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THE EFFECT OF LOW pH ON DIFFERENT PHASES OF NODULE FORMATION IN PEA PLANTS

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INTRODUCTION

In general leguminous plants, with the exception of those of the cowpea and lupine group, fail to nodulate and grow in acid soils. The limiting pH value for nodulation varies with the plant species and the strain of *Rhizobium* used [4 5 10 11 14]. Plants supplied with combined nitrogen are able to grow at a lower pH value than those deriving their nitrogen from symbiosis with the root-nodule bacteria [10 16]. Little is known of the inhibitory effect of low pH on the nodulation process. Failure to form nodules has been ascribed to the inhibition of bacterial growth at low pH [2 14 15]. So far, however, no evidence has been presented which shows that the growth of nodule bacteria in laboratory media is similar to that in the rhizosphere. Furthermore, it is questionable whether the pH in the rhizosphere or on the root surface is identical with that of the medium in which the root is growing.

In the present investigation an attempt has been made to elucidate the effect of low pH on different phases of nodule formation by exposing the plant roots to nutrient solutions of low pH for different periods of time. At the pH value chosen, good plant growth occurred provided combined nitrogen was supplied, but nodule formation was inhibited. A preliminary account of the results obtained was given at the IX International Congress for Microbiology, Moscow, 1966 [10].

MATERIAL AND METHODS

Seeds of *Pisum sativum* cv. Rondo were surface-sterilized by shaking for 20 minutes in 3% H_2O_2 to which a drop of a detergent (Teepol) had been added. The seeds were incubated for 5 to 7 days on water agar (1%) at 25°C in the dark and then transferred aseptically to tubes containing 180 ml nutrient solution of pH 6.5 of the following composition: K_2HPO_4, 0.36 g; KH_2PO_4, 0.12 g; $MgSO_4.7\,H_2O$, 0.25 g; $CaSO_4$, 0.25 g; Fe (III) citrate, 30 mg; $MnSO_4.4\,H_2O$, 1 mg; $ZnSO_4.7\,H_2O$, 0.25 mg; $CuSO_4.5\,H_2O$, 0.25 mg; H_3BO_3, 0.25 mg; $Na_2MoO_4.2\,H_2O$, 0.05 mg; water 1000 ml.

Two weeks after germination, when the roots were susceptible to infection, the seedlings were transferred to nutrient solutions having different pH values and inoculated with the root-nodule bacteria. Nutrient solutions of different pH values were obtained by addition of dilute H_2SO_4 or NaOH. During the experiment the pH was maintained by adjustment once or twice daily, except in the case of Experiment III where the pH was kept constant by using an automatic Titrator (Radiometer).

The effective *Rhizobium leguminosarum* strain PRE and the ineffective strain P8 were used for inoculation. They were maintained on yeast-mannitol-agar slopes. For inoculation, a 5 days old culture was suspended in 50 ml water and 1 ml of the resulting suspension was added to each jar.

The plants were grown in a greenhouse with supplementary light and heating during the winter period. However, Experiment III, in which the pH was kept constant automatically, was conducted in a light cabinet. The numbers of nodules were usually counted twice during the experiment *viz* 7 days after inoculation and again 3 to 4 weeks after inoculation at the time of harvesting. For fresh-weight determination the nodules were detached, the adhering water removed by blotting with filter paper and immediately weight. For dry-weight determination the plants were first air dried and then at 105°C for 30 minutes. Total nitrogen was determined by the Kjeldahl method.

RESULTS

1 *The effect of combined nitrogen and inoculation with* Rhizobium leguminosarum *on nodulation and plant growth at different pH values*

Experiment I. Pea seedlings were transferred at three different stages (*viz* 6 days before (—6), during (0), and 6 days after (6) inoculation with *Rhizobium leguminosarum* strain PRE) to nutrient solutions of pH 4.5, 5.0, 5.5, 6.0, 7.0 and 8.0 respectively. The plants of the third group had small nodule initials at the time of transfer. Simultaneously, plants grown in the presence of combined nitrogen (100 mg N per liter as $Ca(NO_3)_2$) were also transferred to nutrient

solutions having different pH values. The results are presented in Fig. 1, 2 and 3. They may be summarized as follows:

