ABSTRACT OF THESIS ON THE ROOT NODULES OF MYRICA GALE L.

WILLIAM W. FLETCHER, B.Sc.

A study is made of the development and structure of the root nodules of Myrica gale utilising the water-culture technique for the growing of the plants. Observations are also made on field material.

The general cultural methods are described and observations made on the germination and early stages of growth of the plant.

From cytological evidence it is concluded that the organism responsible for nodulation is a member of the Actinomycetes and that it makes its way into the plant via the root hairs which become twisted and distorted in the process. Nodules can be observed some 14 days after inoculation, when the plant is at the 3 leaf stage, and they arise due to meristematic divisions of the parent root pericycle under the influence of the organism. In the young condition they are red, due to the presence of anthocyanin, (not haemoglobin as in the nodules of the Leguminosae). It is characteristic of the nodules that each develops a terminal prolongation termed the "nodule-root" which proceeds to grow upwards. The nodule branches to give rise to fresh lobes which eventually form a close cluster enmeshed by the nodule roots.

Internally the infected cells of the nodule become enlarged and the actinomycete can be seen within them. The actinomycete threads become swollen and are eventually digested sometimes forming a dark clump within the cells (in many ways similar to that seen in the digestive cells of the roots of Neottia.) Stages in digestion are described and figured.

Attempts to isolate the causative organism are described. All of these have proved to be unsuccessful, the isolated organisms failing to induce nodulation/

nodulation. An examination is made of previous authors attempts at isolation and it is concluded, despite claims to the contrary, that the organism has not yet been isolated. A discussion follows on the possible reasons for failure and the evidence examined for the possibility of the organism being an obligate symbiont.

Cross-inoculation experiments, using certain rhizobia and the endophytes of the root nodules of Alnus and Myrica, are described. From these it is concluded that none of the three are cross inoculable.

A comparison is made of the effect of the pH of the rooting medium on nodule formation in two non-legumes (Myrica gale and Alnus glutinosa) with that in a typical legume, namely Trifolium pratense, utilising data obtained by Bond (1951) on Myrica gale and unpublished data on Alnus glutinosa kindly communicated to the author by Mr. T. Ferguson of the Botany Department, Glasgow University. In order to provide a direct comparison, the author has obtained data for Trifolium pratense by growing it, as were the two non-legumes, in water culture at pHs ranging from 3.3 - 7.0 It is shown that the endophytes of all three plants have different pH tolerances - the endophyte of T. pratense cannot survive a pH of 4.2 or lower; the endophyt of A. glutinosa can survive a pH of 4.2 but not of 3.3 or lower. The endophyte of M. gale can survive a pH of 3.3

It is further demonstrated for M. gale and A. glutinosa that the reacti of root and nodule tissue does not vary with the pH of the medium in which the plants are growing. In M. gale the root pH is 5.0 - 5.3 and the nodules pH 5.4 - 5.8 over a solution pH range of 4.2 - 7.0 In A. glutinosa the root pH is 4.9 - 5.2 and the nodule pH 5.8 - 6.0 over a solution/

solution pH range of 4.2 - 7.0

The effect of combined nitrogen (as $NaNO_3$ and $(NH_4)_2SO_4$) on nodulation in Myrica gale is investigated, together with comparative data for Trifolium pratense, and it is shown that whereas a small quantity of combined nitrogen (17.5 mgms. N/litre) markedly depresses nodulation (both as regards size and number of nodules) in Trifolium, combined nitrogen (up to 140 mgms. N/litre) does not affect the number of nodules found on Myrica; and that the nodules of Myrica plants receiving combined nitrogen increase in size much more rapidly than those developing in nitrogen free solution. It is concluded that the effect of combined nitrogen on nodulation is fundamentally different in the two plants and that the Myrica-actinomycete symbiosis is a less highly evolved system than the legume-rhizobium association since in the former there is a superfluous expenditure of raw materials in the formation of nodules in the presence of combined nitrogen.

A STUDY OF THE MOOR NOTELET

OF

ERICA CARN. L.

BEING A THESIS PRESENTED BY

WILLIAM W. FLETCHER, B.Sc.

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE

UNIVERSITY OF GLASGOW.

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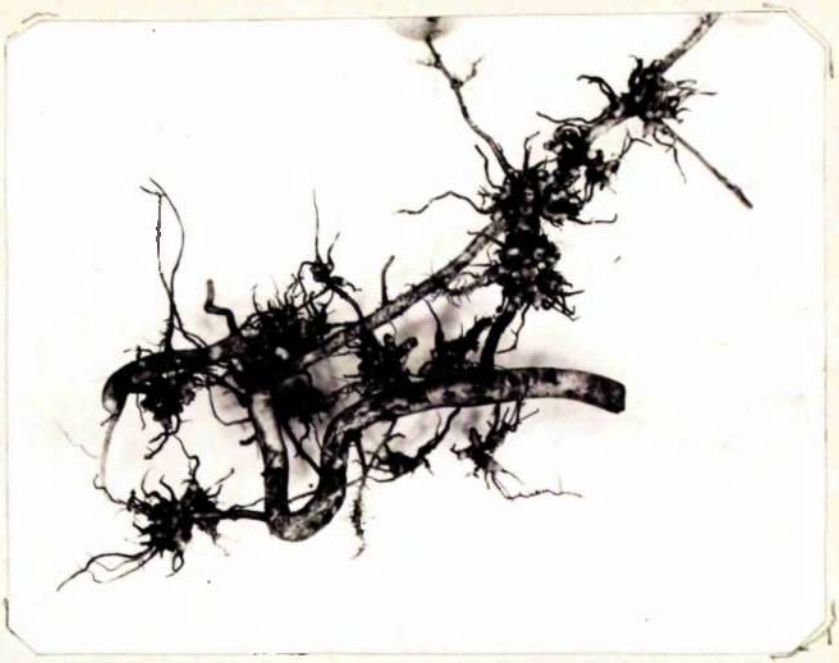

FRONTISPIECE

Root nodules of *Myrica gale* - Natural size.

C O N T E N T S.

C O N T E N T S (Continued).

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I. GENERAL INTRODUCTION.

Myrica gale (Bog Myrtle or Sweet Gale) is a shrubby plant which occurs in bogs and wet moors in Northern and Arctic Europe, in Asia and North America. It is a dominant plant over large areas of wet acid peaty soils in Scotland, Northern England and Ireland. Several other species of *Myrica* occur in North America, but *M.gale* is the only species native to Britain.

According to Bentham & Hooker (1924) the genus *Myrica* may be included in the Amentaceae or Catkin family together with *Alnus*, *Betula*, *Carpinus*, *Corylus*, *Salix*, *Populus*, *Quercus* and *Fagus*, but they remark that most modern botanists distribute these genera into four families, one of which, Myricaceae, is limited to the genus *Myrica*. This latter is the procedure adopted by Clapham, Tutin and Warburg (1952). It is of interest to note that root-nodules, similar in many ways to those occurring on *Myrica*, are also found on the roots of *Alnus*.

Although the presence of nodules on the root system has long been known to be a regular feature of *M.gale*, they (in common with the other non-leguminous root nodules) have attracted relatively little attention, so that Fred , Baldwin and McCoy (1932) could state:-

"There are many errors and confusion in the reports on non-leguminous nodules. For lack of authentic information therefore, it is, in most cases, impossible to give an accurate picture either of the causal agents or the structure and function of such nodules". Despite the fact that twenty years have passed since the above statement was made, the position today is not much improved other than for Bond's (1951) work on nitrogen fixation by the nodules of Myrica gale.

A full account of the investigations of the sixteen or so previous workers (starting with Brunchorst (1886-87)) who have concerned themselves with M.gale nodules will be presented in later Sections of the thesis, but a brief summary will now be given in order to indicate the background of existing knowledge against which the present investigation has been carried out.

Summary of the observations of previous investigators.

The root nodules of Myrica gale (see Frontispiece) take the form of mulberry-like nodule clusters with a clothing of fine roots ("nodule roots"). The manner in which these clusters arise has been studied by several workers, who observed that the simple primary nodule attains a length of

2-3 mm. and is terminated by a nodule-root. Around the base of the latter, secondary nodules arise, usually three in number, and these again terminate in nodule-roots. This process is repeated, with the eventual formation of perennial clusters which occasionally attain the size of a walnut.

The study of the structure of the nodule either of the original simple nodule or of one of the subsequently-formed branches, has led most investigators to the view that it represents a modified lateral root, though differing from a true root in forming no root hairs and (prior at least to the outgrowth of the nodule-root) in having no root-cap to protect the apical meristem. In contrast with the legume nodule, it is in the cortical region that hypertrophy of cells occurs in the Myrica nodule, and it is in these cells that the endophyte occurs. Most authors have agreed that hyphal structures are present in these cells, and have variously concluded the endophyte to be a true fungus or an actinomycete. Final proof of the identity of the endophyte has not been obtained, since no investigator has succeeded in isolating in pure culture an organism which by satisfactory re-inoculation could be shown to be the true endophyte.

Until recent years the question of the function of the nodules of Myrica had scarcely been investigated. There is now good evidence (see the work of Bond below) that fixation of atmospheric nitrogen is associated with the nodules, so that normal growth of nodulated plants can be obtained in a culture solution free of combined nitrogen. It seems then that we have here a state of symbiosis between higher plant and endophyte resembling in many respects that found in legumes. This finding endows M.gale with especial ecological significance, for this species may well play an important part in the fixation of nitrogen in peat bogs where the pH is too low for the normal free living and symbiotic nitrogen-fixing systems to operate. In the same investigation Bond (1951) noticed that the nodule-roots have peculiar tropic properties.

Scope of present investigation.

Despite the volume of work done there are yet many aspects on which further information is desired, and there are some fields which have not as yet, been even touched upon e.g. the method of infection of the root by the causative organism has not yet been investigated; no one has described the early stages in nodule formation

nor the origin of the young nodule in the parent root; there is much divergence of opinion on the nature of the endophyte and as is noted some workers state that there is more than one organism present in the nodule. Even among those who agree that it belongs to a particular group e.g. fungus, there is disagreement as to the genus and species to which it belongs within the group. Again no two authors have isolated the same organism from the nodules of Myrica gale and the re-inoculation tests have been inconclusive. The inter-relationships between endophyte and host, the effect of endophyte on host and host on endophyte have been variously interpreted. The effect of external factors (other than pH) e.g. combined nitrogen, on nodulation is an untouched field. No cross-inoculation experiments have been recorded in order to determine whether or not Myrica is cross inoculable with Alnus or with members of the Leguminosae.

The above are the aspects dealt with and form the subject matter of this thesis.

II. GENERAL CULTURAL METHODS WITH OBSERVATIONS ON THE GERMINATION OF MYRICA GALE.

Many of the omissions of previous workers have undoubtedly been due to the difficulty in obtaining young plants of Myrica. They are seldom observed in the field and the natural method of propagation appears to be by off-shoots. The reason for scarcity was discussed by Bond (1951). He examined the pH of 26 soil samples from Myrica gale stations in Western Scotland and found the mean to be 4.2 with a range of 3.7 - 4.8. From his water-culture experiments he concluded that the species is growing at pH levels below the optimum for nodulated plants. At this pH range there was considerable mortality of seedlings in nitrogen-free culture owing to non-development of nodules. Coupled with the low viability of seed this may explain the rare occurrence of seedling plants.

