

After germination, healthy seedlings were transferred, under aseptic conditions, to the bottles of sand and of nutrient agar. With the exception of species of Phaseolus, which were grown alone, plants of two genera were grown in each bottle. Two seeds or seedlings of large seeded species were included in each bottle, and when the seed was small, three seedlings were planted. (Fig. 12).

Plants were inoculated with a water suspension from a yeastrel agar slant. Two sand bottles and one agar bottle of each pair of host plants were used for each culture treatment. As checks on the technique employed, two sand bottles and one agar bottle of each pair of hosts were not inoculated. After inoculation bottles were transferred to the unheated glasshouse and, to prevent possible heating of the roots, the bottles were embedded to the depth of the sand or agar, in damp sawdust.

Fig. 12. Method of growing plants in bottles under controlled conditions.

The majority of the bottles did not require further attention until harvested; after four weeks the sand bottles containing Lathyrus pratensis and Phaseolus vulgaris plants received from 5 to 8 ml. of sterile water.

Back-inoculation Experiments.

The back-inoculations were carried out in sand bottles in New Zealand, and the technique of the cross-inoculation trials was reproduced as closely as possible. All treatments were carried our in duplicate and uninoculated checks were included with each set of host plants.

CULTURAL AND MORPHOLOGICAL CHARACTERS OF NODULE BACTERIA.

Results.

In general all cultures on the two yeastrel agar media showed similar growth. Slight differences developed in surface appearance, amount of growth of the cultures and of mucilage at the base of the slants. The following description of strain 22, was typical of the majority of the cultures on both media. The slant growth was luxuriant, raised to convex, smooth, moist, shining, mucilaginous, clouded to white, with colourless streaks. The margin of the growth was entire and only a small amount of slime collected at the base of the slant. In the first three days of growth cultures of strains 23, 25, 27, 29, 32, and 36 were similar to 22; 18 and 33 showed white streaks or a general chalky appearance, and 33 had a rough surface; 34 was not so luxuriant

as 22 and was dry on the surface. With increase in age the slant growth of all cultures became whiter; 18, 32, 33, 34 and 36 became distinctly chalky and showed the most abundant growth and 18, 27, 29, 32 and 33 formed a deposit of mucilage at the base of the slant. Cultures of 33 developed horizontal surface folds.

In yeastrel broth all cultures became clouded within three days. A ring was formed by 18 and 27, and a pellicle by 32 and 33. Clouding persisted in the broth for at least three weeks.

In veal broth slight clouding occurred in three days in tubes inoculated with strain 32, and in six days with 33, but no clouding was apparent in tubes inoculated with the other strains. On veal agar slants 32, in three days, and 33, in six days, produced luxuriant growth similar to that formed on yeastrel agar. In three weeks 34 showed a few small colonies, 1 mm. to 1.5 mm. in diameter, on the surface of the slant. The other strains did not produce visible growth.

On potato slice only strains 29, 33 and 36 formed more than a meagre growth. Strain 29 showed a convex, moist, shining, slightly brown growth; 33 a slightly raised, moist, shining, light cream spreading growth; and 36 a raised, dry, light brown growth restricted to the line of inoculation.

In litmus milk a serum zone of 1 to 2 mm. was formed in three days by all strains except 25, and a slight alkaline reaction occurred in cultures of 18, 25, 27 and 33. After ten days the serum zone was deeper in cultures of 18, 27 and 29, but did not

exceed 3 mm. in depth. An alkaline reaction was evident in all cultures except those of 27 and 32, being most marked in 18, 22, 23 and 25. Later, cultures of 27 and 32 developed an acid reaction.

None of the strains liquefied gelatine within three months. Slides from four day old cultures on yeastrel sucrose slants did not show any marked differences in morphology. All exhibited the same irregular forms associated with nodule bacteria, though the relative numbers of the forms varied in the different cultures. Cocci or short rods predominated in all strains except 22, which consisted of comparatively long rods, single and pairs, and a few cocci, club-shaped and Y-shaped rods. In strain 18 unevenly stained oval cells, banded rods, and club-shaped rods were common. Strains 25 and 33 were similar to 18 though the larger irregular forms were less evident. Strains 23, 27, 29, 32, 34 and 36 consisted almost exclusively of cocci or very short rods and small unevenly stained oval cells.

Although a few bacteria in every culture carried up to four peritrichous flagella, the majority of rods with flagella possessed but one or two.