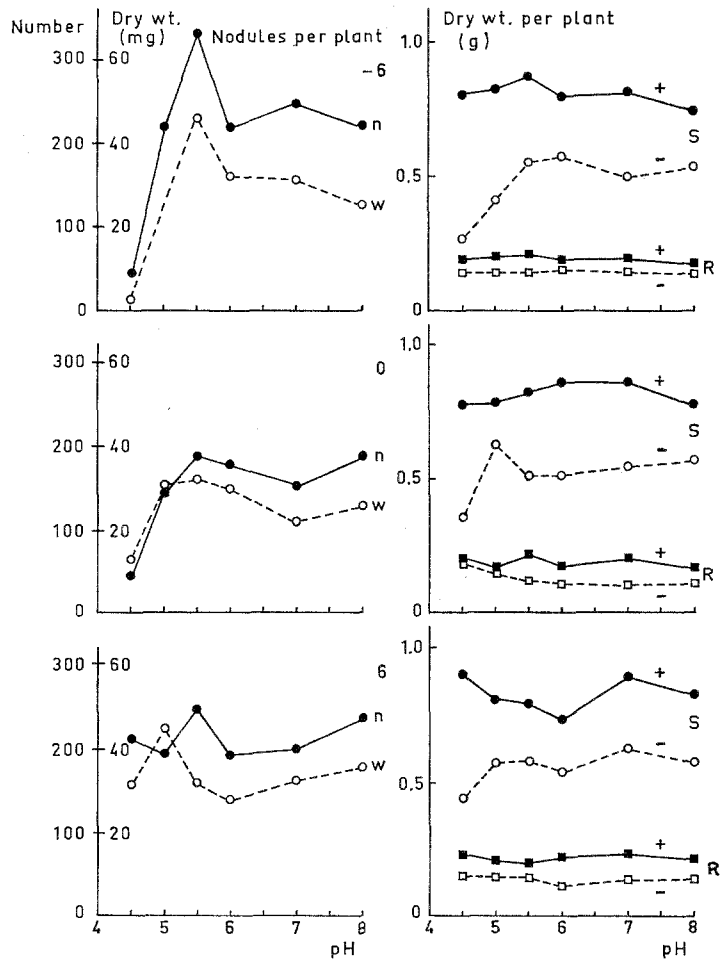


Fig. 1. Effect of pH of the culture solution on nodulation and growth of pea plants when either inoculated with *Rhizobium leguminosarum* strain PRE or supplied with combined nitrogen (100 mg N per liter as $Ca(NO_3)_2$). Plants were grown in a neutral solution and then transferred to solutions of different pH: 6 days before inoculation (−6, *top row*), at time of inoculation (0, *centre row*) and 6 days after inoculation (6, *bottom row*). (n: number of nodules, w: dry weight of nodules from the inoculated plants. S: shoot dry weight. R: root dry weight. +: plants supplied with nitrate, −: plants without nitrate).

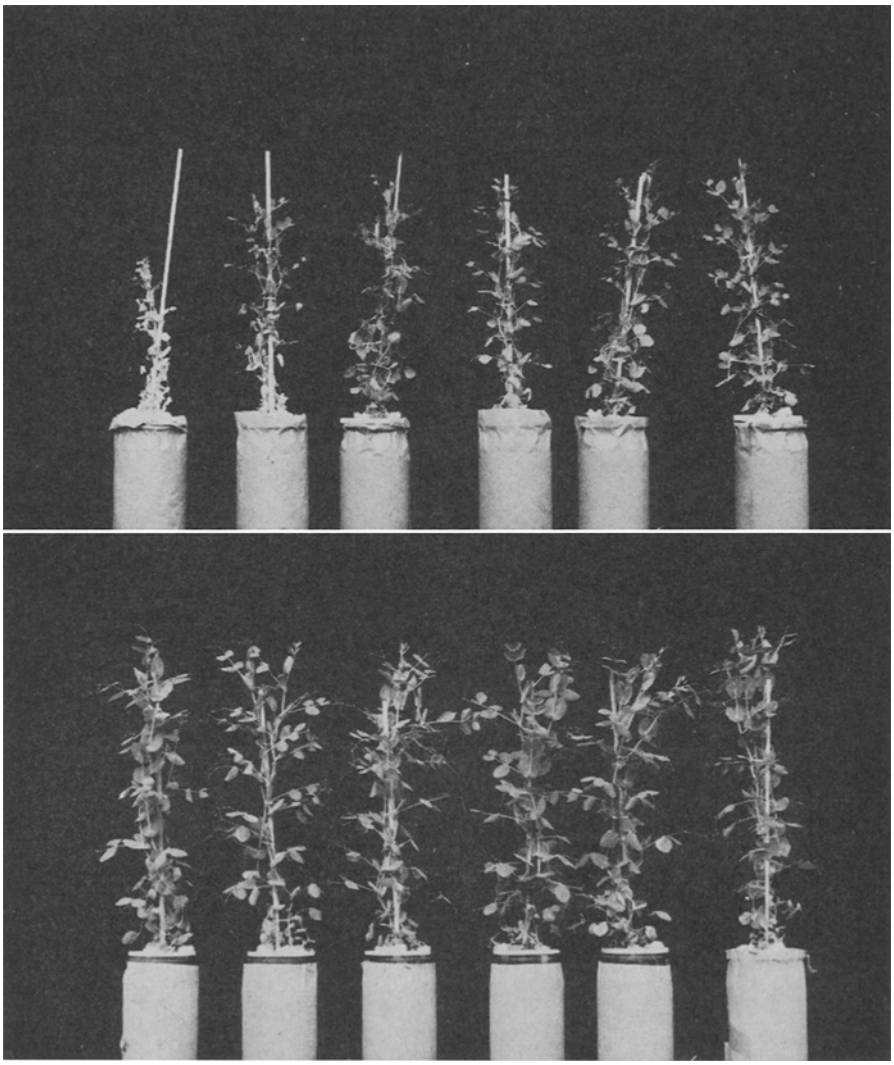


Fig. 2**A** and 2**B.** The effect of pH of the nutrient solution on growth of pea plants. **A** inoculated with the effective *Rhizobium leguminosarum* strain PRE or **B** supplied with combined nitrogen, 100 mg N per liter as $Ca(NO_3)_2$. *From left to right:* pH 4.5, 5.0, 5.5, 6.0, 7.0, 8.0. (Experiment I series 0).