A few workers have raised seedlings of M. gale in the greenhouse but prior to the present investigation no one is on record as having inoculated such seedlings in order to obtain ample material of nodulated plants for the study of nodule development.

Van den Bergh et al (1930) collected two samples of seed in Holland in October. Only 40% of the first sample were full, 90% of the second. The first sample showed 5.5% germination after 49 days on a porous tile

in strong daylight. In diffuse daylight only 1% germination was obtained. From the second of the samples no germination was obtained. Seed was also collected in January from under Myrica gale bushes and this germinated freely in 50-60 days. Two types of seedlings were observed by the authors in the greenhouse. The commoner type, also noted in the field, shows the seed wall not carried up by the cotyledons but remaining attached for shorter or longer time to the top of the root. In the rarer type, the seed coat is carried up by the cotyledons. The radicle bears numerous root hairs and is lightly coloured.

The findings of Bond (1951) confirm and extend those of Van den Bergh. He noted that seed collected in September and stored at room temperature showed only 1.8% germination, whereas similar seed collected in January showed 8.0% germination. This led him to store the seed in moist peat at low temperature for several weeks prior to sowing. Such seed stored for 11 weeks at + 2°C before sowing showed 15.3% germination. ~~As noted above~~ Bond proceeded to inoculate the young plants so obtained and in this way raised large numbers of nodulated plants in water culture providing material for studies of nitrogen fixation. By using the same technique the present author has obtained material for his investigations.

The seeds were pretreated according to the methods described by Bond (1951 ~~see above~~) to increase percentage germination and then sown in horticultural peat in trays in a cool greenhouse. The latter were watered from time to time with tap water and covered with a glass plate to prevent excessive evaporation. Germination occurred in a few days and the hooked hypocotyl emerged from the peat in 1-2 weeks. Germination is epigeal and the two types noted above as observed by Van den Bergh et al (1930) were seen namely (1) the seed coat carried up by the cotyledons and (2) the seed coat left behind on the peat. The radicle is richly clothed in root hairs. It is soon supplemented by adventitious roots which arise from the hypocotyl. These too are densely clothed with root hairs which extend almost to the tip. The first true foliage leaf appears some 5-6 weeks after sowing and the plants were transplanted from the peat some 2-3 weeks later i.e. when the first two foliage leaves had unfolded. Drawings of the various stages in germination are shown in Figures 1 and 2. Occasionally a plant with 3 cotyledons was observed. The plants were transferred from the peat to Crone's N-free solution, prepared as follows:-

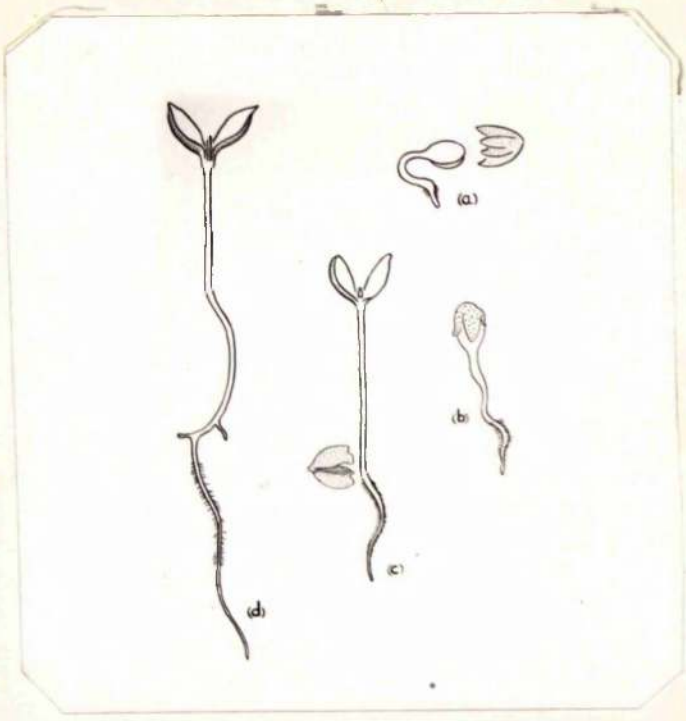


Figure 1.

Stages in the germination of M. gale. Note radicle richly clothed. The pericarp

(a) showing hook expose the cotyledons.rp has been removed to expose the cotyledons.

(b) a further stage of (a)
In (a) and (b) the pericarp is carried up by the cotyledons.

(c) In this type the pericarp is left behind on the peat.

(d) A young plant with cotyledons expanded.

X 3.

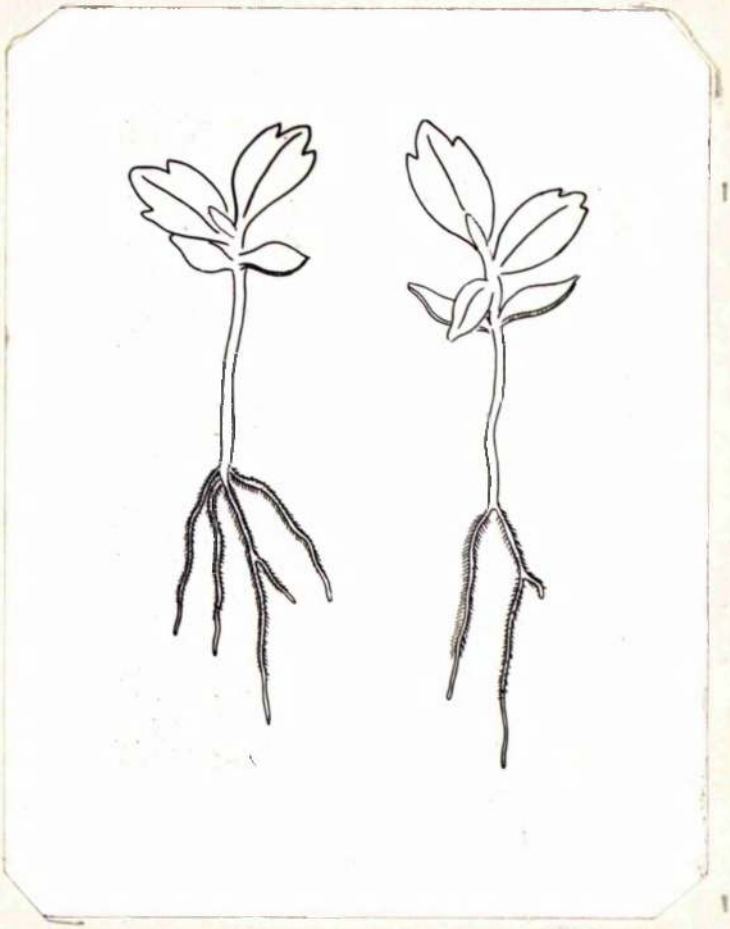


Figure 2.

Showing stage at which the *M. gale* plants were usually transplanted from peat to water culture in the experiments described in this thesis. Note adventitious roots clothed with root hairs which extend almost to the tip. X 1.5.

KCl	7.5 gms.
$CaSO_4.2H_2O$	5.0 gms.
$MgSO_4.7H_2O$	5.0 gms.
$Ca_3(PO_4)_2$	2.5 gms.
$Fe_3(PO_4)_2.8H_2O$	2.5 gms.
Distilled Water	10 litres.

+10 cc. of Hoaglands A-Z solution (Templeman 1941) with molybdenum added as described by Bond (1951).

The pH of this solution is approximately 6.3 and prior to use was adjusted by means of HCl to approximately 5.4. (This latter pH has been shown by Bond (1951) to be a favourable one for nodule production). The roots of the young plants had much peat débris attached to them and before transplanting most of this was removed by brushing the roots in water. The Crone's solution was usually contained in glazed earthenware jars of capacity 2 litres and each jar was covered with a teak top which had been previously thoroughly waxed in order to lessen the possibility of fungal contamination. Each teak top had seven holes in it and the young plants were threaded through these holes and held in position by means of small wedges of rubber.

Alternatively the Crone's was poured into specimen tubes size 3" x 1". Each specimen-tube cork was waxed and a hole bored through the centre to hold

the young plant. The glass tube was covered with black paper to exclude the light from the roots.

Growing the plants in water culture has proved to be very helpful. Using this technique it has been possible to examine plants in the very earliest stages of nodulation and also to examine nodules daily, if required, without any upset to the nutrition of the plant. This was particularly useful in studying the mode of entry of the endophyte. Also, the mineral content of the solution could be controlled and nitrogen salts added if desired.

Two or three days after transplanting the Myrica plants were inoculated. Since no pure culture of the endophyte was available an inoculum was obtained by crushing nodules from the field, or from plants growing in the greenhouse, with pestle and mortar, in distilled water to which was added a little sand. The débris was allowed to settle and the supernatant fluid kept for inoculating purposes. This was carried out by dipping a camel hair brush in the fluid and then applying the loaded brush to the Myrica roots. In addition a few drops of the fluid was poured into each jar or tube. The plants were placed in the greenhouse where they could obtain maximum illumination. The jars were examined from time to time and distilled water added to replace liquid lost through evaporation and

transpiration.

Youngken (1919) stated that the endophyte passes via the pitted vessels of the main root to the stem and is conveyed by the transpiration stream to the flowers, bracts and seeds. But this seed infection has not been borne out in germination tests by Bond (1951) who did not find seed sterilisation necessary. Plants of Myrica have been grown from seed in the Botany Department of Glasgow University for a number of years by Dr. Bond and by the present author. Nodulation has never yet occurred without prior inoculation.

III. THE DEVELOPMENT OF NODULES.

A. THE INFECTION STAGE.

Introduction.

As will be reported in a later Section, (III B) inoculation of the roots of young plants of *Myrica gale*, in the manner already described, is followed by the appearance of nodules in some ten days. This appears to be the indubitable result of the entry of the endophyte contained in the inoculum into the roots, and in the present Section we shall endeavour to establish its mode of entry.

So far as can be determined from the literature no previous observations appear to have been made on this initial stage in the setting-up of the symbiotic relation in *Myrica*. Arzberger (1910) did state that "The tubercles in *Myrica cerifera* originate from small adventitious roots which bear many root hairs and it is quite possible that the fungus in some form makes its inroad through them or by some epidermal cell of the growing root", but this appears to have been pure conjecture.

