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Rhizobium in the Soils of the Rothamsted and Woburn Farms

P. S. NUTMAN and G. J. S. ROSS

Introduction

This paper reports counts made of root nodule bacteria in the classical field experiments (except Broadbalk field, the results for which were given in Part 2 of *Rothamsted Report for 1968*), in the long-term fallow plots and in fields where legumes have been inoculated. Some other counts are summarised in the *Rothamsted Reports for 1960* (p. 68), *for 1962* (p. 79) and *for 1965* (p. 86).

Species of nodule bacteria cannot with certainty be identified by cultural characteristics and so counted by plating. Enumeration depends on the nodulation of an appropriate host, grown in a sterile medium, from inocula of serially diluted samples of soil. The estimate of the number of nodules in the original sample is then obtained from the distribution of nodulated plants in the test, and rests on the assumption, verified by experiments with pure cultures (Date & Vincent, 1962), that a single cell of the appropriate *Rhizobium* introduced into the plant culture can suffice to produce nodules. Technical details and cultural methods are given by Date and Vincent (1962).

Serial dilutions used as inocula usually differed by a factor of 10, but occasionally by 4; 2–4 replicate samples of soil were used and each dilution was inoculated to 2–4 plants. Because test plants are sometimes attacked by fungi introduced in the soil suspension, each plant culture was classified as + (nodulating), − (not nodulating) or blank (dead or severely damaged), and the maximum likelihood method for estimating the most probable number of bacteria (MPN) in the original suspension was modified to take account of blank readings. In the tables and figures results are given as logarithms (base 10) of numbers per g dry soil. Variances of the estimated log densities have two independent components, the dilution series variance and the sampling variance. The dilution series variance depends mainly on the dilution factor and the number of tubes per dilution, and for two tubes in a tenfold dilution series averages 0·15, although the variance exceeds this figure if some plants die. The sampling variance and dilution series variance combined can be estimated from the sub-plot error in a replicated trial, which for Broadbalk over the four species averaged 0·30; results for Barnfield were similar (0·28). Where the range of dilutions chosen missed either the starting or end points for nodulation, numbers are given as fewer than, or more than, specified values. The counts recorded as zero in the tables are to be interpreted as fewer than one organism per 0·5 g dry soil. Rhizosphere counts are related to the dry weight of the soil adhering to the roots.

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The four common species of *Rhizobium* found in arable farmland were separately counted by using different test legumes: viz *Vicia hirsuta* for *Rhizobium leguminosarum*, *Trifolium pratense* for *Rhizobium trifolii*, *Medicago sativa* or *M. lupulina* for *Rhizobium meliloti*, and *Lotus corniculatus* or *L. uliginosus* for *Rhizobium lupini*.

Test plants were grown on a N-free mineral salts agar medium (Jensen, 1942) so that their growth can be used to estimate the effectiveness with which the most numerous representatives of the bacteria present fixes nitrogen. This estimate may be affected by other soil micro-organisms introduced in the soil suspension and by the presence of both effective and ineffective (non nitrogen-fixing) nodules on the same root. For this reason effectiveness is best judged from plants nodulating with the most dilute inoculum or by supplementary tests using bacteria re-isolated from single nodules taken from such plants.

Barnfield

The 0–1 in. soil layer was sampled in February 1967 before Barnfield was sown for the first time to field beans (*Vicia faba*). To count *R. leguminosarum* and *R. trifolii* four samples of soil were taken from each of the original plots (see Table 1). Two of the samples were taken from each half plot sown in the previous year to mangolds (a) or potatoes (b), one from the sub-plot given no fertiliser (N_0) and one from the sub-plot (N_1) given 1·8 cwt ammonium sulphate per acre (except in the 0 and 3 strips which were not subdivided for the new fertiliser dressings). *R. lupini* and *R. meliloti* were not counted in the samples taken from the N_1 sub-plots.

The beans sown in Barnfield after February were inoculated with a peat culture of *Rhizobium leguminosarum* from Dr. D. A. van Schreven, Kampen, Holland, except those for strip 3 which were sown with uninoculated seed. In May 1967 bacteria in the rhizosphere and soil (between rows) were counted in duplicate samples taken from the 3 and 8 (no PKNaMg) and 0 (no nitrogen fertilisers), and A (ammonium sulphate) combinations only, and from winter beans growing in the neighbouring allotment field.

Table 1 shows the average numbers of the four *Rhizobium* species for each plot, combining (a) and (b) and (N_0) and (N_1) sub-plots.

Rhizobium trifolii and *R. leguminosarum* were about equally frequent, with average numbers per plot ranging several hundredfold. *R. lupini* and *R. meliloti* were very scarce; many samples contained none and no sample contained as many as 100/g dry soil. Analysis of variance showed significant differences between plots of each series (1–8 and O–C) and a highly significant interaction between series. FYM seemed to decrease numbers, and nitrate and ammonium sulphate to increase numbers more than did rape cake, but because there is no true plot replication the interpretation of these effects is uncertain. The analysis also showed no effects of previous cropping or of supplementary dressings of nitrogen fertiliser recently applied.

Rhizobium numbers were quite unrelated to the long-term mangold yields, which ranged from 3 to 28 cwt/acre (Watson & Russell, 1943)

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TABLE 1

Numbers of Rhizobium *in Barnfield soil, before and after inoculation of beans, and in neighbouring allotment field*

Log estimated no./g dry soil

February 1967 (before sowing beans)

Plots[1]	*R. trifolii* 1	3	4	8	*R. leguminosarum* 1	3	4	8	*R. lupini* 1	3	4	8	*R. meliloti* 1	3	4	8
O	3·06	1·04	0·70	1·83	1·17	1·92	2·39	2·80	0[2]	0	0·48	0	0·60	0·78	0·48	1·92
N	3·40	1·08	0·60	2·55	2·65	2·61	3·54	2·65	0·42	0	0·78	0	0·30	1·08	0·48	0·70
A	2·35	1·20	2·64	2·06	1·71	3·06	3·67	3·12	0	0·70	1·04	0	0	0	0	0·60
C	1·76	2·06	2·40	3·37	1·86	2·47	2·72	2·86	1·10	0	1·40	1·81	0·78	1·08	0	1·94

(a) after mangolds	2·29	2·63	0·48	0·88
(b) after potatoes	1·77	2·51	0·49	0·46
(N_0) no supplementary ammonium sulphate		2·58		
(N_1) with supplementary ammonium sulphate		2·57		

May 1967, *R. leguminosarum*

Barnfield Plots		Inoculated 3	Not inoculated 8	Allotment beans rhizosphere
O	soil	1·52	1·95	5·18
	rhizosphere	5·18	4·96	
A	soil	2·51	3·47	
	rhizosphere	5·30	5·46	

[1] Key to plot numbering: 1, FYM; 3, nil; 4, PKNaMg; 8, nil; 0, nil; N, nitrate of soda; A, ammonium sulphate; C, rape cake.
[2] Less than one nodule bacterium per 0·5 g soil.
Standard errors of all values approx. ±0·3.

