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SYMBIOTIC NITROGEN FIXATION IN NON-LEGUMINOUS PLANTS

II. THE INFLUENCE OF THE INOCULATION DENSITY AND EXTERNAL FACTORS ON THE NODULATION OF ALNUS GLUTINOSA AND ITS IMPORTANCE TO OUR UNDERSTANDING OF THE MECHANISM OF THE INFECTION

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CONTENTS

I. INTRODUCTION

The formation of a root symbiosis is a complicated process, the details of which are only partially understood. We do possess many data on the influence of external factors on the nodulation of Leguminous plants, while many observations were described regarding the formation of mycorrhiza at different environmental circumstances. It is difficult however to get an impression of the way in which these external circumstances influence the formation of the symbiosis.

E.g. we do know that the nodulation of Leguminous plants is inhibited by the presence of nitrogenous substances in the medium or by a decrease of the light intensity, while other factors like fosfate concentration or the presence of organic substances play a role as well. We refer to the compilations by FRED, BALDWIN and McCOY (1932) and WILSON (1940). We dont know, however, why these factors influence the nodulation. The oldest hypothesis, that these external factors influence the concentration of sugars in the roots appeared to be too simplified. According to WILSON (1939) the C/N ratio might be the determining factor for the formation of nodules, each deviation in either direction from the ideal C/N ratio resulting in a decreased nodulation. Certainly this theory can describe all observations but an explanation of the observations is not possible as long as we do not know which of the carboniferous and nitrogenous substances are especially involved and why nodulation depends on their ratio.

One has to realize that internal factors are also of importance. It is well known that the different strains of *Rhizobium* not only differ as to their efficiency but as to their nodulating capacity as well: inefficient strains as a rule form more nodules though smaller ones than efficient strains. On the other hand NUTMAN (1949) draws attention to the genetic factors of the plants themselves which influence their nodulation. From different kinds of observations he draws the conclusion that the infection does not occur at random on the root surface but is only possible on certain preformed foci which are in some way related to the root initials (NUTMAN 1948, 1949, 1952, 1953).

As NUTMAN (1948) observed a correlation between the number of nodules formed and the initiation of lateral roots in a number of varieties of red clover it might be supposed that the genes which govern the formation of nodules primarily determine the potential number of foci. Other possibilities are that these genes might determine the concentration of food substances which are necessary for the development of nodules. Both the number of foci and the concentration of food substances may in their turn be influenced by the external circumstances.

Moreover the situation may be complicated by the possibility that the roots excrete chemotactic substances which attract the bacteria as the eventual production of such chemotactic substances might be influenced by the internal and external factors as well.

Therefore, when trying to survey the complex relationships which may exist between nodulation, internal and external factors we can classify the theoretically possible mechanisms of the influence of the external factors on the nodule production under the following four categories:

1. they might influence the plants either by changing the concentration of food substances (or their mutual ratio) or by changing the number of foci so that the roots become more or less liable to a subsequently occurring infection.
2. they might influence the infective capacity of the bacteria.

3. they might influence the process of the infection itself e.g. by influencing the production of chemotactic substances.
4. they might influence the growth of the infected foci into visible nodules.

It is no easy task to distinguish between these four categories of possibilities. One can only try to separate the first three categories which all bear directly upon the infection or the preparation of the infection from the fourth possibility which applies to the processes after an infection has occurred. This may be done by counting the number of nodules and not confining oneself to measuring the weight of the total quantity of nodules. However we are not justified in supposing that the number of nodules is not influenced at all by the subsequent growth after the infection has occurred. This is demonstrated by the extreme possibility that after a successful infection no growth at all might be possible.

As will be shown below an analysis of the relation between nodulation and inoculation density might be able to elucidate some of the difficulties described above. This relation, however, has never been analyzed in detail. The few publications dealing with the influence of the inoculation density and the number of nodules clearly demonstrate the cause of this gap in our knowledge (THORNTON 1929, BHADURI 1951). As the Rhizobiums are able to multiply in the vicinity of the roots the inoculation density can not be controled: after some days the number of bacteria in the slightly inoculated experiments is as numerous as in the heavily inoculated ones.

It seemed justified to suppose that better results might be obtained with *Alnus glutinosa* as it has been shown that the endophyte which is responsible for the formation of the nodules in *Alnus* cannot multiply outside the host plant in the usual culture media (KREBBER 1943, BOUWENS 1943, QUISPEL 1954). Even in root extracts, root homogenates and root exudations no growth was observed. If no multiplication of the endophyte in the vicinity of the roots is to be expected the inoculation density can be controled and its effect on nodulation studied.

The main reason why I started this analysis, however, was of another kind. In the first publication of this series (QUISPEL 1954) I stated that it would be very important to have a method of determining in a sensitive way the amount of the endophyte in a suspension or in an inoculated nutrient medium. Such a method might be based upon the relation between the amount of the endophyte in a solution and the number of nodules which can be formed by this solution.

Finally it was thought to be important to study the influence of external circumstances on nodulation in this non-leguminous plant and to compare the results with those obtained with Leguminous plants. The only investigation of this kind with *Alnus* (BJÖRKMAN 1942) already indicated that in *Alnus* the same relationships are found as in Leguminous plants.