Discussion of Results.

Growth of the different strains was similar to that described by Loehnis & Hansen (1921), Stevens (1925), Fred et al. (1932) and Hofer (1935). The chalky condition of cultures on agar media is not a diagnostic character, although regularly associated with some strains. Loehnis & Hansen noted a similar

condition in colonies of cultures from clover and vetch and also in cultures of Bacillus radiobacter, and stated that the presence or absence of a whitish centre could not be used in differentiation. The formation of a dry, rugose surface in strain 33 is unusual in cultures of nodule bacteria, although a somewhat similar condition was noted in strain 18. Growth on veal media of strains 32 (Medicago lupulina) and 33 (Melilotus arvensis) showed close agreement with that obtained by Hofer (1935) who found that only the alfalfa group of nodule organisms developed on this medium. On the other hand liquefaction of gelatine by cultures from M. lupulina and M. arvensis or by cultures by other legumes as reported by Hofer & Baldwin (1932) and Fred et al. (1932) was not observed. In litmus milk differences, other than the acid reaction produced by strains 27 and 32, were only slight. The serum zone was not strongly developed in any culture and its presence was not connected with the grouping of strains indicated by Stevens (1925), Loehnis & Hansen (1921) and Fred et al. (1932). Further, production of acid in litmus milk by strain 32 and not by 33, and the formation of growth on potato by strain 33 and not by 32, suggested that organisms from the alfalfa plant-group are most variable in their reactions. All cultures used were derived from 'single colony' isolations, and all formed typical nodules on their respective hosts.

The morphological differences between organisms of the strains were slight and mainly variations in numbers of different cell forms present.

The relation of morphology and cultural characters of the strains to the species of nodule bacteria is discussed later in this paper.

CROSS-INOCULATION TRIALS.

Introduction.

In June 1939 the strains enumerated on page 90, were used for inoculating seedlings of legumes grown under controlled conditions. With the exception of strain 25 (Cytisus laburnum), they had been isolated from nodules approximately one year prior to the trials and during that period were cultivated and stored on yeastrel sucrose agar slants. Strain 25 was isolated in 1933, and also cultivated and stored on this medium.

Two sand bottles and one agar bottle of each pair of host plants were used for each culture treatment. The host plants were paired as follows:-

Trifolium repens	and	Lathyrus pratensis.
Lotus corniculatus	"	Melilotus arvensis.
Anthyllis vulneraria	"	Medicago lupulina.
Ononis repens	"	Astragalus danicus.
Lupinus nanus	"	Cytisus laburnum.
Ulex europaeus	"	Cytisus scoparius.
Phaseolus vulgaris	"	Phaseolus vulgaris.

Seeds and seedlings were planted in the bottles 17 to 22 June 1939, and on the latter date were inoculated with cultures of the various strains. On 21 August and 8 September, plants were removed from the bottles and examined for nodules. Plants examined

8 September consisted of the slow growing species L. nanus, C. laburnum, U. europaeus and C. scoparius. Some of the plants of C. laburnum, U. europaeus and C. scoparius were so small, not more than 2.5 cm. in height, that they were left for the longer period to permit of further growth.

Results of Cross-inoculation Trials.

Conditions in the closed bottles were not ideal for growth, but the majority of the plants remained healthy for the duration of the trials. Of those plants which did not possess nodules, T. repens, M. lupulina, M. arvensis and L. pratensis, were pale green or yellow in colour and usually stunted. All plants of P. vulgaris were bronzed. The other hosts without nodules did not exhibit any unhealthy appearance. Roots of all plants lacking nodules except T. repens, were normal in colour and healthy in appearance. Roots of T. repens without nodules were brown in colour.

Results of inoculation of the various legumes with strains of nodule bacteria are given in Tables XX and XXI.

TABLE XX. The Formation of Nodules by Nodule Bacteria from Various Leguminous Plants. Agar Series.

