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SYMBIOTIC NITROGEN FIXATION IN NON-LEGUMINOUS PLANTS

III. EXPERIMENTS ON THE GROWTH IN VITRO OF THE ENDOPHYTE OF ALNUS GLUTINOSA

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CONTENTS

I. INTRODUCTION 671

II. METHODS 673
a. Preparation of the inoculation matter 673
b. Nutrient solution 674
c. Incubation period 674
d. Determination of the amount of endophyte 674
e. Critical remarks as to the reliability of the method 677

III. EXPERIMENTS 678
a. Experiments with some usual nutrient media 678
b. Experiments with soil and peat suspensions 679
c. Experiments with suspensions and extracts of alder roots . . . 682
d. Some critical remarks as to the reliability of the results 686

IV. DISCUSSION 687
SUMMARY 689
REFERENCES 689

I. INTRODUCTION

The study of the symbiotic relations in the nitrogen-fixing root nodules of non-leguminous plants like alder is much hampered by the fact that it has been proved impossible up till now to isolate and cultivate in vitro the endophyte which is responsible for the nodulation and the fixation of atmospheric nitrogen. The difficulties which are experienced are not at all restricted to this symbiosis as in certain other cases of symbiosis or parasitism as well the endophyte or the parasite can not be isolated. This certainly is one of the most difficult, yet one of the most fascinating problems, which are encountered by those who study symbiotic or parasitic relationships. It seemed reasonable to assume that the solution of this problem might be somewhat easier in the case of alder as in the case of e.g. obligate parasites. While in nature the obligate parasites can only multiply inside the tissues of the host plant, the endophyte of alder

is so universally present in most soils that the conclusion seems guaranteed that the endophyte does multiply in most soils as well so that its growth requirements will not be as specialized as those of the obligate parasites.

In the first communication of this series (QUISPEL, 1954) some investigations were made into the possible causes of the failures to isolate the endophyte of alder. Four possibilities were distinguished:

1. The endophyte contained in the nodules used for isolation is no longer vital after undergoing the necessary pre-treatments.
2. The endophyte can undoubtedly be grown on certain media, but during the isolation and the following growth in vitro it loses its virulence so that its identity can not be proved by the formation of root-nodules.
3. The type of growth of the endophyte is so slow, so diffuse or so brief, that no visible colonies can be formed.
4. The endophyte requires special nutrient or environmental circumstances which up till now have not been fully provided by the growth circumstances and nutrient media used.

It was shown that the first two possibilities were not the reason for the failures of the isolation. We have to take into account point 3, while point 4 certainly is most important. Now we are in a vicious circle: the endophyte can not be cultivated because we do not know the food requirements, but we can not study the food requirements because we can not cultivate the endophyte. In order to overcome this difficulty we have to satisfy two methodical demands. In the first place we have to possess pure inoculation matter for our culture experiments. In the second place we must be able to demonstrate the growth of the endophyte in a more sensitive way than was hitherto possible as the chance to obtain our first indications of the growth requirements of the endophyte will increase with the sensitivity of our methods to observe an eventual growth. If once we possess such sensitive methods our experiments will not be influenced by the mentioned possibility 3.

In the first communication of this series I described a method that enables us to perform experiments under sterile conditions. When the nodules that remained visibly sterile after an incubation period of six weeks on a suitable nutrient agar, are crushed in sterile water we acquire a suspension of nodules "selected by incubation" that is sterile according to the usual microbiological criteria but yet appears to contain the endophyte in its vital, virulent and efficient form. With this method the first methodical demand is satisfied.

As to the second methodical demand it is evident that the usual method is rather insensitive, as the appearance of a perceptible colony on solid media or the formation of a visual turbidity in liquid media is taken as the criterion for the occurrence of growth. A visible colony or turbidity is only formed when the millions of cells, of which a small colony already consists, have developed out of the small quantity of cells present in the inoculation. More sensitive methods might be found by observation of hanging-drop cultures under the

microscope. However, we do not know which is the most vital stage of the endophyte (hyphae, vesicles or bacteroids), so that we do not know what to isolate and inoculate into the hanging drops. Moreover, the use of cloudy media, such as root- or soil suspensions, which appear to be most apt for starting growth experiments, may cause serious difficulties during the observation of the hanging drops. For the time being it appeared to be more promising to develop a method by which the amount of endophyte in a suspension or inoculated nutrient solution could be determined e.g. by measuring the number of nodules that can be formed by such suspensions. This method could only be developed after an analysis of the relations between inoculation density and nodulation. In the second communication of this series (QUISPEL, 1954 II) was shown, both by reason of theoretical considerations and by experiments, that the relation between inoculation density and nodulation could be described by the formula of Mitscherlich: $y = A(1-e^{-c \cdot x})$, where y is the number of nodules formed after inoculation with the inoculation density x if A is the maximum number of nodules that can be formed under the prevailing conditions of the experiment, while c is a constant. The important observation was made that the environmental factor which is most difficult to control during our experiments, the light, only influences the value of A, while the value of c remains uninfluenced by the light conditions. Most probably the same will be true for some other environmental factors which influence the nodulation. So the relation between y and A is a sufficient criterion for the amount of endophyte in the inoculation matter. It has been shown by experience that this method enables us to determine the amount of endophyte with such an accuracy that an increase by 10 times (corresponding to an average number of cell divisions of 2–3) can be demonstrated. In this communication this method, which satisfies the second methodical demand will be described in detail. The first results which were obtained by the combined application of both methods will be summarized.

