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SYMBIOTIC NITROGEN FIXATION IN NON-LEGUMINOUS PLANTS

IV. THE INFLUENCE OF SOME ENVIRONMENTAL CONDITIONS ON DIFFERENT PHASES OF THE NODULATION PROCESS IN ALNUS GLUTINOSA

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INTRODUCTION

The influence of environmental factors on nodulation in Leguminous plants has been studied by many authors. A survey of these studies may be found in the reviews of FRED, BALDWYN and MC COY (1932), WILSON (1940) and NUTMAN (1956). It has been generally observed that nodulation depends on good light conditions, but is inhibited by the presence of ammonium salts or nitrates. Some studies that were devoted to nodulation in the non-leguminous alder gave evidence for comparable effects in this plant (BJÖRKMAN (1942), BOND, FLETCHER and FERGUSON (1954), QUISPEL (1954[2]) and MC CONNELL and BOND (1957)). It is especially the inhibition by nitrogenous substances that has drawn the attention of many investigators and gave rise to some hypothesis to explain this effect. However, when we consider such effects, we have to realize that nodule formation is a complicated process that consists of different phases, e.g. infection of root hairs, penetration of the cells, host reactions leading to nodule formation. It is very probable that these different phases will be differently influenced by the environmental factors, and it will be important to elucidate which of these phases are particularly influenced. Moreover a better analysis may enable us to differentiate further between the phases of the nodulation process. Unfortunately many authors studied the nodulation process as a whole, and only measured the total weight of the nodules per plant, while only some of them clearly differentiated between nodule formation, as measured by the number of nodules per plant, and nodule growth, as measured by volume or weight of the nodules. A better analysis might be obtained by restricting the comparison of different environmental factors to certain well-defined moments in the nodulation process e.g. the moment of the infection or the period of subsequent nodule development. This only is possible if we are able to restrict the infection to a limited period e.g. by washing away all cells of the infecting organism after the infection has been made possible. In leguminous plants this is rather difficult as some cells of *Rhizobium* that remain adhered to the roots are able to develop again and to give new infections. STALDER (1952) tried to overcome this difficulty by performing some control experiments in which the plants were brought together with a suspension of Rhizobium during a very short period so that no infections were possible and then the bacteria were washed away. Though STALDER obtained very interesting results with this method one might still object that the bacteria are more difficultly washed away if they were in contact with the roots during a longer time and had the opportunity to accumulate in the rhizosphere. His remarkable observations that environmental influences after the infection period may restrict nodule formation might therefore be explained by the influence on eventual infections during these later periods.

It was hoped that the danger of subsequent infections was reduced in experiments with a non-leguminous plant like *Alnus glutinosa* as here the endophyte can not develop outside the host plant (QUISPEL 1954[1], 1955). In one of the former articles of this series (1954[2]) it was shown that nodulation in *Alnus* is inhibited by weak light intensities, by the presence of nitrates or ammonium salts and by the absence of phosphate. The intention of the experiments, that are described in this article, was to study at which moment during nodule formation these environmental influences are most effective and how these observations may further the analysis of the nodulation process.

METHODS

The plants were cultivated and inoculated according to the nonsterile method as described in the first article of this series. The plants were grown in a hot-house with a moderate temperature and illuminated during the winter months with fluorescent lamps giving a light intensity of about 3000 Lux at the level of the leaves from 8.00 to 20.00 o'clock.

The following nutrient solutions were used:
with nitrate and phosphate: 0.126 gr KNO_3; 0.295 gr $Ca(NO_3)_2 . 4H_2O$; 0.012 gr $MgSO_4 . 7H_2O$; 0.034 gr KH_2PO_4; per liter dest. water.
without nitrate, with phosphate: 0.093 gr KCl; 0.139 gr $CaCl_2$; 0.012 gr $MgSO_4 . 7H_2O$; 0.034 gr KH_2PO_4 per liter dest. water.
without nitrate, without phosphate: 0.093 gr KCl; 0.139 gr $CaCl_2$; 0.012 gr $MgSO_4 . 7H_2O$ per liter dest. water.

