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SYMBIOTIC NITROGEN FIXATION IN NON-LEGUMINOUS PLANTS:

V. THE GROWTH REQUIREMENTS OF THE ENDOPHYTE OF ALNUS GLUTINOSA

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INTRODUCTION

Interest in symbiotic nitrogen fixation by non-leguminous plants has increased during recent years (we refer to the recent reviews of ALLEN and ALLEN 1959 and BOND 1958, 1959). The experiments done by Bond demonstrated the fixation of elementary nitrogen in root nodules of many non-legumes. It is, however, still impossible to cultivate the micro-organisms which are responsible for the formation of these nodules, even though they can be easily observed as endophytes in the nodular tissues. Many claims have been made that the endophyte of *Alnus glutinosa* had been isolated, but none of these claims has been able to stand the test of modern criticism. The only exception seems to be a very recent claim by POMMER (1959) that he had isolated an Actinomycete-like organism on plain glucose-asparagine-agar which was able to produce nodules on roots of *Alnus* plants under aseptic conditions. This claim by Pommer is so in contradiction to the experience of so many other investigators that his results are very difficult to reconcile with those of other experiments. We will try to do so after describing our own experiments.

These experiments were based on the idea that a reproducible isolation of the endophyte of *Alnus glutinosa* or those of other non-leguminous root nodules will only be possible when the growth requirements of these endophytes are sufficiently known. One of the previous communications of this series (1956) described a method which makes it possible to determine the increase of the number of infective particles in a nutrient solution during incubation after inoculation with a small amount of a suspension of "pure" nodules. It was then possible to determine the increase in the number of infective particles during a three-week period of incubation at 27° C. in certain peat suspensions, although no increase could be observed in other peat suspensions. It was shown that this difference was due to the presence of growth-inhibiting substances in the latter peat

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suspension and that the increase of the endophyte in the former peat suspension was caused by the presence of an alcohol-soluble growth-promoting substance (or substances). Addition of an alcoholic extract from these peats to a solution of glucose, peptone, yeast autolysate, and salts gave a small development of the endophyte in these solutions as measured by the increase of the infective capacity during incubation. The same result was obtained after addition of an alcoholic extract of N-deficient alder roots. Without these extracts or with water extracts of peats or roots no development was observed.

However, in all these experiments the results appeared to be badly reproducible. This was partly caused by the presence of growth-inhibiting substances in some alcoholic root extracts, but further experiments showed that other causes were responsible as well. Theoretically, three types of factors may be responsible for this unreproducibility:

1. differences in the composition of the basal nutrient solution,
2. differences in the composition of the alcoholic root extracts,
3. differences in the endophyte content and the chemical composition of the nodules used for the inoculation.

It took a long time to unravel the causes of the unreproducibility since so many causes were simultaneously operative that few experiments gave reliable information. However, this information was not only important for elucidation of the causes of the unreproducibility but also for a better understanding of the growth requirements of the endophyte.

In the following article we will give a survey of some crucial experiments which demonstrate the role of growth-promoting and growth-inhibiting substances and the first efforts to identify the chemical nature of the growth-promoting substance in the alcoholic root extracts.

Methods

The methods used for the determination of the growth of the endophyte in vitro were essentially the same as those previously used (Quispel 1954, 1956). We will therefore restrict ourselves to the description of the main principles and certain improvements in the method.

a. Preparation of the inoculation

Inoculation was effected by a suspension of nodules which had been purified by the method of selective incubation. During the selection, most of the nodules had to be discarded because they were infected by contaminating organisms. This number could be considerably reduced by a second disinfection of the nodules after the removal of the outer cell layers. The second disinfection did not affect the vitality of the endophyte inside the nodules. The present procedure is as follows: nodules are sampled in the field, washed with water, soap and alcohol and, after rinsing with water, disinfected

with a bromium solution (0.1 % v/v) for 5 minutes. After four successive washings with sterile water the outer cell layers are removed with a sterile scalpel. The peeled nodules are collected in a petri dish containing sterile water and finally again disinfected with bromium for a few minutes. The nodules then are washed with four successive portions of sterile water and each nodule is put in a tube with glucose-peptone-yeast autolysate-agar. After six weeks incubation at 27° C. the infected nodules are discarded and the nodules which on careful examination appear to be sterile are crushed in sterile water. In all experiments, 10 nodules were crushed in 5 ml sterile water with a sterilized glass rod in a thick-walled culture tube. After crushing, the suspension was allowed to stand for a few minutes so that the heavier particles could sink to the bottom of the tube. The supernatant was then decanted and used for the inoculation of the culture media. One drop of this suspension (0,05 ml) was inoculated with a sterile pipette into 10 ml nutrient solution.

