Plant and Soil XIII, no. 2 October 1960

EFFECT OF pH AND ORGANIC COMPOUNDS ON NITROGEN FIXATION BY RED CLOVER

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INTRODUCTION

Symbiotic nitrogen fixation by leguminous plants depends on a number of different factors *viz* a) presence of cells of an effective Rhizobium strain in the culture medium, b) increase in numbers of Rhizobium cells in the rhizosphere, c) infection of the roots by the bacteria, d) growth of the nodules, e) activity of the nodules, and f) longevity of the nodules. Environmental conditions may affect nitrogen fixation by way of a more or less specific influence on one of the components. Boron deficiency, for instance, inhibits nitrogen fixation by preventing the growth of the nodule tissue. It does not seriously affect the mechanism of nitrogen fixation [5]. Molybdenum deficiency, however, primarily affects the nitrogen-fixing process, but it has only a slight effect on the development of the nodules [4].

As to the pH of the culture medium, it is a well-known fact that most leguminous plants grow less favourably in acid media than under neutral or slightly alkaline conditions [13]. This is mainly due to a reduced nitrogen fixation as may be concluded from the improved growth at low pH upon the addition of combined nitrogen. There exists much variation, however, amongst different species of leguminous plants as to the effect of pH on growth in the presence of combined nitrogen.

The purpose of the present study was to elucidate the observed beneficial effect of added calcium carbonate and of some organic compounds on symbiotic nitrogen fixation by red clover, growing on acid soil. The investigations started in 1957 when mixtures of grass and red clover were sown on a series of plots of varying pH.

The growth of the clover appeared to be luxurious at pH 6.6 but extremely poor at pH values of 5.0 and lower. The latter was due to the absence of root nodules.

On some acid plots which had been treated with stable manure a few years before starting the clover experiments, the red clover was well nodulated and an excellent growth was made, notwithstanding the pH of the soil was somewhat lower than that of untreated plots, where the clover suffered from severe nitrogen deficiency. This demonstrated that the effect of low pH on nodulation was eliminated by some unknown substance supplied by the stable manure.

To investigate the effect of pH and of various organic compounds on symbiotic nitrogen fixation in red clover a number of pot experiments were undertaken with sandy soils and with culture solutions.

METHODS

Field experiments

Red clover was grown on a number of plots with different pH values as a pure culture in 1959 and in mixture with perennial rye grass (*Lolium perenne*) in 1957 and 1958. These plots had been laid down in triplicate more than thirty years before on a poor sandy soil (organic matter 2%) in the garden of the Laboratory of Microbiology by applying calcium carbonate in the following amounts: 1250, 2500, 3750, 5500, and 11000 kg per ha. For maintaining the pH levels calcium carbonate was added periodically. The basic dressing consisted of superphosphate (100 kg P_2O_5 per ha) and potassium sulphate (125 kg K_2O per ha).

No clover had been grown on the plots in the years preceding the experiments and no effort was made to introduce Rhizobium during the 1957–'59 experiments or earlier.

Pot experiments

Soil cultures. Use was made of Neubauer glass jars containing 0.5 kg of sandy soil. The basic dressing for each pot consisted of 300 mg KH_2PO_4, 20 mg Na_2HPO_4 and 125 mg $MgSO_4.7H_2O$. Approximately twenty plants of red clover were grown per pot. In order to prevent the introduction of foreign *Rhizobium trifolii* cells into the soil employed, the glass containers, watering tubes, and nutrients were sterilized. The seeds were desinfected by treatment during 15 minutes with a 3 per cent H_2O_2 solution containing a few drops of the detergent "Teepol" per 100 ml of solution. Subsequently they were washed 5 times with sterile water and transferred to the soil. The pots were wrapped in cellophane paper until the seedlings had reached a

height of about two cm. Then the cellophane was removed and the soil was covered with a one-cm layer of glass sand, which had been paraffined by treating 10 kg sand with 10 g of paraffin wax dissolved in one liter benzene. After evaporation of the benzene this sand had been sterilized for several hours at 100° C. For watering the plants, sterile distilled water was added via a glass tube filled with sterile, coarse sand and sealed with a cotton plug. During autumn and winter the plants were kept in a greenhouse where they were provided with artificial light from 4 a.m. to sunrise and from sunset until 22 p.m. During spring and summer they were kept outdoors during day time and transferred to the greenhouse overnight and in rainy weather.

Cultures in nutrient solution. The culture solution employed had the following composition: KH_2PO_4 80 mg, K_2HPO_4 200 mg, $Na_2HPO_4.2H_2O$ 50 mg, $MgSO_4.7H_2O$ 250 mg, $CaSO_4.2H_2O$ 250 mg, NH_4NO_3 30 mg, $FeCl_3.6H_2O$ 25 mg, $MnSO_4.1H_2O$ 1 mg, $ZnSO_4.7H_2O$ 0.25 mg, $CuSO_4.5H_2O$ 0.25 mg, H_3BO_3 0.25 mg, and $Na_2MoO_4.2H_2O$ 0.25 mg per litre of distilled water.

Counting of Rhizobium trifolii in soil

To estimate the number of Rhizobium cells in soil, 10-g samples were suspended in 100 ml of a sterile solution of the following composition: K_2HPO_4 1 g, $Ca(H_2PO_4)_2$ 0.25 g, $(NH_4)_2SO_4$ 0.25 g and $MgSO_4.7H_2O$ 0.25 g per litre of distilled water. The suspension was shaken vigorously for 5 minutes before being diluted by serial transfer of 1 ml suspension to 9 ml of fresh, sterile medium until a dilution was attained of 10^9. Each dilution was made in duplicate. One-ml portions of the various dilutions were transferred to sterile 20-days old red-clover plants growing on agar slopes in 16 × 155 mm tubes. Each tube contained 7 ml of the nutrient solution, employed in the water cultures, solidified with 0.6 per cent Davis agar.

The plants were incubated during 45 days in the light in a greenhouse before being examined for effective nodules. When no nodules had formed, the plants were small and had an almost yellow leaf colour. Nodulated plants always had a dark-green colour indicating the presence of an effective Rhizobium strain. When effective nodules were formed at a certain dilution, it was assumed that at least one cell of an effective strain had been introduced (cf Hely *et al.*[2] and *Purchase* and *Nutman*[7]). The number of virulent bacteria was calculated according to the method described in "Standard methods of water analysis" [9].

