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SYMBIOTIC NITROGEN-FIXATION IN NON-LEGUMINOUS PLANTS

I. PRELIMINARY EXPERIMENTS ON THE ROOT-NODULE SYMBIOSIS OF ALNUS GLUTINOSA

BY

A. QUISPEL

(*from the laboratory of general botany, plant physiology and pharmacognosy of the University of Amsterdam*)

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INTRODUCTION

Whereas symbiotic nitrogen-fixation in root-nodules of leguminous plants is the subject of a wide-spread research in the field of botany, microbiology, biochemistry and agronomy, little is known about the nitrogen-fixation of root-nodules which occur in non-leguminous plants. This is even more regrettable considering that not only are these symbiotic phenomena extremely interesting in themselves but also that it is not unlikely that a better knowledge concerning the nitrogen-fixation of root-nodules of certain non-leguminous plants might give a clearer insight into the process of nitrogen-fixation in leguminous plants. It would not be the first time in Biology that a change of subject matter with its accompanying possibilities for comparative research would open up new prospects which would never have been discovered within the limitation of the old, well-known subject matter. Up till now, however, research on the symbiotic nitrogen-fixation in non-leguminous plants has not succeeded in increasing the knowledge of symbiotic nitrogen-fixation in general. The fact that literature on the subject of nitrogen-fixation in root-nodules of non-leguminous plants is scarce is not only, and perhaps not even primarily, due to a lack of interest on the part of those working on problems concerning the nitrogen-fixation. The explanation may

be found in the difficulties which are experienced directly at the beginning of the experiments on nitrogen-fixation in non-leguminous plants.

It has proved impossible up till now to cultivate the organisms responsible for the formation of these root-nodules with certainty in vitro. Whereas a symbiosis cannot be studied profitably if one does not succeed in observing the properties of the component organisms separately, everything should be done to conquer the difficulties of isolation of these endophytes. Should this prove impossible, however, one should at least try to obtain some insight into the cause of these difficulties.

SURVEY OF LITERATURE

The initial research on symbiotic nitrogen-fixation of the root-nodules of the alder was carried out by HILTNER (1896, 1904) and HILTNER and NOBBE (1904).

Owing to the fact that the nodule-bearing alder and the sea-buckthorn can be grown in N-free nutrient solutions they had already concluded that a fixation of elementary atmospheric nitrogen occurred in these nodules.

KREBBER (1932) and ROBERG (1934) repeated these experiments, thereby convincingly demonstrating the fact that the growth of alder plants with root-nodules was excellent on N-free nutrient solutions, whereas comparable plants without nodules perish on those solutions owing to lack of nitrogen. The fact that nitrogen was indeed fixed was abundantly proved by the Kjeldahlanalyses of v. PLOTHO (1941), whereas VIRTANEN and SAASKAMOINEN (1936) even observed the secretion of nitrogenous compounds out of roots. More recently FERGUSON and BOND (1954) obtained convincing evidence that the nitrogen is fixed in the nodules of the nodulated roots.

The only objection one might raise with regard to all these experiments is that they could not be carried out in sterile conditions, so that there is a possibility, although an extremely improbable one, that the nitrogen fixation was caused by contaminating organisms e.g. Azotobacters or Clostridiums which had also been inoculated.

The microscopic-anatomical picture of the nodules is very well-known owing to the descriptions of SHIBATA (1902), SHIBATA and THAHARA (1917) and, especially, of SCHAEDE (1933). The infection itself, though experimentally established (the symbiosis is not of a cyclic kind) has never been observed microscopically. In the recently infected cells the endophyte forms clusters of thin actinomycetic hyphae which continually infect the younger cells very near the top. In the older cells vesicular swellings occur which should probably be considered to be the beginning of a degeneration under the influence of the counter-attack of the plant. In the still older cells this counter-attack has been accomplished and nothing is left of the endophyte but a small lump of cell-walls. In some instances a few cells of the endophyte would survive this counter-attack and sub-

sequently multiply in the intercellulars as "bacteroids". SCHAEDE suggests that these bacteroids can leave the root, multiply in the soil and from there again infect new alder plants. It is not altogether certain whether these bacteroids really belong to the endophyte. It is also premature to conclude from the microscopic picture that the endophyte belongs to the actinomycetes. One should realise that one observes the endophyte in the cells of the alder-root, influenced by the defense reactions of these cells. Only cultures on known nutrient media could increase our knowledge regarding the systematic position of the endophyte. For further physiological research such cultures are also indispensable. Attempts hereto have been made and published by several investigators e.g. by PEKLO (1910), BOTTOMLEY (1912), SPRATT (1912), ZIEGENSPECK (1929) and BORM (1931). These authors did indeed isolate organisms but they made no attempt at all to establish the identity of the isolated organisms by their nodulating capacity or they tried to do so by unreliable methods. The fact that notwithstanding this the contentions of these authors are still of force is proved by the fact that one of the leading new books on soil microbiology WAKSMAN (1952) mentions BOTTOMLEY's assertion that the endophyte of the alder belongs to the Rhizobium group. KREBBER (1932), a more critical observer, is forced to the conclusion that the endophyte cannot be isolated on the media used by him. In 1941 VON PLOTHO remarks that an actinomycete can always be isolated on certain media which is even capable of forming nodules, though a long time might elapse after the inoculation. This pronouncement has been critisized by BOUWENS (1943). She pointed out that infections could occur spontaneously, given the cultural conditions used by VON PLOTHO, which might be responsible for the appearance of the nodules. Fruitless efforts at isolation were made by Miss BOUWENS herself. Generally speaking we are forced to the conclusion that, although many organisms have been isolated from the nodules of the alder, none of these organisms can definitely be identified as the endophyte.