Plants inoculated	Source of Inoculum									
	18 Trifolium repens	22 Ulex europaeus	23 Cytisus scoparius	25 Cytisus laburnum	27 Vicia cracca	29 Lotus corniculatus	32 Medicago lupulina	33 Melilotus arvensis	34 Ononis repens	36 Astragalus danicus
Trifolium repens	X	-	-	-	-	-	-	-	-	-
Lathyrus pratensis	-	-	-	-	X	-	-	X	-	-
Lotus corniculatus	-	X	-	-	-	X	-	-	-	X
Melilotus arvensis	-	-	-	-	-	-	X	X	-	-
Medicago lupulina	-	-	-	-	-	-	X	X	-	-
Anthyllis vulneraria	-	X	-	-	-	X	-	-	-	-
Ononis repens	-	-	-	-	-	X	-	-	-	X
Astragalus danicus	-	-	-	-	-	-	-	-	-	-
Lupinus nanus	-	-	-	-	-	-	-	-	-	-
Cytisus laburnum	-	-	-	X	-	-	-	-	-	-
Ulex europaeus	-	X	X	X	-	-	-	-	-	-
Cytisus scoparius	-	-	X	X	X	-	-	-	-	-
Phaseolus vulgaris	-	-	-	-	-	-	-	-	-	-

The presence of nodules is indicated by X sign.
The plants bracketed were grown in the same bottle.

TABLE XXI. The Formation of Nodules by Nodule Bacteria from Various Leguminous Plants. Sand Series.

Plants inoculated	Source of Inoculum									
	18 Trifolium repens	22 Ulex europaeus	23 Cytisus scoparius	25 Cytisus laburnum	27 Vicia cracca	29 Lotus corniculatus	32 Medicago lupulina	33 Melilotus arvensis	34 Ononis repens	36 Astragalus danicus
Trifolium repens	X	–	–	–	–	–	–	–	–	–
Lathyrus pratensis	X	–	–	–	X	–	–	–	–	–
Lotus corniculatus	–	X	–	X	–	X	–	–	X	X
Melilotus arvensis	–	–	–	–	X	–	X	X	–	–
Medicago lupulina	–	–	–	–	–	–	X	X	–	–
Anthyllis vulneraria	X	X	–	X	–	X	–	–	X	X
Ononis repens	X	X	–	X	–	X	–	X	X	X
Astragalus danicus	–	–	–	X	–	X	–	–	X	X
Lupinus nanus	–	X	X	X	–	–	–	–	–	–
Cytisus laburnum	–	X	–	X	–	–	–	–	–	–
Ulex europaeus	–	X	X	X	–	–	–	–	–	–
Cytisus scoparius	–	X	X	X	X	–	–	–	–	–
Phaseolus vulgaris	–	–	–	–	–	–	–	–	–	–

The presence of nodules is indicated by X sign.
The Plants bracketed were grown in the same bottle.

The results contained in Table XX show that in agar most strains formed nodules on two or more hosts. With a few exceptions, the legume from which the strain was obtained and the hosts on which it formed nodules are grouped together in the cross-inoculation groups of Fred et al. (1932), Carroll (1934) and Wilson & Sarles (1939). The exceptions occurred where nodules were formed on plants of L. pratensis by strain 33 (from M. arvensis), on L. corniculatus by strain 22 (from U. europaeus) and strain 36 (from A. danicus), on A. vulneraria by strain 22 (from U. europaeus), on O. repens by strain 29 (from L. corniculatus) and on C. scoparius by strain 27 (from V. cracca). Some of the host plants, C. laburnum, O. repens and A. danicus, have not been assigned to any particular cross-inoculation group by previous workers. Results contained in Table XX indicate that C. laburnum could be included with U. europaeus and C. scoparius.

In the sand bottles the number of successful cross-inoculations was approximately double the number in the agar bottles. In the sand, nodules were formed on A. vulneraria and O. repens by six strains, on L. corniculatus by five strains, on A. danicus and C. scoparius by four strains, and on M. arvensis, L. nanus and U. europaeus by three strains. With the exception of strains from C. scoparius and U. europaeus, and perhaps C. laburnum, which are included in the Cowpea group (Fred et al., 1932), and from A. danicus and O. repens which are not included in any group, the majority of the strains were from hosts belonging to different

cross-inoculation groups. Some of the strains formed nodules on a wide range of hosts. Strain 25 (from C. laburnum) formed nodules on seven genera, representing at least four cross-inoculation groups. Strain 22 (from U. europaeus) showed an almost similar range of nodule formation. In spite of the wide range of plants infected by some strains, and the number capable of forming nodules on some hosts, only a few reciprocal cross-inoculations occurred : M. arvensis X M. lupulina : U. europaeus X C. scoparius : U. europaeus X C. laburnum : L. corniculatus X O. repens : L. corniculatus X A. danicus : O. repens X A. danicus. The first two cross-inoculations are to be expected from association of the hosts in the recognised groups. The genera Ononis and Astragalus have not been classified, but results indicate they belong to the Lotus corniculatus group.