II. METHODS

The methods used are the same as those described in the preceding article (QUISPEL 1954). The inoculation was always performed by using suspensions of crushed young nodules. In order to obtain an equal inoculation of all cultures it is advisable to filter the suspensions through glass wool. Variations in the inoculation density were obtained by dilutions of the original suspension. The nodulation was determined by the non-sterile method, each series consisting of 4 jars which contained 3 plants each. Only in the experiments on the influence of sugars the sterile method was used. In these experiments the sterile plants were inoculated with suspensions of crushed nodules which were "selected by incubation".

The definite count of the number of nodules was always performed 6–7 weeks after inoculation. Though the first nodules are already visible 3 weeks after inoculation most nodules are then so small that they can be easily overlooked, while after 6 weeks all nodules can be easily counted. A prolonged incubation did not increase the number of nodules. The nodules of *Alnus* have a branched form so that wherever an infection has occurred a branched group of nodules develops. If not stated otherwise such groups were counted as one nodule. In some experiments the total weight of the nodules was determined as well and from these determinations the average weight per nodule was calculated which gave an impression of the growth of the nodules.

The variation between the number of nodules of the individual plants of each series was always rather important. This was to be expected as I had to work with genetically heterogenous plants grown from seeds which were collected in the field. Therefore statistical analysis are necessary. In the tables I always mention the average number of nodules with the standard deviation of the average. As the number of experimental values in each series was rather small (12 or sometimes 11) its significance was estimated by using STUDENT's "t" analysis (FISHER 1948). As has been observed by NUTMAN (1952) in Leguminous plants here also the standard error increases with the average. The transformation on a logarithmic scale which is customary in such cases did not seem justified, as in this case as a result of the small values and the comparitively great deviations the resulting deviations from the normal frequency distribution would introduce still greater errors.

III. THE RELATION BETWEEN INOCULATION DENSITY AND NODULATION

a. THEORETICAL PART

Already from the first experiments which are not reproduced here it was evident that a definite relation could indeed be observed between inoculation density and nodulation. At slight inoculations the number of nodules increases with an increase of the inoculation density till a maximum is reached where the number of nodules is independent of the inoculation density. This proves the supposition to be true that in *Alnus* the influence of the inoculation density could be better demonstrated than in Leguminous plants.

The relation between inoculation density and nodulation being experimentally analysable the question had to be asked what could be theoretically predicted about the nature of this relation. On closer examination it appears that the nature of this relation depends on the mechanisms of the infection. If we assume that the infection can occur anywhere on the root surface the relation between inoculation density and nodulation is rather simple. In this case we may assume that a doubling of the number of cells of the endophyte in the vicinity of the root will double the chance of the root surface being hit by cells of the endophyte. Consequently a linear relation between the inoculation density and the number of nodules has to be expected. This will be limited by the fact that the concentration of food substances e.g. sugars in the root does not allow for a further increase of the number of nodules. Moreover the possibility exists that the production of inhibiting substances by the developing nodules (NUTMAN 1952) limits the possible maximum number of nodules. The graphical curve showing the relation between the inoculation density and the number of nodules will more or less take the form of a typical "Blackman" curve consisting of two straight parts: one ascending part which shows the linear relation between inoculation density and nodulation and a horizontal part which shows the region where other factors are limiting a further increase of the number of nodules.

According to several investigators, especially NUTMAN, the infection does not occur at random on the root surface but is restricted to a limited number of certain preformed foci. If this is true the calculation of the probability of the occurrence of an infection must be based on the fact that a limited number of foci is surrounded by cells of the endophyte in the medium while now and then a focus is hit by a cell of the endophyte and infection is possible. It has been shown that in comparable cases (e.g. haemocytometer counts) the chance distribution is the distribution of Poisson: the number of foci which is never hit will be e^{-m} and the number of foci which is hit and infected will be $1 - e^{-m}$ where m is the mean number of hits per focus. As we may assume that the mean number of hits per focus is directly proportional to the number of cells of the endophyte surrounding the foci (x) we may write $m = c.x$ in which c is a constant factor. If y is the number of nodules formed (= the number of foci which is hit) and A is the maximum number of nodules which is possible (= the total number of foci) we obtain the following relation:

$$y = A.\ (1 - e^{-c.x})$$

This is the well-known formula of Mitscherlich.

Of course the possibility exists that the infection is not a mere game of chance but that chemotactic substances play a role. These substances may be excreted either by the whole root surface or by the foci themselves. If the first is true this excretion will result in a greater concentration of the endophyte in the rhizosphere of the roots but in this concentrated rhizosphere the infection will happen in the same way as described above so that the same theoretical considerations hold true. If the chemotactic substances are excreted by the foci each focus will be surrounded by a concentration gradient of these substances. An infection will occur always whenever a cell of the endophyte enters into the attractive zone around a focus and again the Mitscherlich relation will be found. It is unlikely that in our well-shaken experiments the attractive influence of the excreting foci would be exerted over a long distance. If, however, this should occur all cells of the endophyte in the vicinity of the roots will be directly attracted into the foci. Now an influence of the inoculation density will only be observed as long as there are too few cells of the endophyte available to infect all foci; in this case the number of nodules will be identical with the number of cells of the endophyte and the relation between inoculation density and nodulation will be of the "Blackman" type.