The mechanism of infection in other non-leguminous nodule-forming genera has received some investigation, but little definite information has emerged. Hiltner (1903), Krebber (1931-32) and Plotho (1941) observed that the application of crushed-nodule inoculum to the

roots of Alnus resulted in root-hair curling but none of them have figured the deformed root hairs. The curling is of interest since, as is well known, a deformation of root hairs is induced in leguminous plants by inoculation with Rhizobium. Hiltner (loc. cit.) and Plotho (loc. cit.) considered the root hair irregularities in Alnus to be induced by the endophyte, but Krebber (loc. cit.) took a different view. He observed similar root hair features on uninoculated alders growing in nitrogen-free solution and to a lesser extent on plants supplied with nitrate, and thought they merely reflect a disordered nutrition due to nitrogen starvation. Alternatively he suggested that the root hair deformation might be due to the injurious effect of substances (such as tannins) present in the inoculum. This last suggestion is not, however, in accord with his observations on uninoculated plants already mentioned. Peklo (1910) claimed to have detected slimy infection-threads in the root hairs of alder plants inoculated with an organism which he had isolated and claimed as the endophyte, a claim which has not been accepted by subsequent workers.(see Section V). Arcularius (1928), working with Hippophäe rhamnoides, and Krebber (loc. cit.) and Plotho (loc. cit.), working with Alnus, reported after careful search that except for the deformation of root hairs

noted above nothing could be said about the mechanism of infection.

Observations.

As has been already noted, the roots of young plants of *Myrica gale* were found to be richly clothed in root hairs, both in plants taken from peat and those grown in water culture. Inoculation had a marked effect on the root hairs. When roots were examined microscopically four or five days after inoculation it was seen that the hairs had become twisted, contorted, and branched, and these effects became more marked as time went on.(Figures 3 & 4). The root hairs of uninoculated plants in nitrogen-free solution were also examined, in the case of these plants a brushful of sterile distilled water having been applied to the roots instead of inoculum. No, or very occasional, deformation of root hairs was observed here. This difference in root hair features between inoculated and uninoculated plants becomes obvious to the naked eye some three to four weeks after inoculation, as is clearly indicated by Figures 5 & 6.

The next effect of inoculation to be observed in the roots was that some root hairs and many cells of the piliferous layer became filled with dense and somewhat granular contents.(Figure 7). When tested by the method of Vinson (1910), which depends on the use of nitrous

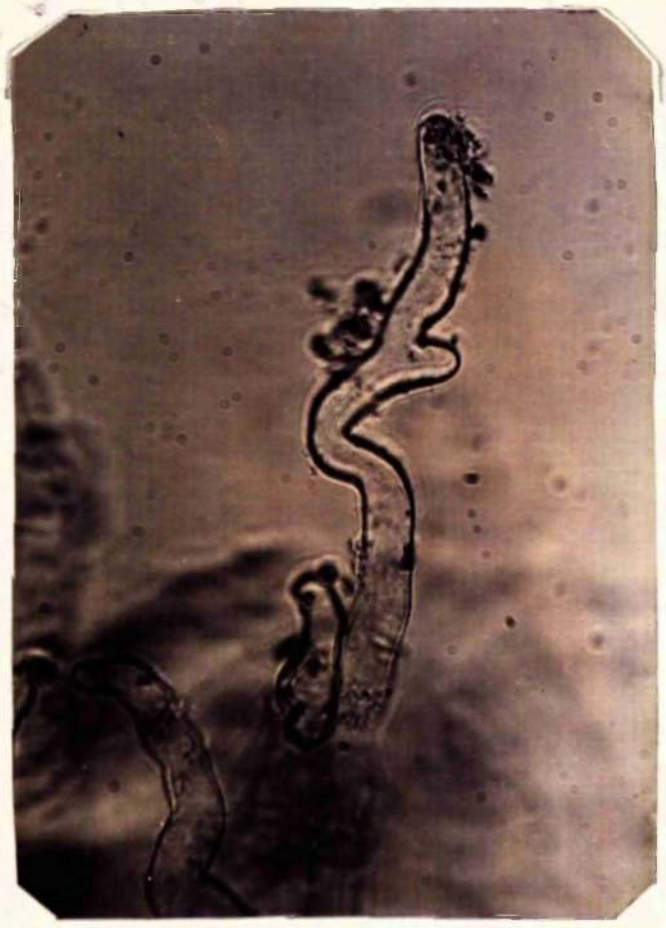


Figure 3.

Photomicrograph of distorted root hairs of *Myrica gale*.
The plant growing in water culture was inoculated with a suspension of *M. gale* nodules.
5 days after inoculation
Fresh preparation.

X 900

Figure 4.

Photomicrograph of portion of root of inoculated plant of *Myrica gale* grown in water culture. Note distortion and twisting of the root hairs. Fresh preparation 2 weeks after inoculation.

X 250.

Figure 5.

Myrica gale plants growing in Crone's solution in specimen tubes. Plant (b) is uninoculated. Plants (a) and (c) inoculated 3 weeks. Nodules are present but not easily visible. Note that root hairs in (b) are long; those in (a) and (c) are not easily distinguishable because the twisting and contortion tends to shorten them. X 2/3

Figure 6.

Myrica gale growing in Crone's solution in specimen tubes. Plant (b) is uninoculated. Plant (a) inoculated 3 weeks.

X 2/3

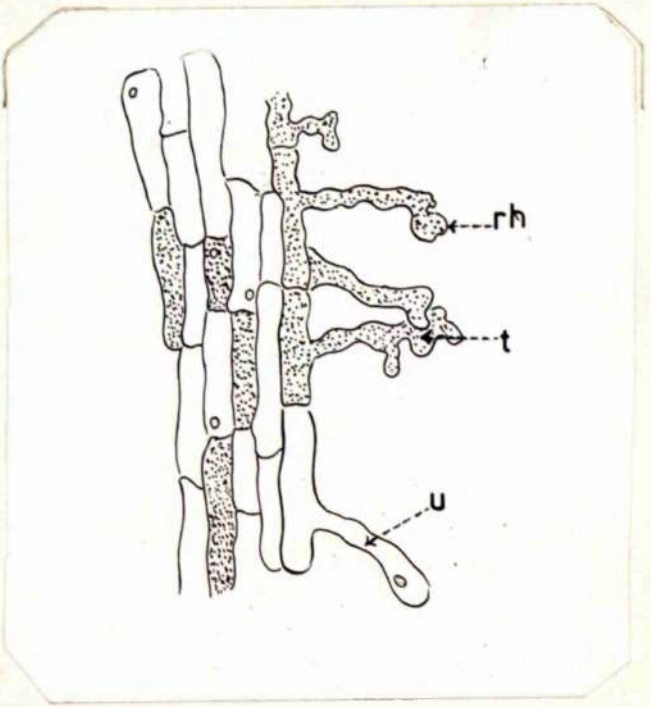


Figure 7.

Piliferous cells and root hairs (rh) of *M. gale* inoculated with nodule suspension. 4 weeks after inoculation. Note contorted hairs filled with tannin (t): normal root hair with no tannin (u).

X 250.

ethers, these contents were found to give the tannin reaction. This tannin was never found in the corres - ponding cells of uninoculated plants, so that it too is taken to be a manifestation of the presence of the endophyte.

A further feature observed several days after inoculation was the presence of many rod-shaped bacteria-like structures attached end-on to the root hairs, giving a curious pin-cushion effect. (Figure 8). In fresh preparations in water, apparently similar organisms, but now actively motile, could be seen in the vicinity of the root hairs. Uninoculated seedlings did not show these organisms, which therefore appear to have originated from the inoculum.

The root hairs of inoculated plants were carefully examined for the presence within them of any invading organism, but the development of tannin, noted above, greatly interfered with this examination. In microtome sections some root hairs appeared to contain infection threads (Figure 9), but further examination of these indicated that they were merely folds in the cytoplasm brought about by slight plasmolysis during fixation. Unlike true infection threads such as are seen in root hairs of inoculated leguminous plants, these "threads" could not be traced beyond the piliferous cell. An extensive search of fresh material, unstained or vitally stained with Methylene Blue or

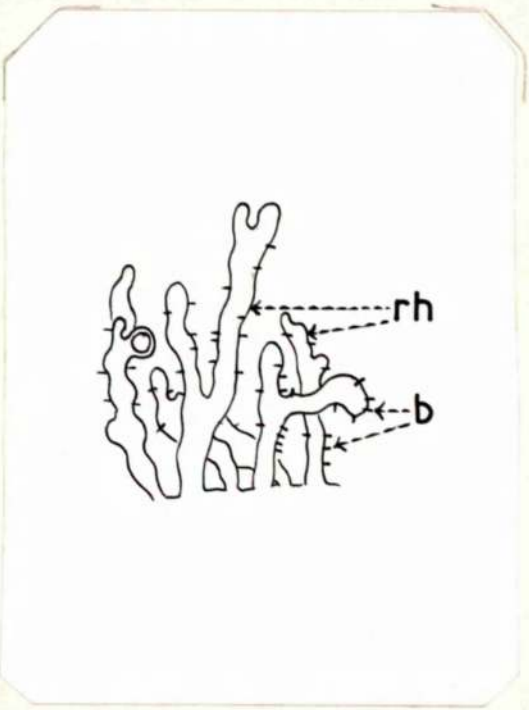


Figure 8.

Contorted and twisted root hairs (rh) of M. gale inoculated with nodule suspension. Note numerous bacteria-like bodies (b) giving "pin-cushion" effect. 6 days after inoculation.

X 250.

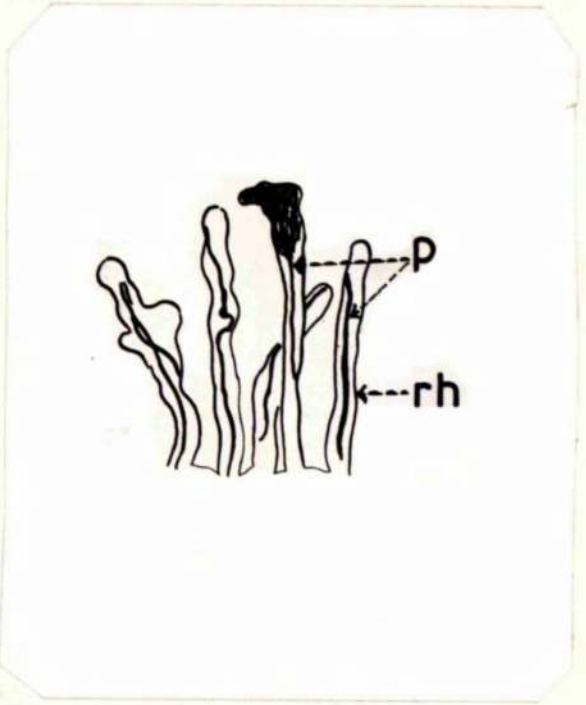


Figure 9.

Root hairs (rh) of *M. gale* with plasmolysed cytoplasm (p) giving erroneous impression of infection threads.

X 250.

Crystal Violet, and microtome sections stained with Safranin and Fast Green and also with Sharman's (1943) stain, failed to reveal any indubitable infection threads within the root hairs. In some cases, however, motile rods of bacteria-like nature were observed within the root hairs and piliferous cells (Figure 10). In appearance they were similar to those previously observed attached to the surface of the root hairs.