Nodule Characters.

Variations in size, shape and number of nodules occurred in different strain treatments. Data on these characters were limited because of the comparatively few cross-inoculations available, but certain observations were possible.

Most of the nodules were small, rarely exceeding 2 mm. in diameter, the majority falling within a range 0.75 mm. to 1.5 mm. Nodules on L. nanus were large and few in number, and those on A. vulneraria and C. scoparius were many, ten or more on each plant, and of average size. On A. danicus the nodules were small, not larger than 0.5 mm., and usually one to five in number.

Three strains, 22, 25 and 27, were generally associated with a large number of nodules, and strains 18 and 33 with few nodules.

The form of nodules on a plant was usually typical of those normally found on that species. The chief exception was the occurrence on some plants, of root swellings of doubtful origin. Swellings were not unlike incipient nodules of Lupinus nanus and Anthyllis vulneraria. They were convex and similar in colour to the surrounding root tissue. On Ononis repens they were small, 0.5 mm. to 1.0 mm. in diameter. On Lathyrus pratensis swellings appeared as longitudinal ridges, sometimes 4 mm. in length and, where a number were formed close together, gave the root a knotted or distorted appearance, as in Fig. 13. On Melilotus arvensis were found conspicuous swellings confined to root junctions. Nodules or swellings similar to those described were not observed on plants in the uninoculated bottles.

Plates were prepared from nodules and root swellings formed in the cross-inoculation trials and, where growth resembling that of nodule bacteria developed, single colonies were picked off. Those root swellings which did not produce bacterial growth were considered of non-bacterial origin, and no further reference is made to them.

Variation in Nodulation.

Some plants from sand bottles did not possess nodules even when other plants of the same host, in the same or duplicate bottle, were well nodulated. Those without nodules on one plant

in a bottle were; A. vulneraria inoculated with strain 22 from U. europaeus and 25 from C. laburnum; and M. lupulina with 33 from M. arvensis.

A few exhibited root swellings on one or two plants only, namely M. arvensis inoculated with strain 27 from V. cracca, O. repens with 33 from M. arvensis, and L. pratensis with 18 from T. repens.

Absence of nodules on some susceptible hosts indicated that individual plants varied in their proneness to infection. Occurrence of root swellings on one or two of the plants in a bottle also indicated variation in host susceptibility. This suggested that if a greater number of plants had been used in each test it is possible that nodules might have been formed on a wider range of hosts.

Variations also occurred in the number of host plants infected by strains from the same cross-inoculation group, (Table XXI). Strain 22 formed nodules on seven and strain 23 on three hosts. Both were from hosts classified in the cowpea group (Fred et al., 1932). Had more strains from each group been used, nodules might have been formed on more hosts.

Small variations in environment, such as texture and moisture content of the substratum, temperature and light intensity may have been partly responsible for these differences in nodulation. The influence of substratum was shown by the greater number of plants infected in the sand series than in those grown in agar. Growth was better in the sand bottles and it is possible that

factors which were responsible for improved plant growth were also responsible for greater nodulation. Formation of typical nodules in one bottle and root swellings in the duplicate could be accounted for by differences in environment in the two bottles.

BACK-INOCULATION TRIALS.

Introduction.

The outbreak of war 3 September 1939, necessitated cessation of this work in Edinburgh. It was resumed at a later date in New Zealand, to which country cultures were carried on yeastrel agar slants held in cool chamber.

Cultures derived from nodules and root swellings produced in the cross-inoculation studies were used for inoculating the original hosts with the object of verifying the results obtained. The two groups of hosts referred to in the cross-inoculation trials are designated "original hosts" and "secondary hosts", the former supplying the original strains, the latter those on which nodules were formed. Cultures from the secondary hosts were isolated 15 to 17 weeks prior to being used in the back-inoculation trials, and in the intervening period were stored on yeastrel sucrose agar.

Seed of L. nanus, A. vulneraria, L. corniculatus, A. danicus, C. scoparius, C. laburnum were taken from the same supply as used in the earlier trials, but those of T. repens, U. europaeus and M. lupulina were of New Zealand origin. Medicago sativa was used instead of Melilotus arvensis, and Vicia villosa instead of V. cracca as seed of these original hosts were not available at the time of planting.