Therefore, depending on the fact whether the infection occurs everywhere on the root surface or is restricted to preformed foci and in the latter case whether the infection is a mere game of chance or is dominated by the activity of chemotactic influences the relation between inoculation density and nodulation will be of the "Blackman" or the "Mitscherlich" type.

b. Experimental Part

It is no easy task to distinguish experimentally between these two possibilities as the variation of the observations is rather large while the difference between the two theoretical curves is rather small. The difference between the "Blackman" curve with its two straight parts and the more smoothly curved "Mitscherlich" curve is most evident at the point where in the "Blackman" curve the transition is found between the ascending and the horizontal part of the curve. Attention has to be focussed on observations in this region and it has to be calculated whether the number of nodules which is observed here is consistent with either the ascending or the horizontal part of the "Blackman" curve which is calculated from the other observations. On the other hand one has to examinate whether the observations are consistent with a "Mitscherlich" relation.

In table 1 and fig. 1 the results are summarized of an experiment in which the relation between inoculation density and nodulation was determined by inoculating a number of jar-cultures of *Alnus* with dilutions from the same suspension of nodules. In fig. 1 the best fitting "Mitscherlich" curve is drawn and it is clear that no significant departures from the theoretical values occur. However, this experiment is not yet conclusive. It is evident that the number of nodules obtained with the inoculation "75 mgr" is far below the value which could be calculated by extrapolation of the best fitting straight line through the values obtained with "0.1", "1", "10"

TABLE 1

Relation between inoculation density and nodule formation

Inoculations were performed with dilutions of a suspension of crushed nodules in water, the inoculation density is expressed as mgr fresh-weight of nodule material inoculated per jar. Each jar contained 2–3 plants, each series consisted of 4 jars. The inoculation was performed 4 II 53, the number of nodules counted 18 III 53

Inoculation density	Number of plants	Number of nodules (average with stand. dev. average)
300	12	29.9 ± 5.8
150	12	32.4 ± 7.4
75	11	20.9 ± 2.9
25	12	19.3 ± 4.8
10	11	7.3 ± 1.8
1	12	2.3 ± 0.7
0.1	12	0.2 ± 0.1

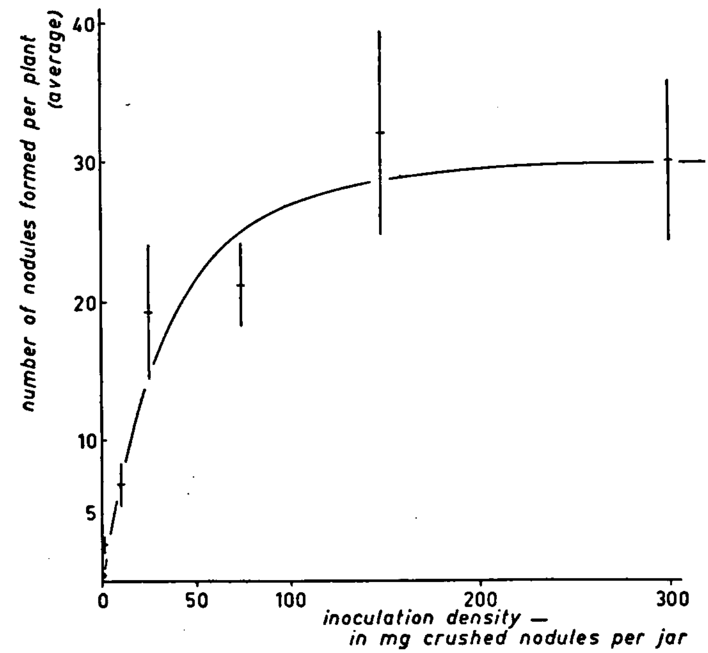

Fig. 1. Relation between inoculation density and nodulation according to the experimental results of table 1

and "25 mgr.". However, at inoculation with "75 mgr." the maximum number of nodules might have been reached already as the difference with the mean of the number of nodules obtained with the inoculations "150" and "300 mgr." is not yet significant. (n = 33, t = 1.427, P = 0.1–0.2 %).

In table 2 and fig. 2 the results are shown of another experiment

TABLE 2

Relation between inoculation density and nodule formation

Inoculations were performed with dilutions of a suspension of crushed nodules in water, the inoculation density is expressed as mgr fresh-weight of nodule material inoculated per jar. Each jar contained 2–3 plants, each series consisted of 4 jars. The inoculation was performed 25 VIII 52, the nodules were counted 8 X 52

Inoculation density	Number of plants	Number of nodules (average with stand. dev. average)
100	10	61.3 ± 11.0
10	10	24.8 ± 4.5
1	12	6.6 ± 1.6
0.1	11	0.5 ± 0.2

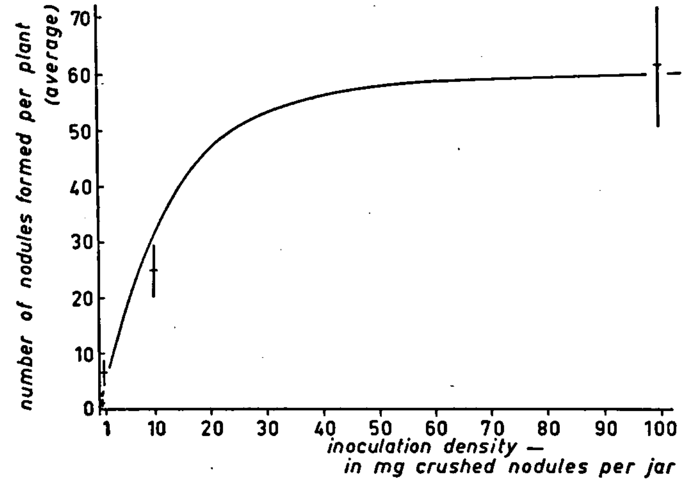

Fig. 2. Relation between inoculation density and nodulation according to the experimental results of table 2

which is less illustrative in consequence of a smaller number of observations. The number of nodules which is obtained with the inoculation "10", however, significantly differs from the "Blackman" curve which can be constructed through the other observations. If we extrapolate the best fitting straight line through the values obtained with the inoculations "0.1" and "1" to the inoculation "10" we obtain a value of 65.6 ± 4.1. This value is of the same order as the value obtained with the inoculation "100" and has to be regarded as the maximum number of nodules which is possible under the conditions of this experiment. The observed number of nodules at incubation "10", however, significantly differs from this maximum ($n = 18$, $t = 2.93$, $P = 0.01$).