(a) Plants supplied with combined nitrogen grew equally well at all reactions between pH 4.5 and 8.0.
(b) Small differences were observed among the inoculated plants

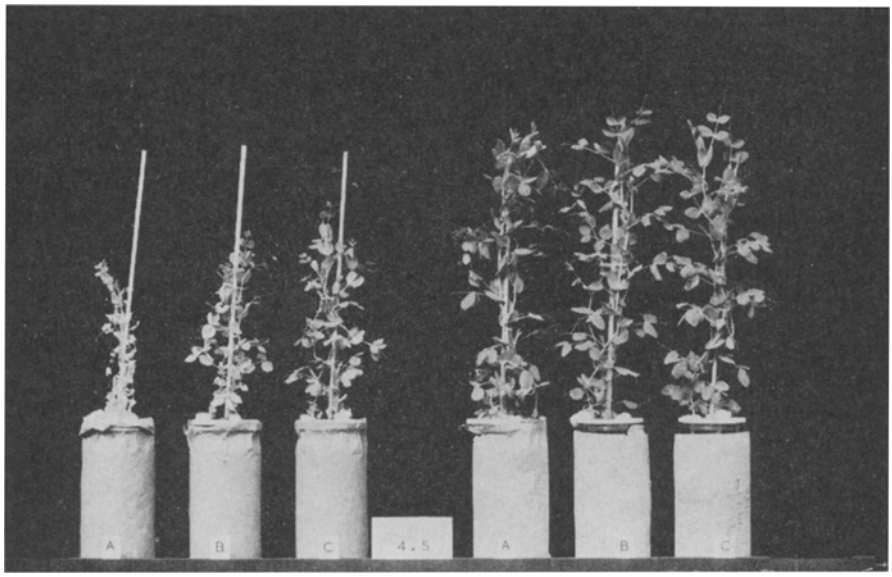


Fig. 3. The effect of low pH of the nutrient solution on different stages of root-nodule formation by pea plants. Plants on the *left* inoculated with *Rhizobium leguminosarum* strain PRE, plants on the *right* supplied with combined nitrogen (100 mg N per liter as $Ca(NO_3)_2$). The plants were grown initially at pH 6.5 and then transferred to a solution of pH 4.5 (A) 6 days before inoculation, (B) at inoculation and (C) 6 days after inoculation (Experiment I).

after growth at different pH values ranging from 5.0–8.0. At pH 4.5 nodulation was markedly inhibited in the case of the plants which had been transferred 6 days before or at the time of inoculation. Inhibition of nodulation was not observed, however, when the transfer was delayed until 6 days after inoculation. Obviously the growth and N-fixing capacity of the nodule were not inhibited by transfer to an acid solution once nodules had been initiated at a neutral reaction. The growth of the shoot was found to be correlated with the development of the nodules but the growth of the roots was very similar in all treatments.

2. *The effect of low pH on different phases of nodulation*

Experiment II. In order to study the effect of acidity on nodulation in more detail, groups of plants were transferred to nutrient

solutions of pH 4.5 at different periods of time (0, 1, 2, 4, 6, and 8 days) after inoculation. Plants grown in a neutral solution served as controls. Two Rhizobium strains, the effective strain PRE and the ineffective strain P8, were tested. The results are shown in Figures 4 and 5, and Plate 1. These results confirm the previous observation (Experiment I) that once nodulation has been initiated, no inhibition of nodule growth and functioning take place.

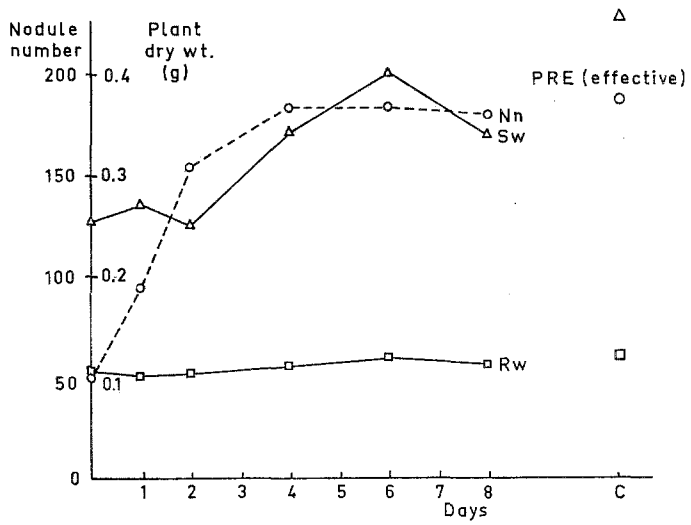


Fig. 4. Effect of low pH of the culture solution on nodulation and growth of pea plants inoculated with the effective *Rhizobium leguminosarum* strain PRE. Plants were kept for different periods of time after inoculation in culture solution of pH 6.5 (as indicated on the horizontal time scale) before being transferred to a solution having a reaction of pH 4.5. (Nn: nodule number; Sw: shoot dry weight; Rw: root dry weight; C: control plants grown for the whole period at pH 6.5).