Results.

TABLE XXII. <u>Inoculation of Original Hosts with Cultures from Secondary Hosts.</u>

"Original Host" and Culture	"Secondary Host" and Culture	Hosts inoculated.											
		Trifolium repens	Vicia villosa	Anthyllis vulneraria	Ononis repens	Astragalus danicus	Lotus corniculatus	Lupinus nanus	Ulex europaeus	Cytisus laburnum	Cytisus scoparius	Medicago sativa	Medicago lupulina
T. repens		X	X										
	L. pratensis	-	?										
	A. vulneraria	X		X									
U. europaeus									X				
	A. vulneraria			X					X				
	O. repens				X				X				
	L. corniculatus						X		X				
	L. nanus							-	X				
C. scoparius											X		
	L. nanus							X			X		
C. laburnum										X			
	A. vulneraria			X					X	X			
	O. repens				X				X	X			
	A. danicus					X			X	X			
	L. corniculatus						X		X	X			
	L. nanus							X	X	-			
V. cracca			X									X	
	M. arvensis		X									X	
	C. scoparius		X								X		
L. corniculatus							X						
	A. vulneraria			X			X		X				
	O. repens				X		X						
	A. danicus					X	X						
M. arvensis												X	X
	M. lupulina											X	X
	O. repens				X							X	
O. repens					X								
	A. vulneraria			X	X								
	A. danicus				X	X							
	L. corniculatus				X		X						
A. danicus						X	X						
	A. vulneraria			X		X							
	O. repens				?	X							
	L. corniculatus					X	X						

X = nodules formed : - no nodules formed : ? doubtful root-swellings.
Blank spaces = no inoculations carried out.

The majority of the inoculations produced nodules on both "original host" and "secondary host". (Figs. 14 to 21). Results verify those obtained in the earlier cross-inoculation trials. A discrepancy exists in the first group where no typical nodules were formed by the culture from L. pratensis when transferred to T. repens, the "original host", and to V. villosa, though root swellings were present on the latter, (Fig. 13). On the other hand nodules were formed in the check bottles of plants of T. repens and V. villosa inoculated with the original strain from T. repens.

DISCUSSION.

Cross-inoculation Groups.

In a recent paper J.K. Wilson (1939a) dealt fully with the relation that exists between leguminous plants and their associated organisms. Growing host plants under controlled conditions, similar to those described in this paper, Wilson demonstrated that the majority of 70 genera of leguminous plants tested by him 'symbiosed' with many strains of nodule organisms.

The work covered by this paper was intended to ascertain the inter-relation of nodule bacteria of the more common legumes of Scotland, and therefore only legumes found in that country were employed in cross-inoculation studies. Consequently only a few tests were made which agree with those of Wilson both in origin of the bacterial strains and the hosts subjected to inoculation.

Fig. 13. Root swellings on Vicia villosa inoculated with culture from Lathyrus pratensis originally inoculated with strain from Trifolium repens.

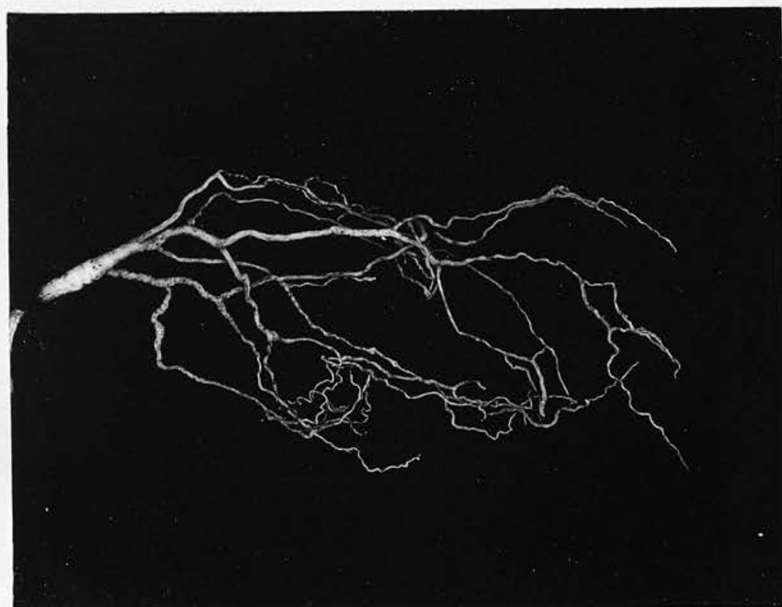

Fig. 14. Ononis repens inoculated with strain from Trifolium repens.