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and so provided very different amounts of roots to indicate any rhizosphere stimulation.

The rhizosphere counts on the first bean crop grown in this field showed that large populations of *R. leguminosarum* developed rapidly in the root zone, where numbers resembled those in the adjoining allotment field where beans have been frequently grown. Inoculation had no effect on the rhizosphere population but slightly increased the soil population, indicating that some of the peat inoculum was introduced into the interrow soil.

No isolates were taken for separate tests of the effectiveness of the bacteria in fixing nitrogen but the responses of the plants used in the counts showed that fewer than 5% of the most numerous bacteria present of *R. leguminosarum* and *R. lupini* were ineffective with *Vicia hirsuta* and *Lotus corniculatus* respectively. However, 15% of the clover bacteria and about 20% of the medick bacteria were poorly effective or ineffective with *Trifolium pratense* and *Medicago sativa* respectively.

Long-term bare fallows

Continuous fallows were begun in 1960 in sections of Highfield (Rothamsted) and in 1959 in Stackyard (Woburn). The site on Highfield was ploughed from permanent grass, the Woburn site from potatoes after an arable sequence without legumes since 1944.

Counts were begun at Woburn in June 1960 and at Rothamsted in December 1960. Because numbers in the surface (0–1 in.) and sub-surface (3–4 in.) soil were similar, counts for the two samples were combined. Table 2 shows the logarithms of the mean numbers per g dry soil at each time of sampling. Initially numbers of *R. trifolii* and *R. leguminosarum* were similar in the two fields but there were more *R. meliloti* at Woburn than at Rothamsted. During 1961, which was very dry during early summer, numbers fell sharply, especially of *R. meliloti* at Woburn, but later the rates of decline of each species were less; at Rothamsted numbers of *R. trifolii* and *R. leguminosarum* tended to fluctuate rather than decline.

At Woburn *R. trifolii* remained slightly more numerous than *R. leguminosarum* whereas at Rothamsted *R. trifolii* was constantly about 100 × more abundant than *R. leguminosarum*. In both fields the *R. meliloti* were few. Many samples from both sites taken since 1961 contained no recoverable cells.

The increase in the numbers of *R. trifolii* and *R. leguminosarum* immediately after the drought in 1961 and in 1963 may have been caused by leguminous and other weeds that grew when cultivations were impossible because of soil conditions (at Woburn 8 cultivations were done in 1960 and 1961, 4 in 1962 and 3 in 1963). The possible effect of weed growth was examined in soil sampled from the plots in August and December 1963 into pots, and kept moist. *R. trifolii* and *R. leguminosarum* were counted in the soil and in the rhizospheres of some of the weeds that developed (and in the rhizoid zone of the moss layer that formed on some of the pots). Table 3 shows the logarithms of numbers in the rhizosphere and the ratios of numbers in the rhizosphere to numbers in soil (R/S). Fifteen of

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TABLE 2

Numbers of Rhizobium *in Long-term Fallow Plots at Rothamsted and Woburn*

Log estimated no./g dry soil

	June 1960	Dec. 1960	April 1961	July 1961	Nov. 1961	Mar. 1962	Nov. 1962	April 1963	June 1963	Sept. 1964	Jan. 1967
ROTHAMSTED HIGHFIELD											
Rhizobium trifolii	—	5·32	5·07	2·93	3·97	4·60	4·93	5·54	5·50	4·86	4·53
Rhizobium leguminosarum	—	3·03	3·22	1·24	2·38	2·96	3·05	3·24	3·05	2·57	2·87
Rhizobium meliloti	—	1·20	1·24	0·22	<0·25	0	0	<0·42	<0·25	0	0
WOBURN STACKYARD											
Rhizobium trifolii	5·14	—	3·86	—	2·81	3·80	2·64	3·39	1·76	1·47	—
Rhizobium leguminosarum	2·55	—	3·54	—	2·50	3·50	2·62	0·69	2·58	1·23	—
Rhizobium meliloti	3·64	—	1·53	—	<0·24	0	0	1·31	<0·26	<0·53	—

Standard error ±0·3

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TABLE 3

The stimulation of Rhizobium trifolii *and* R. leguminosarum *in the rhizosphere of weed species in Woburn and Rothamsted fallow soils*

Log estimated no./g dry rhizosphere soil and ratio of numbers in rhizosphere (R) and soil (S)

		Rhizobium trifolii		*Rhizobium leguminosarum*	
		Rhizosphere	R/S	Rhizosphere	R/S
Experiment 1	August, 1963				
Rothamsted	*Urtica urens*	5·65	14	5·04	180
	Poa annua	5·48	<1	4·48	50
Woburn	*Gnapthalium uliginosum*	3·16	<1	4·88	6300
	Poa annua	3·50	2	2·17	12
Experiment 2	December, 1963				
Rothamsted	*Stellaria media*	4·15	3	2·85	10
	Medicago lupulina	5·82	67	3·17	22
	Moss rhizoid zone	5·31	21	3·29	28
Woburn	*Spergularia* sp.	4·58	27	2·47	7
	Moss rhizoid zone	4·43	19	1·62	<1

the eighteen R/S values were greater than unity, and in most comparisons the rhizosphere effect was appreciable, indicating that some plants other than members of the *Leguminosae* stimulate the multiplication of nodule bacteria. Most *R. trifolii* were found among the roots of the single medick plant that grew in these pots (which, however, was not nodulated, indicating the absence of *R. meliloti*). Most *R. leguminosarum* was found in the rhizosphere of *Urtica urens*. That seeds of leguminous weeds are scarce at the Woburn site was also shown by Thurston, who found only three viable seeds each of *Medicago lupulina* and *Trifolium repens* in 30/Kg of soil sampled between 1960 and 1962, and none in 1963 (personal communication).

Miscellaneous counts in arable fields

Table 4 summarises counts of *R. leguminosarum* and *R. meliloti* made in fields before starting inoculation experiments.

R. leguminosarum. This species occurred in all fields sampled, but numbers were very few in the acid soil of Sawyers III, where beans were not known to have been grown before. The rhizosphere of young bean plants during early spring (in Delafield and Great Field I) already contained as many bacteria as recorded in the much older plant's rhizosphere (Table 1); numbers in soil under cereals after beans were about one-tenth as large as in the bean rhizosphere.