Of each treatment two pots were sampled. In the case of unplanted soil the samples at each sampling time were taken from the same pots, the soil of which was mixed thoroughly. Of the planted pots two were harvested and sampled at each counting date (two samples per pot). To include the rhizosphere soil in the sample, the roots were transferred onto a coarse sieve where the bulk of the adhering soil was removed carefully in such a way that the roots and the nodules were left on the sieve. Thereafter the roots were washed twice with 20 ml sterile water and the washings added to the soil. After thorough mixing the samples were taken.

Preparation of extracts of Rhizobium, yeast, and stable manure

Sterilized Rhizobium cells. Effective and ineffective strains of *Rhizobium trifolii* were cultivated on agar plates in petri dishes of 11 cm in diameter. The agar media had the following composition: K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, $CaCO_3$ 3 g, mannitol 1.5 g, glutamic acid 0.2 g, Difco yeast extract 1 g, cornsteep liquor 1 ml, soil extract 850 ml (prepared from 425 g clay soil), distilled water 150 ml, Davis agar 10 g. After three to four days' incubation at 30° C, the slimy bacteria layer of 16 plates was collected and suspended in 100 ml water. The suspensions were autoclaved twice at 110° C for 10 minutes. Their nitrogen content varied from 0.4 to 0.6 mg per ml.

Yeast extract. 100 g of bakers yeast was heated with 200 ml water for 10 minutes at 90° C. The suspension was centrifuged and the clear solution, containing approximately 1 mg N per ml, autoclaved at 110° C for 10 minutes and used for the pot experiments.

Stable-manure extract. One kg fresh stable manure containing approximately 24 per cent dry matter was cut to small pieces and suspended in 3 l distilled water. The suspension was shaken mechanically for 18 h. Thereafter it was squeezed through cheese cloth and filtered on a Buchner funnel through asbestos. The filtrate was concentrated *in vacuo* to 150 ml. The latter solution contained 0.75 mg N per ml.

RESULTS

1. *Effect of added calcium carbonate on growth and nitrogen fixation of red clover on acid sandy soils*

a. Field experiments. The effect of pH on yield of red clover grown on plots treated with different amounts of calcium carbonate is shown in Table 1. It will be seen that an adequate growth of the clover took place at pH 6.4 and 6.8. At pH 4.7 and 5.0 the development of the plants was poor due to the absence of root nodules. That nitrogen deficiency was the main cause of the restricted growth of the clover on the acid plots may also be concluded from the results obtained with added fertilizer nitrogen. At pH values of 6.4 and higher normal nodulation occurred as a result of which the plants were adequately supplied with nitrogen. Since inoculation of the seed or the soil on these plots had not been performed and no clover crops had been grown there before, this demonstrates that Rhizobium cells were present in the neutral and alkaline soils but in the acid soils they were either absent or unable to initiate the formation of nodules.

To see which component of the symbiotic nitrogen-fixing system

was affected by a low pH, a number of pot experiments with acid soils were conducted.

b. Pot experiments. Acid sandy soil (pH 5.1, organic matter 2%), from one of the above-mentioned plots was mixed with 1 g sterilized calcium carbonate, 1 g per pot, and transferred to the glass containers. Some pots with acid soil and some with calcium

TABLE 1

Effect of pH and fertilizer nitrogen on yield (fresh weight, q/ha *) of uninoculated red clover (first cutting), grown under field-experiment conditions

pH of the soil	No nitrogen added		120 kg N/ha ‡
	1957 ***	1959 †	1959 †
4.7	—	65	193
5.0	4	29	178
5.0 **	242	—	—
5.1	7	70	189
5.6	27	85	182
6.4	200	158	238
6.8	303	169	263

* 1 q = 1 quintal = 100 kg.
** Treated with stable manure some years before starting the experiment.
*** Single values.
† Averages of duplicate values.
‡ In the form of ammonium-nitrate limestone.

carbonate-treated soil were inoculated with a suspension of an effective strain of *Rhizobium trifolii*, the others were left uninoculated. Some further pots with acid soil were provided with 42 mg nitrogen as ammonium nitrate. The soil of the latter pots was not inoculated with Rhizobium. Red clover was grown as described above. The experiment was started on 28 August and finished on 19 December 1957.

In the absence of added calcium carbonate or ammonium nitrate the growth of the uninoculated clover was very poor. The plants were small and yellow-green due to nitrogen deficiency; root nodules were absent. A few plants of these control pots showed a somewhat greener leaf colour and better development than the others. This was brought about by the presence of a few nodules which, however, did not give rise to a much better growth. Apparently the bacteria which had given rise to these few nodules had been unable to spread in the rhizosphere of the clover roots in the acid soil.

The plants on the uninoculated, calcium carbonate-treated soil at first showed a more or less similar appearance. After an initial growth depression owing to nitrogen deficiency some plants turned green and developed vigorously (Plate 1A). The improved growth, which was a result of the formation of nodules, gradually spread throughout the pot. In contrast to the acid soil the few Rhizobium cells which presumably were present originally, now were able to increase in numbers and to give rise to the formation of large numbers of nodules.

In the case of plants inoculated with a suspension of Rhizobium cells, formation of nodules and development of the clover plants on the acid soil were quite satisfactory. This demonstrates that the low pH apparently did not depress the infection of the roots and the growth and the activity of the nodules (Plate 1B).

Uninoculated plants treated with ammonium nitrate initially made much better growth than those of the control pots. The amount of added nitrogen was too low, however, to supply the plants with adequate amounts (Table 2). Inoculated plants dressed with

TABLE 2

Effect of added $CaCO_3$ and NH_4NO_3 on yield (g dry matter per pot) of inoculated and uninoculated red clover, grown on an acid sandy soil

Treatment	Uninoculated	Inoculated
First experiment *		
Control	0.5	3.0
1 g $CaCO_3$ per pot	1.7	2.8
NH_4NO_3	0.6	2.0
Second experiment **		
Control	0.2	—
1 g $CaCO_3$ per pot	11.3	—

* Single values.

** Averages of quadruplicate values.

Plate 1. A. Effect of added calcium carbonate on nitrogen fixation and growth of red clover on acid soil. *Left* no $CaCO_3$ added, *centre* and *right* 1 g $CaCO_3$ per pot. *Left* and *centre* uninoculated, *right* inoculated with *Rhizobium trifolii*. **B.** Acid soil. *Left* uninoculated, *right* inoculated with *R. trifolii*.

