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NODULATION OF *MEDICAGO SATIVA* IN SOLUTION CULTURE

I. ACID-SENSITIVE STEPS

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INTRODUCTION

The sequence of steps which leads to the appearance of root nodules on leguminous plants has been studied in detail by many workers [17]. For the purpose of this paper the following simplified description of the sequence in lucerne is sufficient: (i) development of root hairs, (ii) development of a population of Rhizobium near the root surface, in the rhizosphere, (iii) curling and infection of root hairs, (iv) development of infection threads, (v) formation of nodules.

It is well established that acidity inhibits nodulation, but the nature of the inhibition is not understood [1 9 14 17]. Observations that nodule bacteria can grow at pH too low for nodulation can be interpreted to mean that acidity does not inhibit nodulation by preventing rhizosphere development [8 9 14]. But these observations are not conclusively relevant. Growth of Rhizobium in bacteriological media might not be pertinent to its accumulation in the legume rhizosphere; nor is it certain to what extent a rhizosphere population need develop before further steps in nodulation can be initiated. Apart from this, the possibility that acidity particularly affects any one step in the nodulation sequence has not been tested. This paper describes experiments designed to define acid-sensitive steps in the nodulation of *Medicago sativa* L.

The first two experiments were done to provide preliminary data on range of effective pH, size of inoculum, and rate of nodulation.

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The key experiments exploited the ability of nutrient solutions to be altered rapidly. They tested the effects of imposing chosen pH at chosen times during the course of nodulation on the number and distribution of the nodules which subsequently appeared. Concurrent observations were made to determine the time schedule of production of root hairs, accumulation of Rhizobium, curling of root hairs, development of infection threads, and advent of visible nodules.

EXPERIMENTAL PROCEDURE

The treatments and conditions peculiar to each experiment are described in the Results section. The following is a general description of procedures.

(i) *Glasshouse procedure*

All the experiments except Experiment 6 were done in a glasshouse in daylight and without asepsis. (Experiment 6 was done aseptically under artificial light – see Results, section vii). Air temperature in the glasshouse was maintained at 23°C by day and 19°C by night. The culture solutions reached peak afternoon temperatures of 26°C on clear days.

The seed (var. Hunter River) was soaked for 10 minutes in 0.1% $HgCl_2$, rinsed, soaked overnight in aerated water, and spread on cotton mesh over half-strength Hoagland's solution to germinate. Seedlings were transplanted to the experimental cultures 7–8 days later, when their taproots were usually 5–6 cm long. The culture vessels were either 3-litre beakers or 22-litre steel drums, with polyethylene bags as liners.

The solutions were made with demineralized filtered water and reagent-grade salts, and were aerated continually with filtered air. The pH was adjusted daily with H_2SO_4 or KOH, and stayed within 0.1 units of nominal pH in the 22-litre cultures, 0.2 units in the 3-litre cultures. All the solutions contained 1 m*M* $MgSO_4$, 1 m*M* KH_2PO_4, 20 μM FeEDTA, 10 μM KCl, 2 μM H_3BO_3, 0.4 μM $MnSO_4$, 0.16 μM $ZnSO_4$, 0.04 μM $CuSO_4$, and 0.01 μM H_2MoO_4. Calcium sulphate was provided at 3m*M* concentration in Experiment 1, and at 8 m*M* in the other experiments. Preliminary experiments had shown that variation in calcium concentration had little effect on nodulation in this high range. Combined nitrogen was supplied in the first five experiments to make growth independent of nodulation, to aid pH-control, and to make the conditions more pertinent to soil conditions. These solutions contained 1 m*M* $(NH_4)_2SO_4$ and either 3 or 5 m*M* KNO_3. No nitrogen was provided in the last three experiments, partly to check that the main findings did not depend on the presence of combined nitrogen, and partly because these experiments required precision difficult to achieve with sparsely nodulated plants. The nitrogen-free solutions had 1.5 m*M* K_2SO_4 to replace KNO_3 as the main source of potassium.

(ii) *Inoculation*

Inoculants were two- or three-day-old cultures in yeast–mannitol broth, checked nephelometrically, and diluted where necessary in water at 5°C immediately before use. Counts indicated no significant loss of viability during dilution.

(iii) *Bacterial counts and phase-contrast observations on root hairs*

Plants withdrawn for bacterial counts and microscopic observation were sampled from each replicate of the treatment concerned. The section of taproot within 3 cm of the crown was discarded because nodules rarely appeared in this zone. Bacterial counts were made immediately after sampling. Roots for microscopy were stored up to four days in a saturated atmosphere at 5°C. During this period they were examined one replicate at a time.

Rhizobium cells were counted in Experiment 1 by tenfold serial dilution and infection test [4]. In Experiment 5 total bacteria were plate counted in inoculated and uninoculated treatments and Rhizobium estimated as the difference. This estimate was only possible because Rhizobium greatly outnumbered the contaminants. Experiment 6 was done aseptically to allow direct plate counting of Rhizobium with small inocula. Numbers of bacteria on roots were estimated from counts on washings obtained by shaking two sample roots from each culture vigorously with 20 ml cold sterile water for two minutes [16].