R. meliloti. In fields growing good trefoil (Stackyard A in 1960 and Stackyard C in 1962), *R. meliloti* was abundant, specially in the rhizosphere.

Butt Close field, which is not known to have been sown to trefoil but probably contains medick weeds had a small population of *R. meliloti* in

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TABLE 4

Miscellaneous counts

A *Rhizobium leguminosarum* Field and history		pH	Estimated log no. *Rhizobium* per g soil
Delafield	Feb. 1962. In winter beans after four years cereals	...	6·79
Great Field I	Feb. 1962. In winter beans after two years beans	...	6·36
Long Hoos III	Feb. 1962. In cereal after spring beans	7·8	5·09
Great Field II	Feb. 1962. In cereal after spring beans	7·3	5·79
Butt Close	Feb. 1962. After spring beans	6·9	5·79
Sawyers III	Feb. 1962. No record of beans	5·8	1·76
,,	,,	5·8	2·07
,,	,,	5·6	2·23
,,	,,	5·6	1·16
,,	,,		
B *Rhizobium meliloti*			
Woburn Stackyard A	Oct. 1960. In well established trefoil (*Medicago lupulina*)	...	7·85*
	,,	...	>7·87*
	,,	...	>9·16*
	,,	...	>7·22*
Woburn Stackyard C	Feb. 1962. In trefoil	...	5·31
	,,	...	4·00
	,,	...	4·37
	,,	...	5·06
Butt Close	Feb. 1962 Cottage end	6·7	3·18
	,,	6·6	1·46
	,,	6·7	3·23
	,,	6·6	1·75
	Irrigation end	6·9	2·77
	,,	6·9	3·22
	,,	6·9	2·33
	,,	7·1	3·59
Sawyers I	No record of sown legumes	4·9	0
	,,	5·1	0
	,,	5·2	0
	,,	5·1	0

1962. The field was slightly less acid at one end than the other, and populations were larger in the less acid areas.

Counts were made in Stackyard C and Lansome fields in 1960 to investigate the poor establishment of trefoil grown from uninoculated seed in land not previously known to have grown trefoil. Soil from plots in the experiment growing ryegrass contained either none or very few *R. meliloti*, but the rhizosphere of the trefoil contained *R. meliloti* in numbers ranging from fewer than 100 to more than 100 million per g of dry soil. Stackyard C seemed to be more favourable than Lansome to the bacteria and to nodulation. Numbers of nodules were correlated with rhizosphere populations at Lansome but not at Stackyard C, and in both fields neither nodules nor rhizosphere populations were related to vigour of growth of the plants. The poor growth of individual plants was probably caused by late, and consequently ineffective, nodulation from the small scattered population of *R. meliloti* present in these fields, and did not recur when inoculated seed was sown (*Rothamsted Report for 1962*, p. 79).

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TABLE 4 (*continued*)

Miscellaneous counts

		No. of nodules on plant sampled	Estimated log no. *Rhizobium* per g soil
Woburn Stackyard C	Oct. 1960. Not known to be sown previously to trefoil. In ryegrass	...	0
	,,	...	0·78
	,,	...	0
		...	1·36
	In trefoil (not inoculated). Well-grown plants	62	>6·45*
		6	6·79*
	,,	50	7·85*
	,,	90	>6·57*
	In trefoil. Poorly-grown plants	0	3·36*
	,,	58	7·85*
	,,	50	>8·50*
	,,	36	>8·60*
Woburn Lansome	Oct. 1960. Not known to be sown previously to trefoil. In ryegrass	...	0
	,,	...	0
	,,	...	0
		...	0
	In trefoil. Well-grown plants	9	2·80*
	,,	5	6·00*
	,,	0	1·43*
	,,	0	3·96*
	In trefoil. Poorly-grown plants	1	4·57*
	,,	0	3·36*
	,,	30	>9·00*
	,,	2	4·79*

* Rhizosphere counts; remainder soil counts
Each soil count mean of 4 or 8 samples
... = no observations

Garden Clover

The garden clover plot, measuring 8 × 12 ft, was established in 1854 to grow red clover continuously on rich garden soil at Rothamsted Manor. The equivalent of more than 20 tons/acre of green matter was harvested per year from this plot at first. But yields then declined, sometimes to less than 1 ton/acre, and for many years the plot has been resown or partly resown annually. The plot or part of the plot has been given dressings of lime, fertilisers (including molybdenum) and treated with formalin, carbon bisulphide, clover nodule bacteria and soil extract but without appreciably increasing yields.

Two soil samples were taken in February 1967 from the sub-plot given molybdenum and not treated with formalin. These contained averages of 154 000 cells per g dry soil of *R. trifolii*, 9 cells of *R. leguminosarum*, (capable of fixing nitrogen on *T. pratense* and *Vicia hirsuta* respectively) and none of *R. lupini* or *R. meliloti*. The numbers of clover nodule bacteria have probably been sustained partly by inoculation and partly by the continuing presence of the host, and the poor growth of clover cannot be attributed to lack of effective bacteria.

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TABLE 5

Numbers of Rhizobium trifolii, R. leguminosarum, R. lupini *and* R. meliloti, *and occurrence of their hosts in plots of the Park Grass Experiment*