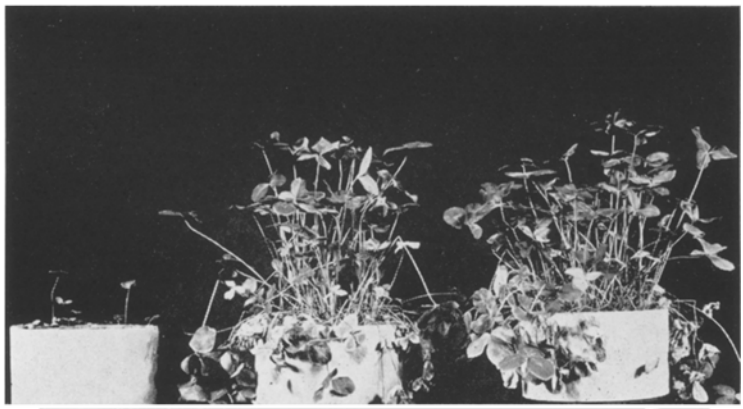
Plate 2. Effect of Rhizobium and yeast extracts on nitrogen fixation and growth of red clover. **A.** Cultures on uninoculated acid soil, (pH 5.1). *Left* control, *centre* and *right* 10 ml per pot of a sterilized suspension of ineffective and effective strains of *Rhizobium trifolii*, respectively. **B.** Uninoculated red clover on acid soil. *Left* (2 pots) control, *right* (2 pots) 2 ml of yeast extract per pot.

A

B

Plate 1

A



B

Plate 2

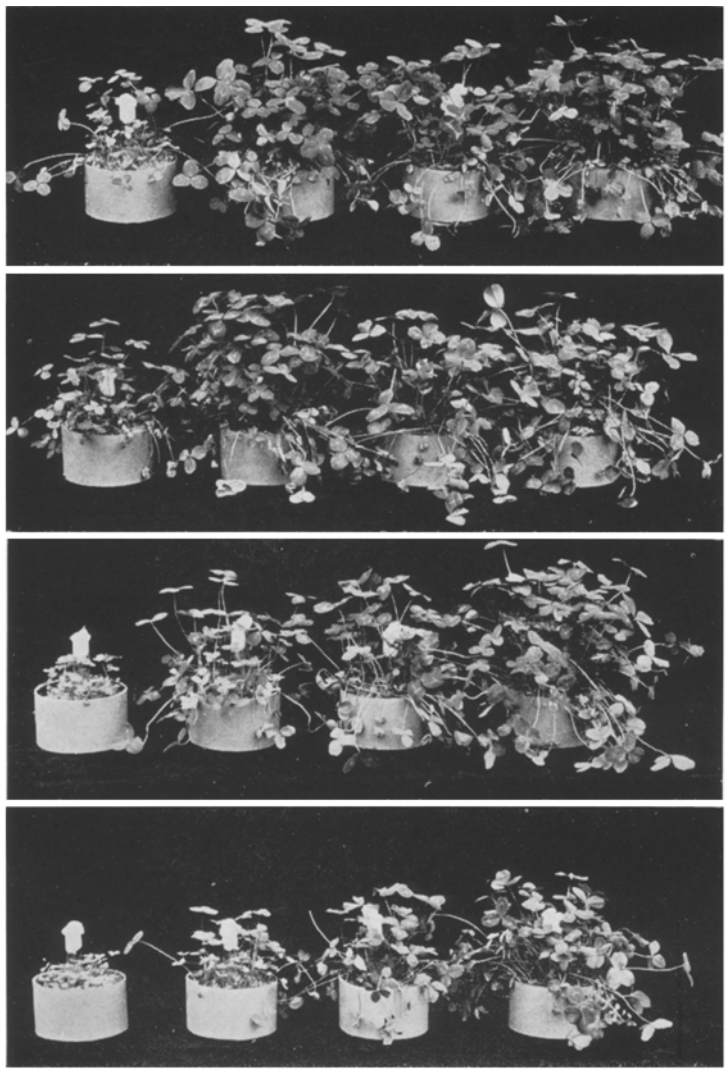


Plate 3

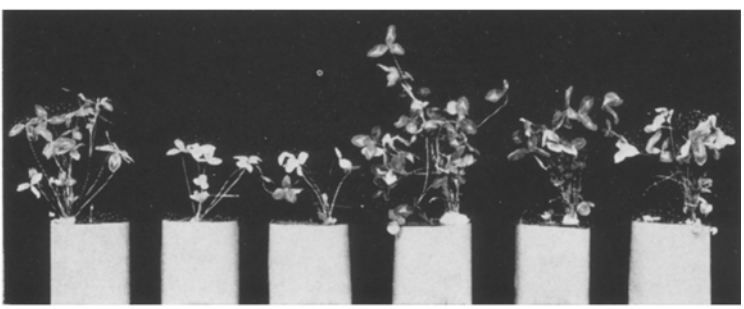


Plate 4

ammonium nitrate gave lower yields than those which had received no combined nitrogen. This was presumably due to the inhibiting effect of ammonium nitrate on nodulation.

A *second* pot experiment was carried out from 18 February to 24 July 1958. The results of this experiment confirmed those of the above one *viz* poorly growing yellow-green clover plants with few small nodules in the control pots and vigorously growing dark-green plants with large numbers of nodules in the pots treated with calcium carbonate (Table 2). The higher yields of the pots with calcium carbonate-treated soil as compared with those of the first experiment were due to the fact that the plants were harvested at a later growing stage.

In a *third* pot experiment which started 18 August and ended 17 November 1958, the effect of added calcium carbonate on nitrogen fixation by red clover was studied with 5 different sandy and peat soils obtained by courtesy of the Institute for Soil Fertility, Groningen. The pH values of these soils varied from 4.3 to 5.0. In this experiment the following treatments were compared: a) control, b) $CaCO_3$, $1\frac{1}{2}$ g per pot in the case of sandy soil and 3 g with peaty soil, c) NH_4NO_3, 120 mg N per pot, d) NH_4NO_3 and $CaCO_3$. One series of pots was left uninoculated, a second series was inoculated with a suspension of an effective strain of *Rhizobium trifolii* which was mixed through the soil. The ammonium nitrate-treated plants were left uninoculated.

The results of this experiment were in agreement with those of the two preceding. On the uninoculated acid soils growth of the red clover was poor with scarcely nodulated yellow-green plants which suffered from nitrogen deficiency. Some plants showed a better appearance due to the presence of a few nodules but there was no tendency of the other plants in the same pot to become nodulated. This was in contrast to the soils treated with calcium carbonate

Plate 3. Effect of calcium carbonate on nitrogen fixation and growth of red clover on four different acid sandy soils. Pots 1 and 3 no calcium carbonate, 2 and 4 with 1.5 g calcium carbonate per pot; 1 and 2 uninoculated, 3 and 4 inoculated with *Rhizobium trifolii* (cf Table 3).