All solutions were provided with 1 ml per liter of a 0.5 % solution of ferric citrate, and adjusted to a pH of 5.4 with HCl or NaOH if necessary. If not mentioned otherwise the solutions were refreshed weekly. Inoculation was performed by mixing the nutrient solution with a suspension of crushed nodules corresponding to approximately 300 mg fresh weight of nodules per jar of 350 ml with three alder plants. During the week of the inoculation the jars were shaken once a day to ensure a thorough contact between infecting organisms and roots. Controls with a double amount of inoculation were used to determine that the inoculation density had not been a limiting factor.

In all experiments the plants were cultivated on the nutrient solution with nitrate and phosphate during the first 5–6 weeks. In some experiments a comparison between the different nutrient solutions was made during the week prior to inoculation. In the tables this week is indicated as week B(before). Then a comparison was made during the week of the inoculation: week I. After this week the plants were thoroughly rinsed with tap water to remove adhering cells of the endophyte. The effect of the environmental conditions on growth was studied by comparing different nutrient solutions or light conditions during the five weeks after the inoculation week (weeks A 1–5). After these weeks the nodules were counted and the fresh weight determined on a torsion balance after removing the adhering water with filter paper.

In the tables the results are expressed as the average number or the average fresh weight per plant with the standard deviation of the average and the number of plants used for the determination. If not all plants had formed nodules the nodule weight was only calculated for those plants that had formed nodules. Tests of significance were performed by Students t-test (FISHER 1948). The levels of significance of the most important differences are added to the tables.

THE INFLUENCE OF THE pH

Though the pH of all fresh solutions was adjusted to the same value of the normal solution 5.4 a drift of pH may occur in the weakly buffered solutions before they are renewed by a fresh adjusted solution after a week. The pH of the cultures was regularly controled and it appeared that the drift of pH was negligible in the solutions without nitrate, while in the solutions with nitrate a drift in alkaline direction was observed after which values of 6.9–7.5 were reached. On solutions with ammoniumsulfate, that were not further used in these experiments, an acid drift occurred to values as low as 3. Before we can consider the results of our experiments we have to know whether these drifts of pH can have influenced and complicated the results of our experiments. In three different experiments some series of plants were cultivated on the nitrogen-free solution during and after inoculation, while the pH of these solutions in the different experimental series was adjusted to values ranging from 3.0–8.5 by addition of HCl or NaOH. In the more acid series the pH remained rather constant but in the alkaline series a drop of the pH was observed. In the experiments that were inoculated 25 IV 50 and 21 VI 57 the pH was only restored to the original value during the weekly renewal of the nutrient solution, but in the experiment of 17 X 57 the pH was adjusted daily by titration with HCl or NaOH. Even then an important daily drift of pH in the alkaline series was observed. Though, therefore, these experiments have to be repeated with nutrient solutions, that are more buffered, some conclusions can be drawn from the results that are summarized in Table I.

It appears that between pH 3.5–6.0 no significant differences in nodule numbers are observed. At pH 3.0 no nodules are formed while above pH 6.0 significantly more nodules are formed. The drift of pH in the alkaline series does not allow us to make any conclusions about

TABLE I

The influence of the pH of the nutrient solution on nodule formation and nodule growth

Experiment 25 IV 50			Experiment 21 VI 57						Experiment 17 X 57					
pH	number of plants	mean number of nodules per plant	pH	pH drift per week	number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg	pH	pH drift per day	number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg
5.4	12	16.3 ± 4.1	3.5	4.5 [1]	11	2.4 ± 0.5	9	14.8 ± 3.4	3.0	3.0	12	0	0	—
7.4	12	33.2 ± 7.1	4.5	5.8 [1]	9	6.0 ± 1.5	9	25.0 ± 5.1	4.0	4.2	12	1.7 ± 0.7	5	5.4 ± 0.9
			5.3	6.7 [1]	12	2.7 ± 0.6	11	19.5 ± 4.7	5.3	5.3	12	2.4 ± 1.1	8	12.1 ± 2.9
			6.3	4.9 [2]	11	5.1 ± 0.9	11	29.1 ± 6.3	6.0	5.6	12	17.1 ± 3.4	12	16.4 ± 3.2
			7.0	6.7	11	13.9 ± 1.7	11	23.5 ± 5.6	6.9	6.0	12	24.7 ± 2.6	12	20.7 ± 2.7
			8.2	7.3	12	12.1 ± 1.7	12	27.7 ± 4.5	7.5	6.9	12	25.0 ± 2.7	12	19.3 ± 2.5
									8.6	6.5 [3]	12	27.3 ± 5.7	12	20.4 ± 3.9

1) drift only observed during the inoculation week.
2) drift not observed during the inoculation week.
3) during the inoculation week the lowest pH was 7.5.