Log estimated no./g dry soil

Plot	Liming	pH 0–3 in.	*R. trifolii* r	*R. trifolii* 0–1 in.	*R. trifolii* 3–4 in.	Occurrence of *Trifolium*	*R. leguminosarum* r	*R. leguminosarum* 0–1 in.	*R. leguminosarum* 3–4 in.	Occurrence of *Lathyrus*	*Rhizobium lupini* r	*Rhizobium lupini* 0–1 in.	*Rhizobium lupini* 3–4 in.	Occurrence of *Lotus*	*R. meliloti* r	*R. meliloti* 0–1 in.	*R. meliloti* 3–4 in.	Occurrence of *Medicago*
3 No manure	L	7·2	>8·51	8·40	6·21	+	4·19	4·56	4·28	++	3·02	5·18	>6·50	++	0	0	0	–
	U	5·1	>9·32	5·90	5·07	+	>3·97	4·55	>4·41	± to –	>4·97	>6·58	>6·40	+	0	0	0	–
5 N 1856–97																		
5/1 No manure since 1898	U	4·4	>8·64	<2·70	<2·79	±	3·28	2·56	<2·42	–	2·73	>6·68	5·55	+	0	0	0	–
5/2 PK since 1898	U	4·9	>9·50	3·64	4·24	±	...	...	...		2·99	4·71	2·39	+	0	0	0	–
7 PKNaMg	L	7·0	6·91	4·37	6·75	+	3·89	5·13	5·78	++	2·44	3·35	2·80	± to –	...	0	0	–
	U	4·8	7·75	5·13	3·94	+ to ±	3·14	3·88	4·56	+	<1·65	3·15	2·68	± to –	...	0	0	–
6 N_3 (1856–62) PKNaMg	U	4·8	>10·85	6·59	<6·47	+	3·62	<4·41	<4·47	±	4·53	>6·55	<5·49	+	0	0	0	–
1 N_1	L	7·2	>7·51	7·06	5·13	±	4·51	>65·9	5·41	+	4·31	3·86	5·44	–	0	0	0	–
	U	3·6	...	0	0	–	...	0	0	–	...	0	0	–	...	0	0	–
9 N_3 PKNaMg	L	5·3	6·28	3·89	4·20	± to –	5·36	4·10	3·47	+	0·92	2·29	>1·60	–	0	0	0	–
	U(A.t)	...	...	0	0	–	...	0	0	–	...	...	...	–	...	0	0	–
	U(H.l)	3·8	...	<2·06	1·08	–	...	0	0	–	...	...	...	–	...	0	0	–
18 N_3 KNaMg	HL	7·6	9·72	5·66	5·44	+	9·86	4·45	4·99	± to –	9·08	<4·41	4·66	± to –	0	0	0	–
	LL	7·3	10·76	4·97	5·12	+	10·29	5·47	6·06	± to –	9·17	4·66	6·21	± to –	0	0	0	–
	U	4·2	...	<1·80	3·39	± to –	...	<1·80	2·01	± to –	...	0·48	<1·60	± to –	...	0	0	–
17 N_1*	L	7·5	>10·58	5·22	5·08	+	>4·36	4·03	3·86	± to –	...	...	...	–	0	0	0	–
	U	5·7	>9·97	4·76	3·77	+	5·73	4·38	2·76	–	...	...	...	–	0	0	0	–
13 FYM and fish guano	L	7·0	7·98	6·51	5·32	+ to ±	6·35	5·90	4·65	+	<4·56	3·47	4·36	±	0	0	0	–
	U	4·7	11·21	3·32	4·77	±	<4·56	2·30	<4·65	± to –	<4·56	4·26	3·38	±	0	0	0	–

SYMBOLS USED

Liming

HL, heavy liming (6788 lb ground lime/acre every fourth year

LL, light liming (3951 lb ground lime/acre every fourth year)

L, liming to plots except plot 18 (2000 lb ground lime/acre every fourth year

U, not limed.

r, Numbers of *R. trifolii*, etc., counted per g of clover rhizosphere soil.

Occurrence of host species on scale: ++, abundant to –, absent.

... No count.

0, less than about 1 organism per 0·5 g.

Counts from areas of plots with dominant *Agrostis tenuis* (A.t) or *Holcus lanatus* (H.l) respectively.

N_1, N_2—43, 86 lb N/acre as ammonium sulphate

N_1*—43 lb N/acre as sodium nitrate

The standard error of each estimate is approximately ±0·30

Park Grass

Samples were taken during the winter from the limed and unlimed sections of certain of the plots given no manure or given nitrogenous, mineral or organic fertilisers (see Table 5). Each sample consisted of either the 0–1 in. (surface) or 3–4 in. (sub-surface) parts of ten half inch cores taken at random from each sub-plot. The ten part-cores were mixed and duplicate 2–3 g sub-samples taken for counting. In addition, some plots were sampled to a depth of 12 in. and samples of clover rhizospheres were taken from plots containing clover.

Table 5 shows the numbers of the four species of *Rhizobium* in the surface and sub-surface soil samples and in the clover rhizosphere (r), and also the occurrence and relative abundance of the leguminous species in each plot and the pH of the 0–3 in. layer of soil.

The most striking result is the absence (i.e. fewer than about 1 organism per 0·5 g soil) of *Rhizobium meliloti* from all plots sampled; this correlates with the fact that none of the hosts of this nodule bacterium—species of *Medicago*, *Melilotis* or *Trigonella*—has been recorded in the plots. Populations of the other three species in the soil of the different plots also depended on the presence of appropriate hosts, except (i) for *R. leguminosarum* in the nitrate plot without lime and in the mineral plot without PK, both of which adjoin plots containing *Lathyrus* or *Vicia* in their herbage, and (ii) for *R. lupini* in the limed halves of plots 1 and 9, both of which contain a few *R. lupini* but not their host plants. The average soil populations of *R. trifolii* were about ten times larger than those of *R. leguminosarum* or *R. lupini*.

Provided there are some host plants present, the populations of nodule bacteria in the soil are only slightly influenced or are unaffected by the abundance of the host. In view of this, the large effect of the clover rhizosphere was unexpected. This effect is restricted to *R. trifolii* except for the limed sections of plot 18 where *R. leguminosarum* and *R. lupini* are also stimulated. On all plots not given nitrogen fertiliser, the rhizosphere of clover seems to have fewer *R. lupini* than the soil. Some rhizosphere populations of *R. trifolii* were very large, and the dilution series used failed to give any negative readings for half of the samples. This rhizosphere stimulation of *R. trifolii* was greatest where the soil was somewhat acid, as in the unlimed half of plot 3 and in plots 5/1 and 5/2, although the largest populations were in the limed plots. The clover nodule bacteria of some of the limed plots were a major constituent of the total bacterial population of the soil counted on a soil-extract agar.

Populations of *R. trifolii* and *R. leguminosarum* but not *R. lupini* were usually 7–8 times larger in surface than in sub-surface soil. Table 6 shows additional counts made in samples from plots 7, 9 and 13 to a depth of 12 in. On the unlimed parts, specially of plots 9 and 13, numbers of *R. trifolii* and *R. leguminosarum* decreased regularly with depth, and at 11–12 in. were about one-hundredth of numbers in the surface soil. *R. lupini* occurred more irregularly through the profile, and showed no clear trend with depth. All species were irregularly distributed and not especially abundant in the top 1 in. of soil.