Concurrently with, or slightly later than, these events in the root hairs, the cortical cells of the root become filled with granular tannin. In the trabecular type of cortex (see ~~Later~~ Section III C) the filaments of cells become twisted and contorted in a manner reminiscent of infected root hairs (Figures 11 and 12). These developments presumably indicate the arrival of the endophyte. They are not confined to one particular side of a root. Thus all the trabeculae in any one section may be filled with tannin and show contortions, suggesting that there is a widespread or mass infection. Subsequent stages in nodule development will be dealt with in a later Section (IIIC)

Another line of approach to the problem of discovering the mechanism of infection was by direct study of the inoculum obtained by crushing nodules, in order to determine whether any characteristic organism was present. An actively-motile rod-shaped organism was

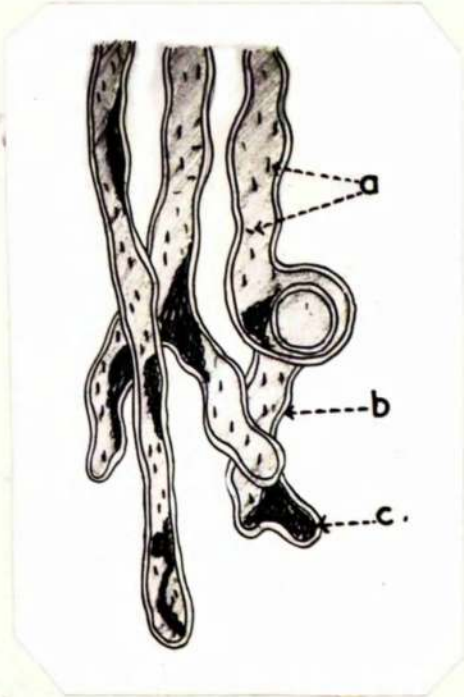


Figure 10.

Contorted and twisted root hairs (b) of M. gale inoculated with nodule suspension. Note numerous bacteria-like bodies within the hairs (a); dark masses of tannin(c), 14 days after inoculation.

X 500.

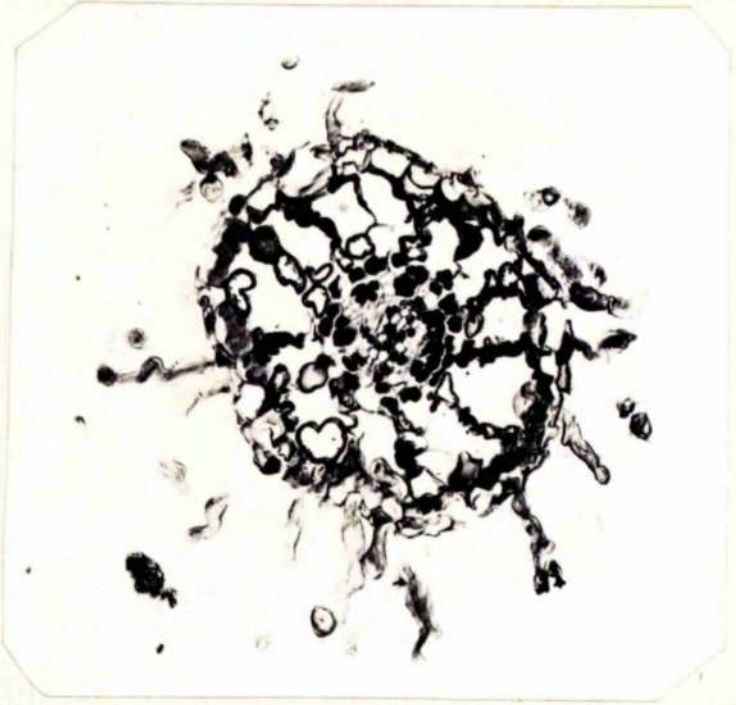


Figure 11.

Photomicrograph of T. S. of inoculated root of M. gale. Note distortion of the root hairs and cells of trabeculae both of which are filled with tannin. Stained Safranin and Fast Green.

X 240.

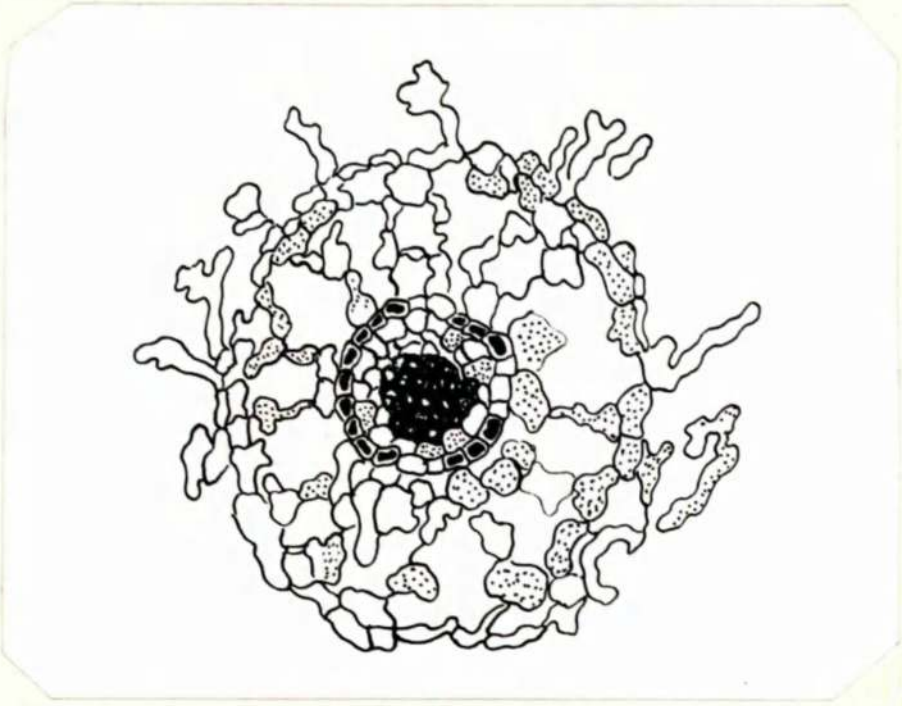


Figure 12.

Drawing of T. S. inoculated root of *M. gale*. Tannin is represented by dots. X 240.

very abundant in a sample of inoculum which ~~had been~~ stood at room temperature for twenty hours after first preparation. Sometimes the organism occurred in short chains or in fascicles; it was particularly abundant around fragments of nodule tissue and in some cases it was present within the cells of such fragments. It is tempting to suggest that this organism represents a stage of the endophyte, particularly since in its general appearance it resembled the organisms previously observed attached to and in the vicinity of the root hairs of inoculated plants. It must, however, be remembered that the inoculum undoubtedly included, besides the endophyte, a considerable variety of other organisms as surface contaminants on the original nodules.

Discussion.

The most positive feature following inoculation of *Myrica gale* plants recorded in the previous Section is the deformation of the root hairs. As noted in the Introduction, this feature has been observed also in *Alnus* by previous workers. Thornton (1952) states that root hair curling is a pre-requisite for infection of legumes by *Rhizobium*, and that it is non-specific since filtrates from other cross-inoculation species of *Rhizobium* will induce the curling, as will also filtrates from other soil organisms (Wilson, 1940). Chen (1938) has shown that root hair curling similar

to that caused by rhizobial inoculation can be induced by indole acetic acid, and has also shown that rhizobia produce this substance. The same substance does not appear to be responsible for the root hair deformation observed in Myrica, since a test showed that inoculation of Myrica roots with rhizobia, besides producing no nodules (see ~~later~~ Section VI of Thesis) caused no root hair response. On the other hand, inoculation of Myrica plants with crushed Alnus nodules or with an unidentified soil Actinomycete did result in root hair deformation, although no nodules were formed.

It seems almost certain that infection must occur through the root hairs. The only other possible route would seem to be via breaks in the cortical tissue at the site of lateral root emergence. Allen & Allen (1940) concluded that this was the method of infection in peanut. In Myrica gale, however, the present author has found nodules appearing on the parent root far removed from the site of lateral roots.

But it is not claimed that the present investigation has enabled the stages in the infection process to be clearly traced. This is because of (a) the obscuration resulting from the gross deposition of tannin at the time of infection, and (b) the drawback that it was not possible to study infection under

aseptic conditions, i.e. with only the Myrica roots and the endophyte present. Thus though the organism giving the "pin cushion" effect may have been the actual invading organism, there is also the possibility that it was a saprophytic form introduced as a contaminant in the inoculum. As stated in the previous Section, bacteria-like organisms were also detected within root hairs of inoculated plants, though not with such regularity nor in such quantity as could have been desired. It is possible that it is in this form that the endophyte enters the root. It will be shown in later Sections of the Thesis that the writer considers the endophyte to be an Actinomycete. It is well known that the threads or hyphae of many Actinomycetes break up into portions which are indistinguishable from bacteria, and indeed in culture many Actinomycetes may become "bacterial". The organisation of the invading organism into an infection thread is not necessarily a feature of Actinomycete infection. Infection in Common Scab of potato caused by Actinomyces scabies is effected by short lengths of hyphae which penetrate and migrate, as such, without any apparent increase in length of the segments.

A final point relates to limitation of infection. Under conditions of water culture the usual sequence is that following inoculation of young transplants

nodules appear at a limited number of foci on the root system, the latter being at this stage very limited in extent. The extension of the root system in subsequent growth is not, however, followed by the development of nodules at many new foci, the mass of nodule tissue being increased rather by proliferation of the original nodules. The same is true of plants carried on into a second and third year, the nodules remaining for the most part in a congested mass on the oldest part of the root system, as will be shown by later illustrations. From one such plant, in its second year, the nodules were excised in another connection. Within a few weeks new nodules appeared at over 100 foci scattered all over the root system. It seemed that nodule excision had removed some restraining influence previously inhibiting further nodulation. It may be noted that Nutman (1948, 1949, 1952), from his studies of nodulation in legumes, has postulated the production in the meristems of existing nodules and lateral roots of a substance inhibitory to further nodulation.

III. THE DEVELOPMENT OF NODULES (CONTINUED).

B. THE EXTERNAL FEATURES OF NODULE DEVELOPMENT.

Introduction.

Although many authors (see ~~Later~~ Section IV) have described the external appearance of the mature nodules there are only a few descriptions of the nodules during early stages of development. Chevalier (1900-02) observed but did not describe nodules on plants which had only opened their two cotyledons. He noted too that frequently a rootlet develops in the interior of a nodule and proceeds to grow like a normal root. Arzberger (1910) working with Myrica cerifera noted that the nodules originate from small adventitious roots which bear many root hairs and that the colour of the nodules varies from light grey to pink in the youngest tubercles changing to flesh colour as they age. Bottomley (1912) worked with Myrica gale. He remarked that the young nodules are visible first as tiny swellings on the sides of the roots which grow until they are two to three millimetres long, resembling at this stage Vicia nodules. From the distal end of each nodule a hair-like rootlet grows out. Each rootlet (nodule root) which arises from the nodules is, according to Bottomley, devoid of a root cap. Bond (1951) stated that in water culture at pH 6.3 and 5.4

nodules become visible to the naked eye 2-3 weeks after inoculation and at this stage formed bright red swellings on the roots. The red pigment was shown by him to be anthocyanin, not haemoglobin such as occurs in the legume nodules. Nodulation was slower and more sparse in more acid solutions. The red pigmentation of the young nodules was gradually replaced by a buff colour, and after a few months few nodules were still red. In a further paper Bond (1952) has noted and investigated the upward growth of the roots (nodule roots) which emerge from the nodules. He has shown it to be due to a negative geotropism and considers that the nodule roots probably have an aerating function. Hawker and Fraymouth (1951) state that, in their experience, naturally and artificially inoculated seedlings of non-leguminous nodule-bearing plants do not develop nodules until they have reached a considerable size, whereas inoculated clover seedlings develop them soon after germination.