Levels of significance for some differences:

		$P > 0.05$	P 0.05–0.01	$P < 0.01$
Numbers:	exp. 25 IV 50:	5.4–7.4		
	exp. 21 VI 57:			6.3–7.0
	exp. 17 X 57:			5.3–6.0
Weights:	exp. 17 X 57:	4.0–5.3		

the influence of pH above 6.0. Nodule weight is decreased at lower pH values in exp. 17 X 57. At lower pH the nodules are only visible with the naked eye after 4–5 weeks, while at neutral to alkaline pH the first nodules can be seen after 2–3 weeks.

As all experiments were performed at pH 5.4 we have to realize that these experiments obviously were performed at a suboptimal pH. However, this pH has the great advantage that this pH remains rather constant during a week. We have to reckon with the possibility that the well-known inhibiting effect of nitrate will have been counteracted by the alkaline drift of pH on solutions that contain nitrates. It is not advisable to use ammoniumsulfate in experiments with these weakly buffered nutrient solutions when pH values as low as pH 3.0 can be expected.

The influence of the light conditions on nodule formation and nodule growth

In a winter experiment the influence of the light conditions was studied by removing some plants from the place under the fluorescent lamps to a place where the plants did not obtain extra light. This was done either during the week before inoculation, during the inoculation week or during the weeks after inoculation. Before ino-

TABLE II

The influence of the light conditions on nodule formation and nodule growth.

Results of an experiment performed in the winter of 1956.

+: plants illuminated with fluorescent lamps from 8.00–20.00 daily.

—: no extra light given.

Exp. No	light conditions during the weeks: B	I	A1–5	number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg
1	+	+	+	12	16.8 ± 3.9	12	65.6 ± 16.3
2	—	—	—	11	1.9 ± 0.6	10	5.3 ± 1.1
3	—	—	+	12	6.6 ± 1.2	12	24.3 ± 4.4
4	+	—	—	12	3.9 ± 0.8	11	6.8 ± 1.2
5	+	+	—	12	9.5 ± 1.4	12	10.4 ± 2.1
6	+	—	+	12	6.3 ± 0.9	12	43.5 ± 11.7
7	—	+	—	12	6.1 ± 0.8	12	7.7 ± 4.5
8	—	+	+	12	11.3 ± 2.0	12	60.0 ± 14.8

Levels of significance for some differences:

	P > 0.05	P 0.05–0.01	P < 0.01
Numbers	1–5	1–6	2–3
	1–8	5–7	1–2
	2–4	7–8	2–7
	3–8		4–5
	4–6		
Weights:		1–3	1–5
			2–3
			4–6
			7–8

culation the plants were cultivated on the solution with nitrogen, during and after inoculation they were cultivated on the solution without nitrogen, but with phosphate. The design of the experiment and the results are given in Table II.

The light conditions during the week B before inoculation have only a small influence on the number of nodules (compare No 1–8, 2–4, 3–6, 5–7).On the other hand the number of nodules is markedly influenced by the light intensity during the inoculation period: the infection is favoured by good light conditions for the leaves (compare No 1–6, 2–7, 3–8, 4–5). It was expected that the light conditions during the weeks after inoculation would only influence the growth of the nodules if the infection had occurred in the same conditions. This, however, is not the case: even after the inoculation the number of nodules is dependent on the light conditions of the leaves. (compare 1–5, 2–3, 4–6, 7–8). Similar after-effects of the environmental conditions were described by STALDER for peas.