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TABLE 6

Distribution of nodule bacteria in the soil profiles of Park Grass plots 7, 9 and 13

Log estimated no./g dry soil

		Sample depth (in.)	*R. trifolii*	*R. leguminosarum*	*R. lupini*
Plot 7	Limed	0–1	4·37	3·89	2·39
		3–4	<1·63	6·07	2·81
		7–8	4·07	4·69	2·56
		11–12	3·81	4·08	1·25
	Unlimed	0–1	4·36	3·80	<0·57
		3–4	2·41	<1·63	<1·63
		7–8	>4·69	1·93	5·48
		11–12	2·76	2·32	1·76
Plot 9	Limed	0–1	3·89	4·05	2·29
		3–4	4·17	3·47	<0·60
		7–8	1·60	2·40	2·75
		11–12	0·86	1·68	1·51
Plot 13	Limed	0–1	5·50	5·01	<2·99
		3–4	4·87	4·83	3·76
		7–8	4·73	4·16	6·16
		11–12	4·25	3·07	2·20
	Unlimed	0–1	3·32	2·30	6·62
		3–4	4·77	<3·40	2·48
		7–8	<4·11	<3·02	1·71
		11–12	3·65	<2·92	2·66

Liming greatly increased *Rhizobium* numbers, probably partly indirectly by its effect in increasing the amount of host species in the herbage. Each host and its bacterium was found only in plots with soil less acid than pH 4·0 and they were usually more abundant on the alkaline plots. Plots sampled were either acid (pH 4·2–5·7) or slightly alkaline (pH 7·0–7·6). Between these groups, but not within them, there was a consistent effect of pH, with more nodule bacteria occurring in the soils (and in the clover rhizospheres) of the alkaline than of the acid group. The alkaline soils averaged 148 times more *R. trifolii*, 13 times more *R. leguminosarum* and 140 times more *R. lupini* than the acid soils.

The principal grasses of the unlimed part of plot 9 are *Holcus lanatus* in clumps, and *Agrostis tenuis*. Soil samples taken from the soil under the *Agrostis tenuis* areas yielded no nodule bacteria whereas soil under the *Holcus lanatus* area contained a few *R. trifolii*, which because of their unusual symbiotic characteristics (see next section) are probably indigenous to this plot.

The symbiotic characteristics of Park Grass strains of *Rhizobium trifolii*. The dominant (most numerous) members of the *Rhizobium* populations in the plots were obtained by isolating from nodules formed on test seedlings inoculated with the most dilute soil suspensions that produced nodules. These came from the limed and unlimed parts (when present) of Plots 1, 3, 5/1, 5/2, 7, 9, 13 and 18. Each isolate was tested on four replicated plants of *Trifolium pratense* grown aseptically on N-free mineral salts

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medium in test tubes kept in a glasshouse. Comparison was made with uninoculated plants, and with plants inoculated with either the effective strain 0403 or the outstandingly effective strain 5 (Rothamsted collection strain numbers). The extent of nodulation was recorded and growth measured by grading for size at intervals and by measuring dry weight at harvest (80 days).

Isolates did not differ in the time at which they formed nodules, but varied considerably in their symbiotic effectiveness and in numbers of nodules produced. Fig. 1 shows the distribution of effectiveness of isolates measured in relation to the dry weight produced by strain 0403 (100%). With the exceptions discussed below, dry weight was distributed normally for each set of isolates; the variation was predominantly of host origin and corresponded to that expected in tests with four replicated plants.

The most effective strains (equal to or more effective than strain 5) came from the limed halves of plots given ammonium sulphate (plots 1, 9 and 18 II), although some strains from other plots (e.g. the unlimed section of the PKNaMg and nitrate plots, plots 7 and 17) were of more than average effectiveness. Strains from limed plots given minerals only and from the heavily limed plot 18 II differed more. Most strains isolated from these plots were as effective (or slightly more effective) than strain 0403, but of 20 strains two were ineffective and 3 poorly effective.

Plot 5, which has not been limed was colonised by bacteria more effective than 0403, except for an ineffective isolate from the rhizosphere of a plant from the section given PK. Only two of the eight bulked samples yielded any rhizobia, and these were of average effectiveness.

Isolates from limed and unlimed halves of plot 17, which is given sodium nitrate, were slightly less effective than those from plots 1 and 9. The small lime dressing applied to plot 18 III had no influence on strain variability, but the heavy liming of 18 II increased it; of the 16 strains examined from 18 II, two were more effective than the average, one less and one very poorly effective.

The acidity of unlimed plots usually either eliminated the clover nodule bacteria altogether or left as survivors only ineffective or poorly effective strains. Of the 8 strains isolated from the very acid (pH 3·8) section of plot 9 (from a *Holcus lanatus* patch) 4 were completely ineffective, and of those isolated from plots given no manure or PKNaMg only (pH 5·1 and 4·8), about one-quarter were ineffective, one-quarter poorly effective and one-half effective.

Nevertheless, neither acidity nor absence of lime were invariably associated with a decline in effectiveness; strains from plot 5/2 (pH 4·4), from the unlimed half of plot 17 (pH 5·7) and from plot 18 I (pH 4·2), were of average or more than average effectiveness. In all plots, isolates from the soil and from the corresponding rhizosphere were of similar effectiveness.

The mean number of nodules formed by each strain provided criteria for further strain differentiation, as shown in Table 7. Ineffective and poorly effective strains from all plots, except 18 II, formed many more and smaller nodules than did their effective counterparts. Differences between effective ones isolated from one plot section were not larger (except in the limed section of plot 17) than those with the strains 0403 and 5, and can be