Fig. 15. Trifolium repens and Anthyllis vulneraria inoculated with culture from A. vulneraria originally inoculated with strain from T. repens.

Fig. 16. Ulex europaeus and Lotus corniculatus inoculated with culture from L. corniculatus originally inoculated with strain from U. europaeus.

Fig. 17. _Cytisus_ _scoparius_ and _Lupinus_ _nanus_ inoculated with culture from _Lupinus_ _nanus_ originally inoculated with strain from _Cytisus_ _scoparius_.

Fig. 18. _Cytisus_ _laburnum_ and _Ononis_ _repens_ inoculated with culture from _O. repens_ originally inoculated with strain from _C. laburnum_.

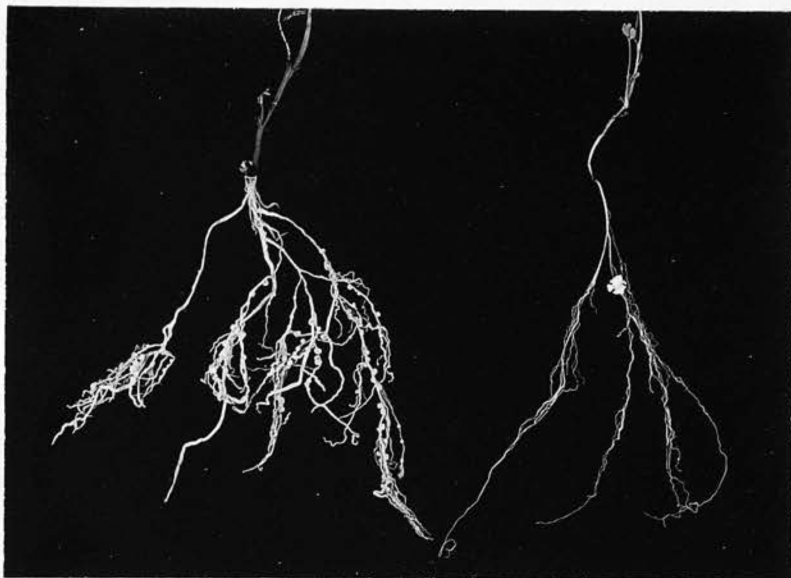

Fig. 19. Vicia villosa and Medicago sativa inoculated with culture from Melilotus arvensis originally inoculated with strain from V. cracca.

Fig. 20. Vicia villosa and Cytisus scoparius inoculated with culture from C. scoparius originally inoculated with strain from V. cracca.

Fig. 21. Lotus corniculatus and Astragalus danicus inoculated with culture from A. danicus originally inoculated with strain from L. corniculatus.

Three strains were from the same host species, i.e., those from Lotus corniculatus, Cytisus laburnum and Cytisus scoparius; and nine hosts were specifically the same, namely Trifolium repens, Lathyrus pratensis, Lotus corniculatus, Medicago lupulina, Anthyllis vulneraria, Cytisus laburnum, Cytisus scoparius, Ulex europaeus and Phaseolus vulgaris. The results obtained by Wilson and those given in this paper are compared in Table XXIII.

TABLE XXIII. Formation of Nodules by Bacteria from Various Leguminous Hosts.

A comparison of results recorded in this paper and those of Wilson (1939a).

Plants inoculated	Source of Inoculum					
	Cytisus laburnum		Cytisus scoparius		Lotus corniculatus	
	Reid	Wilson	Reid	Wilson	Reid	Wilson
Trifolium repens	-	-	-	-	-	-
Lathyrus pratensis	-	-	-	-	-	X
Lotus corniculatus	X	-	-	-	X	X
Medicago lupulina	-	-	-	X	-	-
Anthyllis vulneraria	X	-	-	-	X	X
Cytisus laburnum	X	X	-	-	-	-
Cytisus scoparius	X	-	X	X	-	-
Ulex europaeus	X	-	X	-	-	-
Phaseolus vulgaris	-	-	-	-	-	X

The X sign indicates that nodules were produced and the - sign that nodules were not produced.