METHODS

a. PREPARATION OF THE INOCULATION MATTER

All nodules that were used for the preparation of the inoculation matter were collected in a small alder grove in the neighbourhood of Amsterdam. The clusters of nodules were dissected into groups of 3–5 individual nodules, thoroughly washed with water, shaken with soap-suds during 5 minutes, again washed with water, shaken with alcohol during 5 minutes, again washed with water, shaken with a solution of bromium (0.1 % v/v) and finally washed with sterile water. After the desinfection the nodules were peeled with a sterile scalpel to remove the outer cell layers which are most infected with bacteria and fungi. Then the remaining central part was brought into a sterile test tube with the following nutrient solution: Glucose

2 %, peptone 0.5 %, yeast autolysate 1 %, NH_4NO_3 0.05 %, K_2HPO_4 0.05 %, $MgSO_4.7H_2O$ 0.02 %, NaCl 0.02 %, $CaCO_3$ 0.2 %, agar 1.5 %. The yeast autolysate was prepared from 1 kg bakers yeast and 1 kg water during one night incubation at 55° C, followed by boiling and filtration. This nutrient medium was chosen because it had been shown before that this medium enabled the most abundant development of contaminating microorganisms. The tubes were incubated at 27–30 °C. All tubes in which the colony of a microorganism developed were discarded. It is necessary to continue the incubation 6 weeks before we can be certain that the remaining nodules are free from contaminating microorganisms. The nodules which remained visibly sterile after this incubation period are collected in a test tube of thick-walled glass and crushed with a sterile glass rod in a drop of sterile water and finally diluted with sterile water as necessary. These suspensions which are sterile according to the usual microbiological criteria still contain the endophyte in a vital, virulent and efficient form. They were used for the inoculation of the tubes with nutrient solution in which I wanted to determine an eventual growth of the endophyte.

b. Nutrient Solutions

In all experiments the inoculation was performed in test tubes (18 × 180 mm) with 10 ml of the nutrient solutions. The preparation and composition of the nutrient solutions and suspensions is given at the description of the experiments.

c. Incubation Period

As long as we do not have any indications about the development of the endophyte in vitro we do not know anything about the incubation period of the inoculated nutrient solutions which is necessary to observe a measurable development. As suspensions of the endophyte can be conserved for several weeks without losing their activity a rather long incubation period was preferred. In all experiments to be described below the inoculated media were incubated at 27° C during 3 weeks.

d. Determination of the Amount of Endophyte

The eventual growth of the endophyte in the inoculated nutrient solutions was determined at the moment of the inoculation in the inoculation matter, or better, in the just inoculated nutrient solutions and after the incubation period in the inoculated and incubated media. Therefore the suspensions or inoculated media are diluted with water to a constant volume (mostly 100 ml) and 5 or 10 ml of these dilutions is then inoculated into jars with alder plants. Each determination was performed with four jars with three plants each. The culture of the plants and the inoculation procedure were described in the first communication of this series. If one does not have the slightest idea about the amount of endophyte in a certain sus-

pension it is advisable to inoculate some series of jars with different dilutions of the suspensions. The inoculation density per jar is expressed in the dilution of the original pure nodule suspension with which the test tubes were inoculated. The figures in the tables under the heading "dilution" give the number of these nodules that was present in the inoculation matter per jar with alder plants. Six weeks after the inoculation of the jars the number of nodules can be counted. This gives the value y: the average number of nodules per plant after an inoculation per jar with the mentioned dilution of the original nodule suspension.

This value of y not only depends on the inoculation density, but on the external circumstances and the condition of the plants as well. As we want to eliminate the effect of those external circumstances we have to determine the maximum number of nodules that can be formed under the prevailing external conditions by the plants used in the experiments. This can be estimated after a heavy inoculation of a series of jars with alder plants. In most cases an inoculation with a suspension of 300 mgr fresh nodules per jar yields a number of nodules per plant that approximates the maximum value. A second series of jars is inoculated with half the amount (150 mgr) of fresh nodules per jar to control whether the maximum value was indeed reached. If the last mentioned series yields a somewhat smaller number of nodules the real maximum value can be calculated from the Mitscherlich formula. This gives the value A: the maximum number of nodules per plant that can be formed under the conditions of the experiment.

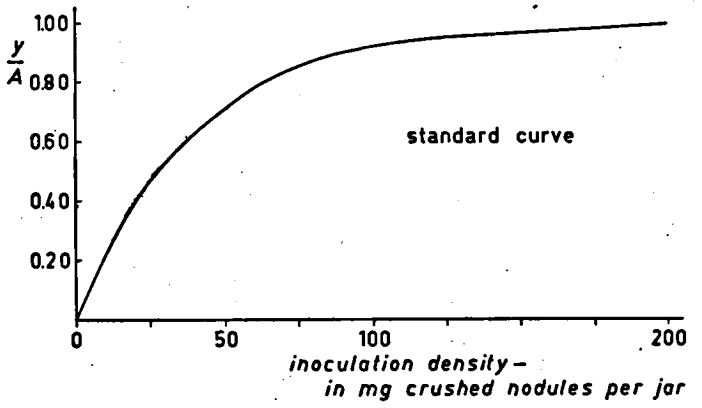

Fig. 1

The value y/A can be taken as a criterion for the amount of endophyte in the inoculation matter per jar. However, there is no direct proportionality between this value and the amount of endophyte in the inoculation matter as this relation is represented by the formula of Mitscherlich: $y/A = 1 - e^{-c \cdot x}$. We therefore have to

construct a standard curve with which all future observations can be compared. This standard curve was constructed from the experiment of 4 II 53 (see table 1 in the second communication of this series). This standard curve that corresponds to an empirical "standard" formula: $y/A = 1 - e^{-0.025 \cdot x}$ is represented in fig. 1. With the aid of this standard curve we can express all observations of y/A as: The mgr of inoculation matter used in the standard experiment which should have given the same value of y/A.