These after-effects of the light conditions on nodule initiation can be explained in two ways:

1. we can assume that even after the infecting cells have been washed away some cells still remain adhered to the roots that can infect the roots during the subsequent weeks. These later infections certainly will be influenced by the then prevailing light conditions.
2. we can assume that the chances of the infections to develop as nodules depend on the environmental conditions after the infection has occurred. These environmental conditions will determine which of the infections will develop as nodules. The experiment of Table II does not allow us to decide on the probability of these two hypothesis.

The influence of the light conditions on growth of the nodules is far more simple. In all series, where extra light was given after inoculation, the nodule weight per plant is significantly greater than in those plants where no extra light was given during the growth period of the nodules. The small influence of the light conditions during the preceding weeks on nodule growth can be easily explained as the development of the plants is inhibited by a period of weak light. Of course we need not be astonished that the growth of nodules is dependent on the light conditions of the leaves.

THE INFLUENCE OF NITRATES ON NODULE FORMATION AND NODULE GROWTH

The well-known inhibiting effect of nitrates and ammoniumsalts on nodulation is more interesting than the obvious influence of the light conditions. In a previous experiment (QUISPEL 1954[2]) it was found that nodule formation in alder is more inhibited by ammoniumsalts than by nitrates. This different influence was imputed to secundary effects and certainly the different drifts of pH will be mainly responsible for the greater inhibition by ammoniumsalts. In the experiments to be described below only nitrates were used. In the nitrogen-free solution the nitrates of potassium and calcium were replaced by equivalent amounts of their chlorides. No experiments were performed to consider the eventual effect of chloride on nodulation. As chloride ions have no effect on nodulation in leguminous plants (DIENER 1950) it is most obvious that the observed effects are caused by the presence or absence of nitrates.

The experimental design and the results of two experiments are given in Table III.

In the experiment that was inoculated 27 IX 54 the number of nodules is reduced by the presence of nitrates during the inoculation week (compare No 1–4, 2–3, 5–6). The presence or absence of nitrate during the week before inoculation has no effect in itself (compare No 1–7, 2–5), but may increase the effect of the absence of nitrate during inoculation (compare No 3–6). This might be explained by a further reduction of the internal amount of nitrogen at the moment of the infection. Here again we observe a decrease of the number of nodules if nitrate is given after the inoculation week (compare No 1–3 and to a smaller extent and insignificant in 2–4, though not in 6–7). Here, too, we can not conclude what the most probable explanation for this after-effect can be. There are some indications that one week of nitrogen deficiency is not sufficient to initiate the maximum number of nodules, the greatest number being found in all series with at least two subsequent weeks of nitrogen deficiency (No 1, 6 and 7) The experiment that was inoculated 19 XII 56 had quite another result as no significant influence of nitrate on the number of nodules was observed. Both experiments are representative for other experiments not mentioned here. The nitrate concentration that was used in these experiments is rather low. MC CONNELL and BOND (1957) only obtained a definite inhibition of nodulation (as measured by nodule weight) at higher concentrations and suggested that in my former experiment the lowering of pH might have been a complicating factor. This certainly applies to experiments with ammonium-salts, but with nitrate the drift of pH might even stimulate nodulation. It is more probable that the nitrate concentration in my experiments is at the border of the inhibitory concentration and that the occurrence of inhibition depends on other physiological conditions. Therefore the experiments with nitrate must be repeated with higher concentrations, but even now show that nitrate may inhibit nodulation if it is present during the inoculation period.

It is very remarkable, indeed, that the effect of nitrate on nodule growth is more evident in the experiment 19 XII 56, where it did not influence nodule numbers, though in experiment 27 IX 54 the same trend is observed. Growth of the nodules is markedly decreased by nitrate during the weeks after inoculation. A more earlier deficiency of nitrate may inhibit nodule growth as a consequence of the smaller development of the plants (No 5, 6 and 7).