Plate 1. The effect of low pH of the nutrient solution on different stages of root nodule formation by pea plants inoculated with *Rhizobium leguminosarum* strain PRE. (Experiment II, Fig. 4).

A. From left to right: C, control plants grown at pH 6.5; plants grown initially at pH 6.5 and then transferred to pH 4.5 at 0, 1, 2, 4, 6 and 8 days, respectively, after inoculation.

B. Roots of plants transferred to pH 4.5 at time of inoculation (0).

C. Roots of plants transferred to pH 4.5 four days after inoculation (4).

D. Roots of plants grown for the whole period at pH 6.5 (C).

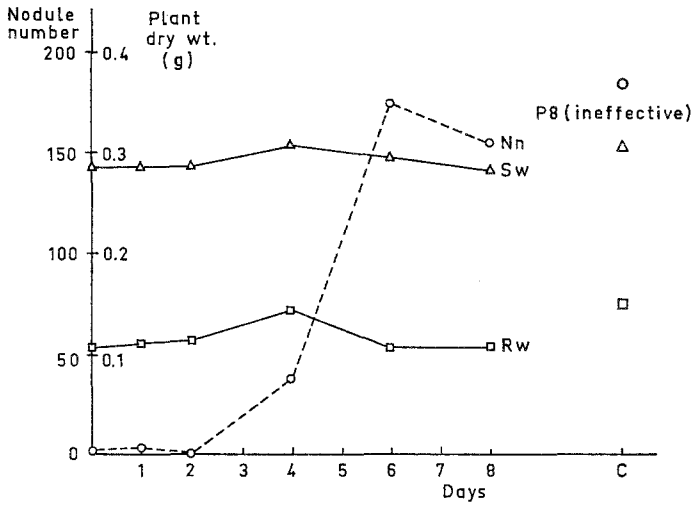


Fig. 5. Effect of low pH of the culture solution on nodulation and growth of pea plants inoculated with the ineffective *Rhizobium leguminosarum* strain P8. Plants were kept for different periods of time after inoculation in culture solution of pH 6.5 (as indicated on the horizontal time scale) before being transferred to a solution of pH 4.5 (Nn: nodule number; Sw: shoot dry weight; Rw: root dry weight; C: control plants grown for the whole period at pH 6.5).

The number of nodules of pea plants inoculated with strain PRE was higher the longer the plants were kept in nutrient solution at neutral reaction before being transferred to the acid solution. No further increase in the number of nodules developed was observed when the transfer to the acid solution was delayed longer than 4 days. Shoot weight did not increase when the transfer took place 1 or 2 days after inoculation but it did increase when transfer took place 4 days or more after inoculation. Since combined nitrogen had not been applied to these plants, the increase in dry weight must have been resulted from nitrogen fixation (see also Fig. 6). Obviously nitrogen fixation takes place in the nodules of pea plants growing in a nutrient solution of pH 4.5 although at a slower rate than in the nodules of plants grown in a nutrient solution of neutral reaction.