Although the nutrient solutions were ordinarily clean, the roots rapidly became so coated with micro-organisms and ferric hydroxide that the root hairs were obscured. (The coating resembled Dart and Mercer's [5] 'rhizosphere' insofar as its structure could be seen). Although shaking the roots with water left them clean, it damaged the root hairs. Consequently, root samples for microscopy were cleaned with the tip of a sable brush in 2% hemosol. (This cleaning procedure was also used as an experimental treatment in Experiment 5 (Fig. 4)).

The results of microscope observations were recorded on scale maps of each root. Mean numbers of root hairs, total and curled, were estimated by counting five to ten random fields within each fairly uniform zone on each root, multiplying mean counts by length of zone, and summing zones over the whole root. Root hairs were considered 'curled' if the tip had deflected 90° or more, or if the hair had a marked protuberance, branch, or convolution.

(iv) *Observations on nodulation*

Plants were inspected daily in the glasshouse to determine when the first nodules appeared. Unless delayed by treatment, nodules appeared within four to seven days of inoculation. The earliest nodulation occurred in summertime, in heavily inoculated cultures free of nitrate. After harvest, the numbers of nodules and nodulated plants were counted in the laboratory, and in experiments 4, 5, 7 and 8 their position on the taproot or laterals was recorded within successive 2 cm intervals from the crown.

RESULTS

(i) *Effects of pH in relation to size of inoculum*

Experiment 1 (Fig. 1): Two pH treatments, 5.7 and 5.1, were tested in factorial combination with five levels of inoculum ranging from 0 to 10^8 viable cells per litre of culture solution. An additional treatment had pH 4.6 and 10^{10} cells per litre. Each duplicate 3-litre culture vessel had 12 plants, in nitrate medium. The cultures were inoculated three days after planting. Numbers of Rhizobium counted in samples of the nutrient solutions six days after inoculation had fallen slightly, but were still within an order of magnitude of the initial number. Nodules appeared on the 7th day after inoculation, and were counted after harvest on the 18th day.

Numbers of nodules increased with increasing inoculum only within a range of small inoculum sizes (Fig. 1). Above 10^4 cells of strain U45 per litre, or above 10^6 of strain SU47, size of inoculum had no effect, and did not modify the effect of pH.

(ii) *Effects of pH on rate of appearance of nodules*

Experiment 2 (Fig. 2): Four treatments were applied, at pH 4.8, 5.1, 5.4 and 5.7. Each triplicate culture vessel contained 22 litres of nitrate medium and received 24 seedlings. Cultures were inoculated with 10^8 cells of strain U45 per litre, three days after planting. The first nodules appeared seven days after inoculation regardless of pH treatment. Eight plants were removed from each culture for nodule counts at each of three harvests, 8 days, 15 days, and 28 days after inoculation.

Acidity inhibited nodulation for the duration of the experiment (Fig. 2). Acidity did not delay the appearance of the first nodules, but fewer nodules appeared at pH 5.1 than at 5.4 or 5.7 and fewer still at 4.8. These differences enlarged over the next three weeks,

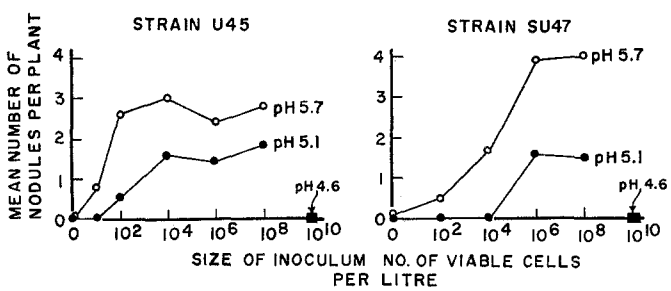

Fig. 1. (Exp. 1). Effects of pH and inoculum size on number of nodules per plant. Plants were inoculated 3 days, and harvested 18 days after planting. Data are means of 2 replicates, each having 12 plants (nitrate medium).

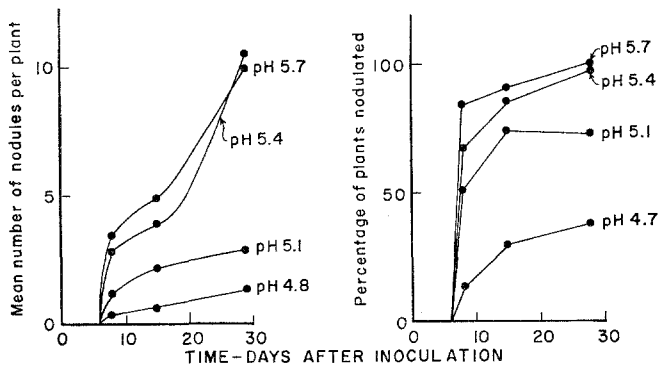

Fig. 2. (Exp. 2). Effects of pH and time after inoculation on nodule number and percentage of plants nodulated. Inoculated 3 days after planting with 10^8 cells of strain U45 per litre. Data are means of 3 replicates, each having initially 24 plants. Eight plants were removed from each culture at each harvest (nitrate medium).

but most of this enlargement occurred between the second and third harvests. The data suggest that early and fairly stable effects of pH on nodule number could suitably be measured between two and seven days after the first nodules appear.