With the exception of nodule formation on "original hosts", and the reciprocal crosses in the Lotus group, there is no agreement in results despite the fact that conditions under which the tests were carried out were apparently similar. It has been shown that strain of organism, host species, basal medium and environment, influence the extent of cross-inoculation. Variation in any one of these could have caused the differences in the two sets of results. Wilson also emphasised the variability of nodule formation, on different hosts and by different cultures, even from the same host species.

In a recent paper Wilson (1939b) suggested that a relationship exists between the degree of cross-pollination and the number of strains of Rhizobia with which a plant will 'symbiose'. He showed that of 32 strains of nodule bacteria used by him, U. europaeus symbiosed with 1; T. repens with 7; Spartium scoparium with 9; M. lupulina with 13; A. vulneraria with 14; L. corniculatus with 15; and P. vulgaris with 27. A comparison of results given in Tables XX and XXI with those of Wilson show that, except for the marked difference in nodulation of P. vulgaris, a similar tendency is apparent. Apart from this comparison the limited number of trials do not permit a more detailed discussion of the results obtained.

The present study was made partly to ascertain the cross-inoculation groups to which the legumes belonged. On the basis of cross-inoculation groups listed by Fred et al. (1932) it is difficult to assign all the legumes to definite groups.

If reciprocal crossing is a requisite then three groups can be arranged from the data in Tables XX and XXI, namely the Ulex-Cytisus group, the Medicago-Melilotus group, and the Lotus-Ononis-Astragalus group. In the last could be included A. vulneraria. In addition there is the equivalent of a reciprocal cross in the Vicia-Melilotus cross-inoculations, though one of the host plants, Lathyrus pratensis, was not the source of one of the cultures. Neither Vicia cracca nor Lathyrus pratensis is included in the groups of Fred et al. (1932) or Wilson & Sarles (1939), but their host relationships suggest they should be placed within the pea group. The first three groups can be readily accommodated in the Cowpea, Alfalfa and Lotus plant-bacteria groups of Wilson & Sarles, but the Vicia-Melilotus cross cannot be so placed. The data concerning the three remaining hosts, T. repens, L. nanus and P. vulgaris, are not sufficient to place these in definite groups.

The above grouping is not satisfactory, for it does not adequately account for overlapping of successful cross-inoculations associated with groups, hosts and strains. This has already been discussed (page 103) and the results suggest that, provided the inter-relation of hosts and nodule bacteria of Scotland be not markedly different to that of other countries, the cross-inoculation groups are not fixed entities, and that nodule formation by strains and hosts of different groups is more general than is usually recognised. Cross-inoculation between some groups - soybean, cowpea, lupin and Dalea - was recorded by Fred et al. (1932), Wilson (1937), and Wilson & Sarles (1939) but in the classification

of plant-bacteria groups this tendency was not considered of practical importance (Fred et al., 1932). J.K. Wilson (1939a) on the other hand showed that cross-inoculation by nodule bacteria and host plants of different groups readily takes place.

Species Differentiation.

The systematic classification of the nodule-bacteria has recently been reviewed by Bergey et al. (1939) whose classification is appended.

1. Litmus milk alkaline.
  a. Formation of serum zone in milk.
    b. Moderate growth, slight acid reaction on yeast water agar plus mono-, di-,and trisaccharides.
      c. Causes formation of root nodules on species of the genera Lathyrus, Pisum, Vicia and Lens. Bacteroids irregular with x, y, star-, and club shaped forms; rods peritrichous when young.
        1. Rhizobium leguminosarum.
      cc. Causes formation of root nodules on Phaseolus vulgaris, P. multiflorus, and P. angustifolius. Bacteroids vacuolated rods, few branched forms; young cells peritrichous.
        2. Rhizobium phaseoli.
      ccc. Causes formation of nodules on species of genus Trifolium. Bacteroids pear-shaped, swollen, vacuolated. Pentoses usually not fermented.
        3. Rhizobium trifolii.
  aa. No serum zone formed in milk.
    b. Scant growth, alkaline reaction on yeast water agar plus most carbohydrates.
      c. Causes formation of nodules on species of genus Lupinus and Ornithopus sativus. Bacteroids vacuolated, rods seldom branched.
        4. Rhizobium lupini.
      cc. Causes formation of nodules on Soja max. Bacteroids long slender rods, seldom vacuolated or branched; young cells monotrichous.
        5. Rhizobium japonicum.
2. Litmus milk acid.
  a. Formation of serum zone in milk.
    b. Moderate growth, slight acid reaction on yeast water agar plus mono-, di-, and trisaccharides.
      c. Causes formation of root nodules on species of the genera Melilotus, Medicago, and Trigonella. Bacteroids club-shaped, branched, young cells peritrichous.
        6. Rhizobium meliloti.