PERSONAL INVESTIGATIONS

From the above it will by now be clear that until now it has by no means proved to be easy to isolate the endophyte of the alder so as to allow us to conclude unconditionnally that the isolated organism is identical with the endophyte. In order to be certain of this identity it is necessary to prove in an effective and convincing way the ability to form nodules. This may be done in two different ways. One way is by using cultures of the alder in open jars as VON PLOTHO did. In that case one has to ascertain statistically by comparing non-inoculated control plants and by using a great many cultures whether the amount of nodules occurring after inoculation is significantly more than that resulting from spontaneous infection. Alderplants may also be grown under conditions such as to preclude spontaneous infections, so that one can be certain that each nodule can be formed only in

the presence of the endophyte in the inoculation matter. In order to ensure a successful infection it is imperative to use healthy plants and therefore not advisable to use completely sterile plants under cotton wool. It is sufficient to use plants whose roots alone are sterile.

a. METHOD TO BE USED WHEN WORKING WITH NON-STERILE CULTURES

The method used was in principle derived from KREBBER (1932), and was the same as applied by VON PLOTHO (1941). Only the nutrient solution according to V. D. CRONE was replaced by a diluted solution according to HOAGLAND, as this provided a better supply of iron. Alder seed is collected yearly in the beginning of December shortly before the alder fruits drop off. In order to preclude the possibility of infection of the seed with endophyte it is cursorily disinfected by shaking for 5 minutes with a 0.1 % (v/v) solution of bromium. Subsequently it is placed in a dish with water and left to germinate. After germinating, which lasts approximately 5 days, the seedlings are removed to a small glass container, the bottom of which is covered up to a height of $\pm$ 4 cm with a good nutrient solution containing $\pm \frac{3}{4}$ % of agar. After about 3 weeks, when the plants have grown to $\pm$ 4–5 cm they are carefully pulled out of the soft agar and fixed with a piece of cotton-wool in pierced corks on jars containing 300 ml. In each jar three plants are placed. Then the jars are filled with the following nutrient solution: 1000 ml of water (dest.), 0.126 grams of KNO_3, 0.295 gr. $Ca(NO_3)_2$. $4H_2O$, 0.012 gr. $MgSO_4$. $7H_2O$, 0.034 gr. KH_2PO_4 to which 1 ml of a saturated solution of ferritartrate and 1 ml of a A–Z solution of the following composition is added; 1000 ml of water, 2.86 gr. H_3BO_3, 1.81 gr. $MnCL_2$. 4 H_2O, 0.22 gr. $ZnSO_4$. 7 H_2O, 0.07 gr. Na_2MoO_4, 0.08 gr. $CuSO_4$. 5 H_2O.

This solution is renewed at regular intervals until the plants have reached a height of about 8 cm in total. Then the nutrient-solution is replaced by a solution of the same composition except for the fact that the nitrates have been replaced by equivalent quantities of KCl and $CaCl_2$. Simultaneously the inoculation-matter (for instance nodule suspensions or isolated organisms) is added to the solution. During a week the jars are carefully shaken once a day, in order to ensure a firm contact between the endophyte and the roots. At the end of the week the solution is renewed by a N-free solution of the same composition, this time without adding inoculation matter. This renewal is repeated once a week until the nodules have grown sufficiently to be counted. This is generally the case after 6 weeks. It did not appear to be necessary to aerate the cultures. As BOUWENS (1943) has shown, this method may induce spontaneous infections. I doubt whether these are aerial infections. They are more likely caused by polluted surroundings. When the jars are placed on clean wooden tables and not on tablets with soil amongst other plants, and one takes care not to touch the roots with one's fingers, the chances of spontaneous infections occurring during brief experiments are practically nil.