b. Nutrient solutions

The composition of the basal nutrient solution is: water 1000 ml, K_2HPO_4 300 mgm, NaH_2PO_4 200 mgm, $MgSO_4.7H_2O$ 200 mgm, KCl 200 mgm, $CaCO_3$ 2 gm, glucose 20 gm, peptone Fleisch Merck 5 gm. In the first experiments, NH_4NO_3 500 mgm and 10 ml yeast autolysate were added but these additions were later omitted. All chemicals were ANALAR quality in double glass-distilled water. Culture tubes of 16 mm diameter were provided with 10 ml of these nutrient solutions and sterilized by autoclaving for ½ hour at 120° C. Where filtration was used for sterilization a Jena G5f or a Seitz filter was employed. In this case the salts and the alcoholic extracts (see below) were dissolved in 5 ml water and sterilized by autoclaving while the glucose and peptone were added in 5 ml portions after filtration.

c. Preparation of the alcoholic root extracts

If not otherwise specified, the alcoholic root extracts were prepared from roots of 14 week old *Alnus* plants which had been cultivated on the normal HOAGLAND nutrient solutions during the first 8 weeks and on a N-free nutrient solution during the last 5–6 weeks (nitrates replaced by equivalent amounts of the chlorides). The roots were separated from the shoots, washed, dried in vacuo at 55° C., powdered in a mill, and stored as a dry powder.

Before the alcoholic extraction the powder was repeatedly extracted by boiling with water and centrifugation of the extract at 12000 g. This procedure was repeated till the supernatant was only faintly coloured. The extracted sediment was again dried in vacuo and then extracted with 95 % alcohol. This alcoholic extract was diluted in such a way that 1 ml corresponded to the equivalent of the dry matter of one root system ($\pm$ 500 mgm).

A 1 ml. aliquot of this extract was added to 10 ml nutrient solution. Because the alcohol must be completely removed and the dissolved

substances finely dispersed in the nutrient solution, the extracts were added in the following way: 1 ml extract was pipetted into a tube with a few drops of water. The tube was put into a boiling water-bath till all the alcohol was evaporated and the dissolved lipids formed a cloudy suspension in the remaining drops of water. The nutrient solution was then added to the tubes. Since the insoluble residue cannot be filtrated, this residue of the extract was always sterilized by autoclaving together with the salt solution.

d. Purification and fractionation of the alcoholic extracts

1. *Preparation of the petroleum ether fraction*

The alcoholic extract was evaporated in vacuo and dried. The dry residue was repeatedly extracted with petroleum ether (boiling point 40–60° C.), and the petroleum ether solutions filtered and combined. The filters were washed with petroleum ether and this petroleum ether added to the other petroleum ether extracts. The petroleum ether solution was evaporated at 55° C. in vacuo and the residue taken up in 96 % alcohol and brought back to the original volume.

2. *Separation of the acid from the non-acid lipid fractions*

A 10 ml aliquot of extract was evaporated in vacuo and dissolved in petroleum ether as described above. The petroleum ether solution was extracted with 10 % KOH in water in a separation funnel. The KOH layers were again extracted with petroleum ether. The separation of the layers was very difficult because a foamy precipitate formed between the two layers. This precipitate was collected in the alkaline water fraction which developed a cloudy appearance. The alkaline layer could only be cleared by extraction with diethyl ether. The diethyl ether solution was added to the petroleum ether layers. The ether-petroleum ether layers were again extracted with distilled water and the water added to the alkaline fraction. The ether-petroleum ether layers were evaporated and dissolved in 96 % alcohol and brought back to the original volume of 10 ml. This solution contained the non-acid lipids.

The alkaline water layers were acidified with HCl and extracted with petroleum ether. The remaining solution was evaporated in vacuo to dryness and the dry residue again extracted with petroleum ether. The petroleum ether solutions were added together, evaporated, and the residue dissolved in 10 ml of 96 % alcohol. This solution contained the fatty acids.

3. *Hydrolysis of the alcoholic extracts*

A 20 ml aliquot of extract was evaporated in vacuo and dissolved in 20 ml 10 % KOH in methanol. The solution was boiled under reflux for 4 hours, 5 ml water was then added, and the boiling continued for another 2 hours. The next morning the precipitation was filtered, dissolved in 6 % KOH in methanol, and boiled for 3 hours. The now clear solution was added to the filtrate, acidified, extracted

with petroleum ether as described above and finally the petroleum ether extract was transferred to 96 % alcohol.