As in both experiments the deviations are observed in the region and in the direction where they have to be expected and as in both experiments a "Mitscherlich" curve may be constructed which shows no significant departures from the observed values the choice between the "Blackman" and the "Mitscherlich" type of curve must be made in favour of the "Mitscherlich" curve. In none of all other experiments, which are not reproduced here, a significant departure from a "Mitscherlich" curve was observed.

I have to mention one possible source of error. As we can only inoculate with dilutions of suspensions of crushed nodules the possibility exists that the results are influenced by the presence of eventual inhibiting substances from the nodules of the inoculums. As the concentration of these substances will be greater in the heavier inoculations the number of nodules produced by these heavier inoculations might be decreased. This possible source of error can only be excluded with certainty when we posses; in vitro cultures of the endophyte. However, it is unlikely that our results were seriously influenced by the presence of inhibiting substances. In one experiment some jars with *Alnus* plants were inoculated with a suspension of crushed nodules while another set of jars was inoculated with the same suspension after removal of all soluble substances by "Seitz" filtration. No significant difference between the number of nodules in these two sets of jars was observed, so that at least no indication was obtained for the occurrence of soluble inhibitors in concentrations as might influence experiments of this type.

Of course the results obtained do not prove that the "Mitscherlich" formula is indeed the exact formulation of the relation between the inoculation density and nodulation. It only proves that the "Mitscherlich" formula is not contradicted by the experimental results while the "Blackman" relation gives more or less significant differences between the theoretical and observed values.

The conclusion may be that the experimental results are in accordance with the theory that the infection occurs at preformed foci. Of course they cannot give any information as to the nature of these foci (root initials, polyploid cells?) nor can they inform us about an eventual activity of chemotactic substances during the infection.

IV. THE INFLUENCE OF SOME ENVIRONMENTAL FACTORS ON THE NODULATION

It may be expected that, as in Leguminous plants, the number of nodules of *Alnus* will be influenced by certain environmental factors. In the experiments to be described below the influence of some of these factors will be shown. In order to eliminate the influence of the inoculation density these experiments were inoculated with such heavy inoculations that the maximum nodulation was approximated. Only in the experiments on the influence of sugars the amount of "sterile" nodules was not large enough to reach this maximum.

a. The Inorganic Composition of the Medium

In the usual experiments the plants are cultivated on a normal Hoagland solution which, at the time of the inoculation, is renewed by a nitrogen-free solution. In the experiments in which the influence of the inorganic composition of the medium on the nodulation was examined the composition of the nitrogen-free solution was changed by the addition of nitrogenous substances (ammonium sulfate of potassium nitrate) or by varying the concentration of fosfate. Care was taken that the total concentration of potassium and calcium remained the same (by addition or omission of their chlorides) and that in all solutions the pH remained at pH = 5.4. I want to stress the fact that the variations in the composition of the medium were only present from the moment inoculation takes place. Contrary to other experiments the nodules of each infection group were counted individually so that the number of nodules is 3–4 times greater as compared with other experiments where only groups of nodules were counted together. The results are given in table 3.

TABLE 3

The influence of the inorganic composition of the medium on nodule formation

Inoculations were performed with a suspension of crushed nodules corresponding with 30 mgr fresh-weight per jar. Each jar contained 3 plants, each series consisted of 4 jars. The inoculation was performed 1 V 51, the number of nodules was counted 18 VI 51

Composition of the medium in milliaeq. N or P			Number of nodules	Weight of the nodules	
NH_4	NO_3	PO_4		Per plant	Per nodule
0	0	0.25	106.5 ± 15.5	56.2	0.53
0	0.375	0.25	176.8 ± 18.8	84.0	0.49
0.375	0	0.25	9.1 ± 3.4	29.2	3.21
0	3.75	0.25	0.4 ± 0.3	0.7	1.6
3.75	0	0.25	0.3 ± 0.3	0.3	1.0
0	0.375	0	14.4 ± 2.9	3.5	0.25
0.375	0	0	0	0	0
0	0	0	18.7 ± 7.1	18.0	0.95
0	0	2.5	98.7 ± 10.9	47.7	0.50

The results are in accordance with what could be expected on the ground of the results which have been obtained with Leguminous plants and the few experiments performed with *Alnus* by Björkman. The presence of bound nitrogen has a definite inhibiting effect on the formation of root nodules. This effect is especially related to the infection itself and not so much to the subsequent growth of the nodules. This may be concluded from the observation that in the presence of 0.375 m.aeq.NH_4 the few nodules which still develop are much heavier than the many small nodules which develop in nitrogen-free solutions. Very small additions of bound nitrogen may result in a small but significant increase of the number of nodules. The difference between NO_3 and NH_4 additions may be caused by secondary effects. The influence of fosfate is very much in evidence: when fosfate is absent the number of nodules is definitely decreased. This is the more remarkable as the plants received enough PO_4 till the moment of the inoculation while the plants did not show any symptoms of fosfate deficiency till the end of the cultivation period. Higher gifts of PO_4 as were present in the usual Hoagland solution had no effect on the nodulation.