b. METHOD ADOPTED WHEN WORKING WITH STERILE CULTURES

The method used for working with sterile cultures is essentially the same as that used for working with non-sterile cultures, differing of course only in the measures taken to ensure adequate sterility. First the seed has to be disinfected. After prolonged experiments by various means of disinfection (e.g. sublimate and concentrated sulphuric acid) the following complicated method proved to be the most satisfactory one. The seed is first shaken with soapsuds, thoroughly rinsed in water, washed for 4 minutes in alcohol 96 %, again rinsed in water, and then disinfected during 15 minutes with a 0.1 % (v/v) solution of bromium. Afterwards the seed is quickly rinsed in sterile water and then placed in a solution of 0.5 % (w/v) of hydroxylamine hydrochloride in water (according to BJÄLVE), see KYLIN (1950). Here it remains overnight and is thoroughly rinsed the following morning with 4 portions of sterile water.[1] Hereafter it is placed in small testtubes filled with sterile water and left to germinate. It is not advisable to let the seeds germinate jointly e.g. in a petridish as, in that case, possibly still infected seeds may infect the others. On the 5th day the germinated seeds are transferred to wide culture tubes filled halfway with a sterile Hoagland solution containing ¾ % of agar and 0.1 % of glucose, in order to be able to recognize infected plants more readily. Three weeks later the plants which have remained sterile are taken out of the tubes in a room disinfected by UV light and wrapped in a strip of sterile cottonwool at the level of the hypocotyle. Thereafter they are placed in tubes of the type shown in fig. 1 with a content of 100 ml. These tubes are thereafter filled with a sterile Hoagland solution via the little tube at the side. Subsequent renewals of the nutrient solution and the inoculation can also take place via this little tube. In order to prevent the contents of the tube from being infected by the no longer sterile leaves during this process the nutrient-solutions are administered by way of siphon flasks whose siphon has a filling apparatus at its extremity, as is shown also in Fig. 1. For long-time experiments the small culture-vessels are no longer suitable. In such cases one should use the culture in suction-Erlenmeyers, as described by LAINE and VIRTANEN (1941). The same drawback as the one already mentioned by GERRITSEN (1935) applies to this method viz. that the fastening of larger plants in the plug of cottonwool facilitates the penetration of aerial infections. Much less trouble is experienced during short-time infection experiments with rather smaller plants. I have never observed spontaneous nodulation to take place during this culture method. As with the non-sterile method the nodules can be counted six weeks after the inoculation has taken place. The appearance of numerous nodules after inoculation with a suspension of root-nodules is certain proof of the usefulness of this method.

[1] The best way to execute all these processes in floating seeds of the alder is to use separation funnels whose tapopening is sufficiently small to prevent seeds from passing through whilst the glassstopper has been replaced by a plug of cottonwool.

All the cultures, whether sterile or non-sterile, were placed in a small greenhouse on the roof of the laboratory which was heated in winter and sufficiently ventilated and screened in summer to keep the temperature from rising too much. During the period of 15 September – 15 March 4 TL fluorescent lamps, each of 60 W, were used for extra lighting from seven in the morning to nine at

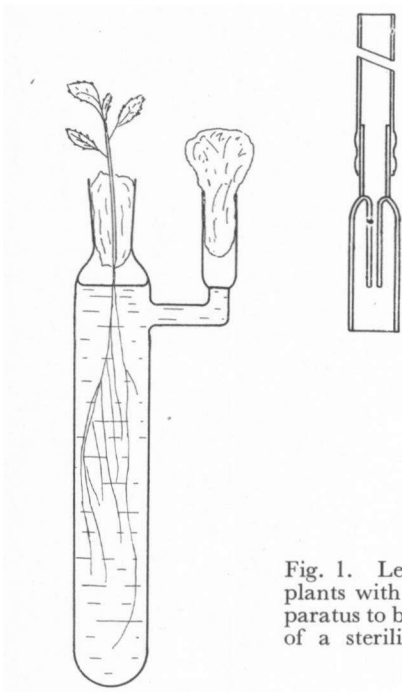

Fig. 1. Left: tube for cultivation of alder plants with sterile roots. Right: filling apparatus to be fixed at the end of the siphon of a sterilized siphon flask with culture solution

night. This enabled us to carry on with the culture of alderplants during the winter months also, although growth was retarded and the capacity to form nodules decreased. The factors which influence the capacity to form nodules will be more fully discussed in a later publication.

c. Endeavours to Isolate the Endophyte

In various ways we have tried to isolate the endophyte from the nodules of the alder:

1. freshly gathered young nodules were disinfected externally by shaking them for 4 minutes with a solution of 0.2 % sublimate in 0.5 % hydrochloric acid and subsequently 3 times rinsed in sterile water. In some instances the outer cell layers were removed at the same time with a sterile knife. Afterwards the nodules were pulverised in sterile water and then spread on nutrient media with 1.5 % agar. Incubation at 30° C.
2. After the same preliminary treatment as described under 1) the

nodules were also pulverised in sterile water. Then a series of dilutions was prepared out of this suspension and ½ ml of each dilution was added to tubes containing 10 ml sterile nutrient media with 1.5 % of agar, which after melting were cooled to 40° C. After mixing thoroughly the tubes were emptied into sterile petri dishes and these incubated at 30° C.