4. *Chromatographic fractionation of the alcoholic extracts*

Chromatographic analysis of the alcoholic extracts was performed with a modification of the methods of FILLERUP and MEAD (1953) and HIRSCH and ARENS (1958). The columns were 2 cm in diameter and 7 cm in height, filled with silicagel (Light 100–200 mesh). The silicagel was purified according to Hirsch and Arens, and activated at 115° C. over night. The dry silicagel was brought into the adsorption tube, washed with petroleum ether and thoroughly mixed with the petroleum ether so that the particles sedimented in the tube. When nearly all the petroleum ether had passed the column so that the surface was nearly dry, 10 ml petroleum ether extract was brought on the column and washed away with another portion of 10 ml petroleum ether. The column was then eluted with successive portions of 100 ml of 1 % ether in petroleum ether (4 portions), 4 % ether in petroleum ether (4 portions), 10 % ether in petroleum ether (4 portions) 50 % ether in petroleum ether (3 portions), pure ether (3 portions) and methanol (2 portions). All eluates were transferred to alcohol in the usual way.

e. Incubation

Unless not otherwise specified, all inoculated tubes with nutrient solution were incubated at 27° C. for three weeks. From time to time the tubes were shaken by hand. The eventual growth of the endophyte in these tubes was determined by the determination of the infective capacity of the tubes before and after incubation.

f. Determination of the infective capacity of the tubes

The infective capacity of the nutrient solutions before and after incubation was determined by bringing known amounts of the solutions into jars with *Alnus* plants which had been transferred to a N-free nutrient solution. The details of this procedure were exactly the same as those described in the first, second and third communication of this series (1954, 1956), The total amount of endophyte in a solution was calculated in relation to the amount used in the standard experiment described in these former communications and expressed as the Endophyte Concentration (EC).

In many experiments it was sufficient to compare the growth in different nutrient solutions which were simultaneously inoculated with the same amount of one pure nodule suspension. If these nutrient solutions are incubated under exactly identical circumstances, it is sufficient to determine the amount of nodules which are formed by *Alnus* plants after inoculation with the same amount of these different inoculated and incubated nutrient solutions. These amounts must be chosen in such a way, that, if no growth of the endophyte occurred during incubation, practically no nodules are formed on the roots of the plants. On the other hand, the amounts used for the inoculation

of these plants must be so large that a considerable number of nodules are formed if some multiplication of the endophyte occured during the incubation period. According to our experience, the following procedure always gave reliable results. The incubated nutrient solution, inoculated with the amounts described above, was diluted to 100 ml, and from this dilution 5 ml was added to jars with 350 ml N-free nutrient solution and three 7 week-old *Alnus* plants per jar. If no growth occurred during incubation the number of nodules counted on these plants never exceeded 1 or 2. If growth of the endophyte occurred during the incubation period, the number of nodules on the plants varied between 5 and 50. In most of the Tables this number of nodules is used as a direct indication for the growth in vitro of the endophyte during incubation.

g. Control of sterility

It appeared that the faint growth in vitro of the endophyte which can be determined with this sensitive method is very susceptible to the presence of contaminating organisms. All tubes with inoculated and incubated nutrient solutions were tested for the presence of contaminating organisms by the inoculation of tubes with peptone-glucose-yeast autolysate-agar, where the endophyte itself does not show any development. All tubes which appeared to contain other organisms were discarded.

Results

a. The composition of the basal nutrient solution

1. *The presence of yeast autolysate*

One of the possible causes of unreproducibility might have been the yeast autolysate with its unknown, complex, and uncertain composition. It appeared that the addition of yeast autolysate could be omitted for at least the first inoculation. Moreover, it was shown by some experiments that sometimes yeast autolysate may exert a growth-inhibiting effect. This is apparent from the results of the experiment described in Table 1.

Table 1

Influence of peptone and yeast autolysate on the growth in vitro of the endophyte.

The nutrient solutions contained the usual salts, glucose, and an alcoholic root extract. The solutions differed as to the presence of peptone and yeast autolysate. The incubated nutrient solutions were used to inoculate 4 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

concentration of peptone	concentration of yeast autolysate	
	0	1 ml %
0	0.2	0
0.5 %	17.8	0.1

Although in other experiments the inhibiting influence of yeast autolysate was not as pronounced as in this one, it was considered advisable to omit the yeast autolysate in the nutrient solution of future experiments.

2. *The necessity of peptone*

The results shown in Table 1, suggest that another conclusion may be drawn because peptone appears to be an indispensable ingredient of the nutrient solution. Since there are important differences between different samples of peptone, the addition of peptone was standardized by always using the same type and quality (peptone Fleisch Merck). This of course does not mean that other samples would give less consistent results.