(iii) *Effects of pH before and after inoculation*

Experiment 3 (Table 1): Preinoculation and post-inoculation pH treatments were tested in the combinations shown in Table 1. The preinoculation treatments were imposed either on the plants or the fully grown broth

TABLE 1

Effects of pH applied before and after inoculation (Expt. 3). Preinoculation treatments were imposed on plants and the fully grown inoculum broth for the 6 days before inoculation. Nodule counts were made 10 days after inoculation. The data are means of 2 replicates, each having 12 plants in a 3 litre culture

pH-treatment			Mean no. of nodules per plant
Pre-inoculation		Post-inoculation	
Host Plant	Rhizobium		
4.9	6.0	4.9	0.7
6.0	4.9	4.9	0.0
6.0	6.0	4.9	1.0
4.9	4.9	6.0	10.5
4.9	6.0	6.0	12.7
6.0	4.9	6.0	13.3
6.0	6.0	6.0	13.2
6.0	uninoculated	6.0	0.0

culture of Rhizobium (Strain SU47), for six days between planting and inoculation. Each duplicate culture had 12 plants in three litres of nitrate medium. Inoculation level was 10^7 cells per litre. Harvests and nodule counts were made ten days after inoculation.

The plants produced 10–13 nodules if the pH was 6.0 after inoculation, but only one nodule or less at pH 4.9. This held regardless of pH imposed on the plants or inoculum during the six days before inoculation (Table 1).

(iv) *Effects of changing the pH after inoculation and of delaying inoculation*

Experiment 4 (Fig. 3): Part (a) of Experiment 3 was devised to test whether acid sensitivity is restricted to a particular stage of the nodulation process. Part (b) was devised to indicate the nature of recovery after an inhibitory pH is raised. Each of the eleven treatments is described with its results in a separate diagram in Fig. 3. The pH treatments are shown on the horizontal bars parallel to the time scale, pH 5.7 where a horizontal bar is

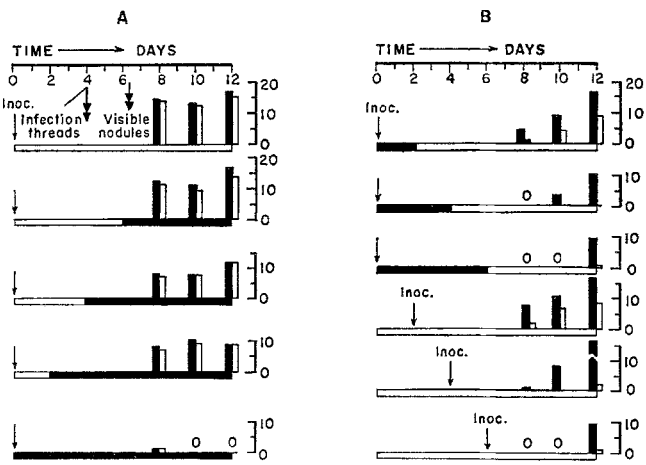

Fig. 3. (Exp. 4). Effects of varying pH during nodulation, and of delaying inoculation, on numbers and distribution of nodules which appeared subsequently.

The pH treatments are shown on the horizontal time scale thus: ▬ pH 4.7–4.8, ⊏——⊐ pH 5.7–5.8. Inoculated with 10^9 U45 per litre at times indicated ↓. Seven plants were removed from each of the 3 replicate cultures at each of 3 harvests, and the mean nodule counts per 7 plants are indicated thus:

▮ for total number of nodules, ⊓ for nodules on taproots. (nitrate medium).

white, pH 4.7 where black. Cultures were planted one day before the day indicated 0 on the time scale, and inoculated with 10^9 U45 per litre either on day 0, 2, 4, or 6, as shown. There were triplicate cultures, each having 22 litres of nitrate medium and initially 24 plants. Seven plants were harvested from each culture at each of three harvests. The times of harvest are shown by the positions of the vertical bars, and the numbers of nodules per seven plants are shown by the heights of the vertical bars. Total nodules and taproot nodules are shown separately, as black verticals and white verticals respectively.

When the pH was held at 5.7, nodules appeared six days after inoculation and nodule numbers had stabilized by day 8 (Fig. 3a). When the pH was held at 4.7 virtually no nodules appeared. However, two days at pH 5.7 following inoculation was enough to induce the subsequent appearance of nodules. After two days at pH 5.7 the pH could be lowered without delaying nodulation or substantially reducing nodule number (Fig. 3a).

Keeping the pH at 4.7 for 2, 4 or 6 days after inoculation, before raising it to 5.7, delayed the subsequent appearance of nodules for a corresponding 2, 4 or 6 days (Fig. 3b). It did not reduce nodule number. The longer the delay the fewer nodules were produced on the taproots and the more on the laterals, which had begun to grow root hairs on day 2. Delaying the provision of the higher pH had almost the same effects as delaying inoculation for an equal time (Fig. 3b). Thus the effect of acidity was demonstrably irreversible only in that it delayed the inception of nodulation. This seemed to allow older sites to become obsolete; and the nodules then formed on newer sites which would otherwise not have been used.

(v) *Effects of varying the time and duration of exposure to high pH, and of disrupting the rhizosphere*

Experiment 5 (Fig. 4): The previous experiment indicated that the critical effects of acidity operate in the early stages of nodulation. This experiment tests pH-treatments of shorter duration, the effect of physically disrupting the rhizosphere, and the effects of acidity on root growth, production and curling of root hairs. The plants were small at the start of the experiment, so that few nodules developed on lateral roots and only total numbers are recorded. Harvests on days 9 and 11 gave similar results which were therefore pooled. There were two replicates. In all other respects the procedure was as in Experiment 4.