3. After the same preliminary treatment as described under A (either only disinfection or peeling or a combination of both, whereby now and again a disinfection with a 0.1 % solution of bromium was used), the nodules as such or small fragments of these nodules were sown on nutrient media with 1.5 % of agar and incubated at 30° C.
4. Nodules which had had no preliminary treatment were pulverished in a drop of sterile water. Under the microscope the endophyte clusters were transferred with the aid of a micromanipulator to other drops of sterile water. This was repeated until the clusters were considered to be freed of attached impurities. Thereafter they were either incubated as hanging-drop cultures in liquid media or transferred on to media with 1.5 % of agar and incubated at 30° C.

With all methods a great deal of media were used. Besides the usual media as maltextract, yeastextract, broth or even simple tapwater, variations of the media, usual to the isolation of Rhizobium and also of the medium recommended by VON PLOTHO were mainly used. The composition of these media is as follows:

Rhizobium culture solution: 1000 ml of water, 0.5 gr. NH_4NO_3, 0.5 gr. K_2HPO_4, 0.2 gr. $MgSO_4$. 7 H_2O, 0.2 gr. NaCl, 2 gr. $CaCO_3$.

Actinomycete culture solution according to VON PLOTHO: 1000 ml of water, 0.5 gr. NH_4NO_3, 0.3 gr. KH_2PO_4, 0.2 gr. K_2HPO_4, 0.2 gr. $MgSO_4$. 7 H_2O, 0.1 gr. NaCl and 0.05 gr. $CaCl_2$.

The variations in these nutrient solutions were provided by the choice of different sources of carbon (glucose 2 % or 0.1 %, mannitol 2 %, glycerol 2 %, starch soluble 2 % and 0.25 %, cellulose soluble 0.25 %, pectin 0.5 %), variations in the salt-composition (replacement of K_2HPO_4 by KH_2PO_4,) omission of NH_4NO_3, omission of $CaCO_3$, dilution of the total salt concentration, addition of iron salts and A–Z solution, addition of organic nitrogenous substances (e.g. albumin 0.025 %, peptone 1 %, 0.5 % or 0.05 %, asparagine 0.1 %), additons of yeast autolysate, or extracts of yeast, broth, alder-roots, soil or peat.

The influence of the following substances was studied auxanographically during isolations according to the method 2 viz.: Ca-lactate, Na-acetate, Na-citrate, Na-succinate, K-tartrate, Ca-glycerophosphate, hexosediphosphoric acid, casein and tannin.

The cultures were incubated under aerobic as well as under anaerobic conditions. Moreover we tried to obtain more subtle differences in redox-potential by auxanographic addition of agar-blocks with ascorbic acid, thiourea, Na-sulphite and K-permanganate to the agar media.

The results of all these experiments can be briefly summarized as follows:

Nowhere did an organism develop when method 4 was used. On applying the three other methods several organisms developed. Only the organisms which appeared with great regularity were kept and examined with regard to their nodulating capacity. During certain experiments when method 3 was used all organisms which developed out of a group of nodules were examined. None of these organisms proved capable of forming nodules whether the sterile or non-sterile method was employed. It should be noted that the organism isolated by VON PLOTHO, which I received from the collection of the Central Bureau for Mould Cultures at Baarn, also proved incapable of forming nodules. It may be possible, however, that this organism has lost its virulence owing to the long culture in vitro. A more serious objection to the correctness of VON PLOTHO's argument is the fact that I have never come across VON PLOTHO's organism amongst the organisms isolated by me. On the strength of my own attempt at isolation I can only subscribe to BOUWENS' criticism and come to the conclusion that the endophyte cannot be isolated in this way. The assertions made that the endophyte is a Rhizobium or a certain actinomycete should therefore be considered of no value whatsoever.

It is well-known that several parasitical or symbiotically living organisms cannot be grown without their host. One has only to remind oneself of the obligate parasitically growing moulds or the endophyte of Lolium temulentum. These are, however, mostly organisms which are also never found in natural surroundings without their host-plant. It is difficult to accept the view that this is also the case with the endophyte of the alder. ROBERG (1934) observed on sowing alderseed on various soils which had never produced alders that nodules were formed all the same. The only way this can be explained is by accepting the view that the endophyte multiplies in these kinds of soil as a normal element of the microflora. We are justified by this in hoping that it will prove possible to find circumstances in which the endophyte will be able to multiply in vitro.

d. INVESTIGATION INTO THE CAUSE OF THE FAILURE OF THE ENDEAVOURS AT ISOLATION

It seemed rather pointless to proceed with our endeavours at isolation, as described in the previous chapter, without first having obtained some insight into the possible causes of these failures. These possible causes can be subdivided into 4 categories.