Observations.

As noted already, these observations on the development of the nodule were made on plants growing in water culture. Inoculation was effected as described in the previous Section. Drawings were made from time to time under the dissecting microscope.

The nodules appeared only on those roots which

were seen to be richly clothed with distorted root hairs. Microscopically the nodules may often be detected some ten days after inoculation as lateral swellings on the parent roots. Although usually these swellings are rounded and each marks the site of a single nodule, (Figure 14) multiple infection also occurs fairly frequently and the swellings may have a tri-lobed appearance. (Figure 23). This should not be confused with the branching of the nodule which takes place at a later stage (see p. 42). The time of appearance of the nodules however varied with the time of the year, sometimes less, sometimes more than ten days. The swellings are seen to cause more disruption of the parent tissues than do the development of lateral roots. To the naked eye the first nodules may be visible some fourteen days after inoculation, when the plant is at the three leaf stage. (Figure 13). This is at a stage similar to that at which clover has been noted by the present author to form nodules. The findings therefore coincide with those of Bond (1951) and are not in agreement with those of Hawker and Fraymouth. (see above).

The young nodules usually early develop a reddish pigmentation (as noted in the Introduction) though this is not invariably so. Thus in roots which are already pinkish in colour the nodule assumes a

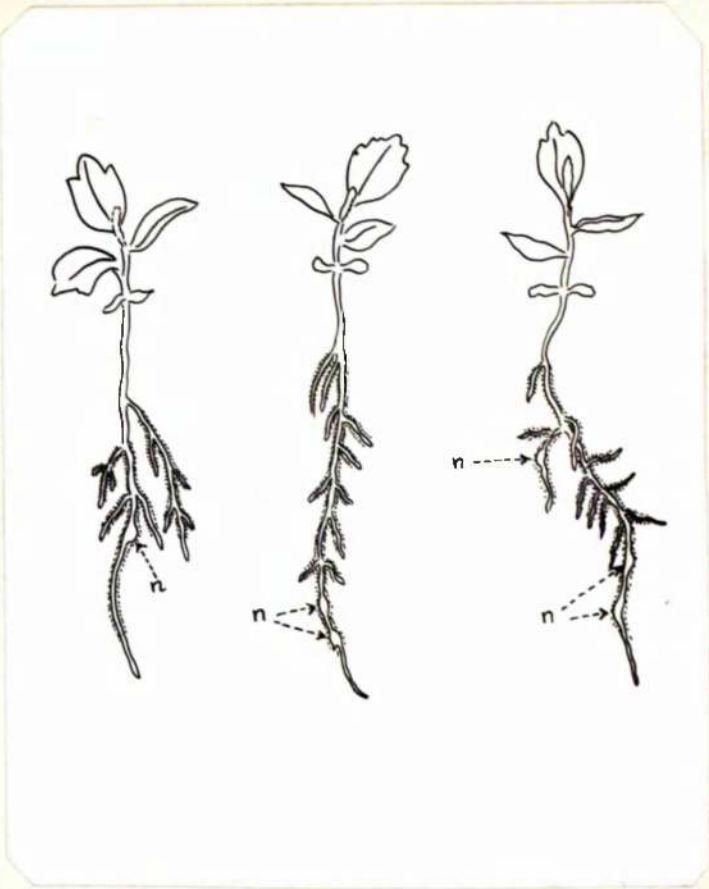


Figure 13.

Young plants of M.gale. Seed sown in peat 25. 2. 52. Transplanted to water culture 1. 5. 52. Inoculated 5. 5. 52. Drawn 30. 5. 52. Note presence of root nodules (n) X1.

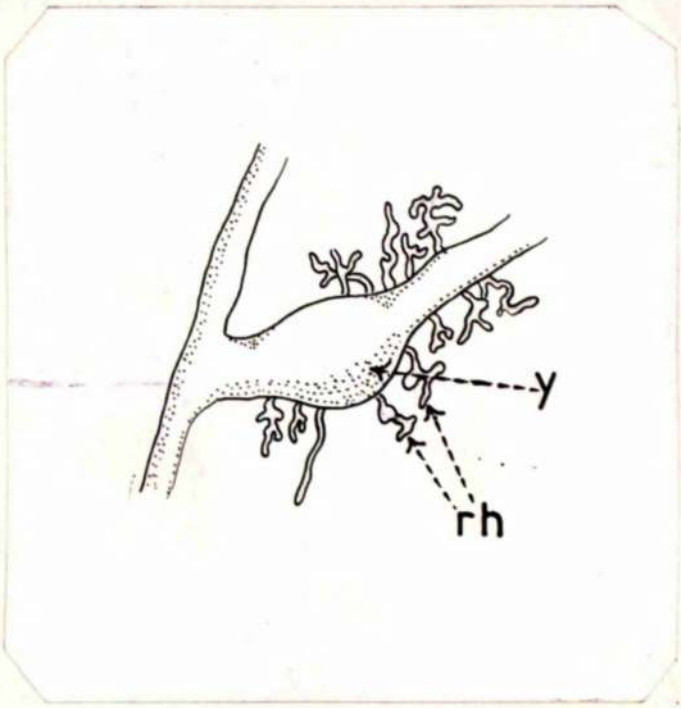


Figure 14.

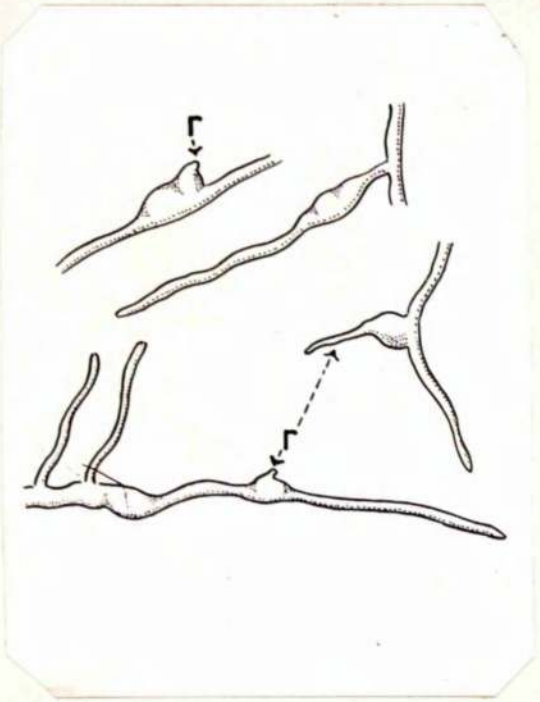
Young nodule (y) of *M. gale* in course of formation. A few root hairs have been drawn (rh) to show typical distortion.

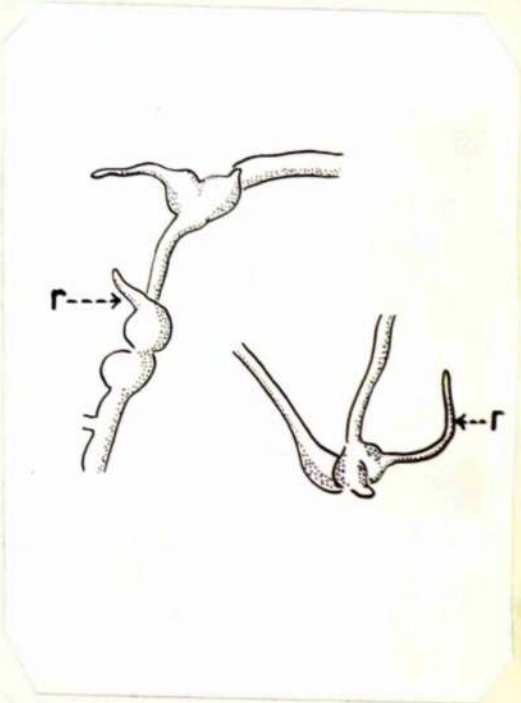
2 weeks after inoculation. X 40.

brilliant red colour. In white roots on the other hand the young nodules may have a pinkish tinge or in some cases be entirely colourless. Examination of sections of fresh material showed that the anthocyanin is in the cell vacuoles and occurs in the root and nodule cortex. No pigment is present in the piliferous layer of the root. Soon, in all cases, the nodules lose their red pigmentation and assume the characteristic light brown tint of the mature nodule. The time for this development to take place is variable.

As the nodule emerges from the parent root it is at first globular but soon it becomes roughly pear-shaped and from its distal pointed end a fine root, the nodule-root, appears early, as noted by Chevalier (1900-02) and by Bottomley (1912), in some cases a month to five weeks after inoculation, i.e.2-3 weeks after first appearance of the nodule and proceeds to grow upwards. (Figures 15-19). The nodule roots are white except at the tip where there is a dark coloured root cap.(Figures 20, 21)

As noted by Arzberger (1910) for Myrica cerifera the nodule roots of Myrica gale also may send off side roots. These have been observed by the present author both in the field and in water culture material. (Figure 22). But it is much more common to find them unbranched. They are typically devoid of root hairs though not invariably so. Hairs have been observed on





Figures 15 and 16.

Early stages in the development of nodules of M. gale. Note formation of nodule-roots (r). 4-5 weeks after inoculation.

The root-hairs have been omitted for the sake of clarity. X 8. and X 12.

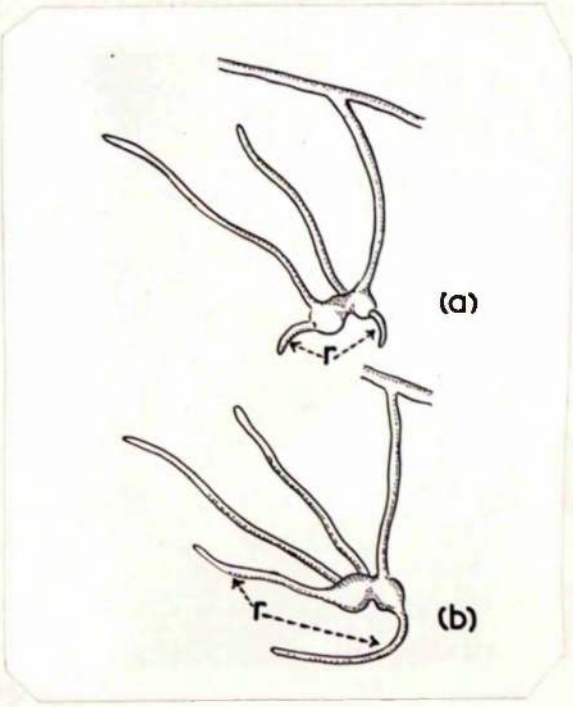


Figure 17.