In the contradictory experiments done by Pommer, asparagin was used instead of peptone. It was therefore interesting to compare the effect of asparagin with the effect of peptone. This was done in the experiment summarized in Table 2.

TABLE 2

Influence of peptone and asparagin on the growth in vitro of the endophyte.

The nutrient solutions contained the usual salts, omitting NH_4NO_3. Glucose and peptone or asparagin were sterilized by filtration. Half of the solutions contained an alcoholic root extract purified with petroleum ether.

The experiment was performed in duplicate. The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Concentration of peptone	Concentration of asparagin	alc. root extr.	Number of nodules in series A	Number of nodules in series B
0.5 %	0	0	0.2	0
0.5 %	0	0.1 ml %	10.2	6.7
0	0.1 %	0	0	0.2
0	0.1 %	0.1 ml %	0.3	0.8

It is evident that asparagin cannot replace peptone. As in all the other experiments, an indication for growth in vitro was only obtained when the alcoholic root extract was added to the solutions.

3. *The source of carbon*

In the basal nutrient solution glucose was used as a source of carbon. Many of the nutrient media used for the cultivation of *Actinomycetes* contain glycerol. According to cytological observations, starch is the main reserve substance in the *Alnus* roots and most probably the natural carbon source for the endophyte. In the experiment shown in Table 3 these three carbon sources are compared.

It is evident that in this experiment only starch is a suitable source of carbon. This is very remarkable, because in other experiments glucose enabled the development of the endophyte.

TABLE 3

Influence of the source of carbon on the growth in vitro of the endophyte.

The nutrient solutions contained the usual salts with peptone. The complete nutrient solution including the sources of carbon was sterilized by autoclaving. The experiment was performed in quadruple. The four series contained different alcoholic root extracts, all purified with petroleum ether.

The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Source of carbon	Number of nodules in Ser. A	Ser. B	Ser. C	Ser. D
2 % glucose	0	0	0	0
2 % glycerol	0	0	0	0
2 % starch (soluble) . .	14.2	16.8	14.5	4.7

4. *The sterilization of the nutrient solution*

The discrepancies between different experiments where glucose was used as a source of carbon might be explained by the formation of inhibiting substances during the autoclaving of glucose. It is possible that in some experiments these substances were present in such quantities that their inhibiting effect was observed, while in other experiments their presence was not injurious. In the experiment shown in Table 4, a comparison was made between solutions sterilized by autoclaving and solutions in which the glucose and peptone were sterilized by filtration.

TABLE 4

Influence of the method of sterilization of the glucose and peptone in the nutrient solution on the growth in vitro of the endophyte.

The nutrient solution contained the usual salts with glucose and peptone. The experiment was performed in duplicate with two different alcoholic root extracts purified with petroleum ether.

The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules per plant.

Method of sterilization	Number of nodules in Ser. A	Ser. B
Autoclaving	0	0
Filtration of glucose and peptone, autoclaving of salts and alc. extract.	24.8	21.8

It appears that sterilization by filtration enabled the growth of the endophyte, while growth was impossible in the solutions where the glucose and peptone had been sterilized by autoclaving. We must conclude that sterilization by autoclaving induces the formation of substances which are inhibitive to the growth of the endophyte. In subsequent experiments glucose and peptone were always sterilized by filtration.

5. *The influence of other factors*

The pH of the nutrient solution after sterilization was 6.8. By addition of NaOH or HCl, slight variations in pH were obtained. A

somewhat more acid pH (6.0) gave the same results, but a slightly alkaline pH (7.6) gave somewhat less growth.

In one experiment, omission of NH_4NO_3 had a favourable effect. Though this was never observed in later experiments no NH_4NO_3 requirement could be demonstrated and in future experiments this salt was omitted.

6. *Conclusion*

The best results were obtained with the basal nutrient solution if yeast autolysate was omitted, peptone was present, and glucose and peptone were sterilized by filtration.

b. The effect of the alcoholic root extracts

In all the nutrient solutions which were used in the experiments described above, growth of the endophyte was only observed if an alcoholic root extract was added to the nutrient solutions. The improvement of the basal nutrient solution was only effective if this extract was present; without this extract no traces of growth were ever observed. The study of this extract is certainly one of the most important aspects of the study of the growth-requirements of the endophyte.

1. *The effect of alcohol*

It is possible that the effect of the alcoholic extracts was caused by the alcohol itself. This supposition was very improbable because the alcohol was damped off in a boiling water bath, but some residual alcohol might have influenced the results. This was checked in some experiments in which a small amount of alcohol was added after sterilization to samples with and without the alcoholic extract. One of these experiments is summarized in Table 5.

TABLE 5

Influence of alcohol on the growth in vitro of the endophyte.