As many nodules were initiated in three days at pH 5.7 as in the control with pH 5.7 for the whole eleven days (Fig. 4). Fewer nodules were initiated in two days at pH 5.7, and fewer still in one day. At the beginning of this experiment root hairs were sparse (see Fig. 5a and section (vi) below). If the one-day exposure to pH 5.7 was applied later, more piliferous root was available and more nodules were produced, with a modal position further down the taproot (Fig. 4). Late inoculation also produced nodules further down the taproot (and was the only treatment to produce a significant proportion (15%) of the nodules on lateral roots).

A large population of Rhizobium accumulated on the roots within the first day after inoculation (see Section (vii) and Fig. 6c below). Brushing the roots to free the coating which contained most of this population of bacteria did not prevent normal production

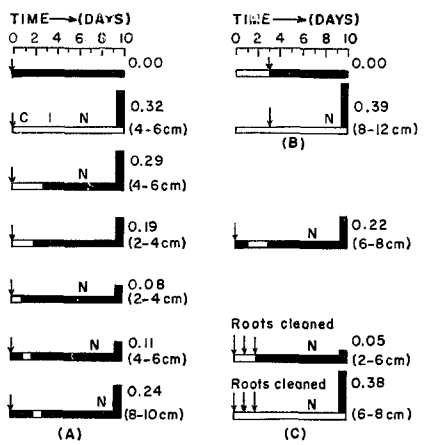

Fig. 4. (Exp. 5) (a). Effects of short exposures to pH sufficiently high for nodulation, on number of nodules which appeared subsequently; (b). Effects of delaying inoculation; (c). Effects of cleaning roots during initial stages. On the horizontal time scale ▬ indicates treatment at pH 4.7, |—| pH 5.7. The symbol ↓ indicates time of inoculation; C, i, and N respectively indicate times at which curled root hairs, infection threads, and nodules were first seen. The mean value of $\log_{10}$ (1 + number of nodules per plant) is shown thus ▮ and also numerically; the least difference significant at p 0.05 = 0.12. The figures in parentheses indicate modal positions of nodules on the taproot, in cm from the crown. Harvests of 10 plants from each duplicate culture on days 9 to 11 yielded the same results, which were pooled (nitrate medium).

of nodules if the pH remained at 5.7; but it suppressed nodulation if the pH was lowered to 4.7 after the cleaning (Fig. 4, lower right).

(vi) *Root hairs, curling, and infection threads*

Experiment 5 and 7 (Fig. 5): Plants were subsampled from glasshouse cultures for microscope examinations of their taproots during the first few days of Experiment 5 (section (v)), and Experiment 7 (section (viii), below). Each datum in Fig. 5a represents a mean of two sample plants from Experiment 5, and each datum in Fig. 5b represents a mean of three sample plants from Experiment 7.

Culture solutions too acid to permit nodulation did not inhibit growth of roots or production of root hairs, but they did prevent curling and subsequent steps. The onset of curling was early enough to comprise one of the events of the acid-sensitive stage. Infection threads, on the other hand, appeared in the root hairs after the acid-sensitive stage had passed. This was particularly clear in Experiment 7, where infection threads could not be seen

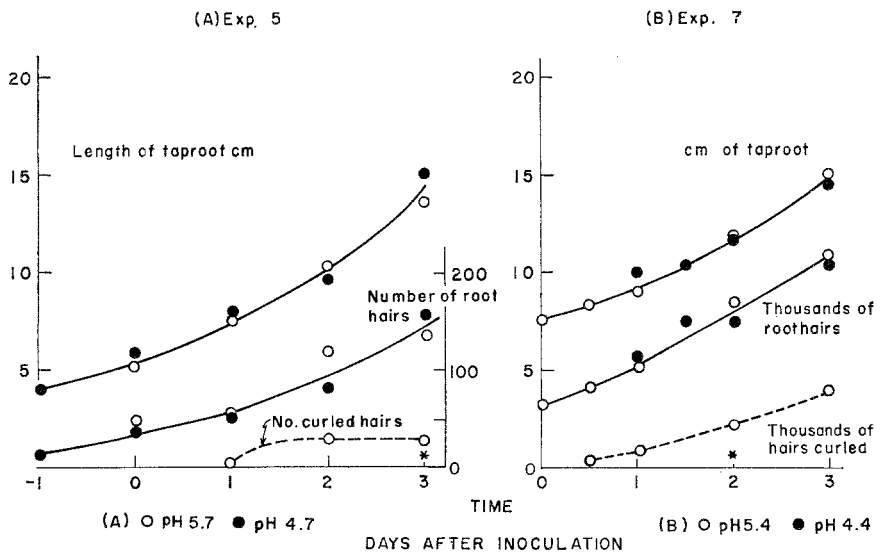

Fig. 5. (Exp. 5, Exp. 7). Growth of taproots, production of root hairs (no. per plant), curling of root hairs, and time of appearance of infection threads. Curling data, and time of appearance of infection threads indicated by *, refer to the higher pH treatments. Each datum in Fig. 5a is a mean from 2 plants subsampled from Exp. 5 (nitrate medium). Each datum in Fig. 5b is a mean from 3 plants subsampled from Exp. 7 (nil-nitrate).

until two days after inoculation (Fig. 5b) although only 24 hours at pH 5.5 was necessary for the subsequent production of 20 nodules per plant (see Table 2 below). (The more numerous root hairs, more extensive curling, and earlier infection in Experiment 7 are probably consequences of the absence of nitrate).