b. The Light Conditions of the Plant

The influence of the light conditions was examined by changing the light intensity and by shortening the day length. The light intensity was changed by putting some plants underneath a box of filter paper which decreased the light intensity to $\pm$ 1/5, while the other plants remained in the full day light of the summer months. The day length was shortened by putting some plants from 17–9 o'clock in absolute darkness. In order to test the possibility that the C/N ratio in the roots might be the determining factor these experiments were performed with plants grown on the normal nitrogen-free solution and plants grown on solutions with the small amount of KNO_3 which had proved to be beneficial in the preceding experiment. All variations in light intensity, day length and nutrient solution were present from the moment inoculation takes place. The results are shown in table 4.

TABLE 4

The influence of the light-conditions on nodule formation

Inoculations were performed with a suspension of crushed nodules corresponding with 30 mgr fresh-weight per jar. Each jar contained 3 plants, each series consisted of 4 jars. The inoculation was performed 15 VI 51, the nodules were counted 8 VIII 51

Light condition		N-content of the medium in m. aeq. NO_3	Number of nodules	Weight of the nodules	
Day-length	Intensity			Per plant	Per nodule
normal	normal	0	58.3 $\pm$ 14.4	88.3	1.5
normal	normal	0.375	78.8 $\pm$ 14.3	125.5	1.6
short	normal	0	50.2 $\pm$ 7.0	38.1	0.8
short	normal	0.375	59.2 $\pm$ 10.1	59.2	0.9
normal	low	0	32.8 $\pm$ 8.5	20.7	0.6
normal	low	0.375	25.0 $\pm$ 5.4	22.4	0.9

The differences obtained in this experiment are for the most part not significant. However, as they agree with the results obtained in other experiments, it is justified to draw the following conclusions. Both the lowering of the light intensity and the shortening of the day length result in a decreased number of nodules. It cannot be decided whether the unfavourable effect of short days is caused by the smaller total amount of light energy per day or by the day length as such. The largest decrease occurs when small amounts of bound nitrogen are present. This might point to the possibility that in *Alnus* as well the C/N ratio is the determining factor in nodulation.

c. The Presence of Organic Substances

In all hypothesis which try to explain the influence of external factors on nodulation attention is paid to the presence of organic food substances, especially of sugars, in the roots, either as such or in relation to the nitrogenous substances. It is generally expected that the addition of sugars in the culture solution might favour the production of nodules and the few experiments with Leguminous plants confirm this expectation. As such experiments must be performed under sterile conditions, at least of the roots, they are rather scarce.

The experiments regarding the influence of sugars on nodulation of *Alnus* were performed with plants with sterile roots and inoculated with "sterile" nodules. In consequence the experimental series could not be as great as in the other experiments. In table 5 the results of three small experiments, performed in the summer and in the winter months, are collected. The addition of glucose was performed at the moment of the inoculation when the plants were transferred to the nitrogen-free solutions while the level of glucose was kept constant by the weekly renewals.

TABLE 5

The influence of glucose on nodule formation

Inoculations were performed with suspensions of crushed "sterile" nodules in cultures of Alnus with sterile root systems. Experiment A was inoculated 8 VIII 51 and counted 11 IX 51, exp. B was inoculated 22 I 52 and counted 11 III 52 and experiment C was inoculated 19 VI 52 and counted 11 VIII 52. The figures represent the counts of nodules in the individual cultures.

Concentration of glucose in %	Number of nodules in the cultures of		
	exp. A	exp. B	exp. C
0	0–4–15–3	2–1–4–0	2–9–14–7
0.1	0–4–0–5	0–0–0–2–0–1	
1	0–0–0–0	0–0–0–0–0–0–0	0–0–0

There can be no doubt that the presence of glucose has an inhibiting effect on nodulation. This was observed in experiments which were performed during the good light conditions of the summer and during the smaller light intensities of the winter months. This is contrary to what might be expected and which indeed had been found with Leguminous plants. The question arises whether this inhibiting effect could not have been caused by the presence of e.g. reductones which were produced during the heat sterilization of the glucose. Therefore the experiment was repeated but now the glucose was sterilized separately by filtration through a Jena G5 filter. The results of this experiment are given in table 6.

With this experiment the inhibiting effect of the glucose on nodulation is not as large as it was in the preceding experiments.

TABLE 6

The influence of glucose (sterilized by filtration) on nodule formation

Inoculations were performed with a suspension of crushed "sterile" nodules in cultures of Alnus with sterile root systems. The inoculation was performed 31 VII 53, the nodules were counted 17 IX 53

Concentration of glucose in %	Number of plants	Number of nodules per plant (average with stand. dev. average)
0	9	3.3 ± 0.6
0.1	7	1.1 ± 0.5
1	9	1.2 ± 0.3

This is an indication that indeed substances, which arise from the glucose during the heat sterilization, were responsible for a part of the inhibiting effect. However, even in this experiment, the inhibiting effect is significant for both concentrations of the glucose ($n = 14$, $t = 2.69$, $P = 0.01$–0.02 and $n = 16$, $t = 3.18$, $P = 0.01$ respectively).