THE INFLUENCE OF PHOSPHATE ON NODULE FORMATION AND NODULE GROWTH

In the second article of this series it was shown that omission of

TABLE III

The influence of nitrate on nodule formation and nodule growth

+ : presence of 3.75 milliequiv. NO_3 per liter nutrient solution
— : absence of nitrates

Exp. No.	Presence or absence of NO_3 during the weeks: B	I	A1–5	Experiment 27 IX 54: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg	Experiment 19 XII 56: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg
1	+	—	—	12	44.8 ± 9.5	12	59.1 ± 9.0	12	35.0 ± 7.2	12	30.6 ± 4.9
2	+	+	+	12	9.2 ± 2.2	9	43.9 ± 7.8	12	35.4 ± 5.1	12	4.6 ± 1.2
3	+	—	+	12	18.3 ± 3.9	12	30.6 ± 8.7	10	38.8 ± 5.1	10	5.3 ± 1.4
4	+	+	—	12	4.9 ± 0.4	12	61.0 ± 12.4	11	26.6 ± 6.0	11	37.0 ± 5.6
5	—	+	+	12	10.1 ± 1.8	12	27.8 ± 6.8				
6	—	—	+	12	55.7 ± 6.8	12	20.9 ± 4.2				
7	—	—	—	12	48.8 ± 3.4	12	26.6 ± 1.9				

Levels of significance for some differences:

	$P > 0.05$	P 0.05–0.01	$P < 0.01$
Numbers: exp. 27 IX 54	2–3 2–4	1–3	1–2 1–4 2–6 3–6 5–6
Weights: exp. 27 IX 54	2–4	1–3 3–4	
exp. 19 XII 56			1–2 1–3 2–4 3–4

KH_2PO_4 inhibited nodulation. Some experiments were performed to investigate at which moment of the nodulation process this effect is most noticeable. As the nutrient solution already contained a relatively important concentration of KCl the omission of KH_2PO_4 was not compensated by an equivalent addition of KCl. As there are no indications that nodulation is influenced by the amount of potassium (DIENER 1950), while the influence of phosphate has been generally observed we may safely assume that the effects that were observed are effects of phosphate, though of course this assumption has to be proved by future experiments.

The design and the results of three experiments are given in Table IV.

The results of the three experiments are very identical. It appears that the presence or absence of KH_2PO_4 during or before the inoculation week does not influence the number of nodules (compare No 1–3, 2–4, 2–5, 3–7, 4–6), even the influence of two weeks phosphate deficiency is insignificant (compare No 1–7, 4–5). However, the absence of KH_2PO_4 definitely reduces the number of nodules if it is absent during the weeks after inoculation. This unexpected result was found in all three experiments (compare No 1–4, 2–3). In this case we can decide between the two hypothesis that were postulated to explain the effect of environmental influences after the infection week on nodule initiation. As phosphate has no measurable effect during or before inoculation it is not probable that it would influence infections that eventually might occur after the infection week. We therefore have to conclude that the absence of phosphate limits the possibilities for further development of the infections on the roots. This might be caused by decreased possibilities for growth or by a more specific reaction. Indeed it was observed that growth was decreased by the absence of the phosphate while there was a definite relation between the number of nodules and the nodule weight per plant. This might indicate that an inhibition of growth causes an inhibition of nodule initiation or that reversibly the total weight of nodules depends on the number of nodules that participate in this total weight, or that accidentally both nodule initiation and nodule growth are influenced in the same way by KH_2PO_4.

This problem may be solved by experiments in which the phosphate was only omitted during the first week after the inoculation week when no visible growth was yet observed. The design of two experiments and the results are given in Table V.

It appears that omission of KH_2PO_4 during the week immediately following the inoculation week inhibits nodulation in exactly the same way as if the phosphate was omitted during the whole growth period. Omission of the phosphate during the second week after infection has no significant effect whereby we have to consider that in this experiment by unknown causes the nodule development was extremely slow. We therefore may conclude that lack of KH_2PO_4 in the nutrient solution only inhibits nodulation during the period that immediately follows the inoculation period! Apparently during this period the nodule development demands phosphate to a special degree. Absence

TABLE IV

The influence of phosphate on nodule formation and nodule growth

+: KH_2PO_4 present in the nutrient solution
—: KH_2PO_4 absent from the nutrient solution