Finally all values have to be calculated on the same unit as the jars that we want to compare may have been inoculated with different dilutions of the original suspension of pure nodules. As a unit we take an inoculation of the dilution 1. If e.g. the jars in which the number of nodules y was determined were inoculated in such a way that every jar contained 0.08 nodules of the original suspension of pure nodules, the value obtained from the standard curve has to be multiplied by a factor 1/0.08. This gives the value that I called the "Endophyte Concentration": EC.

In the following tables the following symbols will be found:

dilution: the number of pure nodules, prepared by selective incubation, that was finally present in the inoculation matter per jar of 300 ml nutrient solution with three alder plants.

number of nodules y: the average number of nodules per plant that was formed in the jars that were inoculated with a suspension of nodules or an inoculated nutrient solution in the dilution as mentioned above.

maximum number of nodules A: the maximum number of nodules that could have been formed by the plants under the conditions of the experiment.

endophyte concentration EC: the mgr of nodulous material used in the standard experiment of 4 II 53 that, after the same dilution as mentioned above, should have produced the same number of nodules y, under the conditions of the experiment where the maximum number of nodules is A.

Example: 10 nodules, selected by incubation, are crushed in sterile water and diluted to 10 ml. One drop of 0.08 ml of this suspension is added to jars with alder plants. So each jar obtained an amount of inoculation matter equivalent to 0.08 nodules. The dilution is 0.08. After six weeks the average number of nodules per plant is counted as 0.8 ± 0.2 (average with standard deviation of the average). The maximum number of nodules was determined as described above and gave a value of $A = 43.1 \pm 6.5$. According to the standard curve the observed relation of $y/A = 0.018$ corresponds to 0.7 mgr of the nodules suspension used in this standard experiment. So 0.08 nodules of our experiment is equivalent to 0.7 mgr of the nodulous material used in the standard experiment. The Endophyte Concentration EC is ± 9.

e. Some Critical Remarks as to the Reliability of the Method

If we compare y with A we have to be aware of the fact that y and A were determined by the inoculation of jars with alder plants with different suspensions. E.g. if y was determined by the inoculation of some jars with an inoculated and incubated nutrient solution, the maximum value of A was determined by the inoculation with a dense suspension of crushed nodules. Both the nutrient solution and the nodule suspension may contain substances that influence the nodulation of the roots so that a direct relation of y to A, as determined in our experiments, is not allowed.

The presence of substances in the nutrient media, that affect the nodulation of the roots, may be easily checked by some control experiments in which the maximum number of nodules is determined in the presence and in the absence of this nutrient solution.

It is more difficult to examine whether the value of A is influenced by substances in the nodule suspensions. Nutman (1953) indeed observed that in Leguminous plants the roots may contain and excrete substances that inhibit further nodulation. As long as we did not possess cultures of the endophyte we could not compare maximum determinations that were determined by the inoculation with nodule suspensions and by the inoculation with suspensions of the endophyte in other media. As soon as I obtained cultures of the endophyte such comparisons were possible.

Some control experiments, in so far as they were necessary for the interpretation of the most important results, are described in Chapter III d. It will be shown that there is no reason to doubt the validity of our results and that for our purposes the method is reliable.

Another difficulty is formed by the peculiar character of our determinations. The number of nodules that we determine depends on the number of infective particles with which the alder plants are inoculated. If we want to determine the growth of the endophyte we must determine the increase of the number of infective particles. A growth of the hyphae without any cell divisions will not be observed. On the other hand a simple division of the hyphae into small cells e.g. a kind of spore formation, without any further development, will be observed as growth of the endophyte. Our method is nothing more than a determination of cell divisions, that lead to the formation of new infective particles.

Finally I have to mention the accuracy of our method. This accuracy depends on the standard deviations of the determinations of y and A, on the accuracy of our samples and inoculations. Moreover the accuracy depends on the question whether in all circumstances the Mitscherlich relation is the most exact description of the relation between inoculation density and nodulation and if so whether the value of c is indeed as constant as was shown by the experiment mentioned in the second communication of this series. However, it has been shown by experience that an increase of the value EC

by 10 times is a completely significant indication of a real increase of the endophyte in the nutrient solution. This means that an average number of cell divisions of 2–3 per cell of the endophyte can be determined with certainty.