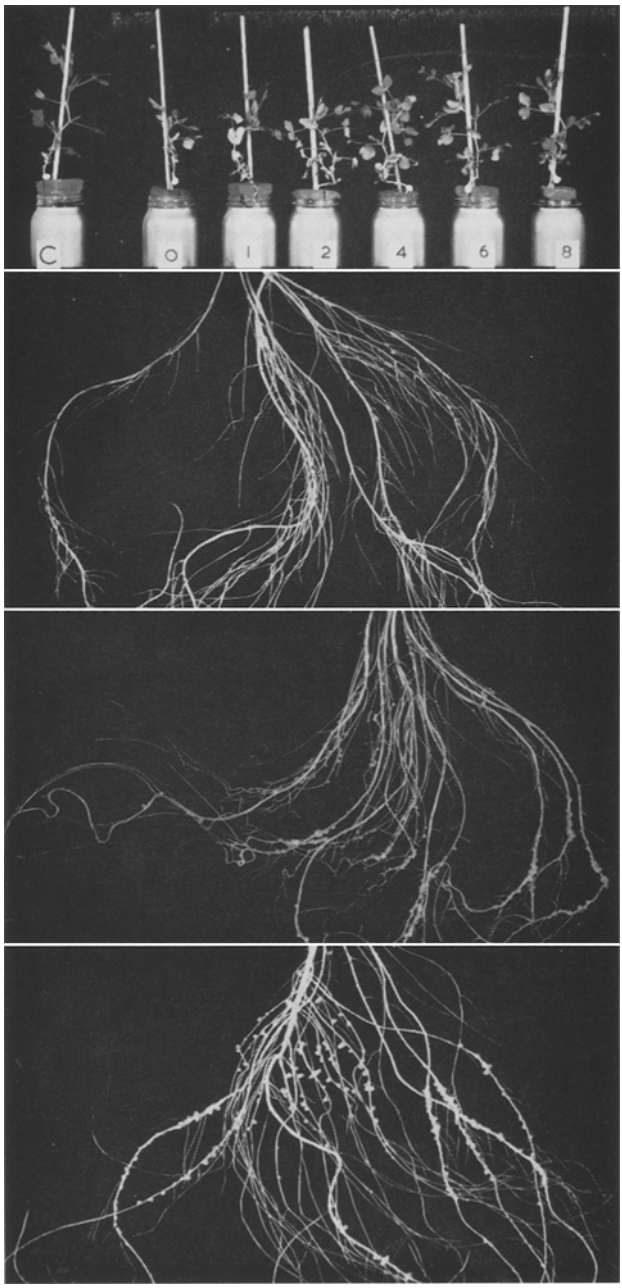
In contrast to the plants inoculated with strain PRE, those inoculated with strain P8 showed no increase in the number of nodules when the time of exposure to low pH was delayed up to 2 days. With increasing interval of time between inoculation and transfer a

gradual increase in nodulation was observed which reached a maximum when the interval was 6 days. Since nitrogen fixation does not take place in pea plants inoculated with the ineffective strain P8, root and shoot growth was the same in all treatments.

Experiment III. The previous experiment, as far as it concerned the effective strain PRE of Rhizobium, was repeated except that an automatic titrator was used to maintain the pH of the nutrient solution constant during the experimental period. This was necessary since the pH of the solution tended to rise even when the pH was adjusted twice daily. The plants were grown in a light cabinet having a day temperature of 22°C and a night temperature of 16°C. Light was supplied by fluorescent tubes (30.000 ergs/cm^2 sec) for 16 hours per day. The relative humidity was 70%. The nutrient solution was aerated vigorously and the utmost care was taken to keep the whole root system immersed in the nutrient solution to prevent the formation of nodules on that part of the root which was not in contact with the acid solution.

The results given in Figure 6 are in general agreement with those of the previous experiment. The number and the weight of nodules increased with increasing time of exposure up to 7 days to the neutral solution. However, the effect on nodulation of an interval of 2 days between inoculation and transfer to a solution of pH 4.5 was less pronounced than that in Experiment 2 (Fig. 4). Shoot weight and the amount of nitrogen fixed increased with increasing amount of nodule tissue. It is of interest to note that the amount of nitrogen fixed per unit of nodule fresh weight was very similar for plants transferred to nutrient solution of pH 4.5 after different intervals of time after inoculation. These values were significantly higher than those for plants grown for the whole period in a nutrient solution of pH 6.5 (Table 1). It may be assumed that under these experimental conditions the nodules of the plants grown in the neutral solution fixed less nitrogen per unit of nodule tissue, because of the large amount of nodule tissue present on these roots.

Experiment IV. In the preceeding experiments nodule initiation was allowed to take place in a neutral solution, but subsequent development had to take place under acid conditions. In these experiments it was observed that the adverse effect of low pH on nodulation was confined to the early stages of this process and was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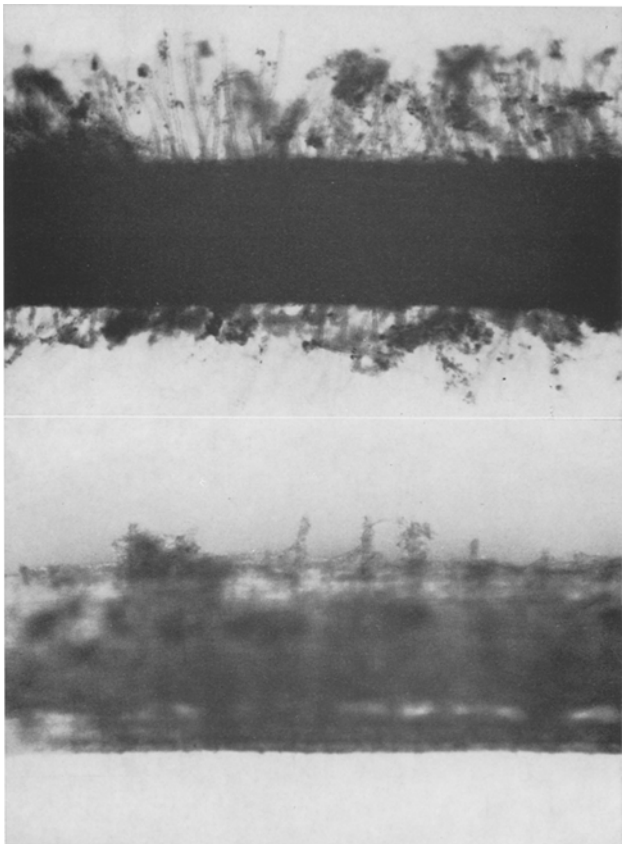

Plate 2. The effect of pH on root-hair formation and the production of a mucous layer. **A**, plants grown at pH 6.5 and **B**, plants grown at pH 4.5. In both cases the plants were inoculated with *Rhizobium leguminosarum* strain PRE (Experiment III).