1. The endophyte contained in the nodules used for isolation is no longer vital after undergoing the necessary pre-treatment.
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 cannot 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 nutrition and/or environmental circumstances, which up till now have not been fully provided by the growth-circumstances and nutrient media used.

ad 1. The possibility that the endophyte contained in the nodules used for isolation purposes should no longer be vital can by no means be ignored. Examples of other symbioses are known. E.g. the bacterium causing the leaf-nodules of Ardisia cannot be isolated from these nodules, but this isolation is possible from the vegetation-top (e.g. see DE JONGH 1938). The so-called bacteroid stage of the Rhizobium in leguminous plants cannot develop after having been isolated with the micromanipulator on media where the Rhizobium is capable of showing marked development. (ALMON 1933). The nodules of the alder undoubtedly contain vital stages of the endophyte, otherwise these nodules could not be used for infection purposes. However, when the main body e.g. the vesicular stage would no longer prove vital and the vital cells would lie superficially close to the top, these vital stages would be quite likely to be either killed or removed by the pre-treatments (disinfection and peeling). Most authors, when using isolation methods took into consideration the possibility of the endophyte being killed by a too strong disinfection, but no one has proved experimentally whether this was indeed to be feared. This can be easily done by checking whether the nodules are still capable of forming nodules after undergoing pre-treatments.

Exp. 1. A number of freshly gathered nodules was divided in approximately equal parts. Each part was subjected to another pre-treatment. These pre-treatments consisted of disinfection with a solution of 0.2 % (w/v) $HgCl_2$ in 0.5 % HCl during 5 or 15 minutes, or with a solution of 0.1 % (v/v) bromium for 5 minutes, whether in combination with the removal of the outer cell-layers or not. Each group of nodules was then pulverised in sterile water and a quantity of these suspensions added to non-sterile alder cultures large enough to provide each jar with a quantity equivalent to 100 mg fresh weight of the original nodules. With each suspension 3 jars, each containing 3 plants, were inoculated. The result is shown in table 1.

TABLE 1 (Exp. 1)

The influence of the petreatment of the nodules on the vitality of the endophyte

Means of disinfection	Length of disinfection	Peeling	Number of nodules produced per plant (average with stand. dev. of the average)
0.1 % $HgCl_2$	5 minutes	—	7.1 ± 1.3
0.1 % $HgCl_2$	5 minutes	after disinf.	14.6 ± 5.2
0.1 % $HgCl_2$	15 minutes	—	1.1 ± 0.4
0.1 % Bromium	5 minutes	—	18.4 ± 4.0
0.1 % Bromium	5 minutes	before disinf.	18.0 ± 1.0
Untreated	—	—	4.3 ± 1.3
Control (not inoculated) .			0

When considering the results of exp. 1 one must be sure to take into consideration the fact that the disparities in the observation series are not only the result of the disparities in pre-treatment, but also of those which originally existed between the groups of various nodules. At any rate these experiments prove that after all kinds of pre-treatments the nodulous matter was still clearly of an infectious kind. There is, therefore, no reason to suppose that failure of our endeavours at isolation was due to these pre-treatments.

On the strength of the microscopical research of the nodules it is reasonable to suppose that the vitality of the endophyte can be found only in certain parts of the nodule and that principally the vesicular cells, as also the bacteroids of the leguminoses, are not capable of further development. Whereas according to the description of the endeavours at isolation fragments of nodules were often used, the failures may perhaps partly be ascribed to the use of non-vital parts of nodules.

Exp. 2. A number of young root-nodules averaging a length of 2 mm and a total fresh weight of 1 gram was sliced at 1 mm distance from the top and thereby divided into a top- and basal fragment. Both series were pulverised in 10 ml of water each and a $^1/_{10}$ and a $^1/_{100}$ dilution was moreover made of these suspensions. Out of each dilution 4 jars containing 3 alders were inoculated with $\frac{1}{2}$ ml. The result may be seen in table 2.

TABLE 2 (Exp. 2)
Comparison of the vitality of the endophyte in various parts of the nodules

Fragment of nodule	Dilution of the suspension	Number of nodules produced per plant (average with stand. dev. of the average)
Topzone	undiluted	3.4 ± 1.4
	$^1/_{10}$ diluted	0.9 ± 0.4
	$^1/_{100}$ diluted	0.1 ± 0.1
Basal zone	undiluted	4.2 ± 1.5
	$^1/_{10}$ diluted	0.6 ± 0.2
	$^1/_{100}$ diluted	0

Contrary to all expectation there appears to be no difference either qualitatively or quantitatively in the capacity of both zones to induce infection. It would be premature to conclude from this that the vesicular stage is as vital as the "actinomycete" stage in the topzone, as hyphae may still be found between the vesicles also. We may however draw a practical conclusion from this experiment viz. that one need have no fear of using non-vital zones of the nodules during the efforts at isolation.

The experiments concerning the formation of nodules and the efforts at isolation are not entirely comparable inasmuch as regarding the first experiments the endophyte is able to contact the alderroots directly after suspension, whereas regarding the second experiments

this suspension is maintained during the incubation period. One may question whether the negative result of the efforts at isolation may not depend upon the dying off of the endophyte owing to the suspension in water or nutrient media.