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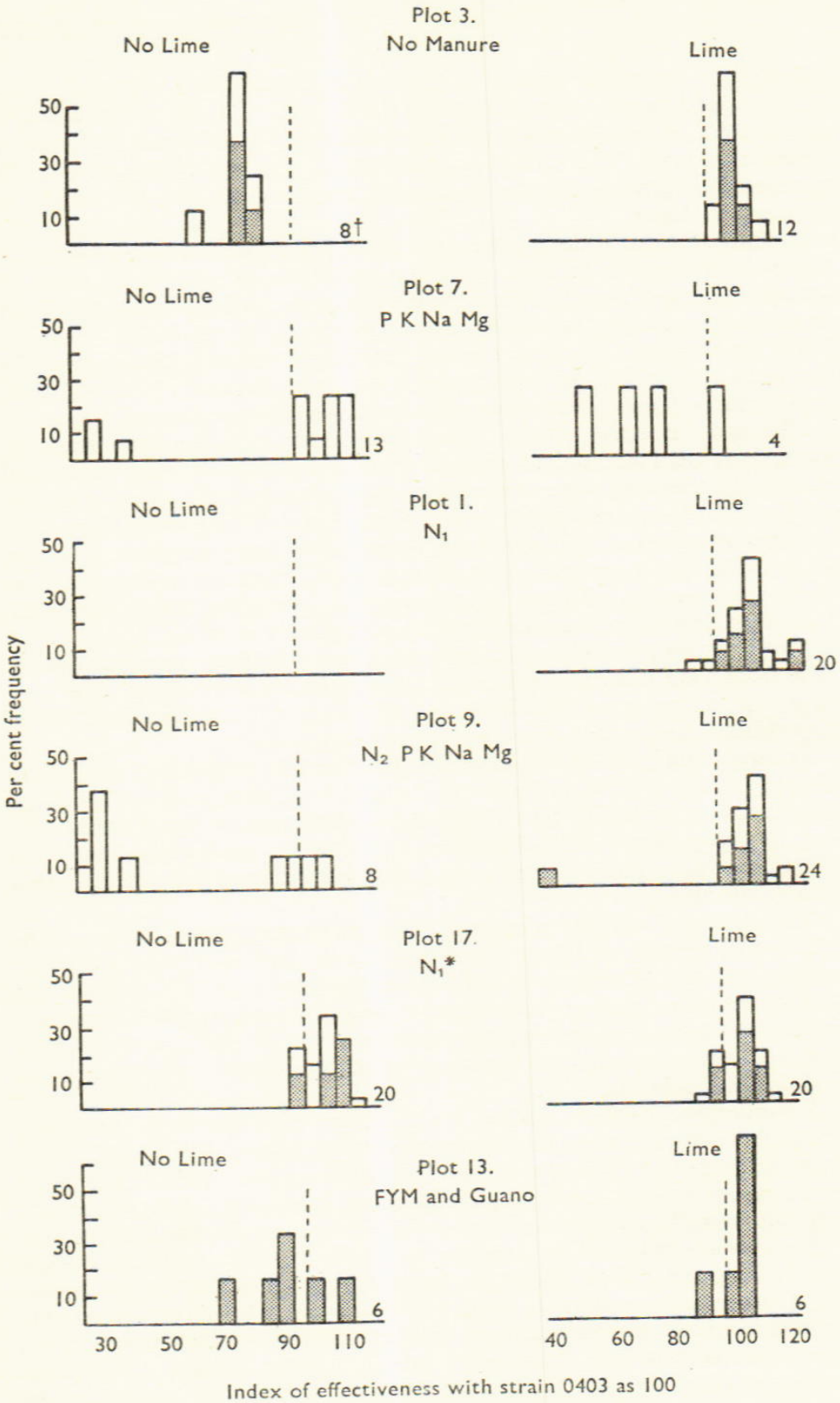

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attributed to host variation. However, there were large and significant differences in numbers between effective strains from different plots. Many more nodules were formed by effective strains from plot 5/2 (PK and no lime) and plot 7 (PKNaMg and lime) than by effective strains from the other plots. Strains from the limed sections of plot 13 (FYM) and plot 18 formed fewer nodules than strains from other plots. The single ineffective strain from plot 18 II was unique in its very sparse nodulation.

Exceptions to the general similarity in nodule number produced by effective strains from within a plot were three strains from the limed half

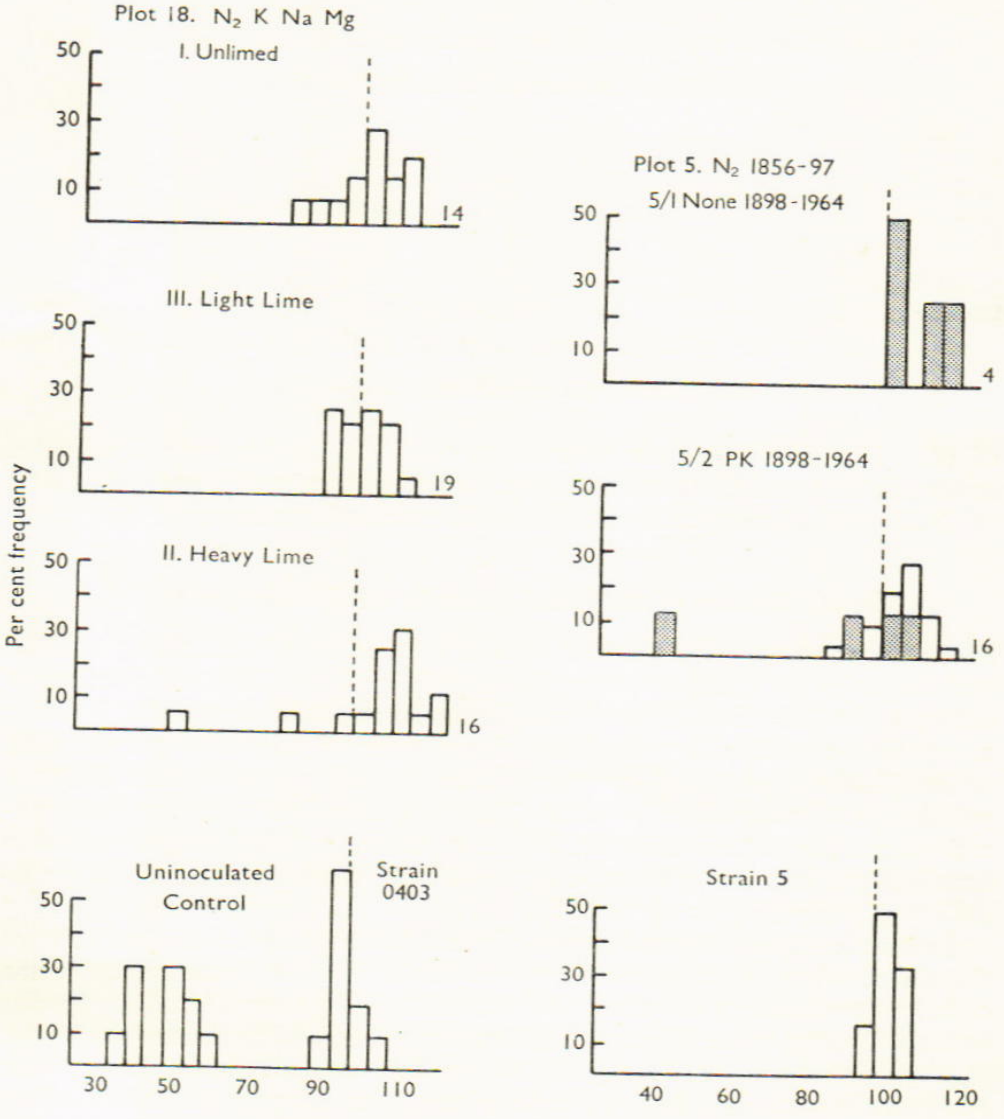

Fig. 1 Distribution of effectiveness of isolates of *Rhizobium trifolii* from Park Grass, tested on *Trifolium pratense*. Shaded areas refer to isolates from rhizospheres.

† Number of isolates tested.