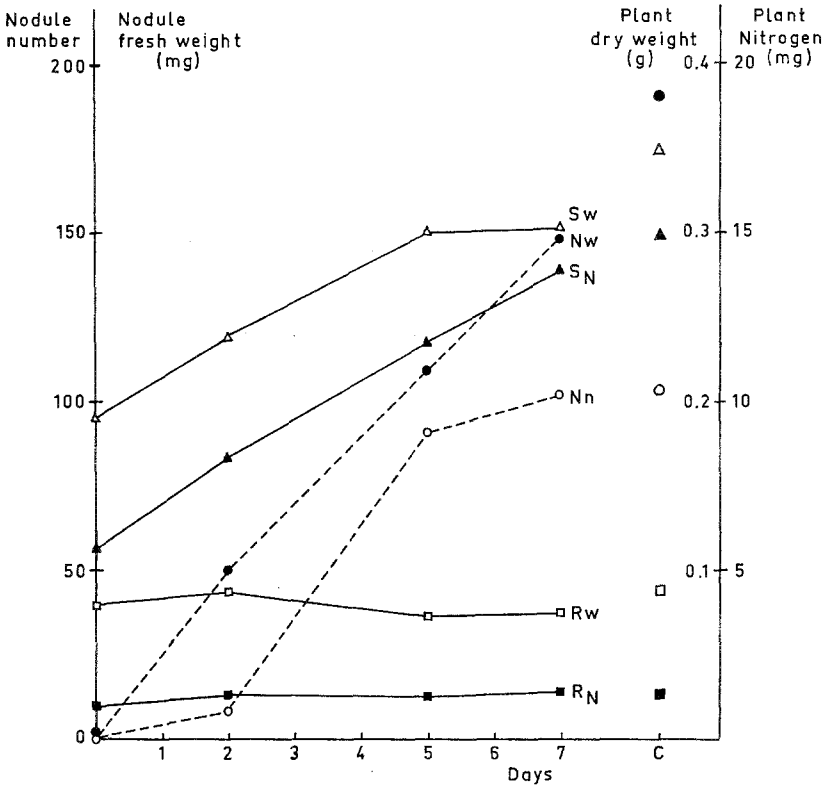

Fig. 6. Effect of low pH of the culture solution on nodulation, nitrogen fixation and growth of pea plants inoculated with the effective *Rhizobium leguminosarum* strain PRE. Plants were kept for different periods of time after inoculation in a solution of pH 6.5 (as indicated on the horizontal time scale) before being transferred to a solution maintained at a reaction of pH 4.5 by an automatic titrator. (Nn: nodule number; Nw: nodule fresh weight; Sw and Rw: dry weight of the shoot and root, respectively; S_N and R_N: total nitrogen of the shoot and root, respectively; C: control plants grown for the whole period at pH 6.5).

limited to a period of 4 to 6 days after inoculation. To determine more precisely which part of the nodulation process was affected by the acidity of the culture medium, plants precultivated for two weeks in a neutral solution were subsequently exposed to the acid solution for a short period varying from 1 to 10 days and then returned to the neutral solution. In series I these treatments started directly after inoculation. Since the nodules developing on the plants

TABLE 1

The effect of exposure to a nutrient solution of low pH, at different times after inoculation with *Rhizobium leguminosarum* strain PRE, on nodulation * and nitrogen fixation * of *Pisum sativum* cv. Rondo

	Plants transferred from a nutrient solution of pH 6.5 to a solution of pH 4.5 at .. days from inoculation				Control plants pH 6.5
	0 days	2 days	5 days	7 days	
Nodule number per plant	0	8.2	90.8	104.6	104.0
Nodule fresh weight per plant (g)	0	0.052	0.118	0.146	0.203
Amount of nitrogen fixed mg N per mg fresh nodule tissue	0	0.656	0.619	0.616	0.438

* Mean values of 10 plants at the time of harvesting.

of series I could have been derived from infections taking place in the neutral solution, after the exposure to the acid solution, a second series of plants (series U) was included to test this hypothesis. The plants of the latter series were treated in the same way as those of series I, except that inoculation was postponed until the plants were returned to the neutral solution. If the nodules formed in series I arose from infections whilst the plants were in the neutral solution, after the exposure to the acid medium, then the plants of series I and U might be expected to have the same nodulation picture. The results from this experiment (Table 2) demonstrate the following points:

(a) In general more nodules developed on plants of series I than on those of series U. This suggests that the nodules formed on plants of series I were not only derived from infections taking place after exposure to the acid solution as was the case with plants in series U. Presumably some of the early stages of nodule formation take place at low pH.