Nitrate present during week B, absent during weeks I and A

Exp. No.	Presence or absence of KH_2PO_4 during the weeks: B	I	A1–5	Experiment 27 IX 54: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg	Experiment 26 I 56: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg	Experiment 16 V 56: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant in mg
1	+	+	+	12	44.8 ± 9.5	12	59.1 ± 9.0	12	8.3 ± 1.7	12	24.3 ± 7.0	12	10.2 ± 1.8	12	15.1 ± 4.0
2	+	—	—	12	17.9 ± 5.1	12	26.0 ± 6.3	12	1.9 ± 0.3	11	14.7 ± 2.7				
3	+	—	+	10	35.0 ± 5.6	10	35.8 ± 10.0	12	4.9 ± 2.1	11	18.6 ± 5.5	12	10.7 ± 1.1	12	7.2 ± 0.9
4	+	+	—	12	18.5 ± 2.9	12	21.1 ± 2.9	12	2.0 ± 0.5	10	10.4 ± 1.8	12	6.5 ± 1.4	12	6.7 ± 2.7
5	—	—	—					12	1.4 ± 0.4	8	8.9 ± 3.8				
6	—	+	—					12	3.3 ± 0.3	11	17.0 ± 3.0				
7	—	—	+					12	4.7 ± 0.6	12	14.6 ± 2.3				

Levels of significance for some differences:

		P > 0.05	P 0.05–0.01	P < 0.01
Numbers:	exp. 27 IX 54:		1–2 1–4	
	exp. 26 I 56:	1–3 1–7 3–4	1–6 5–6	1–2 1–4 1–5 5–7
	exp. 16 V 56:	1–4		
Weights:	exp. 27 IX 54:		1–2	1–4
	exp. 26 I 56:	1–2 1–4 1–5		
	exp 16 V 56:	1–3 1–4		

TABLE V

The influence of phosphate after inoculation on nodule formation and nodule growth.

+: KH_2PO_4 present in the nutrient solution
—: KH_2PO_4 absent from the nutrient solution

During the weeks I and A 1–5 no nitrates were given.

Exp. No.	Presence or absence of KH_2PO_4 during the weeks: I	A_1	A_2	A_{3-5}	Experiment 16 V 56: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant	Experiment 13 IV 57: number of plants	mean number of nodules per plant	number of plants with nodules	mean weight of nodules per plant
1	+	+	+	+	12	10.2 ± 1.8	12	15.1 ± 4.0	12	6.1 ± 1.3	12	6.8 ± 3.6
2	+	—	+	+	12	6.5 ± 0.7	12	5.6 ± 0.9	12	0.8 ± 0.3	5	7.2 ± 2.6
3	+	—	—	—	12	6.5 ± 1.4	12	6.7 ± 2.7	12	1.9 ± 0.5	8	4.1 ± 2.3
4	—	—	—	—	12	2.8 ± 0.8	7	2.9 ± 0.8	—	—	—	—
5	—	+	+	+	12	10.7 ± 1.1	12	7.2 ± 0.9	—	—	—	—
6	+	+	—	+	—	—	—	—	12	3.3 ± 0.7	12	5.8 ± 1.4
7	+	+	—	—	—	—	—	—	12	3.3 ± 0.5	11	7.3 ± 1.2

Levels of significance for some differences:

	$P > 0.05$	P 0.05–0.01	$P < 0.05$
Numbers: exp. 16 V 56:	1–2 1–3	2–4 3–4	1–4 4–5
exp. 13 IV 57:	1–6 1–7		1–2 1–3
Weights: exp. 16 V 56:	3–4 1–3	1–2 4–5	1–4

of the phosphate during the later weeks does not effect the number of nodules or even the nodule weight (compare No 1–7, 6–7). This shows that the presence or absence of KH_2PO_4 in the nutrient solution does not influence the growth of the nodules in a way that might explain the inhibition of nodule numbers. The effect of the phosphate in former experiments was most probably of a secundary nature and was a result of the smaller number of nodules that could participate in the determination of nodule weight. Though certainly phosphate will be required for the growth of the nodules the demand of phosphate then can be supplied by the phosphate that already is present in the roots. The remarkable effect of the phosphate during the week after inoculation must be explained by the special requirements of a phase of nodule development that occurs shortly after inoculation.

Experiments are in progress to investigate whether the after-effects of other environmental factors on nodule numbers are especially observed during the first week after inoculation. The first indications show that for nitrate this indeed is the case.