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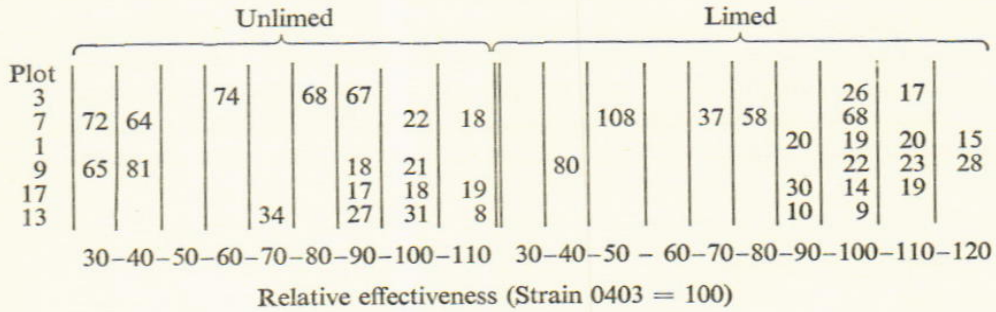
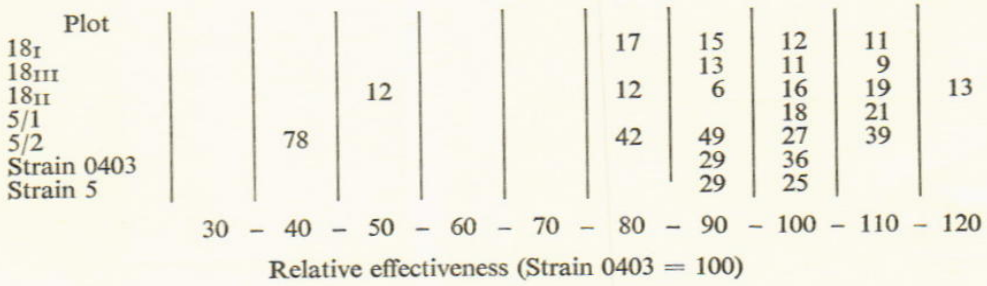
TABLE 7

Numbers of nodules formed by Park Grass isolates in relation to effectiveness





of plot 17; although these seemed to conform to the normal effective group, as its least effective members, they produced many more nodules, *viz* 37, 38 and 52 compared with 16 for the remaining member of the group.

The distribution of nodulated plants in the dilution series

In the above counts no adjustment was made for 'skips', i.e. plants without nodules at lower dilutions than plants with nodules. The distribution of skips in the Barnfield, Broadbalk, Long-Term Fallows and Park Grass counts was examined by tabulating the number of skip tubes per dilution series (Table 8). A 'skip' tube is defined as any negative tube above a positive one in the dilution series. This simple definition somewhat overestimates their true incidence, because where a 'skip' negative and a terminal positive are produced by adjoining dilutions, its normal probability of happening is up to 50%.

Because of the different lengths of the dilution series, statistical tests of the distribution of skips could not be made, but the results show some definite trends. Skips occurred in the counts of the four *Rhizobium* species in the following order of increasing frequency (neglecting manurial effects): *R. leguminosarum* or *R. trifolii*, *R. meliloti* and *R. lupini*. This order occurred in each field and was also observed in other experiments where results were too few to be tabulated. Skips were least prevalent in the Broadbalk samples. Long-term fallowing at Rothamsted and Woburn decreased the incidence of skips in the counts of *R. meliloti*, but seemed not to affect their frequency in counts of the other species. In Broadbalk,

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TABLE 8

Number of skip negatives per dilution series

A. Species differences

	Broadbalk	Barnfield	Rothamsted Fallow	Woburn Fallow	Park Grass
R. trifolii	0·23	0·56	0·24	0·57	0·47
R. leguminosarum	0·19	0·29	0·57	0·66	0·51
R. meliloti	0·50	0·67	3·30	3·08	—
R. lupini	0·66	—	—	—	0·96

B. Effect of duration of long-term fallowing

	1960–61	1962–63	1964–67
R. trifolii	0·78	0·25	0·40
R. leguminosarum	0·22	0·66	0·69
R. meliloti	4·87	1·87	1·50

C. Effects of manuring (combining test species)

Broadbalk

Plots		Sections	
3 (Unmanured)	0·44	I	0·22
5 (PKNaMg)	0·54	II	0·52
8 (PKNaMg and ammonium sulphate)		III	0·29
	0·31	IV	0·42
16 (PKNaMg and sodium nitrate)	0·15	V (fallow)	0·33

Barnfield

Sections		Sections	
1 (FYM)	0·56	O (nil)	0·31
3 (No manure)	0·16	N (sodium nitrate)	0·50
4 (PKNaMg)	0·36	A (ammonium sulphate)	0·36
8 (No manure)	0·54	C (rape cake)	0·34

Park Grass

Plots	Unlimed	Limed
3 (Unmanured)	1·4	0·30
7 (PKNaMg)	1·2	0·55
1 (2 cwt ammonium sulphate)	—	0·22
9 (4 cwt ammonium sulphate)	—	0·24
17 sodium nitrate	0·13	—
13 FYM and guano	0·71	0·59

neither one year's fallow nor applying herbicide affected the number of skips.

Combining results for the different *Rhizobium* species, both Broadbalk and Park Grass show very similar differences caused by manuring. Plots given nitrate provided dilution series with fewest skips, followed by those given ammonium sulphate or PKNaMg or left unmanured. On Park Grass FYM increased the incidence of skips and liming greatly decreased it in all except one set of the paired plots where skips were few. The results for Barnfield showed no consistent plot differences, but in view of the Park Grass results it is of interest that the FYM plot gave most skips.

Discussion

The counting method. The method gave consistent results but with large differences between replicates; the standard errors commonly ranged from 10 to 100% of the mean most probable number.

Estimates of numbers were affected by skips in the expected sequence of

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positives and negatives which led to sporadic and sometimes gross underestimation of actual numbers present in the original suspension, and also increased replicate variation. Thompson and Vincent (1967) showed that skips in dilution tubes used for counting *R. trifolii* were unaffected by conditions of growth of the test seedlings, but were commoner with soil containing few bacteria than with soil containing many. Adding soil to a dilution series of a pure culture of nodule bacteria decreased the count, especially when the soil was added to the tubes some time before the bacteria. Thompson and Vincent also showed that the contents of skip tubes when used to inoculate fresh seedlings sometimes produced nodules and sometimes did not, indicating that the nodule bacteria may be suppressed or eliminated, presumably by microbial antagonism, lysogeny or predation. There is much evidence that rhizobia are subject to microbial antagonism in soil and in pure culture studies (Hely, Bergersen, & Brockwell, 1957; Wieringa, 1963; van Schreven, 1964; Visona & Tardieux, 1964; Hattingh & Louw, 1966a, 1966b; Holland & Parker, 1966; and Robinson, 1968).