Two stages in the development of nodules of M.gale (a) 4 weeks after inoculation.

(b) same nodules 10 days later.

Note nodule-roots (r)The top of the solution is to the left of the photograph.

Root hairs omitted for the sake of clarity.

X 5.

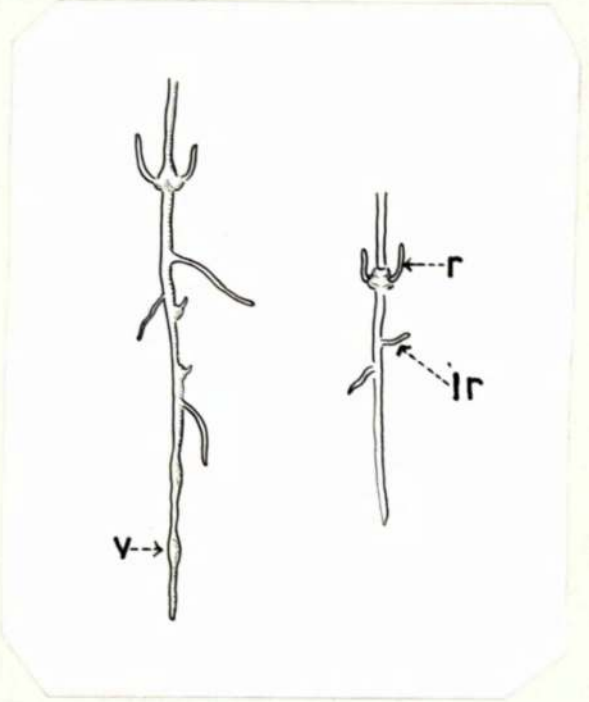


Figure 18.

Inoculated roots of M. gale. Note very young nodule (v), nodule-root (r) and lateral root (lr) Root hairs omitted for sake of clarity. X 1 1/3.

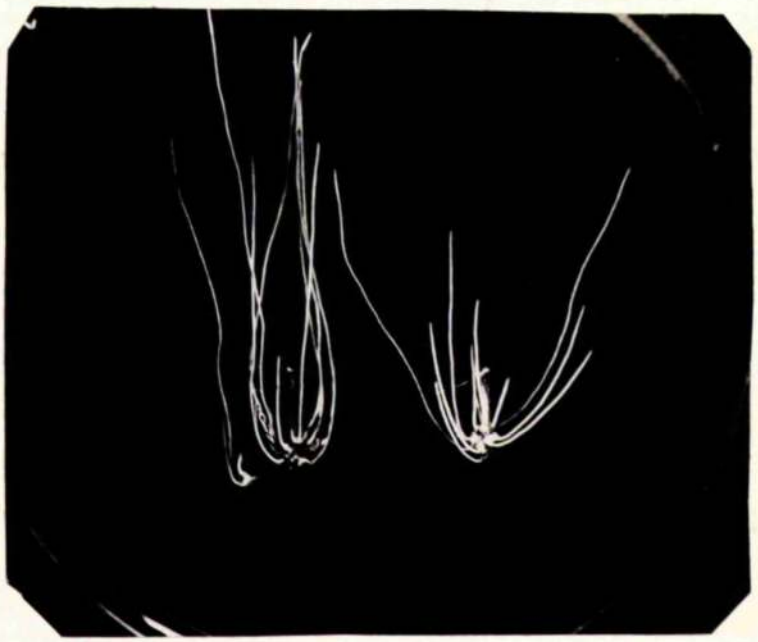


Figure 13.

Young nodules from water-culture plant. Note long upward growing nodule-roots.

Natural orientation. Nat. size.

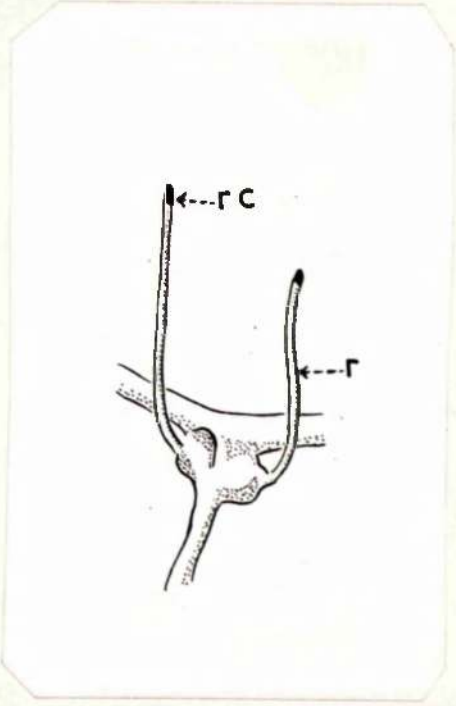


Figure 20.

Young nodules of M.gale showing nodule-roots(r) with prominent root-caps (rc) 3-4 weeks after inoculation. Natural orientation. X 10.

Figure 21.

Photomicrograph of terminal portion of nodule-root of M.gale showing prominent root cap. Fresh preparation. X 240.

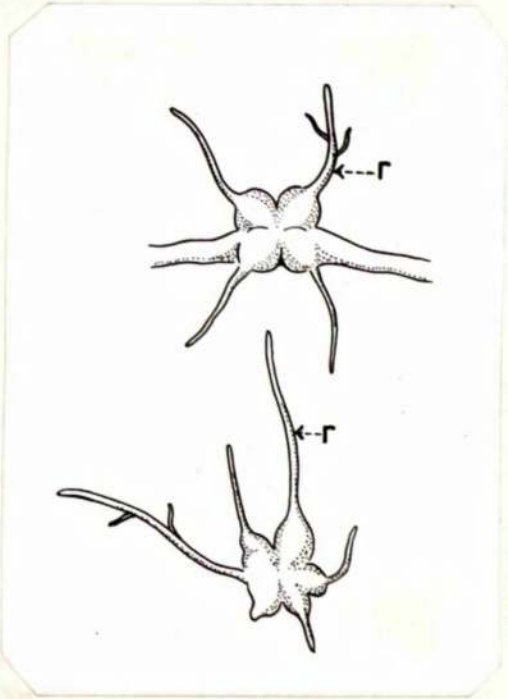


Figure 22.

Field-nodules of *M.gale*. Note prominent nodule-roots (r) which sometimes branch. X 5.

a few nodule roots when in the very young condition (Figure 23) and also some mature nodule roots may be sparsely provided with them. But never are they thickly clothed with hairs as are the roots. In this connection it is of interest to note that the nodule-roots never bear nodules.

The branching of the nodule will be dealt with in a later Section.(IV)

Discussion.

The only aspect of this early development of the nodule that calls for discussion here is the pigmentation.

It is of interest to note that there is often a fair concentration of anthocyanin in the roots of those plants, growing in nitrogen free solution, which have not been inoculated, and that this increases in concentration the longer the plants are allowed to grow. It is deep red particularly at the meristematic zones e.g. the root tips. Presumably nitrogen shortage becomes more acute as time goes on and as the plant is actively synthesising carbohydrates then the C/N ratio will widen and it is postulated that the reddening is due to the widening of the ratio. As stated above the red pigmentation of the nodule is more marked on roots which are themselves reddish in colour. These roots are already deficient in nitrogen

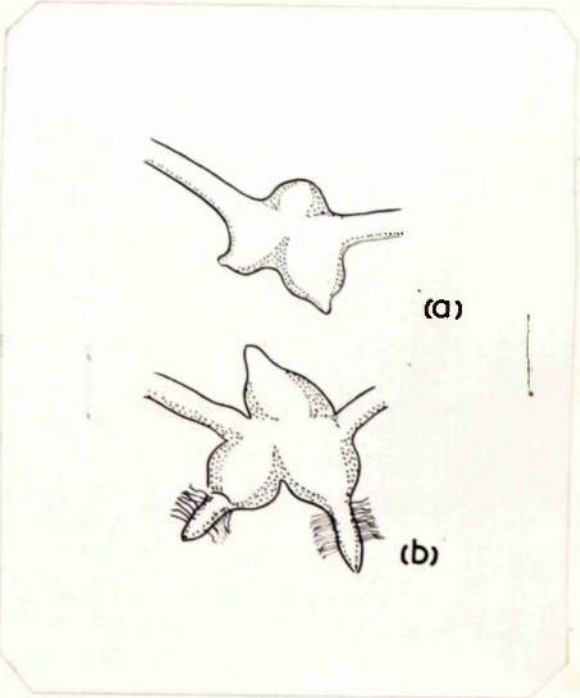


Figure 23.

Two stages in the development of tri-lobed nodules of M.gale (a) drawn 4 weeks after inoculation (b) the same nodules 2 weeks later. Note nodule-roots bearing root hairs. This is an unusual feature. X 15.

and the increased meristematic activity due to nodule formation further depletes the store and leads to a deepening of the colour.

In white roots the nodule may have a pinkish tinge or in some cases be colourless. Presumably in the white roots the C/N ratio is of such an order to prevent reddening but the advent of the endophyte (which will also require nitrogen for its growth and multiplication) and the consequent meristematic activity leads to a reddening in that area due to a widening of the C/N ratio. At this early stage in nodule formation it is presumed that the endophyte is not fixing enough nitrogen to offset the temporary shortage. The white roots which produce non-red nodules have enough nitrogen to meet the demands of the endophyte and the demands of the meristematic activity until such time as the endophyte or the nodule starts fixing nitrogen from the air. Consequently these nodules are never red in colour. During experiments on the effect of combined nitrogen on nodulation (see later Section VII B) it was observed that plants growing in N-free solution formed bright red nodules. Those supplied with small amounts (say 7 mgms N per litre of solution) had pinkish nodules while those growing in solutions containing 35 to 70 mgms had nodules which were colourless or of a very pale pink.

The coloration then appears to be primarily

due to a nitrogen shortage. But light, quite apart from its effect on photosynthesis, may also play a part. Nodules developing on roots which were exposed to light from time to time, were of a much more brilliant red than those developing on roots in darkness. The loss of red coloration is no doubt due to the fact that the endophyte within the nodule fixes nitrogen in sufficient quantities required for its own and for the nodule's growth. Red-coloured roots also become white as the nodule supplies the host plant with nitrogen compounds.

A discussion on the morphological nature of the nodule-root will be reserved until the anatomy of the nodule-root has been examined.

THE DEVELOPMENT OF NODULES (CONTINUED).

C. THE INTERNAL FEATURES OF NODULE DEVELOPMENT.

Introduction.

The study of the early development of the nodule within the parent root appears to be almost an neglected field , probably due to the lack of material. Chevalier (1900-02) from a microscopical examination of mature nodule sections, because of their internal structure and location, considered that they were modified side roots. Dangeard and Trnka (1929) on the other hand stated that "The formation of nodules is due to the proliferation of the cortical cells (presumably of the parent root). Under the influence of the parasite it thus produces regions of contaminated cells which can completely enclose the vascular cylinder". Hawker and Fraymouth (1951) stated that the nodules were modified lateral roots.