(vii) *Establishment of Rhizobium populations on the root*

Experiment 6 (Fig. 6a, 6b): Aseptic conditions were maintained so that Rhizobium could be directly plate-counted. The experiment was done under artificial light at 20–26°C. The seed was immersed 30 minutes in 0.1% $HgCl_2$, and the rinsed seed was sown onto sterile plates of yeast mannitol agar so that seedlings with microbial contamination could be detected and rejected. The seedlings were transplanted to solution cultures when the roots were only 2 to 3 cm long, to minimize transfer of nutrients from the agar. After four days of growth with daily pH check and adjustment, the roots had lengthened to 7 to 8 cm. Samples of the solutions, and of roots cut off 4 cm below the crown, were then taken from each culture for initial plate counts to ensure that asepsis had been maintained. The cultures were then inoculated with either 5×10^5 or 1×10^8 cells of strain U45 per litre. Further counts and pH-adjustments were made during the course of the experiment. The solution cultures and reagents had been autoclaved 1 hour at 15 lb pressure. Aeration air was filtered, and the electrodes of the pH-meter were rinsed with alcohol and sterile water. Uninoculated cultures remained aseptic. Each triplicate culture initially had 10 plants, in 3 litres of nil-nitrate solution.

Experiment 5 (Fig. 6c): Estimates of numbers of Rhizobium in the rhizosphere were also available from Experiment 5, described previously (Sections (v) (vi)). This experiment was not aseptic, but the inoculum was heavy enough for Rhizobium to outnumber contaminating bacteria by approximately 20 times. Plate counts were made on inoculated and uninoculated cultures, and Rhizobium can be estimated by difference. These estimates serve to support extrapolation from the aseptic, artificially lit conditions of Experiment 6 to glasshouse conditions.

When the inoculum was small (5×10^5 cells/litre) an initial rapid, pH-independent accumulation of bacteria on the roots was followed by a pH-dependent multiplication (Fig. 6a). Thus after 1 to 2 days at pH 5.4, but not at 4.8 or 4.4, the root population developing from the small inoculum had approached the magnitude of the population on heavily inoculated roots (Fig. 6b). The Rhizobium multiplied somewhat in the culture as a whole, possibly because the experiment was done aseptically and no other bacteria were competing for whatever small energy source was available

(In Experiment 1, which was not aseptic, Rhizobium numbers had decreased slightly).

From the large inoculum (10^8/litre, Fig. 6b) a root-population of 10^3 cells per cm was reached by two hours and 10^4 per cm by seven hours regardless of the pH. A relatively slower, smaller increase followed. The data for the glasshouse experiments using nitrate medium were consistent with this (Fig. 6c). The initial development of the rhizosphere population in the first seven hours was too rapid to be accounted for by multiplication with a mean generation time of the order of four hours (as found by Bergersen[2] for shaken cultures); but the later slower phase is compatible with a generation time of this order. Thus the rhizosphere population probably developed as a result of an initial mechanical accumulation followed by some bacterial multiplication at the root surface.

The volume occupied by the bacteria on the roots can be estimated variously, and only approximately, as the 1 μl of water adhering per cm of root, or the 2 to 3 μl root volume per cm, or the 5 μl per cm hollow-cylinder space containing the root hairs. Any of these estimates indicates that the population of Rhizobium

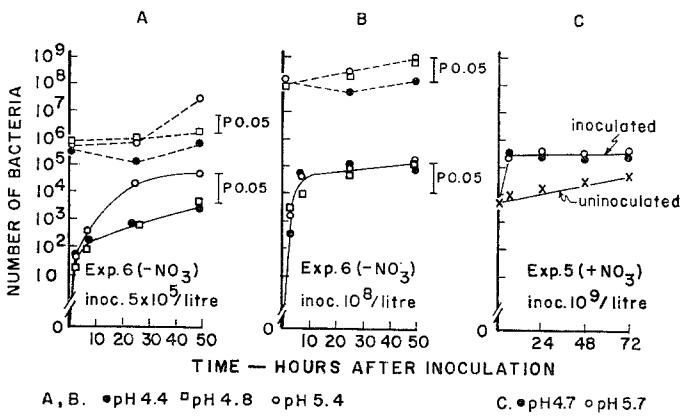

Fig. 6. Accumulation of Rhizobium on roots, in relation to pH and time. Counts on root washings, expressed per cm of root are joined by solid lines, and counts on culture solutions, expressed per litre, by broken lines.

Fig. 6a and 6b represent Exp. 6, done aseptically so that all bacteria counted were Rhizobium.

Fig. 6c represents data from Exp. 5 done in the glasshouse nonaseptically.

in the rhizosphere was 50 to 100 times denser than in the bulk nutrient solution, despite continuous stirring by the aerators.