The nutrient solution contained the usual salts plus glucose and peptone sterilized by filtration. Half of the solutions contained an alcoholic root extract purified with petroleum ether. The alcohol was added after sterilization. The experiment was performed in duplicate. The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Concentration of alcohol	with alc. root extract		without alc. root extract	
	Ser. A	Ser. B	Ser. A	Ser. B
0	15.8	25.5	0.8	1.0
0.001 ml %	7.7	12.0	1.5	1.2
0.01 ml %	3.7	3.5	0.5	1.3

There is no indication that alcohol promotes the growth of the endophyte. This is the more evident because in nutrient media containing inhibitive concentrations of alcohol, growth was nevertheless

stimulated by the addition of the alcoholic root extract. Clearly the growth-promoting influence of the alcoholic root extract is not caused by retention of small amounts of alcohol in the nutrient solution. Small amounts of alcohol definitely inhibit growth and might have been partly responsible for the unreproducibility of some experiments. It is advisable to remove the alcohol as thoroughly and carefully as possible.

2. *The removal of inhibiting substances*

In a previous communication of this series, experiments were described which demonstrated the possible presence of toxic substances in the alcoholic root extracts. This was certainly one of the main reasons for the unreproducibility of the first experiments. Therefore it was very important to separate the growth-inhibiting substances from the growth-promoting factors. It was possible to do this very simply, by fractionation with petroleum ether. A comparison between the effects of normal and purified extracts is given in Table 6.

TABLE 6

Effect of the purification of the alcoholic root extracts with petroleum ether on their promotion of growth of the endophyte in vitro.

The nutrient solutions contained the usual salts plus glucose, peptone, and yeast autolysate, all sterilized by autoclaving.

The experiment was performed in duplicate with two different alcoholic extracts. Both extracts were added either directly or after purification by transfer to petroleum ether.

The incubated nutrient solutions were used to inoculate 4 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Fraction of extract	Number of nodules in Ser. A	Ser. B
complete extract	17.3	0
petroleum ether-soluble fraction	29.1	18.0
water soluble fraction	0.6	0

Table 6 shows that the growth-promoting substances or substance are still present after the fractionation in the petroleum ether solution. Moreover, it appears that an inactive extract may become active after this purification. Obviously the growth-inhibiting substance or substances are not soluble in petroleum ether. This observation has considerably increased the reproducibility of later experiments. Moreover, an important conclusion may be drawn: the growth-promoting substance is lipophilic. This was further confirmed by experiments where the solution in petroleum ether was extracted with water in a separation funnel without loss of activity.

3. *Further chemical fractionation of the extract*

Since it was shown that the growth-promoting substances belongs to the lipids, the next step was to find out whether the growth-promoting substances belonged to the hydrolysable lipids, to the fatty

acids, or to the neutral lipids. This was done with the usual methods of hydrolysis and extraction with alkali. The results of an experiment of this type are shown in Table 7.

TABLE 7

Effect of alkali-extraction and alkaline hydrolysis of the alcoholic root extracts on their promotion of growth of the endophyte in vitro.

The nutrient solutions contained the usual salts excepting NH_4NO_3, plus glucose and peptone sterilized by filtration, and different fractions of the alcoholic root extract.

The incubated nutrient solutions were used to inoculate 2 jars containing 2 plants each. The figures refer to the average number of nodules formed per plant.

	Normal extract	Saponified extract
unfractionated	1.3 (?)	8.7
petroleum ether layer after KOH extraction	44.2	13.9
KOH extract	0	—

For some unknown reason, in spite of purification with petroleum ether the growth in the tube with the untreated extract was very small. However, it is evident that the growth-promoting substance is resistant to hydrolysis and is not extracted with alkali either before or after hydrolysis. The growth-promoting substance therefore belongs to the non-acid, non saponifiable lipids.

4. *Chromatographic separation*

Though the efforts to isolate the growth-promoting substance are still in a very early stage, it is worthwhile to give a preliminary account of the first results.

Some older experiments have shown that the growth-promoting substance is not adsorbed to the kation exchanger Imac C 12 and the anion exchanger Imac A 17 (Activit, Amsterdam) from a 70 % alcohol solution. This is in accordance with the conclusion from Table 7, that the substance belongs to the neutral lipids.

Some preliminary experiments were performed with Aluminium-oxyde Brockmann (Merck). The substance is adsorbed to this adsorbens from petroleum ether and eluted with 50 % ether in petroleum ether.

The most extensive experiments were performed with silicagel as adsorbing substance. The result of one of these experiments is given in Table 8.