Finally it may be mentioned that in another experiment sucrose appeared to inhibit nodulation in the same way. So we have to conclude that the number of nodules is decreased by the presence of sugars in the culture solution. Further experiments are necessary before we can give an explanation for this remarkable and unexpected effect.

d. Conclusion

The conclusion drawn from this part of the investigation can be that the presence of bound inorganic nitrogen, fosfate and the light conditions exert the same influence on the nodulation of *Alnus* as they exert on Leguminous plants. The necessity of good light conditions, the inhibiting effect of ammonium salts and nitrates as well as the promoting effect of these salts in very small concentrations agree with the idea that the number of nodules depends on the ratio between carboniferous and nitrogenous substances in the roots. However, the inhibiting effect of glucose and sucrose is not in accordance with this idea. A final explanation can only be given when we know which carboniferous and nitrogenous substances are especially concerned and at which moment in the chain of processes leading to nodulation they exert their influence.

V. THE INFLUENCE OF THE INOCULATION DENSITY ON NODULATION IN ITS RELATION TO THE INFLUENCE OF EXTERNAL FACTORS

a. Theoretical Part

It is very difficult to ascertain what is the crucial moment in the nodulation process where the external factors exert their effect on the formation of the nodules. In the general introduction the possibilities could be divided into four categories. If any of these four possibilities would be realized certain consequences regarding the relation between inoculation density and the number of nodules and the way in which this relation itself is affected by the influence of the external factors would result. We shall now consider the consequences with respect to all these possibilities.

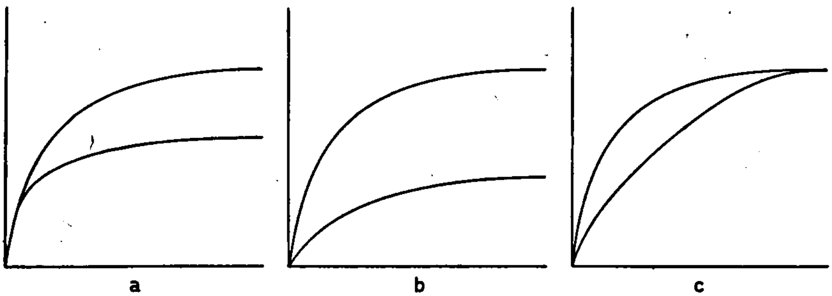

Fig. 3. Theoretical relations between inoculation density and nodulation at different external circumstances (explanation in text)

Possibility 1. a. *The external factors influence the plant by changing the concentration of certain food substances in the roots so that they become more or less liable to the occurrence of an infection.*

If indeed the concentration of food substances is the crucial point we may assume that the higher the number of nodules the sooner the lack of food substances will be evident. After heavy inoculations the lack of certain food substances will sooner be of importance than after slight inoculations when the small amount of food substances is still sufficient for the small number of nodules. The influence of the external factors which govern the amount of food substances will be especially apparent at the heavier inoculations while at the slightest inoculations no effect will be observed. The curves which show the relation between inoculation density and nodulation will change as is schematically shown in fig. 3*a*. If we compare the Mitscherlich formulae at different external circumstances the values of both A and c will be different. At the most unfavourable circumstances definite departures from a Mitscherlich relation will be found.

When considering the possibility of the formation of nodules being limited by mutual excretion of inhibiting substances by nodules while the formation of these substances is influenced by external factors we arrive at the same results as the effect of the amount of these inhibitors will be most manifest if the nodules are close together (after heavy inoculations).

Possibility 1. b. *The external factors influence the plant by changing the number of foci which can be infected by the endophyte.*

The consequence of this possibility is the following: The total number of foci is identical with the maximum number of nodules, so that after heavy inoculations when the maximum number of nodules is approximated the number of nodules will depend on the external factors which influence the number of foci. After slight inoculations the chance of the cells of the endophyte to hit a focus will be decreased if the number of foci is decreased, so that at this stage the number of nodules will depend on the external circumstances as well. The actual mechanism of the infection, however, will remain unaffected. The curves of the relation between inoculation density and nodulation will change with the external factors as is schematically illustrated in fig. 3*b*. In the Mitscherlich formula A will change with the external factors, but c will remain the same.

Possibility 2. *The external factors influence the infective capacity of the bacteria.*

Though the presence of nitrogenous or organic substances in the medium of the roots might possibly influence some properties of the endophyte so that their infective power is affected it is absolutely inconceivable that e.g. the light conditions influence the cells of the endophyte in the surroundings of the roots in a direct way. This possibility can never be considered to be the sole or even the main cause of the influence of these external factors.

Possibility 3. *The external factors influence the process of the infection itself e.g. by influencing the formation of eventual chemotactic substances.*

If the process of the infection has to be considered as a mere game of chance it is unlikely that it is influenced by the external factors observed. If, however, chemotactic substances play a role, the external factors might influence the excretion of such substances. This will affect the chance of the foci being hit and infected, but the total amount of possible infections will remain the same. The influence of the external factors would, in this case, occur according to the scheme of fig. 3*c*. In the Mitscherlich formula c will be influenced while A remains unchanged.

Possibility 4. *The external factors influence the growth of the infected foci into visible nodules.*

As in all our experiments the number of nodules was primarily concerned this possibility can only have been of secondary importance. In those experiments where both the number and the weight of the nodules was measured it was observed that the influence on the number of nodules sometimes far exceeded that on the weight of the nodules. Of course we must reckon with the theoretical possibility that in some experiments the infection had succeeded but could not be observed as none of the infected foci developed into nodules. Certainly the growth of the nodules will depend on external factors but the design of our experiments was such that the main attention was focussed on the influence of external factors on the events prior to and during the infection itself.