III. EXPERIMENTS

As was mentioned in the introduction the experiments of the first communication of this series left two possibilities for the explanation of the failures at isolation of the endophyte. One of these possibilities was that the endophyte can develop on the usual nutrient media but that the development is too slow or too diffuse to be perceptible by the usual criteria of development. This possibility could only be excluded by the use of a more accurate and sensitive method. Now that we possess a more sensitive method the first task will be to examine whether indeed no development at all occurs on the media that were mostly used up till now. If indeed no development occurred another possibility remained. This possibility is that the growth of the endophyte depends on peculiar nutrient or environmental requirements. Then it is obvious that we have to look after media that make possible at least a small development in vitro. As a starting point two media were used in which the endophyte occurs in nature: suspensions of crushed roots of alder plants, especially of plants that were grown under conditions of nitrogen deficiency, and suspensions of soils, especially of the peat soil where I always sampled the nodules.

a. Experiments with Some Usual Nutrient Media

The medium that was most used in our initial experiments had the following composition (standard medium): glucose 2 %, peptone 0.5 %, NH_4NO_3 0.05 %, K_2HPO_4 0.03 %, KH_2PO_4 0.02 %, $MgSO_4.7H_2O$ 0.02 %, NaCl 0.02 %, $CaCO_3$ 0.2 %, yeast autolysate 1 %. This medium gave a very good development of the many contaminating organisms in the nodules. The experiments of table 1 show, however, that the endophyte did not grow at all on this solution.

TABLE 1

Growth experiments in the standard medium

10 pure nodules were crushed in 3 ml sterile water. One drop of this suspension (0.08 ml) was inoculated into 10 ml standard medium. Before and after the incubation ½ ml of this inoculated medium was inoculated per jar with alder plants. Further explanation of the symbols in the text (Chapter IId).

Inoculation matter	Dilution	Number of nodules y	Maximum number of nodules A	Endophyte concentr. EC
inoculated standard medium before incubation	0.008	0.3 ± 0.2*)	18.5 ± 3.7*)	60
inoculated standard medium after incubation	0.008	0	34.0 ± 2.6*)	—

*) average with standard deviation of the average.

In this experiment and in all other experiments with this nutrient solution the Endophyte Concentration decreased in a more or less significant way during the incubation period. The same results were obtained when the pH was changed by replacing the mixture of phosphates either by 0.05 % K_2HPO_4 or 0.05 % KH_2PO_4, when the glucose was replaced by mannite, when the peptone and/or the yeast autolysate were omitted, or when these additions were replaced by mixtures of the most important growth factors (per 10 ml nutrient solution 0.1 ml of a solution in 500 ml water of 50 mgr m.inosite, 10 mgr Ca.panthothenate, 10 mgr thiamin, 10 mgr nicotinic acid, 10 mgr pyridoxin, 2 mgr p.amino-benzoic acid, 25 μgr biotin, in some experiments with the addition of 0.25 μgr vitamin B 12 per 10 ml nutrient solution), amino acids (per 10 ml nutrient solution 2 mgr of each of the following amino acids: alanine, arginine, cystine, glycine, histidine, leucine, iso-leucine, nor-leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine and 4 mgr of asparagine and glutamic acid), and nucleic acid components (per 10 ml nutrient solution 0.1 ml of a solution in 100 ml water of 30 mgr adenosine, 20 mgr adenine, 50 mgr hypoxanthine, 10 mgr 'guanosine, 50 mgr uracil, 5 mgr thymine) and all groups of nutrilites together. In other experiments additions of 0.1 mgr and 1 mgr of indole-acetic acid, vitamin D 2, vitamin D 3, oestron, ergosterol, or cholesterol, or of 10 or 50 mgr oleic acid (or 0.2–10 mgr Tween 80), to 10 ml of the complete standard medium had no effect. It did not make any difference whether the standard medium was sterilized by autoclaving or by filtration through a Jena G5 filter (the water soluble nutrilites were all sterilized by filtration). Experiments with some other media like malt extract too gave a decrease of the endophyte concentration.

All these experiments demonstrate with certainty that the failures to isolate the endophyte on the media used were not caused by a too faint development of the endophyte. It is evident that no development at all does occur. This failure is not caused by a deficiency of one of the well-known growth substances, though we have to reckon with the possibility that the growth-promoting effect of one substance was antagonized by the growth-inhibiting effect of another growth substance as was observed in some other organisms (e.g. McNUTT, 1952; NORKRANS, 1950).

b. EXPERIMENTS WITH SOIL SUSPENSIONS

Most experiments were performed with a peat soil from the alder grove where I mostly sampled the nodules. Suspensions were prepared by mixing ± equal parts of peat and water in a "MSE Atomix" Blendor. The resulting suspension was distributed over broad test tubes (10 ml per tube) and sterilized. The normal period of sterilization (½ hour at 120°.) appeared to be insufficient. A reliable sterilization is only obtained by autoclaving during 1 hour at 130° C.

In one of the first experiments an indication was obtained that the endophyte had developed in this peat suspension. In two transfers a

further multiplication seemed evident. However, this experiment was performed at a moment when the method of the determination of the endophyte concentration was not yet fully developed and in consequence this experiment could not be considered to be convincing. On the other hand the results of this experiment stimulated further research in this direction. Such stimulation was indeed very necessary as all subsequent experiments with peat suspensions yielded completely negative results. In spite of several time consuming attempts to obtain better results by changing the way of preparation, time and temperature of sterilization, concentration of the suspension, addition of glucose, salts and nutrilites, the effect of eventual contaminating organisms prior to or during the incubation period, the place of sampling the peat, and so on, the early experiment could not be repeated. In the winter of 1953, however, I obtained quite unexpectedly a new peat suspension, in which the growth of the endophyte could be demonstrated in a convincing way. The result of this experiment is given in table 2. The "good" peat suspension "A" is compared with an unusable peat suspension "B".

TABLE 2

Growth experiments in peat suspensions

45 pure nodules were crushed in 5 ml sterile water. One drop of this suspension (0.08 ml) was inoculated into tubes with 10 ml peat suspensions.