The consistent differences between *Rhizobium* species in liability to this counting aberration, and of effects of manuring and liming, are interpretable in terms of microbial antagonism encouraged by dung and acidity and discouraged by nitrogen fertilisers. The fields at Rothamsted and Woburn provide valuable soils for further study of this phenomenon.

Populations of *Rhizobium* and their effectiveness. Populations of the four species of *Rhizobium* studied occurred widely in the arable fields of the Rothamsted and Woburn farms but in numbers that varied many-fold, usually in relation to the presence or recent presence of their respective host plants, and to soil acidity. *R. trifolii* was most widespread and usually most abundant, followed by *R. leguminosarum*, with *R. meliloti* and *R. lupini* much less abundant and more restricted in distribution.

At the time Broadbalk was surveyed (*Rothamsted Report for 1968*, Part 2, p. 180), this field had been cropped with nothing but wheat since 1843. Numbers were unaffected by manurial treatment or by fallow the previous year or by herbicide. Numbers were not simply related to the distribution or abundance of leguminous weeds, although the absence of clover may have been a contributory cause of the fewer *R. trifolii* found in Broadbalk than in other arable fields. There are no records of leguminous weeds on the plot area of Barnfield (J. M. Thurston, private communication), but clovers, vetches and medick are common on the field boundaries and in neighbouring fields.

In the continuously fallowed fields, numbers declined progressively in the early years but later more erratically, particularly at Rothamsted. That the host plant is not entirely necessary for multiplication of nodule bacteria in soil was shown by experiments on their stimulation by some non-leguminous weeds. Such multiplication was sporadic and uncertain and is probably unimportant in maintaining populations. This is also indicated by the absence of any correlation of populations with wheat yields on Broadbalk, or of mangolds or potatoes on Barnfield. The counts from all the arable fields indicate that nodule bacteria from areas where

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they are abundant are transferred to neighbouring fields, probably in soil blown by wind or brought on implements. Such agents probably suffice to provide inocula for clovers and beans, and possibly lotus, but not lucerne or trefoil grown on the farms. Adverse physical conditions, such as drought and heat accelerate decline of the soil population but when these do not operate, biological factors may be important, and it may not be coincidental that the species of *Rhizobium* most liable to decline in numbers are also most prone to show skips in their MPN count.

Development of appreciable populations from bacteria in the soil or from artificial inoculation, seems mainly to be restricted by soil acidity, with the four species again showing differential susceptibility to this factor in the environment. *R. trifolii* was occasionally isolated from soil more acid than pH 4·0, but *R. meliloti* very rarely from soil more acid than pH 6·0. Alkalinity stimulated rhizosphere increase, though whether directly or by increasing root exudation is not known. Loneragan and Dowling (1958) and Mulder and van Veen (1960) also showed that acidity was important in restricting multiplication.

The symbiotic effectiveness of strains occurring in the arable areas was not studied in detail but the response of the count-test plants showed that most of the rhizobia were effective in fixing nitrogen on their test hosts.

The distributions and relative abundance and effectiveness of *R. trifolii* and *R. meliloti* at Rothamsted and Woburn parallel those reported by Jensen (1969) for more than two hundred arable soils of Denmark, where abundance was clearly related to presence of host and to soil pH. By experiment Jensen also showed that *R. trifolii* only survived well in soil above pH 4·9 and *R. meliloti* only above pH 5·9.

The partial survey of the Park Grass plots demonstrated the dominating influences on rhizobia in pasture of host and soil reaction, for there was an almost exact correspondence between the occurrence of each species of *Rhizobium* in the soil and of its host, and indicated that a continuous plant cover prevents movement of bacteria from plot to plot. The survey also showed that the prolonged differential manuring had some effects on the symbiotic properties of *R. trifolii*. Where manures had not been given but lime had, strains were effective, but in plots without lime, and where pH had fallen to 5·1, strains were only poorly effective. Mineral fertilisers without nitrogen decreased effectiveness, even with liming. The use of nitrogenous fertilisers (nitrate of soda, ammonium sulphate or organic manures) over very long periods of time did not lessen ability of strains to fix nitrogen, provided that lime was also applied to the plots given ammonium sulphate.

Acidity tended to favour ineffective or poorly effective strains, although even acid plots contained some fully effective strains. Completely ineffective strains occurred irregularly and some were found in plots otherwise occupied by populations of strains of more than average effectiveness. Thornton (1954), Masterson (1961), Jones *et al.* (1964) and Jones (1966) showed that strains from hill pastures in the U.K. and Eire were often poorly effective. This was usually, but not always, associated with acidity. Holding and King (1963) attributed the poor effectiveness of strains from Scottish hill pastures to the soils being deficient in bases.

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The classical fields at Rothamsted and Woburn provide unique sites for ecological studies on the relationship between nodule bacteria, their host species and the soil, and for the collection of strains adapted to acidity and to nitrogen fertilisers, which could have agricultural value, but this still has to be assessed.

Summary

Rhizobium trifolii, *R. leguminosarum*, *R. meliloti* and *R. lupini* were counted in some of the arable fields of Rothamsted and Woburn, and in selected plots of the Park Grass experiment. All species were widely distributed throughout the arable areas, with *R. trifolii* and *R. leguminosarum* usually much more abundant than *R. meliloti* or *R. lupini*, especially in fields cropped by the host. When the host plants were not grown, numbers decreased in a few years from tens or hundreds of thousands per g dry soil to very few or none (as for example *R. meliloti* and *R. lupini* in most plots of Barnfield). Numbers were unaffected or only slightly affected by mineral or nitrogenous fertilisers or by moderate infestation with leguminous or other weeds but were reduced by acidity. The results suggest that numbers in arable soil without a recent legume crop are maintained by transfer by natural agents or farm implements from areas of abundance. Most nodule bacteria from these fields were effective in fixing nitrogen with the test hosts used.

The Park Grass survey showed striking correspondence between the occurrence of *Rhizobium* in the soil of each plot and the presence of a host species in the herbage. Numbers were few or absent in the acid plots and were more on the limed plots but were unaffected by mineral fertiliser. *R. trifolii* was strongly stimulated in the clover rhizosphere, even after more than a century of clover cover. The numbers of nodules produced and the nitrogen-fixing effectiveness of isolates of *R. trifolii* differed between plots and were affected by liming and fertilisers. The most effective isolates came from limed plots given nitrogen fertiliser and the least effective from unmanured or somewhat acid plots.

The MPN estimate of numbers varied in reliability according to the species enumerated and soil treatment and was best for limed soil given nitrogenous fertilisers and worst for acid plots or those given organic fertilisers.

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