So these theoretical considerations offer a possibility of experimentally distinguishing the way in which the formation of nodules is influenced by external factors. As the possibilities 2 and 4 can be excluded we can distinguish between the possibilities 1*a*, 1*b* and 3 by determining the relations between inoculation density and nodulation at varying external circumstances. If the Mitscherlich formula is the best description of this relation we must determine whether the values of A, c or of both are influenced by the external factors.

b. Experimental Part

A consideration of the experiments of chapter III already shows that those possibilities according to which the factor A remains constant are not in accordance with the facts. E.g. in the experiments of table 3 and 4 the inoculation has certainly been so heavy that the number of nodules can be considered to approximate the maximum value of A. Nevertheless great differences due to the external influences are observed. So the possibilities 3 (influence on the production of eventual chemotactic substances) can be excluded. The question remains whether the value of c remains constant or is influenced as well.

A practical difficulty here arises. It might be justified to compare the values of c obtained from different experiments if in all those experiments the inoculation was performed with absolutely known quantities of endophyte. However, as we dont possess cultures of the endophyte we have to inoculate with suspensions of crushed nodules of which the absolute content of endophyte is unknown. Therefore it is only possible to compare experiments which are inoculated with dilutions of the same nodule suspension, while it is impossible to compare experiments which are inoculated with dilutions from different nodule suspensions. In consequence: if we try to determine the value of c in the Mitscherlich formula we cannot obtain absolute values. We can only obtain values of the product c.x, but we cannot determine which part of it belongs to x and what part of it belongs to c. If we want to examine the effect of external factors on c we must be sure that x is the same in all experiments. In consequence we can only compare experiments which were inoculated at the same moment but at different external circumstances with dilutions from the same suspension of crushed nodules.

The first experiments which allowed for such a comparison did not answer the question in a convincing way. Though there was no reason to doubt the constancy of c the variation of the observations was too great to establish this constancy without any doubt. I therefore performed a new experiment with more plants per series (24) so that the influence of the variation was restricted. Three inoculation densities were compared. Unfortunately these densities were obtained by dilutions of a suspension of nodules which appeared to be only slightly active, so that in the heaviest inoculation the maximum number of nodules was not yet obtained and the value of A had to be calculated from the other values. Half of the plants remained in the normal light conditions of summer (the experiment was inoculated 26 V 53), the other half of the plants was cultivated at lower light intensities by putting the plants underneath a box of filter-paper which reduced the light intensity to $\pm\,^1/_5$. The results are shown in table 7 and fig. 4.

TABLE 7

The influence of inoculation density on nodule formation at different light conditions

Inoculations were performed with dilutions of the same suspension of crushed nodules in water, the inoculation density is expressed as mgr fresh weight of nodule material inoculated per jar. Each jar contained 3 plants, each series consisted of 8 jars. The figures between brackets are calculated from the Mitscherlich formulae (see text). The inoculation was performed 26 V 53, the number of nodules was counted 16 VII 53

Inoculation density	Number of nodules (average with stand dev. of average)	
	Normal light intensity	reduced light intensity
300	25.3 ± 1.8 (23.7)	4.1 ± 0.6 (4.2)
50	4.5 ± 0.6 (5.6)	0.9 ± 0.2 (1.0)
10	1.2 ± 0.2 (1.2)	0.5 ± 0.1 (0.2)

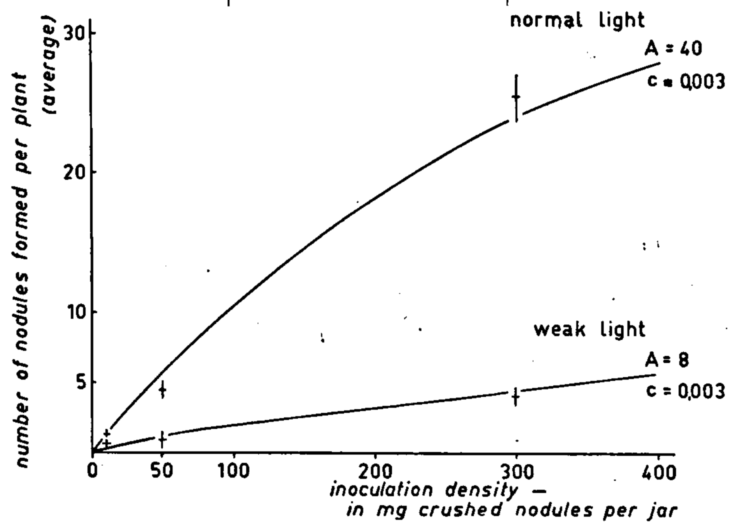

Fig. 4. Relation between inoculation density and nodulation at different light conditions according to the experimental results of table 7

In the first place it is again evident that the law of limiting factors in its simplest shape does not hold true. The influence of the light intensity is already manifest at the slightest inoculations. Again this experiment gives additional and convincing evidence that the relation between inoculation density and nodulation cannot be described by a simple „Blackman" relation as the influence of the external factor already is manifest in a region where the inoculation density is a limiting factor. If we calculate the best fitting constants of the Mitscherlich formula we obtain for the high light intensity A = 40 and c = 0.003, for the low light intensity A = 7 and c = 0.003. The theoretical values for the three inoculation densities which are calculated from these constants are inserted between brackets in table 7. The agreement between the theoretical and experimental values requires no further comment. There is no reason to assume that the value of c is influenced by the light conditions. Only the value of A is changed. Of all possibilities mentioned in the beginning of this chapter only the possibility 1 a is in accordance with the facts. The experimental results may be entirely explained by assuming that the light conditions alter the number of foci on which the infection has to occur, the infection itself being a mere game of chance. Though the possibility is not excluded that chemotactic substances play a role, the production of these substances is certainly not changed in such a way that it is experimentally demonstrable.