Exp. 3. 2 grams of fresh nodulous matter was pulverised in 600 ml of water. This suspension was distributed over 6 Erlenmeyers of 100 ml, each of which therefore receiving 100 ml suspension. With HCl and NaOH the pH in 4 Erlenmeyers was regulated to resp. 3, 5, 8 and 10. The other two were set at pH 5 and conserved under anaerobic conditions over alcalic pyrogallol. One of these moreover was made extra-anaerobic by adding 0.1 % of cysteine. All these Erlenmeyers were thus kept at very different pH and Eh for 17 days at 27° C. At the end of this period of conservation 10 ml per jar of each Erlenmeyer were inoculated in 4 jars containing 3 alderplants. It is noteworthy to record that these concentrated nodulous suspensions were not spoiled by bacterium-or mould development during conservation (antibiotic influences e.g. on account of the high degree of tannin?). The result is shown in table 3.

TABLE 3 (Exp. 3)

Tenability of nodulous suspensions in water under various ways of conservation

Way of conservation		Number of nodules produced per plant (average with stand. dev. of the average)
pH 3	aerobic	73.6 ± 8.6
pH 5	aerobic	54.3 ± 9.8
pH 8	aerobic	58.2 ± 7.9
pH 10	aerobic	67.0 ± 8.5
pH 5	anaerobic	80.5 ± 9.5
pH 5	anaerobic + 0.1 % cystein	52.3 ± 10.1

A very large amount of nodules is formed in all cultures. We shall not discuss the cause of this high rate of nodules now, as we intend to discuss this in a later publication. At this stage it is only of importance to record that the endophyte loses none of its vitality owing to the conservation in suspension under any of the conditions, even those of rather extreme pH's and Eh's. There is no reason therefore to fear that, owing to suspension under conditions at variance with those in the nodules, the endophyte would be lost. The resistance to conservation even appears to be remarkably great.

One would almost suspect the endophyte to form resistent spores. There is no reason, however, to think this. This is namely proved by experiments during which I have subjected the nodulous suspensions to the influence of higher temperatures. The infectuous capacity of such a nodulous suspension was seen to have completely disappeared after heating the suspension for an hour at 60° C. This is indeed a very strong proof that no resistent spores are present. Furthermore,

it's logical conclusion in practise is, that one has to be careful during the pre-treatments in using high temperatures. It is better to avoid methods of disinfection during which the nodules are externally flamed e.g. with alcohol.

The above mentioned experiments might be critisized by pointing out that the nodules formed were not produced by the endophyte, but by a nodulating substance. On microscopical investigations, however, the nodules appeared to show the usual microscopical picture of cells filled with the endophyte. Moreover, after filtration through a Seitz bacterium filter the nodulous suspensions appeared to form no nodules whatsoever, whereas on the other hand the filter residue retained the same nodulating capacity as the non-filtrated suspension. This altogether rather improbable criticism seems to me to be adequately disproved hereby.

Summarising we may draw the conclusion that not a single reason exists to suppose that the inoculation matter used for isolation purposes would no longer contain the endophyte in a vital condition. The cause of the failing of the isolation endeavours mentioned sub. 1. cannot therefore be the real cause.

ad. 2. The infectuous capacity of several parasitically existing organisms is known to diminish after a certain period of culture in vitro. Cultures of Rhizobium also show similar symptoms, first in the form of a diminishing of the nitrogen-binding capacity of the formed nodules (efficiency), later as a diminishing of the nodule-forming capacity itself (virulence). It might be that the endophyte possesses this quality to such a marked degree, that the virulence disappears directly during the isolation. This would mean that one of the organisms, isolated up till now, is definitely identical with the endophyte but that one is unable to prove this identity as the isolated organism is not able to form nodules after the isolation. The following observation shows this to be untrue.

When trying to isolate the endophyte from a group of freshly gathered nodules according to method 3, after disinfecting and removing the outer cell-layers, the nodules are sown on a good nutrient agar (e.g. Rhizobium-salt solution with 2 % of glucose, 0.5 % of peptone and 1 % of yeast autolysate). Micro-organisms are seen to develop out of most nodules. None of these organisms, however, are able to form nodules after isolation. A small percentage of nodules (variating from 2–20 %), however, remains sterile. The question now arises whether these sterile nodules were indeed completely sterile at first or possessed the endophyte as did the other nodules. If this were the case one may expect on the strength of the experiments mentioned under ad. 1, that they will also still contain this endophyte in vital condition after the incubation period from 4–6 weeks. The last supposition proved to be true. When such "sterile" nodules are pulverised in sterile water and used for inoculation of sterile cultured alder plants several nodules may be seen to appear after a few weeks. By spreading the suspension simultaneously over various nutrient

media (broth agar, pepton-glucose agar, yeast agar etc.) this suspension may be observed to be truly sterile according to the usual microbiological criteria. Yet, notwithstanding this, the endophyte appears still always to be present in a vital condition.