Plate 4. Effect of a suspension of sterilized Rhizobium cells on nitrogen fixation and growth of *inoculated* red clover, growing in culture solution. *Left* 3 pots control, *right* 3 pots 20 ml of Rhizobium extract (containing approximately 8 mg N) per pot.

where all the plants after a lag period of approximately four weeks became adequately nodulated and gave normal green plants (Plate 3 and Table 3).

TABLE 3

Effect of calcium carbonate and ammonium nitrate on yield * (g dry weight per pot) of uninoculated and inoculated red clover, grown in pots on a number of acid soils

Type of soil	Ironstone peat (A)		Sandy (B)		Sandy (C)		Sandy (D)		Sandy (E)	
Organic matter content %	36		7.8		9.5		6.7		2.9	
Treatment	Yield	pH**	Yield	pH**	Yield	pH**	Yield	pH**	Yield	pH**
Uninoculated, control	4.6	5.3	0.9	4.7	1.1	4.3	0.4	4.2	0.2	4.5
Uninoculated + $CaCO_3$	5.4	5.8	3.5	5.1	5.1	5.0	1.2	5.0	1.1	6.1
Inoculated, control	4.1	5.3	2.5	4.5	4.1	4.4	2.7	4.2	1.7	4.2
Inoculated + $CaCO_3$	4.0	6.1	5.2	4.9	4.8	5.1	5.3	4.8	3.4	5.4
Uninoculated + NH_4NO_3	5.4	5.2	3.4	4.5	4.8	4.3	3.1	4.0	1.0	4.5
Uninoculated + NH_4NO_3 and $CaCO_3$	6.6	5.8	6.7	5.0	6.9	5.2	3.9	4.9	2.9	5.7

* Averages of duplicate values.

** At harvest.

Inoculation of the acid soils in general brought about a much improved nodulation as a result of which considerably higher yields were obtained. Inoculation of the calcium carbonate-treated soils in some cases gave also higher yields. In general, nodulation of these plants was still more heavy than when inoculation had been omitted.

Ammonium nitrate gave green plants with relatively high yields. Nodulation of these plants was poor. On calcium carbonate-treated soil ammonium nitrate gave still higher yields. Initially nodulation of the plants was poor, but the young white roots which appeared after the combined nitrogen had been used up contained many small nodules.

From the results of the above three experiments it may be concluded that the poor growth of red clover growing on acid soil is largely due to nitrogen deficiency as a result of an inadequate nodulation. However, infection of the roots and growth and nitrogen-fixing capacity of the nodules are not severely depressed by the low pH of the culture medium. This may be concluded from the heavy nodulation and the relatively high yields of plants, grown on acid soil inoculated with a suspension of *Rhizobium trifolii*. Although the latter result might suggest that living Rhizobium cells are not

present in the acid soils, this suggestion is refuted by the fact that treatment of the soils with sterile calcium carbonate brought about, after a retardation of from three to four weeks, a normal nodulation and nitrogen fixation. Apparently Rhizobium cells were present in small numbers in every acid soil tested, but they were unable to spread in the rhizosphere as they did in the soils treated with calcium carbonate. As a result formation of nodules was absent or restricted to a few localized areas of the root system in the case of acid soil as contrasted to the large numbers of well-distributed nodules occurring on the root system of red clover growing on neutralized soil. It is not known whether this depression of the spread of Rhizobium in acid soil depended on an effect of pH on the growth and the multiplication of the bacteria or on an effect on growth of the plant roots or on excretion by the roots of substances required for growth and multiplication of the bacteria.

c. Effect of various calcium compounds and neutralizing substances on nodulation. To investigate whether the observed effect of calcium carbonate on nodulation and growth of red clover on acid sandy soil was due to its neutralizing effect or to its calcium content, three pot experiments with different calcium compounds and with trisodium phosphate and sodium carbonate, respectively, as the neutralizing agent were carried out. Although trisodium phosphate gave a similar rise in pH as calcium carbonate, it affected nodulation and growth of the clover plants less favourably. The same was true of sodium carbonate. These results might indicate that the observed beneficial effect of calcium carbonate on acid soil was due partly to its calcium content. This conclusion was not corroborated, however, by the fact that a mixture of mono- and dicalcium phosphates which did not alter the pH, did not affect nodulation and plant growth. The same was true of added calcium sulphate. However, application of the latter lowered the pH slightly.

d. Counting of Rhizobium cells in acid soil upon application of calcium carbonate. To study the effect of added calcium carbonate on numbers of Rhizobium cells in acid soil, the following experiment was carried out. Acid sandy soil from the garden plots was transferred to Neubauer glass jars with and without the addition of 2 g of calcium carbonate per 500 g soil. For comparison pots filled with soil from neutral plots were also studied.

Some pots of each series were left unplanted, others were sown on 8 June 1959 with red clover in the usual way. Counts of Rhizobium cells in the soil were made after various periods of time in the way described above. In the case of unplanted soil the same pots were used for every sampling. Of the planted pots two were harvested at each sampling time.

The first counting was made on 18 June when the clover plants were approximately 2 cm high and the roots about 5 cm long. Nodules were not yet visible.

The second counting took place on 1 July. The plants were approximately 5 cm high. Nitrogen deficiency had not yet occurred. The control plants on acid soil had no nodules; two of the twenty plants growing on calcium carbonate-treated soil had a few nodules, the other plants in this pot and also all plants in the second pot of this treatment had no nodules. The plants growing on the neutral soil had from 4 to 8 nodules per plant.

The third counting was carried out on 17 July. The plants growing on the acid soil were approximately 6 cm high; they showed severe symptoms of nitrogen deficiency. Root nodules were absent. The plants of the calcium carbonate-treated soil were slightly taller. They also were severely nitrogen deficient. Some plants showed a darker green leaf colour, however. Many nodules occurred on the roots, but the majority of them were small and white. Plants with green leaves had a greater proportion of pink nodules. The plants growing on the neutral soil were approximately 15 cm high, they had many pink nodules.

The fourth counting was made on 9 August. The control plants growing on acid soil were extremely nitrogen deficient. A few plants of one pot showed a better appearance with green leaves. These plants had a number of pink nodules. In the case of calcium carbonate treatment the red clover had now made vigorous growth, due to the presence of many pink nodules. The yields of dry matter were still lower, however, than those of the clover grown in the neutral soil and the leaf colour was somewhat lighter green.