The substance is adsorbed to silicagel from the petroleum ether solution. In 1 % and 4 % ether in petroleum ether, no elution is observed. Small amounts are eluted with 10 % ether, 50 % ether, and 100 % ether, but the bulk of the substance is eluted by methanol. Most probable the results are influenced by secondary adsorption and elution, since the growth-promoting substance is certainly present together with far greater amounts of other lipids. Better results may be obtainable by chromatography after a previous chemical purification.

TABLE 8

Growth-promoting effect of eluates from a silicagel column, on which the petroleum ether solution of an alcoholic root extract had been adsorbed, on the growth of the endophyte in vitro.

The nutrient solutions contained the usual salts excepting NH_4NO_3, glucose and peptone sterilized by filtration, and equivalent amounts of the eluates. The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Eluate	Number of nodules in successive fraction 1	2	3	4	Moment of elution of some pure lipids
1 % ether/petr. ether .	0	0	0.3	0.3	carotene
4 % ether/petr. ether .	0.5	0	0.7	0	
10 % ether/petr. ether .	6.0	4.7	8.7	6.6	palmitic acid cholesterol
50 % ether/petr. ether .	12.8	6.7	5.3	—	
ether	10.8	1.5	2.8	—	
methanol	36.5	2.3	—	—	

5. *The physiological condition of the roots before the extraction*

All alcoholic extracts were prepared from roots which had been cultivated on a N. free nutrient solution for at least 5 weeks. This procedure was inspired by the suggestion that the growth-promoting substances might be most abundant in N-deficient roots because in vivo the formation of root nodules, and thus the growth of the endophyte, is promoted by N-deficiency of the roots. Some experiments were carried out to compare the growth-promoting activity of extracts prepared from roots with different degrees of N-deficiency with roots cultivated exclusively on the normal nutrient solution. It appeared that the growth-promoting substances can be extracted from all roots, while the differences between the growth-promoting effect of extracts from normal and N-deficient roots were too small to allow of any conclusions.

6. *Addition of known substances*

In the course of the experiments many efforts were made to replace the alcoholic extract by known alcohol-soluble substances. No effect was obtained with cholesterol, ergosterol, vitamin D1, vitamin D2, tocopherol, menadion, lecithin, indole acetic acid, oleic acid or olive oil.

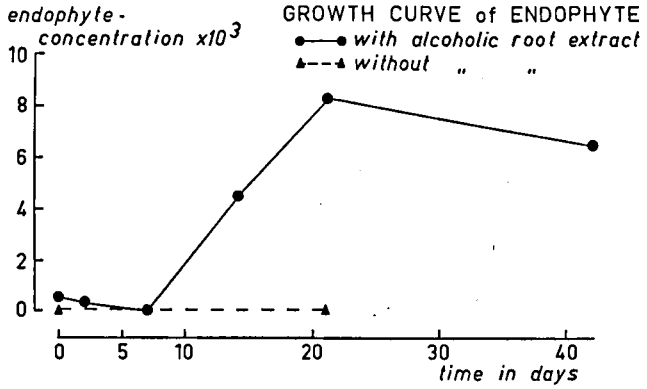
7. *Conclusion*

Reliable and reproducible growth-promoting effects are obtained by alcoholic extracts of *Alnus* roots if these alcoholic extracts are fractionated by transfer to petroleum ether. The active substance belongs to the non-acid, non-saponifiable lipids, is not adsorbed to ion-exchangers, but is adsorbed to aluminiumoxyde and silicagel. The activity could not be replaced by some known lipids.

c. The growth curve of the endophyte in vitro

In all previous experiments growth was determined after an arbitrarily chosen incubation period of three weeks. As soon as the

growth requirements were sufficiently analyzed to make reproducible experiments possible, the growth curve of the endophyte in vitro was determined in a nutrient solution without NH_4NO_3, without yeast autolysate, with the glucose and peptone sterilized by filtration, and with the petroleum ether fraction of an alcoholic root extract. A number of tubes containing this nutrient solution were simultaneously inoculated, and at intervals one of these tubes was used for the determination of the Endophyte Concentration. The results are given in Fig. 1. The same Figure gives a growth curve drawn from an experiment in which the alcoholic root extract was omitted.