Discussion

When the experiments were started it was suggested that all environmental factors that influence the infection process would be especially active during the week of the inoculation or shortly before inoculation, while those factors that influence the subsequent growth phase would be especially effective during the weeks after inoculation. As to the latter assumption the experimental results are according to expectation: the inhibiting effect of weak light conditions or the presence of nitrates on nodule growth is especially evident if these influences are exerted during the weeks after inoculation, while the analysis of the KH_2PO_4 effect questioned the direct importance of external phosphate for nodule growth. The number of nodules was influenced by the light conditions and by the presence of nitrates during or shortly before the inoculation period, while lack of phosphate had no effect if it occurred during these weeks. Contrary to expectation it was observed that the factors light and nitrate influenced the number of nodules after the inoculation week, while lack of KH_2PO_4 inhibited nodulation only if it was absent during the week immediately following the inoculation week. STALDER concluded that the growth possibilities might limit the number of nodules. This conclusion only is warranted if we are certain that the effects are not caused by an influence on subsequent infections that might occur after the inoculation week notwithstanding our experimental precautions. As to the phosphate effect, however, we may safely assume that no subsequent infections were influenced as the normal infections during the inoculation week were not influenced by lack of phosphate. A closer analysis made probable that the lack of KH_2PO_4 did not effect the number of nodules by an influence on their growth, but that a more specific phase of nodule development was concerned.

How can we insert these conclusions into the general theories on the phases of the nodulation process? According to NUTMAN (1948) the infection of leguminous plants by Rhizobium would only be possible at certain preformed foci. In the second article of this series (QUISPEL 1954[2]) it was shown that the relation between inoculation density and nodule numbers in alder was in accordance with theoretical conclusions based on NUTMAN's theory. The infection itself was considered as a chance contact between the infecting cells and the postulated foci according to the chance distribution of POISSON. As in these experiments the infecting cells only were present during the inoculation week this contact must have occurred during this period. The possible role of chemotactic attractions was not considered, though we have to reckon with this possibility. It now appears that the mere contact between foci and infecting cells as occurring during the infection week is not sufficient for nodule development as the environmental conditions after this contact has been established may determine which infections develop into nodules. The fact that the absence of KH_2PO_4 in the nutrient solution only inhibits nodule formation during the week immediately after the infection week shows that during this period the fate of the developing nodule is decided. We may call this phase of nodule development the phase of nodule initiation. We then might consider the following phases in the proces of nodule formation:

1. formation of foci by the roots of the plants.
2. chance contact between infecting cells and these preformed foci.
3. nodule initiation.
4. growth of the nodules.

About the nature of this initiation phase nothing can be said with certainty. This only will be possible if we are better informed about the nature of the other phases. The first penetration of the roots only is possible at the root hairs as was first shown for leguminous plants and observed in alder by POMMER (1956). The postulated foci of NUTMAN certainly are of a more restricted occurrence and are in some way related to the root initials. Moreover there is some evidence that the penetration depends on the presence of polyploid cells (WIPF and COOPER 1940, BONNIER 1954). It is most appropriate to consider the phase of nodule initiation as the period that the contact has been made, the infection thread enters the root hair and the struggle between infecting cells and the host reactions has started. However, a better insight into the complicated nodulation process will only be possible after more experiments and observations.

SUMMARY

1. The influence of external circumstances on nodulation in alder is studied by restricting the comparison of different environmental circumstances to certain periods of the nodulation process.
2. The number of nodules depends on the light conditions and the presence of nitrates during or shortly before the inoculation period, but even after this period these environmental factors may inhibit the formation of nodules.
3. The growth of the nodules especially depends on the environmental conditions (light, nitrate) after the infection has occurred.

4. The absence of KH_2PO_4 in the nutrient solution has no effect during or before inoculation, but inhibits nodule formation during the week immediately following the inoculation period. The absence of phosphate during the later weeks does not influence either the number of nodules or their growth.
5. It is suggested that the number of nodules not only depends on the infection of preformed foci but that during a subsequent phase of nodule initiation it is decided which of the infected foci will develop into nodules.

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