(b) Pea plants exposed for one day to the acid solution had the lowest number of nodules if the plants had been exposed during the second or third day (I_{1-2} and I_{2-3}) after inoculation. Only slight reduction in nodule number was observed when the plants had been exposed at the first (I_{0-1}) or fourth (I_{3-4}) day after inoculation. These results suggest that the first stages of the nodulation process (bacterial multiplication, infection?) are less strongly affected by

TABLE 2

The effect of a short exposure of the roots to nutrient solution of pH 4.5, for varying times, on nodulation of pea plants inoculated with *Rhizobium leguminosarum* strain PRE. Plants of series I were all inoculated at zero time (2 weeks after germination) before transfer to the acid solution. Plants of series U were inoculated after the exposure to low pH, when they were returned to the neutral solution

Period of exposure to acid solution †	Number of nodules *		Period of exposure to acid solution †	Number of nodules *		Period of exposure to acid solution †	Number of nodules *		Period of exposure to acid solution †	Number of nodules *		Period of exposure to acid solution †	Number of nodules *	
	I_0	U_0		I_1	U_1		I_2	U_2		I_3	U_3		I_4	U_4
0– 1	**175**	91												
0– 2	124	87	1– 2	**136**	78									
0– 3	87	—	1– 3	141	—	2– 3	**142**	83						
0– 4	64	77	1– 4	107	77	2– 4	—	81	3– 4	**175**	84			
0– 6	77	98	1– 6	62	66	2– 6	161	67	3– 6	148	73	4– 6	180	—
0–10	59	—	1–10	60	—	2–10	161	—	3–10	—	—	4–10	206	—

Control, 205 nodules per plant, grown for the whole period at pH 6.5.

* Mean values of 6–9 plants.

† 0 is time of inoculation of plants from series I, 1 is 1 day, 2 is 2 days after inoculation etc.

acidity than the processes occurring during the second or third day. In agreement with the preceeding experiments it was found that once nodule initiation had taken place at a neutral reaction, nodule growth and functioning were not inhibited by low pH.

(c) Nodule numbers of plants, transferred to the acid solution directly after inoculation or one day thereafter (I_0 and I_1) decreased with increasing time of exposure to low pH. The decrease was less or absent in plants in which nodule initiation was allowed to take place under neutral conditions for two or more days, before being transferred to the acid solution (series I_2, I_3 and I_4).

3. *The effect of low pH on the nodule bacteria growing in the root medium*

From the above-mentioned experiments it is evident that a relatively short period of contact (4 to 6 days) between the legume roots and the rhizobial cells at a neutral reaction is sufficient to ensure optimal nodulation in pea plants in an acid medium. To investigate whether this effect is due to inhibition of multiplication of the bacteria in the rhizosphere in acid media an experiment was carried out in which the acid nutrient solution (pH 4.5) was inoculated with different numbers of nodule bacteria. The highest number of bac-

teria added was approximately 5.10^7 per ml and the lowest 10^2. However, addition of larger numbers of nodule bacteria did not increase nodulation under acid conditions, showing that poor nodulation in acid nutrient solution is not due to inhibition of multiplication of the rhizobia.

This finding is in agreement with the results of experiments in which rhizobia were grown in exudates of isolated pea roots, which had been grown according to the Raggio technique [13], at pH 6.5 and 4.5 respectively. Although exudates from roots grown at pH 6.5 supported a heavier growth than exudates from roots grown at pH 4.5, there was considerable increase in the number of the bacterial cells in both cases.

The roots of pea plants which were kept, after inoculation, for different periods of time in a neutral solution before being transferred to the acid solution (Experiment III, Fig. 6), were subjected to microscopical examination 12 days after inoculation. It was found that roots which had been growing throughout the experimental period at pH 6.5, were surrounded by a thick mucous layer, consisting almost entirely of bacteria (Plate 2 A). The long root hairs were also covered with this layer. The roots of plants grown in a culture solution of pH 6.5 for 5 days before being transferred to the acid solution, had almost the same appearance as those grown for the whole period at neutrality. However, the roots of plants kept for only two days in the neutral solution before being transferred to the acid medium, had shorter root hairs and only a thin mucous layer. The development of root hairs and the mucous layer were still further reduced in the plants grown for the whole period in the acid nutrient solution (Plate 2 B).

To ascertain whether the rhizobial cells, added to the acid nutrient solution have lost the capacity of inducing root nodules, samples of the culture solution of pH 4.5 were removed at different periods of time after inoculation of the roots, and assayed for the presence of nodule forming bacteria by inoculating pea plants, growing in a neutral culture solution. All the pea plants became well-nodulated indicating that at least some of the bacteria were still alive and capable of inducing root nodules.