Inoculation matter	Dilution	y	A	EC
before incubation	0.45	0.3 ± 0.2	20.8 ± 3.8	10
	0.045	0	20.8 ± 3.8	—
after incubation in:				
peat suspension "A"	0.023	49.7 ± 5.8	49.6 ± 7.9	max.
	0.0023	20.6 ± 2.2	49.6 ± 7.9	9000
peat suspension "B"	0.023	0	49.6 ± 7.9	—
	0.0023	0	49.6 ± 7.9	—

This is the first experiment demonstrating that the endophyte from the root nodules of alder is able to multiply outside the host plant in vitro. Herewith, however, the problem is not solved at all. A peat suspension is a very complex medium. The remarkable result is obtained, that one peat suspension enables the growth of the endophyte in such a convincing way, while in the other peat suspension no growth at all is observed. Both peat suspensions were prepared from the same peat soil in the same way though the two samples were collected at different times of the year. All peat samples that were sampled later on were again unusable as media for the endophyte. It is obvious that the results depend on certain accidental properties of the peat samples, that can not be controled for the time being. Alas the situation is such that the "usable" peat samples are far in the minority as compared with the "unusable" peat samples.

As I still possessed a small number of tubes with the "usable" peat "A" some other experiments could be performed. First of all I wished to get an impression of the cause of the differences between

the peats. It might be that the usable peat contains a growth-promoting substance that is absent in the unusable peats. Another possibility is that the unusable peat contains a growth-inhibiting substance that is absent in the usable peat. Therefore an experiment was made in which the growth in each of the two suspensions was compared with the growth in a mixture of equal parts of both suspensions. The growth in the mixture was compared with the growth in each of the peat suspensions after dilution with equal parts of water. This was done to exclude the possibility that the necessary dilution of the eventual growth-promoting or growth-inhibiting substances at the mixing of the two suspensions might influence the results. The results are given in table 3.

TABLE 3

Growth experiments in mixtures of peat suspensions

10 pure nodules were crushed in 5 ml sterile water. One drop of this suspension (0.08 ml) was inoculated into tubes with peat suspensions.

Inoculation matter	Dilution	y	A	EC
before incubation	0.008	0.3 ± 0.2	18.5 ± 3.7	60
after incubation in:				
5 ml peat "A" with 5 ml water	0.008	24.0 ± 2.6	34.0 ± 2.6	12000
5 ml peat "B" with 5 ml water	0.008	0	34.0 ± 2.6	—
5 ml peat "A" with 5 ml peat "B"	0.008	0.6 ± 0.4	34.0 ± 2.6	150

Though the amount of endophyte in the mixture did not decrease no significant growth was observed in the mixture in contrast with the highly significant growth in peat "A" alone. It is evident that the unusable peat contains a growth-inhibiting substance(s). Though the jars that were inoculated with the incubated mixture do contain some nodules, while the jars that are inoculated with the incubated peat "B" do not contain nodules, the difference is too small to conclude that the peat "A" contains growth-promoting substances. However, this conclusion is justified if we consider the result of the next experiment.

The question was asked whether the development of the endophyte in the standard medium could be made possible after the addition of extracts of the usable peat "A". For this purpose the peat "A" was repeatedly extracted with boiling water and centrifuged in the multispeed attachment of an International Centrifuge at ± 17000 g. The sediment was extracted with alcohol 96 % in an extraction apparatus of Haanen and Badum through a Jena 55G3 filter. By centrifugation the alcoholic extract was clarified and the residu that remained after both extractions was resuspended. All three fractions were added in amounts equivalent to 10 ml of the original suspension to 10 ml standard medium (of course the alcohol and the excess water were evaporated). These media were inoculated and incubated. The results are given in table 4.

TABLE 4

Effect of peat extracts on the growth of the endophyte in the standard medium.
10 pure nodules were crushed in 5 ml sterile water. One drop of this suspension (0.08 ml) was added to the tubes with nutrient solution.

Inoculation matter	Dilution	y	A	EC
before incubation	0.008	0.3 ± 0.2	18.5 ± 3.7	60
after incubation in:				
standard medium with water extract peat "A"	0.008	0	34.0 ± 2.6	—
standard medium with alcoholic extract peat "A"	0.008	11.7 ± 2.5	34.0 ± 2.6	4000
standard medium with residu peat "A"	0.008	0.2 ± 0.1	34.0 ± 2.6	60

While the additions of the water extract and the residu do not enable the growth on the standard nutrient solution, an addition of the alcoholic extract enables the growth in a convincing way. We may conclude that the addition of this alcoholic extract of the peat "A" that was prepared after a previous water extraction, enables the growth of the endophyte on a nutrient solution, which contains peptone and yeast autolysate and that will probably contain most of the ordinary nutrilites. For the growth on this medium the presence of an alcohol-soluble substance (or substances) appears to be indispensable.