(viii) *Effect of inoculum size on the length of time required at high pH*

Experiment 7 (Table 2): At pH 4.5 a large rhizosphere population can develop within a few hours following heavy inoculation, but root hairs remain uncurled and uninfected. This indicates that a step following rhizosphere development is acid-sensitive; but it does not indicate whether a large rhizosphere population of Rhizobium is necessary for nodulation. If a large population must develop before the acid-sensitive step can proceed, then the completion of the acid-sensitive step will be delayed if the inoculum is small. Experiment 7 compares the length of time that the pH must be kept at pH 5.4 to produce nodules, following a heavy or a light inoculation. Each triplicate culture had 24 plants in 22-litres of nil-nitrate medium. On day 1 of the experimental period, two days after planting, the cultures were inoculated to provide either 10^9 or 10^4 cells of strain U45 per litre. (Observations of root hair development and curling were made on plants subsampled from the heavily inoculated treatments, and have been presented in section (vi), Fig. 5b). Nodule numbers were pooled from harvests taken 9 and 13 days after inoculation (Table 2).

Given nine days at pH 5.4, inocula of 10^4 or 10^9 cells per litre both yielded about 20 nodules per plant (Table 2). The smaller

TABLE 2

Effects of short-term pH treatments and inoculum size on nodule number and distribution (Expt. 7)

Cultures were held at pH 4.4 except when indicated * in first column. All cultures were inoculated on day 1, 2 days after planting. Nodulation data were pooled from harvests made 9 and 13 days later, and are means of 3 replicates, each having 24 plants.

Treatment	Mean no. of nodules per plant		Mean distance between topmost and lowest nodule on taproot (cm)		Mean distance from crown to topmost taproot nodule (cm)	
Inoculum, cells/litre	10^9	10^4	10^9	10^4	10^9	10^4
pH 5.4 starting day 1:						
for 9–13 days	23.7	20.2	5.1	8.0	5.6	7.4
for 48 hr*	24.7	2.7	5.0	2.7	5.3	6.9
for 24 hr*	20.0	0.3	3.4	—	5.8	—
for 12 hr*	13.0	0.2	2.6	—	6.3	—
pH 5.4 starting day 2:						
for 24 hr*	19.9	0.1	4.4	—	7.7	—
for 12 hr*	12.7	0.0	3.4	—	7.5	—
Control pH 4.4	0.0	0.0	—	—	—	—
Least difference significant, *P*0.05	4.8		1.0		0.5	

inoculum had produced nodules later (in five days rather than four); and the nodules developed further down the taproot and were spread further apart (Table 2). With the large inoculum, a pH of 5.4 had to be provided for only twelve hours to allow an average 13 nodules per plant to develop subsequently at pH 4.4. Doubling the time at pH 5.4 to 24 hours allowed nodulation on a greater length of developing root, and a full complement of about 20 nodules was produced, not greatly exceeded by longer treatments. Delaying the high-pH treatments from day 1 to day 2 did not affect nodule number; it merely delayed the appearance of nodules and shifted their distribution downwards. With the small inoculum, nodulation did not proceed until the pH had been at the high level for 48 hours after inoculation. This delay corresponds reasonably with the time a large Rhizobium population took to develop on the roots from a similarly small inoculum in Experiment 6 (Fig. 6a).

(ix) *Relationship of the acid-sensitive stage to rhizosphere development and curling of root hairs*

Experiment 8 (Fig. 7): The pH was raised from pH 4.5 to pH 5.5 for short intervals ranging from 4 to 24 hours and then returned to 4.5. These short exposures to pH 5.5 followed either of two equally heavy inoculation treatments, applied at different times. In one, the inoculum (10^9 U45/litre) was added as the pH was raised, so that the rhizosphere population would have to develop during the exposure to pH 5.5. In the other, the inoculum was added 16 hours earlier, to allow the rhizosphere to develop at pH 4.5 before the exposure to pH 5.5 began. Each triplicate culture solution had 24 plants in 22 litres of nil-nitrate medium. One plant was subsampled from each culture for microscope observation, at the time the pH was returned to 4.5 to end the exposure to pH 5.5. One more plant was subsampled the following day, also for microscope observation. The results of the microscope observations are in Fig. 7a. Nodules were counted on half the remaining plants in each culture after 7 days and on the rest after ten days, with results which were similar and have been pooled (Fig. 7b).

The two inoculation times were included to test the supposition that a rhizosphere population which accumulates from a heavily inoculated culture at pH 4.5 is competent to induce curling of root hairs and subsequent nodulation; i.e. that the stage of bacterial accumulation in the rhizosphere contains no acid-sensitive events essential to nodulation when the limitation on increase in bacterial numbers is overcome by heavy inoculation. If this supposition is correct, the earlier inoculation should advance the onset of curling

and shorten the minimum exposure to the high pH necessary for nodulation to ensue. The time 'saved' in this way by the earlier inoculation should equal the time needed for the later inoculation to establish a competent rhizosphere population. A second purpose of the experiment was to test indications from earlier experiments that root hairs can continue curling at low pH after a short treatment at pH high enough to initiate curling. A third purpose was to determine whether nodulation remained sensitive to pH after root hairs had curled.