When the fifth counting took place (27 August) most plants growing in the control pots had died. In one pot of this series two plants had a better appearance. Root nodules were not detected on these plants, however. On the calcium carbonate-treated soil the clover plants had made a vigorous growth and they had a dark-green leaf colour. Large numbers of pink nodules occurred. The neutral soil gave still a somewhat higher yield of clover, presumably due to the earlier start of nitrogen fixation.

The results of the bacteria countings and the yields of tops and roots are given in Tables 4 and 5. It will be seen that no Rhizobium cells were detected in 0.1 g of acid soil. Treatment with calcium carbonate without planting gave approximately one cell per 0.3 to

TABLE 4

Number of root nodules per pot in red clover and number of *Rhizobium trifolii* cells per 0.1 g of moist soil * as affected by calcium carbonate, yeast extract, and planting of the soil with red clover

Soil treatment	Days after sowing									
	10		23		39		60		79	
	Nodules	Cells	Nod.	Cells	Nod.	Cells	Nod.	Cells	Nod.	Cells
Acid soil										
Unplanted	—	0	—	—	—	0	—	—	—	0
Planted	0	0	0	0	0	0	93	110	0	780
$CaCO_3$, unplanted	—	0	—	—	—	0	—	—	—	1
$CaCO_3$, planted	0	0	2	17	139	490	556	13,000	1,120	28,000
Yeast extract, unplanted	—	0	—	—	—	0	—	—	—	0
Yeast extract, planted	0	0	0	0	5	1	2	0	266	78
Neutral soil										
Unplanted	—	49	—	—	—	3	—	—	—	17
Planted	0	170	115	330	367	3,300	742	7,900	1,327	4,600

* Water content approximately 16 per cent.

TABLE 5

Effect of added calcium carbonate and yeast extract, respectively, on yield * (g dry weight per pot) of red-clover tops

Soil and treatment	pH of soil at 3rd counting	Days after sowing		
		39	60	80
Acid soil	4.7	0.3	0.3	0.3
Acid soil + $CaCO_3$	6.6	0.3	1.4	4.7
Acid soil + yeast extract	4.7	0.4	0.6	1.3
Neutral soil	6.7	0.7	3.0	8.3

* Averages of duplicate values.

0.5 g of soil, a number which did not vary very much in the course of approximately two months. This demonstrates that *Rhizobium trifolii* did not increase in numbers upon liming of the acid soil. In the neutral soil the number of Rhizobium cells was much higher than in the acid soil *viz* about a hundred per gram of soil. This value varied somewhat during the experimental period.

Planting with red clover resulted in an enormous rise in numbers of Rhizobium cells. In the neutral soil this rise started at an earlier date than in the neutralized acid soil, presumably owing to the larger numbers of cells initially present. In the acid soil a distinct increase of bacteria numbers was observed in a few pots.

It must be stressed that the number of Rhizobium cells in the planted neutral and $CaCO_3$-treated acid soil had increased con-

siderably between the first and the second countings *i.e.* during a period in which nodulation was still restricted and liberation of bacteria from the nodules was unlikely to occur. Apparently the increase of the number of bacteria depended on favourable nutritional conditions in the rhizosphere.

e. Effect of number of added Rhizobium cells on nodulation of red clover growing on an acid soil. If it is assumed that the poor nodulation of red clover growing on acid soil is due to an inadequate growth of *Rhizobium trifolii* in the rhizosphere, it may be supposed that relatively large numbers of the symbiotic bacteria have to be introduced into the soil to attain an adequate nodulation. Therefore an experiment was conducted in which the inoculation of the acid soil was made with various dilutions of a suspension of *Rhizobium trifolii*.

The results of this experiment are recorded in Table 6. It will be seen that more than 60,000 bacteria had to be introduced into 500 g of acid soil to attain an optimal nodulation. This was in contrast to the results obtained with sterile clover plants growing on an agar medium in culture tubes, where introduction of one Rhizobium cell resulted in the formation of a normal number of nodules. This demonstrates that conditions for growth and multiplication of the bacteria in the latter case were much more favourable than in the acid soil.

TABLE 6

Effect of number of added Rhizobium cells on nodulation and yield of tops of red clover grown on acid soil

Number of introduced *Rhizobium trifolii* cells.		Tops, g dry weight per pot (duplicates)	Nodulation
Per pot	Per g of soil		
6×10^6	12,000	2.45; 2.50	Many small nodules on most roots
6×10^4	120	1.07; 2.88	,, ,, ,, ,, ,, ,,
6×10^2	1	0.47; 0.89	Few, large nodules, concentrated in a few areas
6	0	0.28; 0.29	No nodules

f. Behaviour of *Rhizobium trifolii* cells added to various soils. To study the effect of pH and organic matter on behaviour of *Rhizobium trifolii* in soil, a dense suspension of this bacterium was added to three different sandy soils from the garden plots of the Laboratory of Microbiology. These soils were the acid

one of pH 5.0 which was employed in the above-mentioned pot experiments, a neutral one of pH 7.5, and a slightly acid one (pH 5.6) which had been treated with stable manure for several years.

At different periods of time after the addition of the bacterium suspension, the numbers of effective Rhizobium cells in the soils were estimated by the above-described technique. The results of these countings are recorded in Table 7. It will be seen that during the first 10 days the numbers of effective Rhizobium cells in the

TABLE 7

Numbers of effective *R. trifolii* cells per 0.2 g of moist soil different periods of time after the addition of a suspension of the bacteria * to the soil

Incubation time	Acid soil		Neutral soil		Soil + stable manure	
	Control	Inoculated	Control	Inoculated	Control	Inoculated
2 hours	—	4.9×10^7	—	—	—	—
1 day	—	2.3×10^8	—	3.3×10^8	—	1.3×10^8
2 days	1	—	3.3×10^3	—	4.6×10^3	—
3 ,,	—	1.7×10^8	—	4.9×10^7	—	4.9×10^7
7 ,,	—	7.9×10^7	—	2.3×10^6	—	7.9×10^7
10 ,,	—	3.3×10^7	—	3.3×10^7	—	1.2×10^7
15 ,,	—	2.3×10^6	—	1.1×10^7	—	2.3×10^6
23 ,,	—	9.5×10^5	—	4.9×10^6	—	2.3×10^6
37 ,,	0	4.9×10^5	1.3×10^4	1.3×10^6	3.3×10^3	1.1×10^6
64 ,,	—	3.3×10^3	—	2.2×10^5	—	2.8×10^5

* 1.3×10^8 cells per 0.2 g soil.

three soils approximated that of the number added. After an incubation time of 15 days considerably lower numbers were counted in all three soils. After 64 days, the acid soil was found to contain a much lower number than the other two soils.