In the solution without root extract, no growth was observed, as usual. In the solution containing alcoholic root extract, growth started during the second week of incubation and reached an optimum during the third week. The small decrease during the first week and after the third week may be regarded not significant. The determination of growth after three weeks' incubation appears to have been a very lucky choice.

d. The transfer from incubated nutrient solutions

In the Introduction, another possible cause of the unreproducibility of the growth experiments was mentioned: the nodules with which the tubes were inoculated. These nodules were always sampled in the field and, in consequence, must have been different from one experiment to another. There may have been differences in the content of endophyte and in the vitality of the suspension used for the inoculation. Since a certain amount of nodule material is always inoculated together with the cells of the endophyte, substances may be present that either decrease or increase the possibilities for growth in the nutrient solutions. Finally, the preparation of pure nodules by the method of selective incubation has the disadvantage that some slow-growing contaminating organisms may still be present. All these factors remain causes of unreproducibility which can only be overcome by duplicate or triplicate experiments, or by inoculations from nutrient solutions in which growth of the endophyte has been demon-

strated. Unfortunately, transfers from growing cultures into new nutrient solutions failed in nearly all cases. Only in a very few experiments could growth after transfer be demonstrated.

This failure of the transfers can be explained in several ways. It is possible that the nodule material which is inoculated together with the endophyte contains some indispensable growth substances. In that case transfers will be possible if the nutrient solutions contain endophyte-free nodule material. In a preliminary experiment which must be repeated for further evidence, an indication was obtained that this is indeed the case. In this experiment, which is summarized in Table 9, a number of tubes were each inoculated with one drop from an inoculated and incubated nutrient solution. Some of these tubes were provided with a water extract of peeled nodules, centrifuged at 16000 g., and sterilized by filtration through a Jena G5f filter. Other tubes were provided with some well-known vitamins.

TABLE 9

Growth of the endophyte after transfer from one culture to another nutrient solution.

Nutrient solutions with the usual salts excepting NH_4NO_3, with glucose and peptone sterilized by filtration, and with the petroleum ether fraction of an alcoholic root extract, were inoculated with a pure nodule suspension. After three weeks' incubation, one drop of this solution was inoculated in fresh nutrient solution with the same composition but either with or without the alcoholic root extract. Some tubes of the latter solution were provided with 1 ml of an extract from peeled root nodules (10 gram fresh weight in 100 ml water) sterilized by filtration.

Other tubes were provided with 0.1 ml per 10 ml of the following vitamin solution: 500 ml water, 50 mgm inositol, 10 mgm Ca panthothenate, 10 mgm thiamin, 10 mgm niacin, 10 mgm pyridoxin, 2 mgm p. aminobenzoic acid, 0.025 mgm biotin.

The last series of nutrient solutions was used after incubation to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

	with alc. root extract	without alc. root extract	not inoculated
normal medium	0	0	0
with nodule extract	8.8	0	0
with vitamin solution . . .	0	0	0

It is evident from the results of this experiment that growth in a nutrient solution after transfer from another culture was only possible when the second solution contained the alcoholic root extract together with the water extract of the nodules. Since the same solution inoculated with a drop of sterile nutrient solution did not give any development, it is improbable that this result was caused by the presence of vital cells off the endophyte in the filtered nodule extract. Moreover, in former experiments no indications were obtained that the endophyte can pass bacterial filters. The conclusion seems warranted that the nodules contain an indispensable, as yet unidentified, growth substance which is present in the nodule material of the first inocula-

tion but has to be added to the nutrient solution if transfers to fresh nutrient solutions are made. This substance is not identical with one of the added vitamins.

e. The direct microscopic observation of the endophyte

The time-consuming method used in the experiments described above is only necessary when the growth of the endophyte in vitro is so small that a more direct observation is impossible. In recent experiments the growth as measured by the increase of the infective capacity, was so remarkable that it was asked whether the cells of the growing endophyte could not be seen under the microscope. In a very infective culture, clusters of very thin hyphae were observed. These *Actinomycete*-like organisms have hyphae with a diameter of less than 1 μ, with a granulated appearance. In one case it was observed that these hyphae grew out of a piece of nodule tissue. Though we must be very cautious because contaminations are always possible, it is very probable that these clusters of hyphae are indeed the cells of the organisms which are responsible for the infectivity of the solution.

Discussion

When considering the results of our experiments we must thoroughly realize the special character of the method used as described in a previous communication (1956). We should realize that this method is an indirect determination of growth in which the growth of the endophyte in vitro is measured by means of the increase of the infectivity of an inoculated nutrient solution during incubation. This infectivity is measured by counting the number of nodules formed in water cultures of *Alnus glutinosa* when these cultures are inoculated with known amounts of the inoculated and incubated nutrient media. The number of nodules depends on the number of infective particles. In consequence, the method determines the increase in the number of infective particles during incubation. We must realize that no growth is observed if the endophyte loses its virulence during growth: only the infective cells are determined. Moreover, the method is essentially a method for the determination of cell divisions. A mere fragmentation of hyphae will be measured as growth, while an extension of cells without any cell divisions can not be observed.