When one of the organisms which had grown out of the other nodules would indeed be identical to the endophyte, one fails to understand why this endophyte should be produced by one nodule and not by the other, given the same medium and completely similar circumstances. Mechanical obstructions, such as impermeable cell layers, cannot affect this as no development is possible even after the "sterile" nodules have been pulverised and suspended in water. The most likely conclusion is that the organisms which were produced by the nodules had nothing in common with the endophyte. Failure up till now to carry out these isolation endeavours cannot be explained by, or imputed to, loss of virulence during the isolation.

ad. 3. During our endeavours at isolation as carried out by us up till now the appearance of a perceptible colony was taken as criterion for the occurrence of growth of the endophyte. In doing so heavy demands indeed are made upon the multiplication in vitro viz. that millions of cells of which a small colony already consists will develop out of the small quantity of cells present in the inoculation matter (and this specially applies to methods by which the inoculation matter is suspended in the medium or where micromanipulator-isolations are used). Possibly on certain media growth of the endophyte has really occurred, but has been too insignificant to lead to the formation of a visible colony. Certain microorganisms are also known to show a type of growth which does not at all produce visible colonies, and where the position of the colonies can only be recognised by the appearance of solution of suspended particles e.g. chalk, cellulose in the agar. The endophyte also might be able to show a similar type of growth on agar media. It is however unlikely that the failures are caused by this alone. E.g. if one examines the sterile nodules during the experiment mentioned under sub. 2 after 6 weeks incubation on agar media, one sees that although these nodules are markedly shrunken the microscopical picture of the endophyte remains the same as before incubation takes place. In the shrunken cells one can still observe the clusters of the endophyte without any indication of the endophyte extending its boundaries. This is not complete proof in itself as one cannot be certain even after the experiments discussed in chapter *d*, whether these clusters, and this especially applies to the vesicular stage, represent the most vital parts of the endophyte present in the nodules.

There are other reasons also why we should pay attention to this matter. However likely it may be that the failure of the isolation endeavours carried out up till now is mostly due to special food or environment requirements of the endophyte which the media used up till now have not supplied, the chance of success during the research will undoubtedly increase if one possessed a method to

determine the eventual growth of the endophyte in a more sensitive way than is possible on the strength of the formation of colonies on agar plates.

e. THE PREPARATION OF PURE INOCULATION MATTER

One of the greatest difficulties when working on the endophyte of the alder consists of the fact that not only does it prove impossible to let the endophyte grow in vitro but one even fails to liberate the endophyte from other micro-organisms. Also, after disinfection of the nodules combined with the removal under sterile conditions of the outer cell layers, several fungi, actinomycetes and bacteria appear to be present. None of these may be considered identical with the endophyte. This prevents working under sterile conditions. From the experiment described in chapter *d* under 2 we can now derive a method which enables us to acquire the endophyte in pure conditions. When one crushes in sterile water the nodules, which have remained visibly sterile after an incubation period of 6 weeks on a suitable nutrient agar, we acquire a suspension which is sterile according to the usual microbiological criteria, yet appears to possess the endophyte still in its vital, virulent form. Such matter, selected by incubation, is eminently suitable for use as inoculation matter in future growth experiments, and in general for work under sterile conditions. Although, to be sure, one has not got any pure cultures, such suspensions can be called pure suspensions.

f. DEMONSTRATION OF NITROGEN-FIXATION UNDER PURE CONDITIONS

Although the experiments by KREBBER and ROBERG show that very probably elementary nitrogen is fixed in the root-nodules of the alder, it seemed desirable in the first place to investigate this once again under pure conditions, now the possibility hereto existed. It was desirable moreover because it was important to know whether nodulous matter selected by incubation really contained the endophyte in a completely healthy condition, and whether especially during the incubation its efficiency was not impaired. To this purpose some sterile aldercultures were inoculated with pure nodulous suspensions, which had been prepared according to the method described in chapter *d*, ad. 2. As these experiments took rather a long time, the plants were conveyed a few weeks after the infection from the usual tubes to suction Erlenmeyers of 500 ml according to the method of LAINE and VIRTANEN (1941). Inoculation matter, as well as the alderroots, were examined with regard to the possibility of existing infections by spreading on a peptone-glucose agar and malt-agar. Only shortly before the end of the culture period some mould infections were seen which could, however, no longer influence the result. Directly at the first sign of these infections the experiment was terminated. The nitrogen analyses were carried out according to Kjeldahl by destruction with 12.5 ml of concentrated sulphuric acid, 750 mg Na_2SO_4 and 350 mg HgO, followed by destillation in a Parnas-Wagner apparatus in a 2 % boric acid solution. The

nitrate-containing solutions were analysed by also heating them slightly in a Parnas-Wagner apparatus for ½ an hour with Devarda metal (1 gr. per 50 ml solution), followed by destillation in a 2 % boric acid solution. The average nitrogen content per seed was analysed, the amount of nitrogen taken out of the nutrient solutions, (agar, nitrogen-containing solution and nitrogen-free solution) the amount of nitrogen in the inoculation matter and, at the termination of the experiment, the nitrogen content of the plants and the surrounding liquid. The result is given in table 4. 21 VI 51 was inoculated, 31 X 51 harvested.