2. *Effect of organic compounds on nitrogen fixation and growth of red clover*

The effect of organic matter on nitrogen fixation by red clover was noticed in the above-described field experiments. Some of the acid plots had been treated with stable manure a few years before starting the experiment. On these plots nodulation and growth of the clover were excellent notwithstanding the fact that the pH was not higher than on similar plots without manurial treatment where no nodules were formed (Table 1).

The results of this observation might be explained in various

ways: 1) The stable manure could have introduced some inorganic factor which promoted in some way nitrogen fixation. 2) It could have introduced some organic component. 3) It could have formed pockets of higher pH in which the bacteria could have survived. 4) It could have improved the carbon dioxide content of the soil. Therefore a number of pot experiments were carried out to elucidate the observed effect of stable manure.

a. Pot experiments with acid soil in Neubauer glass jars

In the *first* experiment the following treatments were compared: 1) Nitrogen, 42 mg per pot, in the form of ammonium nitrate. 2) Casamino acids (Difco), enriched with 20 mg tryptophane per 2.1 g, in amounts equal to 42 mg nitrogen per pot. 3) Concentrated yeast extract, 4.75 ml, containing 42 mg nitrogen, added per pot. 4) Stable manure, 10 g per pot, containing approximately 42 mg nitrogen. 5) Sterilized stable manure, 10 g per pot. 6) Ash of 10 g stable manure incinerated for 4 hours at 300° C. 7) KH_2PO_4, 0.5 g per pot. 8) Sterilized cultures of an effective and an ineffective strain of *Rhizobium trifolii*, respectively. These bacteria were grown as described above. Ten-ml aliquots of suspensions of the bacteria, containing 0.4 mg nitrogen per ml, were added per pot. Two pots of each treatment were left uninoculated, two further pots were inoculated with an effective strain of *Rhizobium trifolii*.

The experiment was started on 28 August and finished on 19 December 1957.

The results are recorded in Table 8. The following conclusions may be drawn from the pots with uninoculated soil: – In the untreated soil the yield of the clover was very low due to nitrogen deficiency. Ammonium nitrate gave an improved growth but the amount applied apparently was too low to produce much higher yields. Nitrogen supplied in the form of amino acids gave slightly better results. In both cases the formation of nodules was poor. Yeast extract gave better yields than was in accordance with its nitrogen content. This was presumably due to the fact that a considerable number of nodules were present on the roots. Stable manure initially did not affect the growth of the plants. At harvest time, however, the leaf colour and the appearance of a number of plants were clearly improved, presumably due to the nodules which had developed. Sterilized stable manure had a less favourable effect whereas ash of stable manure had no effect at all. In the case of phosphate treatment the yields were even lower than those of the

control plants. The most remarkable fact was the favourable effect of dead cultures of effective and ineffective Rhizobium strains. After a retardation period of several weeks, many nodules were formed which brought about a vigorous growth of the plants (Plate 2 A).

TABLE 8

Effect of different organic and inorganic substances on yield of tops (g dry weight per pot) of uninoculated and inoculated red clover grown on acid soil (Averages of duplicate values)

Treatment	Uninoculated	Inoculated
Control	0.5	3.0
NH_4NO_3 *	0.6	2.0
Casamino acids *	0.9	2.7
Yeast extract *	1.0	1.9
Stable manure *	0.5	2.3
Sterilized stable manure *	0.4	3.0
Incinerated stable manure	0.4	1.4
KH_2PO_4	0.1	2.9
Sterilized effective-Rhizobium extract **	2.5	2.4
Sterilized ineffective-Rhizobium extract **	2.0	2.4

* Corresponding with 42 mg N per pot.
** Corresponding with 4 mg N per pot.

In the case of inoculated soil no clear effect of the various treatments on nodulation and yield of the red clover was observed.

This was in contrast to the results of a *previous* experiment in which stable manure and extract of stable manure were found to have a pronounced effect on yield of inoculated red clover. That experiment was conducted from 28 June to 30 September 1957. Stable manure was applied in amounts of 5, 10, and 20 g per pot, corresponding with 21, 42, and 84 mg N respectively. An extract of stable manure, prepared as described above, gave similar results. Twenty ml of this extract, prepared from 130 g of stable manure and containing 0.75 mg N per ml, was applied per pot. A treatment of 2.5 mg sodium molybdate per pot which was also included in this experiment gave a slightly improved growth. The results of this experiment are given in Table 9. The observed effect of stable manure was not brought about by its nitrogen content. This may be concluded from the fact that a concentrated extract of stable manure, containing only 15 mg N, gave the same results, whereas ammonium nitrate (supplied in amounts of 21, 42, and 84 mg N per pot) depressed nodulation and gave lower clover yields.

TABLE 9

Effect of added stable manure and stable-manure extract on yield (g dry weight per pot) of inoculated red clover, grown on acid soil

Treatment (per pot)	Yield of tops
Control	3.6 *
$Na_2MoO_4.2H_2O$ 2.5 mg	4.4 *
Stable manure, 5g (21 mg N) . .	4.7 **
,, ,, , 10g (42 mg N) . .	5.4 **
,, ,, , 20g (84 mg N) . .	6.3 **
,, ,, extract (15 mg N).	6.0 ***

Average values of the yields of four *, two **, and six *** pots, respectively.

A *third* experiment with sterilized cultures of *Rhizobium trifolii* and with yeast extract added to acid soil was started on 18 February 1958. A treatment with sodium molybdate was also included in this experiment. Each treatment was present in quadruplicate. Inoculation with *Rhizobium trifolii* did not take place and precautions as described above were taken to prevent the contamination of the soil with this bacterium.

Without treatment and in the presence of added molybdate the growth of the clover plants on the acid soil was extremely poor owing to nitrogen deficiency. Treatment with the sterilized Rhizobium suspension and with yeast extract, respectively, gave an improved growth due to the development of root nodules. The response of the plants in the four replications of each treatment was quite irregular, however, as may be seen from Plate 2B and from the yield data in Table 10 where the weight of tops of each individual pot is recorded. This irregularity was also observed in the number of plants per pot which responded to treatment. In general no more than from 2 to 6 of the 20 plants originally present in each pot showed an improved growth and were responsible for the higher yields. No explanation can be given of the irregular response to the added extracts. The beneficial effect of these extracts on nodulation was, however, undoubted (see Table 10).