With these restrictions in view, it appears that the experiments have again shown the absolute necessity of an alcohol-soluble substance for the growth of the endophyte. This necessity was evident in all nutrient solutions used, in no case was any trace of growth observed if no extract was present. The activity of this extract is caused by a non-acid, non-saponifiable lipid. The bad reproducibility of the first experiments appeared to have been due to the possible presence of inhibiting substances in the unpurified root extracts, in the yeast autolysate, and in the autoclaved glucose solutions. Better results were obtained if the alcoholic extracts were purified by transfer

to petroleum ether, when the yeast autolysate was omitted, and when the glucose and peptone were sterilized by filtration. Some indications were obtained for the presence of another indispensable substance in the root nodules used for the inoculation. Peptone is indispensable and can not be replaced by asparagin.

The difficulties with which so many investigators have struggled in their vain efforts to isolate the endophyte appear to have been caused by the influence of the inhibiting substances together with the need for the growth-promoting lipoid substance, and by the very slow growth rate and minimal development even when the circumstances are favourable for growth.

These results are completely contradictory to those which POMMER (1959) published after the experiments described above were finished. According to Pommer, the endophyte grows on plain glucose-asparagin-agar without further additions, though growth is stimulated by the addition of a water extract of peeled *Alnus* nodules. When pieces of nodules, disinfected with phenole, were inoculated on glucose-asparagin-agar, growth of an *Actinomycete*-like organism was observed after several days. This claim has been made by many authors, but Pommer has demonstrated the nodulating capacity of this organism in a reproducible way under aseptic conditions. The description of his experiments does not allow of any criticism, they deserve serious consideration and urgently need repetition.

On some preliminary experiments we have tried to repeat his experiments and have in some cases indeed observed organisms of the type he described, though only on media with nodule extract and after a much longer incubation period. The very thin hyphae resemble the very thin hyphae which were found in our infective nutrient solution.

There would be no difficulty in accepting Pommer's results if the growth requirements of his organism were not much simpler than those indicated by our experiments. If, indeed, his organism turns out to be the endophyte, how is it then possible that his organisms grows on plain glucose-asparagin media while our experiments have without exception demonstrated the necessity for both a lipophilic substance and peptone for even a very faint development? It seems reasonable to explain this discrepancy in terms of the difference in the methods used. Pommer directly observed growing hyphae while we measured the increase in infective capacity. He determined growth of cells while we could only observe the increase in number of infective particles. As has been stressed above, we might have overlooked growth of the endophyte if no cell-divisions had occurred. It is improbable, however, that this is the reason for the discrepancy. If clusters of hyphae grow in length, the chance for subsequent fragmentation during shaking of the suspension of these clusters will certainly increase (before inoculation of the *Alnus* cultures the tubes were heavily shaken). A more reasonable explanation may be found in the infective capacity of the growing cells. According to a personal communication from Pommer, he has observed still more rapidly growing cultures

of an *Actinomycete* which might be identical with the non-infective type of organism that has been isolated many times before. It then might be possible that the organism occurs in different forms (mutants? modifications?): a rapid-growing, non-infective form with simple growth requirements and a slow-growing, virulent form with complex growth requirements (or requirements to remain in the virulent form). In our experiments only the growth requirements of the latter form could be determined. Pommer may have isolated a kind of intermediate form with simple growth requirements but which has retained some of its virulence. Another explanation might be that his cultures consist of a symbiosis between the rapid-growing *Actinomycete* with the virulent endophyte. These are of course mere speculations, but these speculations may be fruitful working-hypotheses for attempts to reconcile Pommers' results and ours, and to stimulate further research in this field.

SUMMARY

The growth in vitro of the endophyte of *Alnus glutinosa* is studied by determining the increase of the infective capacity of inoculated nutrient solutions during incubation. Growth is only possible if an alcoholic extract from *Alnus* roots is added to the nutrient solutions. This extract may also contain inhibiting substances but these substances are removed by transfer to petroleum ether. The growth-promoting substance belongs to the non-acid, non-saponifiable lipids. The substance is not adsorbed to ion-exchange resins but is adsorbed from the petroleum ether solution by aluminiumoxyde or silicagel. The growth of the endophyte is inhibited by substances formed during autoclaving of glucose and by yeast autolysate. Peptone is indispensable and can not be replaced by asparagin. Some indications were obtained that growth is stimulated by a water-soluble factor present in *Alnus* nodules. Even under favourable conditions the growth rate is very low, maximum growth being obtained after 2–3 weeks. In an infective culture, clusters of very thin hyphae were microscopically visible.

The results are discussed with regard to the conflicting results obtained by POMMER and a working-hypothesis is suggested to reconcile the different observations.

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