Methods.

The material for the study of the internal features of nodule development was again obtained mainly from plants growing in water culture. Where field material could be obtained it also was examined. One month after inoculation the Myrica gale roots growing in water culture showed various stages in nodulation ranging from incipient nodules showing as mere red swellings of the root to easily discernible

nodules with prominent nodule-roots. Samples of the various stages were collected. Since nodulation is a continuous process, at least in the growing season, it was hoped that some very early stages in nodulation not externally visible microscopically, would also be revealed in microtome sections.

The roots and nodules were fixed in Bouin's fluid (Sass (1951)) for twenty-four hours under some negative ~~pressure~~ tension. Bouin's fluid was invariably used in these anatomical investigations and gave consistently good results, there being relatively slight plasmolysis after its use on even the most fragile ~~tissues~~ cells, e.g. root-hairs.

The material was embedded in paraffin wax, and microtome sections were cut. These were generally 6-7 μ in thickness except for those stained for suberin which were 26 μ. Hand sections of fresh material were also cut.

Early in the investigation the stain employed for microtome sections was ~~[illegible]~~ Haematoxylin and Orange G. (Chamberlain 1915). This was later replaced by Safranin and Fast Green (Sass 1951) which gave good results for general anatomical purposes, but for cytological examination the best stain was found to be Tannic Acid and Iron Alum with Safranin and Orange G. (Sharman (1943)) which gave a greater degree of differentiation and enabled one to

pick out the various features more easily. For the investigation of the distribution of suberised tissues the stain employed was Sudan 111 and the method employed was that described by Bond(1931). It was as follows:- A Collodion Clove Oil fixative was employed consisting of equal parts of the two constituents. Sections were cut 26μ in thickness in order to make for ease of handling and manipulation. The sections were stretched in the usual way on a slide smeared with glycerine and transferred to slides smeared with the Collodion Clove Oil fixative. The slides were transferred to a 30° C incubator and left there for 24 hours to effect fixation of the sections to the slides. They were then dipped in xylol to dissolve the wax and transferred through the alcohols to water. The slides were then transferred to half strength "Milton" for ten minutes to clear, washed in running water for thirty minutes, then to 50% alcohol and finally into a solution consisting of equal parts absolute alcohol and glycerine. The sections were stained with Sudan III heating gently over a bunsen flame. The Sudan III stain consisted of 0.01 gms. Sudan III dissolved in 5.8 cc. 95% alcohol, 5 cc. of glycerine being then added. The sections after staining, were washed in glycerine and final mounted in glycerine jelly.

Gram's stain was sometimes used but the results were very variable, and it was considered unsuitable for this material. The distribution of woody tissues was determined by staining sections with phloroglucinol and HCl.

Observations and Discussion.

As seen in transverse section (Figures 24, 25) the normal, uninfected root of Myrica gale growing in water culture consists of:-

(1)Piliferous layer consisting of flattened irregularly shaped cells which in the young roots are prolonged to form root-hairs.

(2)Cortex - the appearance of the cortex depends upon the maturity of the root. Thus in the young condition it consists of parenchymatous cells with small air spaces between the cells. In the more mature condition the cortex is made up mainly of trabeculae. Each trabecula is usually only one cell in width and between the strands there are large air spaces. The endodermis consists of small closely-fitting cells, in which in the young condition, the Casparian strip can be clearly seen. As the root becomes older the endodermis passes into the secondary condition and its cells become impregnated with tannin and with a substance referred to by Chevalier (1900-2) as gummy lignin or wound gum, and he lists the various

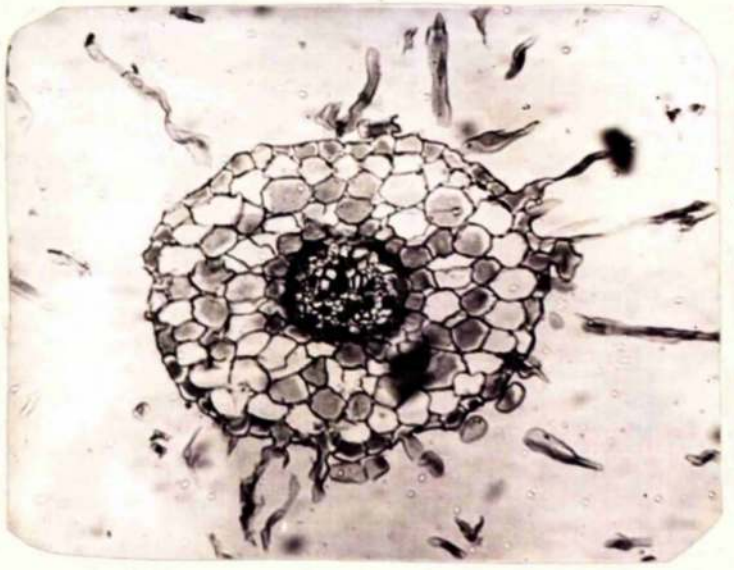


Figure 24.

Photomicrograph T.S. of uninoculated root of M.gale. Note root hairs, cortex, endodermis, central cylinder.

Stained Safranin and Fast Green. X 250.

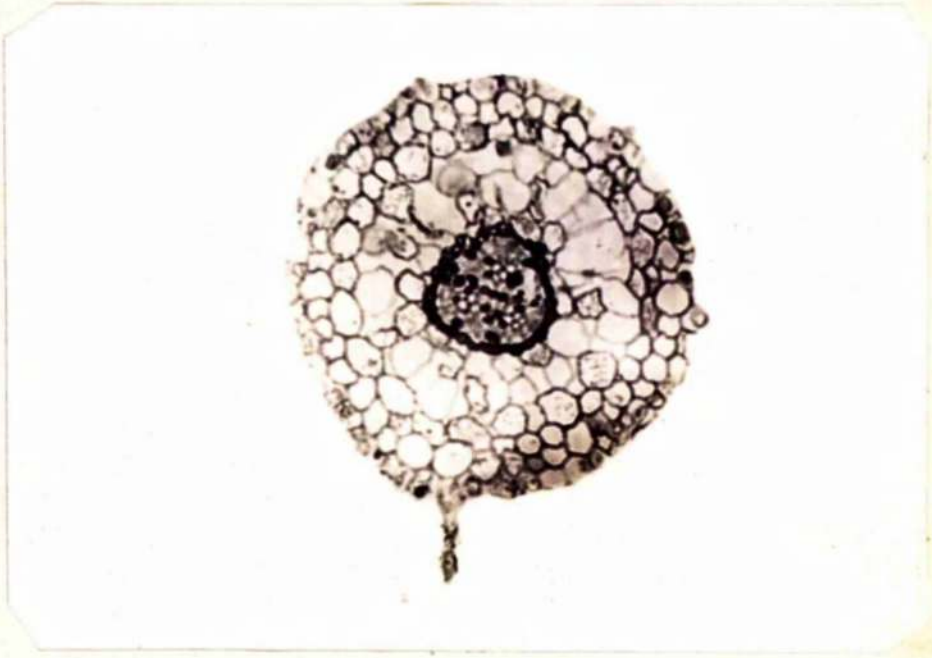


Figure 25.

Photomicrograph T.S. of uninoculated root of M.gale (more mature than that shown in Figure 25).

Note that in this case the cortex is made up largely of trabeculae.

Stained Safranin and Fast Green. X 250.

reactions by which it is identified.

(3) The Vascular Cylinder consisting of xylem, phloem, cambium and pericycle. The xylem is roughly tetrarch in structure but at times may appear almost circular. The xylem elements are never heavily lignified in the water culture roots and are thickened mainly with cellulose with deposits of lignin at the corners. The phloem is not easily distinguishable from the pericycle in transverse section.

The earliest stages that have been found in the development of the nodules are those shown in Figures 26-29 when the nodule is seen as an almost spherical mass of undifferentiated cells enclosed by the parent root endodermis. The cells arise by divisions taking place in the pericycle. The presence of the endophyte in the cells is not easily determined due to the heavy deposits of tannin within most of the cells. An occasional cell may be noted however in which the endophyte can be seen as meshes of extremely fine hyphae, less than 1 μ diameter, together with the nucleus and cytoplasm of the cell.

Even at this very early stage the young nodule may be easily distinguished from a young lateral root (Figures 28, 29) because of their respective shapes. The lateral root is cylindrical or perhaps even conical and its emergence causes little disturbance

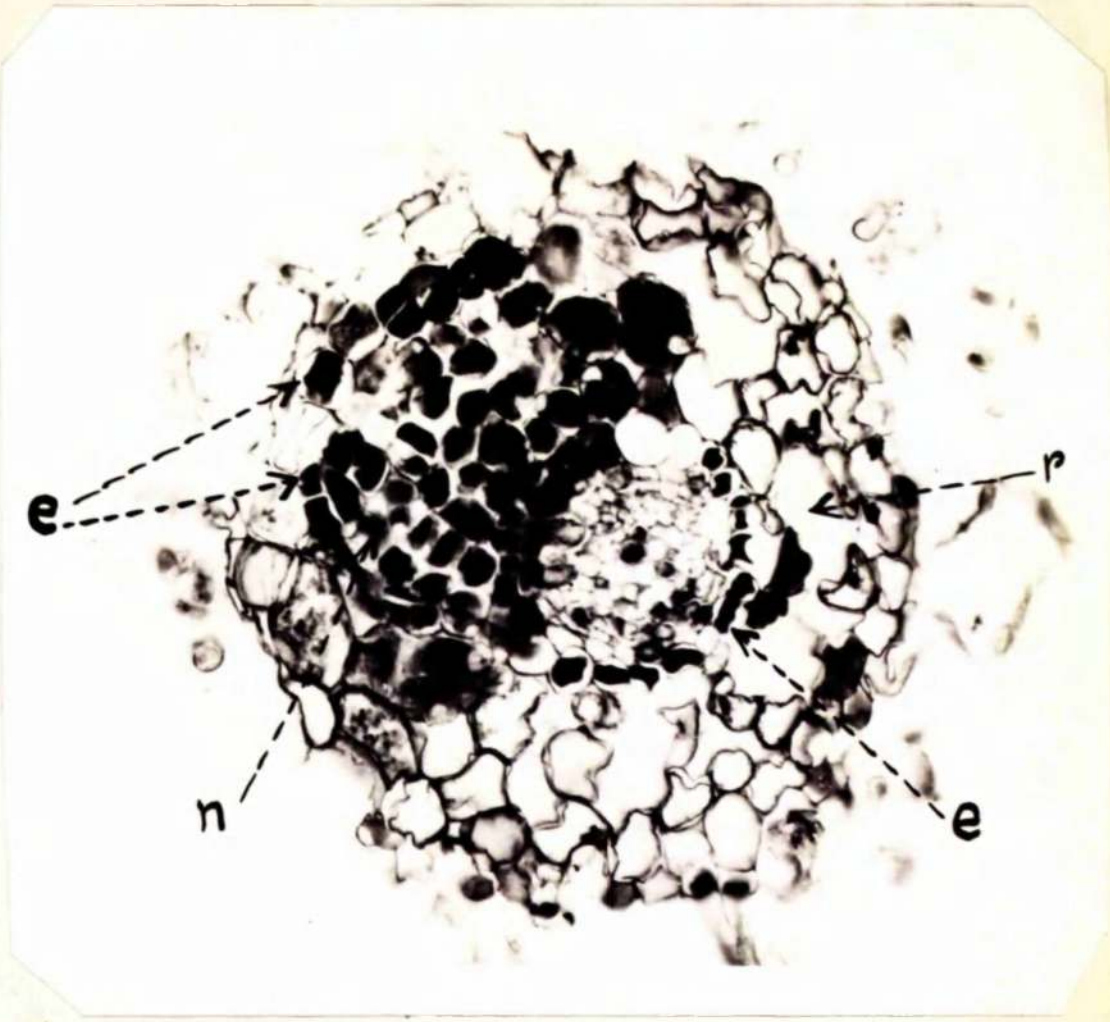


Figure 26.