TABLE 4

Nitrogen balance of two pure grown alderplants which were inoculated with pure nodule suspensions

(nitrogen expressed in m. aeq. N per plant)

	Plant 1	Plant 2		Plant 1	Plant 2
Present in seed	0.004	0.004	Found in plant	1.856	2.917
Taken from agar . . .	0.010	0.015	Found in culture solution	0.024	0.005
Taken from nutrient solution	0.092	0.151			
Present in inoculation .	0.001	0.001			
Present in N-free nutrient solution	0.002	0.002			
Nitrogen increase . . .	1.771	2.749			
	1.880	2.922		1.880	2.922

The result is very clear. In both plants a convincing increase of nitrogen is observed. This proves that, elementary nitrogen can be fixed by the nodulated roots. Secondly, it proves that the nodules selected by incubation contain the endophyte in an efficient form.

DISCUSSION

Up till now it has not proved possible to cultivate the endophyte from the root-nodules of the alder apart from the nodules in vitro. Neither the attempts described in the literature, nor our own attempts which I made as an introduction to this investigation could produce any result at all. All pronouncements to be found in literature concerning successful attempts at isolation are unreliable, as the identity of the isolated organisms was either not established at all or was established with the aid of a method which could not be considered to be conclusive. Much attention was therefore paid by me to the developing of a method which would undeniably demonstrate the faculty of the isolated organisms of forming nodules. It proved impossible however to prove by this method the formation of nodules by one of the isolated organisms.

A detailed examination of the possible causes of the failure of these attempts at isolation was made. The inoculation matter used proved

to contain the endophyte still in a vital condition, so that the cause lay not herein. Both after the application of external measures of disinfection, such as disinfection with bromium or sublimate, as after removal of the outer cell layers the nodules proved still to be considerably infectuous. Also after prolonged suspension in solutions of divergent pH and Eh the endophyte endured remarkably well. The endophyte only proved to be very sensitive to high temperatures. There is also no reason to suppose that one of the isolated organisms would be identical with the endophyte, but that it would have lost its virulence during the isolation and the following incubation, so that the identity of the isolated organism with the endophyte could no longer be proved with the aid of the capacity to form nodules. For nodules which stayed visibly sterile after remaining for 6 weeks on a good nutrient medium appeared to contain the endophyte at the end of this incubation period still in a vital form. The endophyte was therefore clearly not able to develop on this nutrient medium. For further development of the investigation there are two factors of considerable importance. By sowing nodules, after first having disinfected them and having removed their outer cell layers, on a good nutrient medium and, by selecting the nodules which have remained visibly sterile after an incubation period of 6 weeks, one can gather nodulous matter which is pure according to the usual microbiological criteria, but still contains the endophyte in a vital, virulent and efficient form. This inoculation matter, selected by incubation, enables one therefore to conduct experiments under sterile conditions. By means hereof the appearance of elementary nitrogen fixation under "sterile" conditions has been proved already. For further growth experiments of the endophyte similar pure inoculation matter may be likewise used.

Besides this it will be of great importance to elaborate a method which will enable us to demonstrate the growth of the endophyte in a more sensitive manner than was usual up till now. In the first place it is not certain that the endophyte will ever be able to grow so quickly in vitro and in such a manner as to form visible colonies within a reasonable time limit. Secondly, even if one succeeds in finding such media and cultural conditions resulting in a good formation of colonies, the finding hereof will be made easier if one was already able to observe a lesser growth under less ideal conditions. More sensitive methods might be found by observation of hanging drop cultures through the microscope. The disadvantage of these is however, that one does not know which is the most vital stage of the nodules and what one must therefore insert into these drops e.g. by means of a micromanipulator (hyphae, vesicles or bacteroids). Moreover the use of cloudy media, such as root- and soil suspensions, may cause difficulties during observation. The possibility of estimating growth by means of the increase of a property of the organism may also be born in mind. As only known property only the capacity to form nodules can be considered. In that case, however, one will have to know more about the factors which influence this nodulation

and, especially, one will have to examine whether a relation exists between the amount of endophyte in the inoculation matter and the number of nodules that can be formed and, in that case, what kind of a relation exists. In a subsequent publication in this series this item will be more closely studied.

SUMMARY

1. It is impossible to cultivate the endophyte from the root-nodules of the alder on the usual media in vitro.
2. Methods are described by which one can demonstrate with certainty whether certain organisms are capable of forming root-nodules in the alder.
3. The difficulties in connection with isolation are not caused by a dying off or removal of the endophyte during the required pre-treatments (disinfection with sublimate or bromium, removal of outer cell layers, suspension in water or nutrient media).
4. The endophyte is rather sensitive with regard to the influence of higher temperatures. During 1 hour at 60° C. the capacity to infect is completely lost.
5. Nodules which have remained visibly sterile after remaining for 6 weeks on a good nutrient medium, contain the endophyte still in its vital, virulent and efficient form.
6. Such nodules are very suitable as inoculation matter for work under sterile conditions.
7. With the aid of such nodules as inoculation matter elementary nitrogen fixation could be confirmed under pure conditions.

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