The earlier inoculation made the root hairs curl two hours earlier (Fig. 7a); most of the hairs that curled did so within 6 hours after the pH was raised from 4.5 to 5.5 in the plants inoculated early, but not until 8 hours when the inoculum was added as the pH was raised. Apparently the later inoculation took two hours at pH 5.5 to establish a rhizosphere population as competent to induce curling as the population which had already established from the

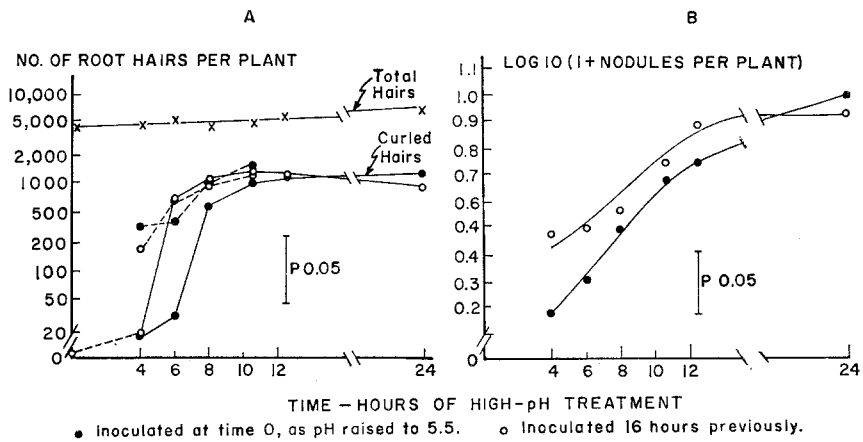

Fig. 7. (Exp. 8). Effects of time of inoculation and duration of treatment at pH 5.5 on (a) number of root hairs curled, and (b) number of nodules produced subsequently. Cultures held at pH 4.5 except during the high pH treatment.

Inoculum, 10^9 U45 per litre, was added either 16 hours before the pH was raised to 5.5, or at the same time as the pH was raised. The root hair data (Fig. 7a) are means of counts on taproots of 3 plants, one sampled randomly from each replicate culture. The broken lines indicate counts on plants sampled the day following high-pH treatment. The nodulation data (Fig. 7b) are means of counts on the remaining plants, pooled from harvests at 7 days and 10 days. (Nitrogen-deficient medium).

earlier inoculation. The early, rapid, mechanical phase of bacterial accumulation would be sufficient for this. To curl some root hairs required only 4 hours at pH 5.5. On plants that got this treatment, more hairs curled after the pH was dropped to 4.5, as shown by observations on plants sampled the following day. Nodule number increased gradually as the exposure to pH 5.5 increased from four to twelve hours (Fig. 7b). This made it impossible to define a minimum exposure necessary for nodulation to ensue. However, the early inoculation did increase the number of nodules developed after the short exposures. The statistical main-effect of the early *vs* late inoculation treatments on nodule number was significant at p 0.01. The interaction between inoculation treatments and duration of exposure to pH 5.5 was not significant. It was difficult to get precise nodulation data from pH treatments as short as these. Nevertheless, it is clear that the number of nodules continued to increase as the exposure to pH 5.5 extended up to twelve hours, *i.e.* nodulation remained sensitive to acidity for a few hours after curling was completed.

DISCUSSION

In these experiments, nodulation began on any part of the root system which bore young root hairs and a sufficient Rhizobium population at the time suitable pH was provided. If suitable pH was maintained indefinitely, initiation of nodules on new roots ceased or slowed after two or three days. This phenomenon appears to be common, and several workers have offered explanatory hypotheses [6 12 15]. The results in this paper do little to test these hypotheses, except to make it clear that plants restrained from nodulating do continuously produce new roots which are inherently capable of nodulating, and that this capability is transitory.

The distribution and number of nodules was further restricted when adequately high pH was provided for only a short interval. Nevertheless, plants treated in this way tended to continue initiating nodule production, even on sections of root produced after the pH had been lowered. This tendency was particularly clear in the final experiment. The data on nodule position together with the root lengths measured during the microscope examinations in the first 24 hours indicated that many plants bore some of their nodules on

roots which could not have existed when the pH was high. Though small, this tendency was erratic enough to vitiate attempts at high precision. The implication that effects of previous treatment are transmitted to new roots tissue is puzzling.

The progress of nodulation into the curling stage had to await the development of a large Rhizobium population in the rhizosphere, and was prevented if the rhizosphere was disturbed. These indications that nodulation requires a large rhizosphere population are consistent with findings of some other workers. For example, Bhaduri [3] showed that nodulation of Phaseolus depended transiently on inoculum size until multiplication obliterated the differences. Purchase and Nutman's [16] evidence that the rhizosphere need contain few virulent Rhizobium to initiate nodulation of lucerne is not contradictory. Their experiments were designed to test the supposition that in a large population, only a single virulent bacterium is needed to produce one infection. Their virulent bacteria were therefore diluted with large numbers of an avirulent strain. This leaves the possibility that a large population is needed to provide the environment at the root which enables the virulent members to infect.

When the bacteria have to multiply extensively from a small inoculum, acidity could conceivably limit nodulation by slowing the development of the rhizosphere population enough to prevent adequate numbers developing at a site before the site becomes obsolete. This might explain benefits to nodulation from heavy inoculation in acid soils.

Nevertheless, even when a large Rhizobium population accumulates rapidly, nodulation remains pH-dependent, perhaps over a slightly lower critical range of pH. In the special case of the heavily inoculated solution culture, dense rhizosphere populations develop quickly, and independently of pH and of extensive multiplication. They are capable of initiating nodulation as soon as a high enough pH is provided, and only then. The requirement for the high pH continues until a short while after the root hairs curl. Subsequently, the pH can be lowered without preventing development of infection threads and growth of the nodule; and Jensen [10] has shown that established nodules on *Medicago sativa* can fix nitrogen at pH 4.5 to 4.8.