Alas our experiments with the usable peat "A" had to be stopped as the available quantity was used up and I did not succeed in sampling a new quantity of usable peat! As it had been shown, that the unusable peats contain growth-inhibiting substances it might be possible that these peats contain yet the growth-promoting substance though the effect of this latter substance is masked by the simultaneous presence of the growth-inhibiting substances. It might be possible that an alcoholic extract, prepared after a previous water extraction, might contain the growth-promoting substance while the growth-inhibiting substance(s) were removed by the water extraction or remained in the residu. The results that were obtained with alcoholic extracts of unusable peat were negative. This means that either the growth-promoting substance is absent from the unusable peat or that the growth-inhibiting substance is alcohol soluble as well. Our conclusion from this part of our work can be that the growth of the endophyte in vitro is possible if an alcohol-soluble substance is present. This substance is either not universally present or its effect is antagonized by the presence of growth-inhibiting substances.

c. Experiments with Suspensions and Extracts of Alder Roots

Simultaneously with the experiments using soil suspensions, experiments were performed with suspensions and extracts of alder roots. Special attention was given to roots of plants that had grown on nitrogen-free nutrient solutions during the last weeks before suspension

or extraction. This was done because we knew that the endophyte only develops in roots (by root nodule formation) if no nitrogenous substances are present in the nutrient solution. It might be that the substances that are necessary for the development of the endophyte are only present in nitrogen-poor roots. In the beginning I used suspensions of roots prepared in the blendor that were sterilized by autoclaving. To be certain that the sterilization by autoclaving does not destroy growth-promoting substances or does not produce toxic substances I made another experiment in which roots were used, that had been cultivated under sterile conditions, according to the method described in the first communication of this series. The plants were cultivated till they were 10 weeks old, while part of them had been cultivated on nitrogen-free solutions during the last three weeks. In a room, that had been sterilized by UV radiation, the roots were crushed in sterile water with sterile sand. In some experiments the roots were suspended in Hoagland solution. Each root system was suspended in 10 ml water or nutrient solution so that every tube with suspension contained an amount of suspension that was equivalent to one root system. Before the inoculation the tubes with the root suspensions were incubated for a week at 27° C to control the sterility. The sterility after this incubation was controled by inoculating a drop of the suspension on peptone-glucose agar. The tubes that were sterile were inoculated in the usual way and incubated. The results are shown in table 5.

TABLE 5

Growth experiments with root suspensions

20 pure nodules were crushed in 10 ml sterile water. One drop of this suspension (0.08 ml) was added to tubes with root suspensions.

Inoculation matter	Dilution	y	A	EC
before incubation	0.08	0.8 ± 0.2	43.1 ± 6.5	10
	0.008	0.2 ± 0.1	43.1 ± 6.5	25
after incubation in:				
suspension of crushed nitrogen-poor roots in water	0.016	0.1 ± 0.1	40.0 ± 9.6	< 10
	0.0016	0	40.0 ± 9.6	—
suspension of crushed nitrogen-poor roots in Hoagland solution	0.016	0	40.0 ± 9.6	—
suspension of crushed nitrogen-rich roots in water	0.016	0	40.0 ± 9.6	—
	0.0016	0	40.0 ± 9.6	—

In the root suspensions no development of the endophyte occurs. Further experiments with these suspensions were not performed as all attention then was focussed on the more promosing experiments with peat suspensions. After I had found that the usable peat suspensions contained a growth-promoting substance that could be extracted with alcohol after a previous extraction with water, the

question arose, whether the same procedure might give extracts of alder roots that possessed this growth-promoting effect. If this substance is indeed essential for the development of the endophyte the substance must be present in the roots of nitrogen-poor alder plants, where the endophyte does develop in nature.

A great number of extracts from alder roots were prepared. In all experiments roots were used from plants that had been grown on a nitrogen-free nutrient solution for at least three weeks before the extraction. The roots were grinded with sand, extracted with boiling water, the residu was dried in vacuo at 55° C and extracted with alcohol 96 %. The alcohol was evaporated till a small drop of water with suspended particles remained and this suspension was brought into tubes with standard nutrient medium. Inoculation and incubation occurred in the usual way. The first experiment of this kind (15 X 53) gave a very promising result. Without any doubt the extract used in this experiment contained a growth-promoting substance. However, all subsequent experiments performed during 1954 gave completely negative results, while only at the end of 1954 again extracts were obtained that enabled the growth of the endophyte in a standard nutrient solution.

TABLE 6

Effect of root extracts on the growth of the endophyte

All experiments were inoculated with one drop (0.08 ml) of a suspension of nodules containing 2 pure nodules per 1 ml. These drops were inoculated in tubes with standard nutrient solution provided with different amounts of alcoholic extracts from nitrogen-poor alder roots. In these extracts 1 ml extract was equivalent to 1 root system of the 10 weeks old plants. The figures represent the total number of nodules formed in the 12 alder plants that were inoculated with ½ ml per jar of the incubated nutrient solutions.

Date of experiment	Number of root extract	Number of nodules formed after inoculation with incubated nutrient solutions that contain per 10 ml solution:				
		0 ml	0.1 ml	1 ml	10 ml	Root extract
15 X 53	1053/1	0	21	173	—	
29 XII 53	1253/1	0	0	0	0	
28 V 54	454/2	0	0	0	—	
	1253/1	0	0	0	—	
28 VI 54	654/1	0	—	0	—	
28 IX 54	954/1	0	—	0	—	
	954/2	0	—	1	—	
	954/3	0	—	18	—	
28 I 54	954/3	0	—	8	—	
	155/10	0	—	31	—	
	155/11	0	—	74	—	
26 III 55	155/11	0	0	4	17	

Some of these experiments are summarized in table 6. In these experiments my only intention was to examine whether the addition of alcoholic root extracts promoted the growth of the endophyte on the standard solution. Therefore it was not necessary to determine the EC value, as the determination of the number of nodules formed

already enabled the necessary comparison between the nutrient solution with and without extracts. In table 6 the figures represent the total number of nodules in the 12 plants that were inoculated with the incubated nutrient solutions. As the maximum number of nodules were not determined the different experiments are not mutually comparable. This table only shows which extracts had a growth-promoting effect and which did have no effect at all. Only in one experiment (28 IX 54) the EC values were determined. Before the incubation the EC value was 3–4, after the incubation the EC value was 370, so that the general conclusions that certain alcoholic extracts enabled the growth on the standard medium seem warranted.