DISCUSSION

Pea plants growing in an acid culture solution of pH 4.5 were found to be almost devoid of root nodules in spite of the presence of a large number of nodule bacteria. It was shown that the failure to nodulate under such conditions was due to the inhibitory effect of acidity on the early stages of the nodulation process. Nodule development and nitrogen fixation proceeded at low pH once nodule initiation had taken place in a neutral medium. Total nitrogen fixed by these plants was less, but calculated per unit of nodule tissue (efficiency) was considerably higher than that of plants growing in a neutral culture solution. These results are in partial agreement with those obtained by Jensen [6] for lucerne and subterranean clover; in this case it was demonstrated that nitrogen fixation was possible at a pH too low for nodule formation. In contrast with the results of the present investigation, however, the efficiency of nitrogen fixation was lower in the case of lucerne plants and unaltered in subterranean clover.

The above-mentioned failure of nodulation, associated with the acidity of the culture solution, is apparently different from the findings described in experiments using acid soils [9 15]. In the latter case the poor nodulation was found to be due to the restricted survival of the nodule bacteria and their inability to increase in number in the rhizosphere of leguminous plants. Inoculation of the acid soils with a large number of rhizobial cells, without altering the pH of the soil, resulted in well-nodulated plants.

Inhibition of the growth of the nodule bacteria was not the cause of the failure of nodulation of pea plants growing in acid culture solution. This may be concluded from the following facts:

(a) Addition of much larger numbers of nodule bacteria to the acid nutrient solution did not improve nodulation.

(b) Root exudates from pea roots growing in an acid nutrient solution supported the growth of nodule bacteria.

(c) Acid nutrient solution inoculated with nodule bacteria and in which pea roots failed to form nodules, still contained active nodule bacteria capable of inducing root nodules.

In the present investigation the adverse effect of low pH on nodulation presumably depends on more than one factor in the initial stages of nodulation. The pronounced difference in the appearance

of the root surface of plants grown in acid and neutral nutrient solution, indicate the importance of root hairs and a slime layer in the nodulation process. The roots of pea plants growing in a neutral solution, inoculated with the nodule bacteria were covered with a slime layer or bacterial cells, which resembled the 'rhizosphere' of Dart and Mercer [3]. In the acid nutrient solution only a thin slime layer was present, but the thickness of the layer increased when the roots were kept for increasing periods of time in a neutral solution before being transferred to the acid medium. It is of interest to know whether such a mucous layer protects the roots from the injurious effect of H-ions when the plants are transferred from the acid to the neutral culture solution. If this were to be the case the pH of the root surface would be different from that of the nutrient solution.

Exposure of the roots to the acid solution for selected periods of time revealed that the strongest inhibition of nodulation was excerted on the second day or third day after inoculation. Since inhibition was less pronounced when the plants were exposed to the acid solution for one day starting immediately after inoculation, some stages in nodule formation, subsequent to bacterial multiplication and presumably also subsequent to root-hair infection may also be affected by low pH. Once these stages have been completed no inhibition of nodulation by an acid reaction was observed. It is of interest to know whether the acid-sensitive period coincides with the commencement of cell division in the roots prior to nodule formation. In crown-gall, caused by *Agrobacterium tumefaciens*, a sensitive period immediately after infection has been demonstrated [1]. Such a sensitive period was also detected in alder plants by Quispel [12], who found that an exogenous supply of phosphate, during the period after infection, was indispensable for nodule formation.

The demonstration that nodule formation and nitrogen fixation may take place at low pH, provided that an initial period of neutral conditions is maintained, may be one of the explanations of the successful use of lime-pelleted seeds in acid soils [8].

SUMMARY

A number of experiments was carried out with pea plants growing in a nutrient solution of low pH, inhibitory to nodule formation but not to plant growth when combined nitrogen was available. It has been found that on

transferring plants from a neutral culture solution to an acid solution of pH 4.5 at different periods of time after inoculation, nodulation is reduced when the exposure to low pH takes place during the first days after inoculation, and particularly on the second or third day. Once nodulation has been initiated, nodule growth and nitrogen fixation proceed normally in an acid solution. Failure of nodulation at low pH, under the conditions of these experiments, was not due to the inhibition of bacterial growth. From these results an acid-sensitive period, occurring during the second or third day after inoculation, was demonstrated.

Note added in proof

After finishing this paper, a paper by D. N. Munns appeared in this journal (D. N. Munns, Nodulation of *Medicago sativa* in solution culture I. Acid sensitive steps. Plant and Soil **28**, 129–146 (1968). Munns obtained results using lucerne plants which are in agreement with our findings for pea plants that a short period of exposure, immediately after inoculation, in nutrient solution of neutral reaction, is required to enable nodulation in an acid culture solution. According to Munns, only a short period, even less than 12 hours, is requirred for the curling of the root hairs to take place. When the latter process had been induced, development of the infection thread and the nodule proceeded even at low pH. However, Munns concluded that the roots of plants, exposed for different periods of time to an acid solution and then returned to a neutral solution, only possessed nodules which had developed from infections taking place during exposure to the latter solution. This is at variance with the results recorded in the present paper (Table 2, Experiment IV).

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