In the description of the species Bergey also stated that Rhizobium trifolii was motile with peritrichous flagella and R. lupini motile with flagella 1-4, usually 2 or 3.

All cultures obtained from the legumes of Scotland showed organisms with peritrichous flagella.

The physiological characters of the organisms from Trifolium and Medicago agree with those given in the above key; but those from other hosts do not agree with the key characters. The organism from Vicia cracca produced acidity in milk and therefore should be included with Rhizobium meliloti, but on the basis of host specificity would be designated R. leguminosarum. Strain 33, from Melilotus arvensis, produced an alkaline reaction and a serum zone in milk, and would be included with the first three species, but its host is included with R. meliloti. The host genera Lotus, Astragalus, Ononis, Anthyllis, Ulex and Cytisus are not included in the key, but the cultural characters of their organisms in milk place them with the first three species. Strain 25, from Cytisus laburnum, did not form a serum zone in milk, and accordingly would be classified with R. lupini or R. japonicum; but as the key indicates that R. japonicum has monotrichous flagella, strain 25 cannot be placed with this species.

The classification of Bergey et al. is based on species erected by Baldwin & Fred (1929b) and Eckhardt et al. (1931). In discussing the characters necessary for differentiation of species of nodule bacteria the former authors stressed the importance of the ability of bacteria to produce nodules upon differential species of Leguminosae. The data contained in Tables XX and XXI show that host differentiation of species is untenable.

As examples, strain 18, from T. repens, formed nodules on T. repens and L. pratensis, hosts according to Bergey et al. of Rhizobium trifolii and R. leguminosarum respectively. Strain 27, from V. cracca, formed nodules on L. pratensis, a host of R. leguminosarum, and on M. arvensis, a host of R. meliloti. Strain 33, from M. arvensis, formed nodules on L. pratensis and on M. lupulina.

The position of the Ulex-Cytisus group is equally indefinite. Ulex europaeus and Cytisus scoparius were included by Wilson & Sarles (1939) in the Cowpea cross-inoculation group, the organism of which had not been given specific rank. Strains 22 and 23 from these hosts formed nodules on Lupinus nanus and therefore could be designated R. lupini; but these strains formed a serum zone in milk and, because of this characteristic, cannot be classified as this species. In addition, strain 22 and the related strain 25, formed nodules on the Lotus group of hosts, organisms from which would be included with the first three species of Bergey's classification.

It is evident that the key characters of Bergey's classification are unsatisfactory for differentiating the nodule bacteria of some legumes of Scotland. Neither physiological characters nor ability to form nodules on specified hosts provided reliable differential features. Those cultural, physiological and morphological characters dealt with in this paper have proved inadequate as bases for segregation of species. It is evident, therefore, all the organisms are strains of a single species, Bacillus radicicola Beijk.

SUMMARY OF SECTION III.

A detailed study was made of the morphological and physiological characteristics of the nodule bacteria of some legumes of southern Scotland. All cultures showed similar growth forms and contained organisms with peritrichous flagella. Growth on laboratory media exhibited cultural characters which varied considerably between organisms from hosts of different cross-inoculation groups and those from hosts of the same cross-inoculation group. Cross-inoculation studies, under controlled conditions, showed that many strains not only produced nodules on hosts of the same cross-inoculation group but also were capable of forming nodules on plants belonging to other cross-inoculation groups. Organisms from different genera of the same group varied in their ability to form nodules on hosts of other groups and individual plants of the same species varied in susceptibility to infection. The type of nodule present on a plant was generally typical of that found on the same species of plant under natural conditions. Root swellings - non-typical nodules - occurred on plants of some cross-inoculations. Cultures prepared from both nodules and root-swellings formed nodules on the original hosts. Nodules were formed on more hosts of different groups grown in sand than on hosts grown in an agar medium.

The results of cross-inoculation trials indicated that production of nodules by cultures and hosts of different cross-inoculation groups is more general than is usually recognised.

Some strains formed nodules on plants belonging to four different groups; and some hosts were susceptible to infection by strains from four different groups.

It is shown that the key characters chosen by Bergey et al. for delimitation of species are untenable for the organisms studied and it is suggested that all be regarded as strains of a single species.

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