How acidity interferes with events of the acid-sensitive stage

remains unclear. Microscope observations might help elucidate this if Fahraeus's [7] slide technique could be coupled with adequate pH-control. In the present experiments, the only readily visible event during the acid-sensitive stage was the curling of root hairs. But acidity only prevented the onset of curling. If curling had begun, the number of curled hairs could continue increasing even if the pH was lowered. Also, in Experiment 8 nodule production remained sensitive to acidity for a few hours after curling had completed. Thus curling appears to be merely contemporary, and only approximately contemporary, with some acid-sensitive event which is necessary for the subsequent production of nodules.

The timing of infection in relation to the other known events in nodulation has not been established, except that it must precede the appearance of an infection thread within the body of the root hair. Perhaps infection and curling are roughly concurrent symptoms of changes in the cell walls of root hairs, induced by products of the rhizosphere population if the pH is high enough. The significance of such products need not be pure supposition. There is evidence that indole-3-acetate and bacterial inducers of polygalacturonase production play a part in the curling and infection of root hairs [11 13 18].

SUMMARY

Acidity reduced nodule numbers at pH below 5.5, and virtually prevented nodulation at pH 4.5. In this range (pH 5.5 to 4.5) it did not affect root growth or the number of root hairs.

In lightly inoculated solution cultures, acidity inhibited the extensive multiplication of Rhizobium which was necessary to establish a rhizosphere population sufficiently large to induce nodulation.

In heavily inoculated solutions, containing 10^8 to 10^9 Rhizobium cells per litre, Rhizobium rapidly accumulated in the rhizosphere by a pH-independent process which seemed to be largely mechanical and to involve little multiplication. Thus large rhizosphere populations accumulated within a few hours even at pH 4.4. Nevertheless, acidity still prevented root hairs from curling and becoming infected. Raising the pH from 4.4 to 5.4 at this stage allowed curling and subsequent steps to proceed without delay.

Shortly after curling was completed, the pH could be lowered to 4.4 without hindering the development of infection and the normal completion of nodulation. Hence the prevention of nodulation by acidity, in heavily inoculated solution cultures, can be attributed to prevention of a step which approximately coincides with the curling of root hairs. This step occupied less than 12 hours of the 4 to 7 days required for visible nodules to appear.

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REFERENCES

1 Albrecht, W. A., Inoculation of legumes as related to soil acidity. J. Amer. Soc. Agron. **25,** 512–522 (1933).
2 Bergersen, F. J., Growth of *Rhizobium* in synthetic media. Australian J. Biol. Sci. **14,** 349–360 (1961).
3 Bhaduri, S. N., Influence of numbers of *Rhizobium* supplied on subsequent nodulation of the legume host plant. Ann. Botany N.S. **15,** 209–217 (1951).
4 Brockwell, J., Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii*. Applied Microbiol. **11,** 377–383 (1963).
5 Dart, P. J. and Mercer, F. V., The legume rhizosphere. Arch Mikrobiol. **47,** 344–378 (1964).
6 Dart, P. J. and Pate, J. S., Effects of delaying inoculation on seedling symbiosis of barrel medic. Australian J. Biol. Sci. **12,** 427–444 (1959).
7 Fahraeus, G., The influence on clover root hairs by nodule bacteria. J. Gen. Microbiol. **16,** 374–381 (1957).
8 Jensen, H. L., Nitrogen fixation in leguminous plants I. Proc. Linn. Soc. New South Wales. **67,** 98–108 (1942).
9 Jensen, H. L., Nitrogen fixation in leguminous plants IV. Proc. Linn. Soc. New South Wales. **68,** 207–220 (1943).
10 Jensen, H. L., Nitrogen fixation in leguminous plants VII. Proc. Linn. Soc. New South Wales. **72,** 265–291 (1947).
11 Kefford, N. P., Brockwell, J. and Zwar, J. A., The symbiotic synthesis of auxin by legumes and nodule bacteria. Australian J. Biol. Sci. **13,** 456–467 (1960).
12 Lim, Gloria, Influence of size of rhizosphere population of nodule bacteria on root-hair infection in clover. Ann. Botany N.S. **27,** 55–67 (1963).
13 Ljunggren, H. and Fåhraeus, G., Role of polygalacturonase in root-hair invasion by nodule bacteria. J. Gen. Microbiol. **26,** 521–528 (1961).
14 Loneragan, J. F. and Dowling, E. J., Interaction of calcium and hydrogen ions in nodulation of subterranean clover. Australian J. Agric. Res. **9,** 464–472 (1958).
15 Nutman, P. S., Studies on the physiology of nodule formation II. The influence of delayed inoculation. Ann. Botany N.S. **13,** 261–269 (1949).
16 Purchase, Hilary F. and Nutman, P. S., Studies on physiology of nodule formation VI. Influence of bacterial numbers in the rhizosphere on nodule initiation. Ann. Botany N.S. **21,** 439–447 (1957).
17 Raggio, M. and Raggio, Nora., Root nodules. Ann. Rev. Pl. Physiol. **13,** 109–128 (1962).
18 Thimann, K. V., The physiology of nodule formation. Trans. Third Comm. Intern. Soc. Soil Sci. Vol. A. pp. 24–28 (1939).