The effect of added yeast extract on numbers of Rhizobium cells in unplanted and planted acid soil was studied in the experiment described on page 101. The results of the countings are recorded in Table 4 and the corresponding yields of the clover plants in

TABLE 10

Effect of sterilized suspensions of *Rhizobium trifolii* and of yeast extract on yield of tops (g dry weight per pot) and nodulation of red clover, grown on acid sandy soil

Treatment (per pot)		Yield	Nodulation
Control	a *	0.18	Small numbers of small white or brown nodules.
	b	0.05	
	c	0.35	
	d	0.21	
$Na_2MoO_4.2H_2O$, 2.5 mg	a	0.13	Similar to control plants.
	b	0.35	
	c	0.22	
	d	0.32	
Yeast extract, 2 ml	a	9.12	Large numbers of normal nodules.
	b	0.56	
	c	1.10	A few very large composite nodules on the roots of a few plants which had a better appearance.
	d	0.47	
,, 5 ml	a	0.52	Poor.
	b	0.92	Many small white nodules and a few large composite ones on the roots of plants with a better appearance.
	c	0.85	
	d	0.97	
,, 10 ml	a	0.68	Poor.
	b	0.65	
	c	0.60	Many small nodules.
	d	1.30	Many small white nodules and a number of large ones.
Sterilized Rhizobium suspension 5 ml	a	5.70	Large numbers of normal nodules.
	b	0.48	Moderate, with white and brown nodules.
	c	0.48	Poor.
,, 10 ml	a	3.15	Large numbers of normal nodules.
	b	6.90	
	c	0.30	Small numbers of small white and brown nodules.
	d	0.25	

* a, b, c, and d, replicates of one treatment.

Table 5. It will be seen that yeast extract had no influence on the number of bacteria in unplanted soil and a slight effect in the case of acid soil planted with red clover.

b. Effect of various organic compounds on nodulation of inoculated red clover growing in culture solution

Sterile plants of red clover were transplanted on 23 August 1957 to large tubes containing approximately 150 ml of a nutrient medium of the above-mentioned composition. On 12 September the plants were inoculated with an active strain of *Rhizobium trifolii*. Two days later the following substances were added: a) 15 ml of a suspension of killed Rhizobium cells containing 0.42 mg N per ml. b) $3\frac{1}{3}$ ml of conc. yeast extract containing 3 mg N per ml. c) 10

mg nitrogen in the form of amino acids derived from hydrolysed caseine supplied with 1 mg tryptophane per tube. d) 10 mg nitrogen in the form of glutamic acid, aspartic acid and α-alanine, respectively. e) 10 mg nitrogen in the form of ammonium sulphate and potassium nitrate, respectively. Each treatment was present in quadruplicate.

One week after inoculation the first root nodules appeared. Two weeks later pronounced differences in nodulation between different treatments were observed. Plants provided with a suspension of killed Rhizobium cells were well nodulated with large, pink nodules. This contrasted with the untreated control plants where the nodules were similar in number but much smaller and white in colour. The pH values of both series were similar, *viz* approximately 5.9. Added yeast extract gave also relatively large, pink nodules. The pH of the latter solution had increased to 7.6, presumably due to bacterial decomposition of some compound of the yeast extract. In the case of added amino acids, nodulation was more or less depressed.

The results of this experiment indicate that some unknown component present in the Rhizobium or yeast extracts may promote the development of root nodules (Table 11). That amino acids are responsible for this effect is improbable in view of the results obtained with the addition of these compounds.

TABLE 11

Effect of various organic and inorganic compounds on nodulation of red clover grown in culture solution

Treatment	Dry weight of nodules from four different plants, mg per plant			
Control	1.1,	1.7,	4.4,	1.4
Sterilized Rhizobium extract *	3.1,	0.7,	4.0,	3.4
Yeast extract **	4.3,	3.0,	4.7,	1.8
Casamino acids **.	0.0,	0.3,	0.4,	0.9
Glutamic acid **	0.4,	0.8,	0.7,	0.3
Aspartic acid **.	0.2,	0.4,	0.7,	1.3
α-Alanine	0.1,	0.2,	1.0,	0.0
$(NH_4)_2SO_4$ **.	0.0,	0.3,	0.3,	0.0
KNO_3 **.	0.3,	0.2,	0.5,	0.3

* Corresponding with 6.3 mg N and ** with 10 mg N, respectively.

The beneficial effect of sterilized suspensions of Rhizobium cells on nodulation and growth of red-clover plants was shown in two

further *culture-solution* experiments. Inoculated plants treated with these suspensions were more heavily nodulated and fixed considerably larger amounts of nitrogen than untreated control plants. This was particularly true of plants growing at pH 4.8; at pH 6.8 the differences were less pronounced. Plate 4 shows the effect of Rhizobium extract at pH 4.8. Yield of tops (mean dry weight of 5 plants): control 0.58 g, treatment 1.46 g, and of roots 0.18 and 0.37 g, respectively.

c. Effect of added vitamins on nodulation. To see whether vitamins could affect nodulation of red clover plants, two experiments were carried out.

The *first* experiment lasted from 14 December 1957 to 29 March 1958. Sterile red-clover plants were grown in tubes containing an agar nutrient medium of the above-mentioned composition. A comparison was made between Rhizobium and yeast extracts and a mixture of four vitamins (0.05 to 0.4 ml of a solution containing 10 mg thiamine, 10 mg riboflavin, 10 mg β-alanine, and 4 mg biotin per 100 ml H_2O). Although the plants treated with the extracts and with the vitamin solution tended to be darker green than the control plants, no significant increase in nitrogen content of the plants due to treatment was found.

In a *second* experiment which lasted from 3 April to 28 July 1958, the agar used in the culture tubes had been purified by washing it daily with distilled water for a period of 14 days. The following vitamins were tested singly: thiamine, nicotinic acid, riboflavin, pyridoxine, pantothenic acid, β-alanine, p-amino benzoic acid, and biotin. The vitamins were supplied in amounts of: 10, 20, and 30 μg per tube except biotin which was given in amounts of 4, 8, and 12 μg per tube. The results of this experiment were inconclusive as to the importance of the added vitamins for nodulation.