It will be evident from the results of these experiments that at least some of the root extracts contain a growth-promoting substance. The presence of the growth-promoting effect could be demonstrated in a reproducible way. In some other extracts this effect was completely absent and the demonstration of this absence was reproducible too. In two experiments with usable extracts (15 X 53 and 26 III 55) a definite relation was found between the development of the endophyte and the amount of root extracts that was added to the nutrient solution.

Here the same difficulty is encountered as was encountered in the gase of the soil suspensions. The success of the preparation of the crowth-promoting extracts depends on certain till now unknown factors. One factor might be the preparation of the extracts. In this respect the comparison betweeen the extracts used in the experiment of 28 IX 54 is very instructive. All extracts were prepared by alcoholic extraction of the vacuum-dried roots. The extract 954/1 was prepared without a previous water extraction, the extract 954/2 was prepared after one extraction with boiling water, while the extract 954/3 was prepared after two extractions with boiling water. This shows that a thorough extraction with water before the alcoholic extraction may be very important. This, however, can not be the main reason for the differences between the extracts as in all other experiments the roots were extracted with alcohol after repeated extraction with water. The physiological state of the roots will certainly be of importance. Experiments are in progress to find the conditions for the reproducible extraction of the growth-promoting substance(s).

The similarity between the root extracts and the peat suspensions appears to be still more evident. Just as the differences between the usable and the unusable peat suspensions were at least partially attributable to the presence of toxic substances in the unusable peats, the unusable root extracts appear to contain a toxic factor too. There is no need to be too astonished about this similarity because the peats that were used in the experiments with peat suspensions were collected in an alder grove and consisted of little more than decayed alder roots. The effect of the growth-inhibiting substances is shown by some experiments that are summarized in table 7. No EC values were calculated here either; the figures represent the total number of

nodules in the 12 plants that were inoculated with the incubated nutrient media.

TABLE 7

Demonstration of the presence of growth inhibiting substances in alcoholic root extracts

All experiments were inoculated with one drop (0.08 ml) of a suspension of nodules containing 2 pure nodules per 1 ml. These drops were inoculated into tubes with 10 ml standard nutrient solution provided with 1 ml of the growth promoting extracts 155/11, 155/14, and 954/3 respectively with or without 1 ml of the extract 954/1. The figures represent the total number of nodules formed in the 12 alder plants that where inoculated with $\frac{1}{2}$ ml per jar of the incubated nutrient solutions.

Number of the growth promoting extract added to the nutrient solution	Total number of nodules formed	
	Without other extracts	Together with extract 954/1
155/11	8	0
155/14	11	0
954/3	5	0

d. Some critical remarks as to the reliability of the results

In chapter II e some critical remarks were made as to the reliability of the methods used in these experiments. Now we have to ask whether the results that were obtained are reliable.

It was emphasized that the results might be influenced by other factors than the inoculation density. Though this difficulty is avoided by the determination of the maximum number of nodules, this method is insufficient in those experiments where the presence of substances from the incubated nutrient media might have influenced the number of nodules formed, as these substances were not added to the jars in which the maximum number of nodules was determined. Therefore some control experiments had to be made to demonstrate whether or not substances from the incubated media could have influenced our results. In table 8 some results are given that show that substances from the nutrient solutions, nor substances from the alcoholic root extracts, might have influenced the results. The small differences in the maximum number of nodules with or without these nutrient media (in the concentration used in the experiments) are not significant.

Another difficulty was that the maximum number of nodules was determined by dense inoculations with nodule suspensions. These nodule suspensions might contain substances that influence the number of nodules. Then we might expect that the number of nodules which could be produced after inoculations with incubated nutrient media exceeded the maximum number of nodules which was determined by heavy inoculations with nodule suspensions. An examination of the tables (e.g. table 2) shows, that such differences were not observed. The only indication that the determination of the maximum nodulation after inoculation with nodule suspensions might give too low values was obtained in a recent experiment where this maximum nodulation

was determined as 9.2 ± 2.1 while an inoculation with an incubated nutrient medium gave 17.8 ± 5.6 nodules per plant. Even this difference, however, can not be regarded to be significant.

There is no reason to doubt the validity of our conclusions.

TABLE 8

The influence of nutrient solutions and root extracts on the determination of the maximum number of nodules

All jars were inoculated with the same amount of a suspension of crushed nodules in water. A control series of jars was inoculated with half this amount to control whether the maximum number of nodules indeed was approximated. The additions were only added during the week after the inoculation of the jars together with the inoculation matter.