VI. DISCUSSION

A theoretical discussion of the relation between inoculation density and nodule formation shows that the nature of this relation is different whether we base our considerations on the assumption that the infection may occur at random everywhere on the root surface or on the assumption that the infection can only occur in a limited number of preformed foci. In the first case a linear relation between inoculation density and the number of nodules has to be expected which will only be limited at heavier inoculations when food substances become limiting factors or when the mutual secretion of inhibiting substances limits a further increase of the number of nodules. In the second case an application of the theory of chances shows that the relation between the number of nodules y and the number of cells of the endophyte in the medium x can be described by a Mitscherlich formula $y = A\ (1 - e^{-c \cdot x})$ where A is the number of foci = the maximum number of nodules and c is a constant factor. While the relation between inoculation density and nodulation cannot be studied in Leguminous plants as the number of *Rhizobium* cannot be effectively controled as they multiply in the medium around the roots, this study is possible with plants of *Alnus* as here the endophyte cannot develop in vitro. The experimental results obtained by inoculating a number of *Alnus* plants with different dilutions from a suspension of crushed nodules shows that the assumption of a Mitscherlich relation is better in accordance with the facts. This may be used as another argument for the theory of NUTMAN, that the infection can only occur in a limited number of preformed foci. Of course the inoculation density is not the only factor which influences the formation of nodules as the number of nodules is influenced by a number of external factors. Nitrates and ammonium salts inhibit the nodulation completely though in very small concentrations a weak stimulation is observed. Moreover the formation of nodules depends on the presence of fosfate and good light conditions. There are a few indications that, as in Leguminous plants, it is especially the C/N relationship in the roots which governs the formation of nodules. The inhibiting effect of added sugars is unexpected and, for the time being, unexplained.

If the inoculation density and the light intensity are simultaneously varied it appears that the relation between the inoculation density and the number of nodules at two light intensities can be described by the same Mitscherlich formula with the same value for c but different values for A. This is in accordance with the expectation when we assume that the light intensity only influences the formation of nodules by influencing the number of preformed foci on which the infection can only occur. The infection itself is determined by the chance that a focus is hit by a cell of the endophyte. If the influence of changed external conditions depends mainly on the presence of available food substances or on the formation of eventual chemotactic substances the value of c might be expected to change as well. Though of course we have to be aware for premature generalizations there are reasons to suppose that the same will be true with regard to the influence of nitrogenous substances as there are definite relations between the effect of light and the effect of nitrogenous substances. Moreover it has been observed that after addition of nitrogenous substances a few nodules develop, the weight of these nodules exceeding the weight of the nodules on nitrogen free media, so that the decrease of eventual food substances could never account for the decrease of the number of nodules.

On the ground of the described experiments we can form the following notion of the process of the infection. The endophyte cannot enter the roots everywhere on their surface but can only enter the roots on a limited number of preformed foci. The number of these foci is influenced by genetical factors as well as by some external conditions like light conditions and most probably also by the presence of nitrogenous substances, fosfate and so on. The infection itself is a mere game of chance, the number of foci which is infected depending on the inoculation density as may be expected according to the chance distribution of Poisson. It cannot be concluded from our experiments whether chemotactic substances play a role either by concentrating the endophyte in the rhizosphere or by attracting the endophyte within a short distance of the foci. Once the focus is infected the growth of the nodule will certainly depend on the available food substances.

I wish to stress the fact that this notion regarding the infection process cannot pretend to be more than a hypothesis most in accordance with the experimental results. The truth of this hypothesis must be verified by other experiments. It is impossible to say anything more about the nature of the postulated foci. NUTMAN supposed that a relation exists between such foci and the root initials. Indeed the formation of root initials as well is influenced by light conditions while it is a common observation that nitrogen starvation results in extensive root formation. Certainly, however, these foci cannot be identified with the root initials as in the presence of normal amounts of nitrates a normal root development is observed while no nodules can be formed. Perhaps the foci are identical with the few polyploid cells in the neighbourhood of the initials (WIPF and COOPER 1940).

Finally I have to mention a practical consequence of the results which is very important for the further study of the root nodule symbiosis of non-leguminous plants. The definite relation between inoculation density and nodulation and the simple way in which this relation is influenced by the external factors provides a possibility of determining in a semi-quantitative way the amount of endophyte in unknown suspensions and inoculated culture media. By using this method it should be possible to measure an eventual growth of the endophyte in such media in a fairly accurate way. The practical execution of this method and its results will be described in the next article of this series.

SUMMARY

1. The relation between inoculation density and the number of nodules in watercultures of *Alnus glutinosa* is in accordance with the hypothesis that the infection occurs through a chance contact of the endophyte with a limited number of preformed foci.

2. The influence of some external factors on the formation of nodules is principally the same as has been observed in Leguminous plants. Nitrates and ammonium salts inhibit nodule formation while fosfates and good light conditions are necessary.

3. The formation of nodules is inhibited by the presence of sugars in the nutrient solution.

4. A further analysis of the mutual effects of inoculation density and light intensity on the formation of nodules suggests that the light intensity influences the number of foci.

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