DISCUSSION

Nodulation of red clover and other leguminous plants, growing on acid soil, in general is poor or absent. As a result development of these plants is restricted and yields are low, mainly due to nitrogen deficiency. It could be suggested that the poor nodulation is a result of the absence of Rhizobium cells or of the inability of either the plant root or the bacteria, or of both, to develop effective

nodules. In the present study inoculation of a number of acid soils with an effective strain of *Rhizobium trifolii* gave well-nodulated red-clover plants which grew vigorously as contrasted with plants growing in uninoculated soil which were poorly nodulated and gave low yields. This shows that the infection of the roots and the formation and functioning of the nodules may be quite normal on such soils, when sufficient numbers of the symbiotic nitrogen fixers are brought into the soil. By using bacterial suspensions of various densities it was found that at least 60,000 Rhizobium cells had to be introduced into 500 g acid soil to obtain adequate numbers of nodules on the clover roots.

Although the results of the inoculation experiments might suggest that no Rhizobium cells occurred in the acid soils, the experiments with calcium carbonate and with various mixtures of organic compounds have shown that *Rhizobium trifolii* was not completely absent from the soils tested. The bacteria may occur in very low numbers, but they increase rapidly when conditions are made more favourable for their multiplication. This may be concluded from the experiments with planted and unplanted soil in which numbers of Rhizobium cells were counted. A pronounced increase took place in planted, acid soil which had been treated with calcium carbonate. Without planting there was no such effect. Planting of acid soil as such did hardly affect the numbers of Rhizobium cells.

From these experiments and from the observation that nodulation, when it occurs at low pH, is restricted to a few, localized areas from which it practically does not extend, it may be concluded that the poor nodulation of red clover growing on acid soils is due to the inability of *Rhizobium trifolii* to grow and spread in the rhizosphere. Whether this depends on a direct effect of pH on multiplication of the bacteria or on an indirect effect, dependant on a limited growth of the plant roots, or on a limited excretion of organic substances required for the growth of the bacteria, is not yet known. The results obtained by Loneragan and Dowling [3] indicate that the critical pH value for the multiplication of *Rhizobium trifolii* in a yeast-extract/mannitol medium lies between 4.5 and 5.0. Vincent [12] found that *Rhizobium trifolii* added to acid soils did not survive when the pH of the soil was below 5. This result was not in agreement with the behaviour of *Rhizobium*

trifolii added to the acid sandy soil employed in the present study. Up to ten days after the addition of a heavy suspension of the bacteria to the acid soil no decline in number of Rhizobium was found. The values reported by Loneragan and Dowling [3] approximate those found for the acid soils used in the present study. This fact could indicate that the inhibited growth of the bacteria in the acid soils is the result of a direct effect of the low pH on the growth of the bacteria. This is not in agreement, however, with the beneficial influence of suspensions of killed Rhizobium cells and of yeast extract on nodulation as it has been observed in the present study with acid soils as well as with culture solutions. Although the possibility of the occurrence of a slight temporary rise in pH following the addition of the extracts to the acid soils can not be excluded, the fact that the effect of these substances was also observed with clover plants growing in culture solutions where the pH was kept at a constant level, renders it improbable that it depended on a change of pH.

The experiments of Albrecht and Davis [1] and of Loneragan and Dowling [3] have demonstrated that the inhibiting effect of a low pH on nodulation might be counteracted to a large extent by increasing the calcium supply of the culture medium. Calcium would function in the formation of nodules and high hydrogen-ion concentrations would depress calcium uptake. In the present study induced calcium deficiency apparently was not responsible for the poor nodulation of red clover in acid media. This may be concluded from the fact that added calcium sulphate, calcium phosphates, and incinerated stable manure had no effect on nodulation. On the other hand neutralization with trisodium phosphate and sodium carbonate worked less favourably than calcium carbonate.

No explanation can be given of the observed effects of the extracts of yeast, sterilized Rhizobium cells, and stable manure. These extracts not only may promote multiplication of *Rhizobium trifolii* and infection of the roots but also the growth of the nodules. The stimulative effect of these substances when added to acid soils is less consistent than that of calcium carbonate, however.

The nature of the component, responsible for the activity of the extracts, is unknown. Experiments with a number of added vitamins (thiamine, nicotinic acid, riboflavin, pyridoxine, pantothenic

acid, β-alanine, *p*-aminobenzoic acid, and biotin) did not conclusively answer the question whether the effect of the extracts depended on the presence of some vitamin.

The existence of unknown unspecific substances, secreted by plant roots, particularly those of legumes, which may stimulate the growth of Rhizobium strains and nodule initiation is recorded by Nutman [6]. In higher concentrations these substances inhibit nodulation. Treatment of nutrient media containing these inhibitors with charcoal might remove them and result in an improvement of nodulation (Turner [10]). Vantasis and Bond [11], growing pea plants in "used" culture media, found that treatment with charcoal gave lower numbers of pea nodules. Growth of these nodules and nitrogen fixation were considerably improved, however. Rudin [8] obtained more nodules in pea plants treated with extracts of leaves of pea or maize plants than in control plants without this treatment.

SUMMARY

1. Red clover grown on acid soil under field conditions and in pot experiments was found to be poorly nodulated or to lack nodules. As a result of this the plants suffered severely from nitrogen deficiency and gave low yields.

2. Inoculation of the acid soils with a suspension of an effective strain of *Rhizobium trifolii* gave well-nodulated plants which grew vigorously. More than 60,000 Rhizobium cells had to be introduced into 500 g soil to attain a normal nodulation.

3. Calcium carbonate added to the acid soils in amounts of one or two grams per 500 g of soil brought about, after a retardation of approximately four weeks, a normal nodulation of the clover plants. Since precautions had been taken that no foreign Rhizobium cells were introduced into the soil after neutralization, it was assumed that the bacteria had been originally present in the acid soil in low numbers.

By counting the numbers of effective Rhizobium cells, it was found that neutralization of the acid soil without planting of red clover had no effect on the multiplication of the bacteria. In the presence of red clover plants, however, an, enormous rise in *Rhizobium trifolii* was found to occur in neutralized acid soil but not in acid soil. This demonstrates that inability of the bacteria to grow and spread in the rhizosphere presumably is responsible for the poor nodulation of the clover plants on acid soils.

4. Treatment of acid soils with yeast extract or a sterilized suspension of effective or ineffective *Rhizobium trifolii* promoted nodulation of red clover growing on these soils. This effect was less regular than that of added calcium carbonate. Stable manure and stable-manure extract in some cases reacted similarly. So far the substance responsible for the stimulative effect has not been found.

ACKNOWLEDGEMENT

The authors are indebted to Dr. J. L. Harley, Department of Botany, University of Oxford for grammatical corrections.

Received September 1, 1959

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