Additions	Maximum number of nodules
no additions	55.7 ± 8.2
+ 0.5 ml standard nutrient solution	38.6 ± 4.4
+ 0.5 ml standard nutrient solution containing root extract 155/14	38.8 ± 4.4
+ 0.5 ml standard nutrient solution containing root extract 954/3	55.4 ± 7.1

IV. DISCUSSION

The endophyte of the root nodules of alder has never been isolated and cultivated in vitro. All efforts to isolate this organism from the root nodules have failed. In the first communication of this series I showed that this failure could not have been caused by the fact that the nodules that were used for the isolation did not contain the endophyte in a vital state. Moreover it was shown that there was no reason to suppose that one of the organisms that had been isolated was identical with the endophyte but that this identity could not be proved because the endophyte had lost its virulence after the isolation. On the other hand we have taken into account the possibility that the methods used were too inaccurate to observe a very faint development of the endophyte on nutrient media. It seemed to be very important to develop a method that enables the measurement of the growth of the endophyte in a far more accurate way than was hitherto possible. Such a method might give an answer to the question whether no growth at all occurred on the usual nutrient media or whether a small development did occur that, however, could not be observed by the usual criteria of e.g. colony formation. In the second communication of this series I showed that a definite relation exists between the inoculation density and the number of nodules that is formed after inoculation. The well-known Mitscherlich relation appeared to be the best description for this relation. Now a method could be developed to determine the amount of endophyte in a suspension of nodules or an inoculated nutrient solution by counting the number of nodules that is formed on the roots of alder plants after inoculation with known amounts of this suspension. Though this method is still rather inaccurate it enables the determination of

an eventual growth of the endophyte in a more accurate way than was possible by the usual methods of colony formation, turbidity measurements and so on. An increase of the amount of endophyte by 10 times (an average number of cell divisions of 2–3) could be measured with certainty. As in the first communication a method was described to prepare pure suspensions of the endophyte by a method of selective incubation experiments could be performed under sterile conditions.

It was shown that even this more sensitive method did not enable us to determine a development of the endophyte in some usual nutrient media. On the contrary in most cases the amount of the endophyte that was present after the inoculation decreased during the incubation period. The addition of the most important growth-substances (vitamins, amino acids, nucleic acid components) did not have any effect. Very irregular results were obtained in peat suspensions, where in most cases no development could be observed. In some suspensions, however, a definite development was measured. Herewith the ability of the endophyte to develop in vitro was established. The difference between the usable peat suspensions appeared to be caused by the presence of a toxic substance(s) in the unusable peat suspensions. On the other hand the usable peat suspension contained a growth-promoting substance that could be extracted with alcohol. An addition of this alcoholic extract, that was prepared after a previous water extraction, to a nutrient solution (glucose-peptone-salts-yeast autolysate) enables the growth of the endophyte in this nutrient solution.

While I could not observe the development of the endophyte in suspensions of alder roots I succeeded in making alcoholic extracts of alder roots that contain the growth-promoting factor. Here, too, some extracts have growth-promoting properties, while other extracts have no effect. Just as in the case of the peat suspensions it was shown that these unusable extracts contain growth-inhibiting substances. The failures to isolate the endophyte up till now appear to have been partially caused by the fact, that the endophyte requires a special growth-substance(s) which either is not universally present or, where present, is masked by the presence of growth-inhibiting substances.

Though we have to be glad that at least some indications could be obtained as to the growth and the growth-requirements of the endophyte in vitro we must realize that these results can only serve as a first orientation and that they are only important in so far as they open up possibilities for further research. These possibilities for further research can be outlined in three different directions:

1. One of the first tasks still is the isolation of the endophyte. Though the method used in these experiments enables us to study the growth requirements of the endophyte, the method is time consuming. A rapid increase of our knowledge of the physiology of the endophyte can only be achieved when we possess visible cultures of the endophyte in which the growth can be studied in experiments of

shorter duration. Now it has been shown that the growth of the endophyte in vitro is possible, one of the first tasks will be the further improvement of the nutrient media, till we obtain so abundant a growth of the endophyte that this growth can be observed in a direct way.

2. Experiments have to be performed about the chemical nature of the growth-promoting and growth-inhibiting substances that were present in the peat and root extracts. The greatest difficulty still is formed by the fact that I still do not possess a procedure to prepare growth-promoting extracts in a reproducible way. All attention has to be focussed upon the standardisation of the cultivation of the plants and the extraction procedure of their roots so that in the future the progress of our experiments need not be delayed by lack of growth-promoting extracts.

3. One of the most important aspects of the study of symbiotic relations is the activity of growth-promoting and growth-inhibiting substances. It would be premature to suggest that the growth-promoting and growth-inhibiting substances that were present in the root extracts play a role in the symbiosis. As they are found in the roots of alder, these substances deserve all attention for the further study of the symbiotic relations.

SUMMARY

1. A method is described that enables the determination of the amount of endophyte in a suspension of root nodules or an inoculated nutrient solution in a semi-quantitative way.

2. This method is used for the study of the development of the endophyte in several nutrient solutions.

3. The usual nutrient solutions do not enable any development of the endophyte. The development is not promoted by the addition of the well known growth substances, amino acids and nucleic acid components.

4. While most soil- and peat suspensions do not enable the development of the endophyte, in some peat suspensions a definite development was measured.

5. The difference between the usable and the unusable peat suspensions is caused by the presence of growth-inhibiting substances in the unusable peat.

6. Alcoholic extracts of the usable peat, prepared after a previous water extraction, contain a growth-promoting factor that enables the growth of the endophyte on the usual nutrient solution.

7. The endophyte does not grow in suspensions of alder roots.

8. Alcoholic extracts of nitrogen-poor alder roots, prepared after a thorough water extraction sometimes contain the growth-promoting substances, while other extracts contain growth-inhibiting substances.

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