Photomicrograph of young nodule (n) of M.gale within the parent root (r) which is seen in transverse section.

Note that nodule cells are filled with darkly staining tannin. Note endodermis (e).

Stained Safranin and Fast Green. X500.

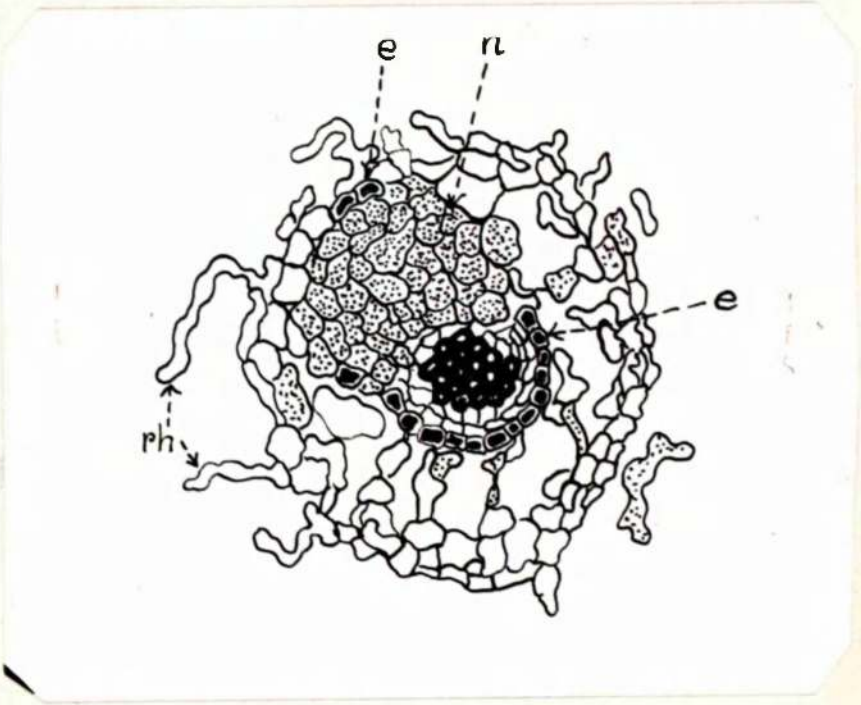


Figure 27.

T.S. Root of M.gale showing young nodule forming (n) Note endodermis (e) and root hairs (rh). X240

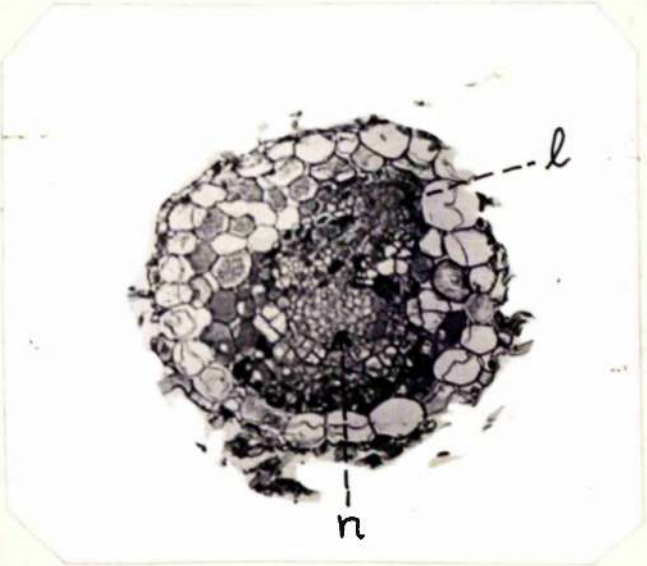


Figure 28.

Photomicrograph of T.S. of root of M.gale showing lateral root forming at (l) and nodule forming at (n).

Stained Sharman's method. X240.

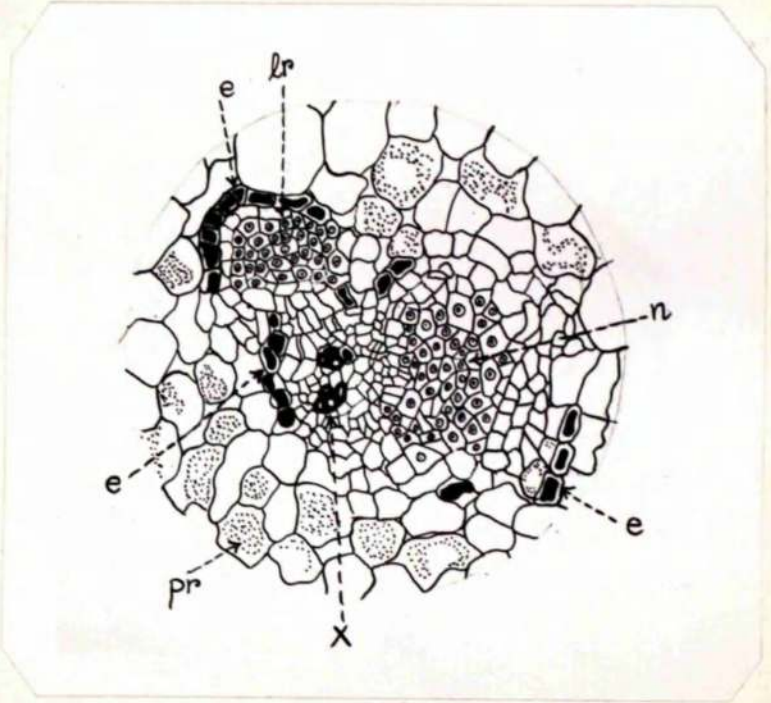


Figure 29.

Drawing from Figure 28 to show details of nodule formation (n) and lateral root formation (lr). Note endodermis (e) xylem of parent root (x) and parenchyma of parent root (pr). X450.

of the surrounding tissues of the parent root. (Figure 30). The nodule, on the other hand, is almost spherical (Figure 26) and there is fairly well marked disruption of the surrounding tissues even, as previously noted, in some cases leading to an obvious splitting of the parent root. (Figure 31). And of course the nodule cells are mostly filled with tannin precipitate which is absent from the cells of the young root.

The young nodule has no vascular strand or recognisable plerome. Neither has it a protective cap. Eventually an apical meristematic zone is established and gradually a centrally placed vascular strand is differentiated which links up with the vascular tissues of the parent root. (Figure 32). The apical meristematic cells are small and densely filled with tannin so that it is difficult to decide whether the endophyte is present within them. But further back towards the base of the young nodule, where the cells are enlarging, the endophyte can be clearly seen within some of the cells of the cortex as a closely coiled mass of hyphae, the diameter of the hyphae being less than 1 μ ~~in diameter~~. These infected cells are enlarged and distributed without order through the cortex. They will be dealt with in more detail in a later Section. The remaining cells of the cortex are

(IV B)

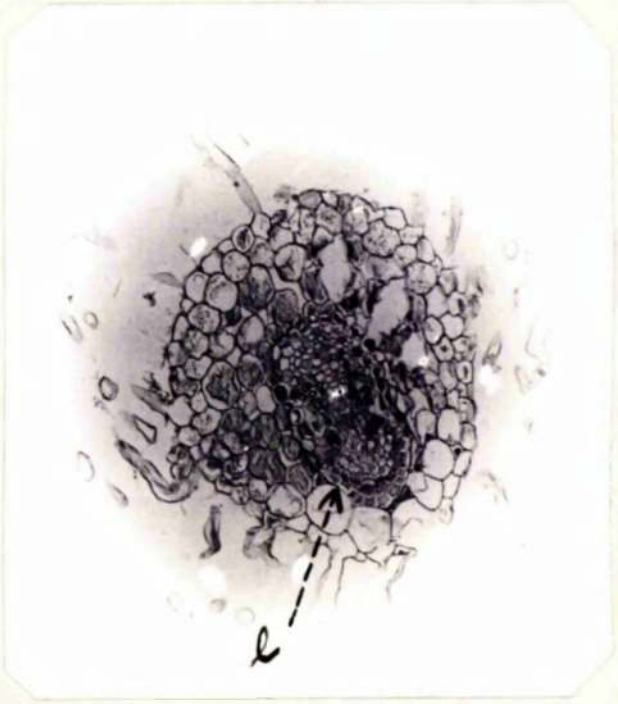


Figure 30.

Photomicrograph of lateral root (l) emerging from the parent root which is seen in transverse section.

Stained Sharman's method. X240.

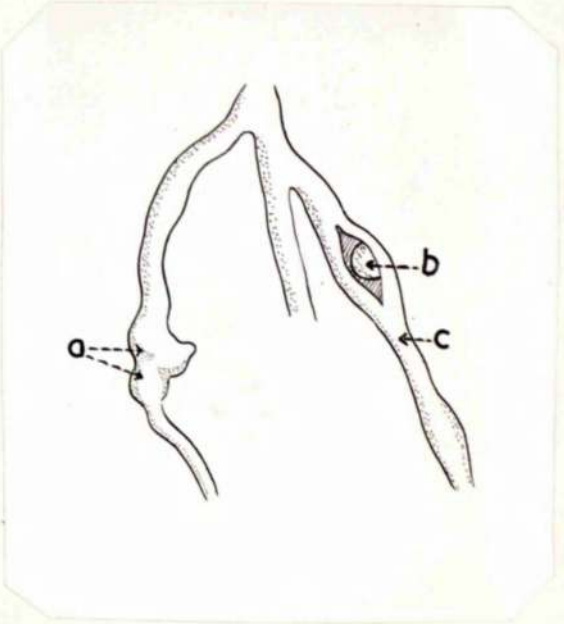


Figure 31.

Early stages in nodule formation of M.gale.
Triple nodule in course of formation at (a).
Young nodule (b) showing through split cortex of parent root (c).
Root hairs omitted for sake of clarity.
5 weeks after inoculation. X12.

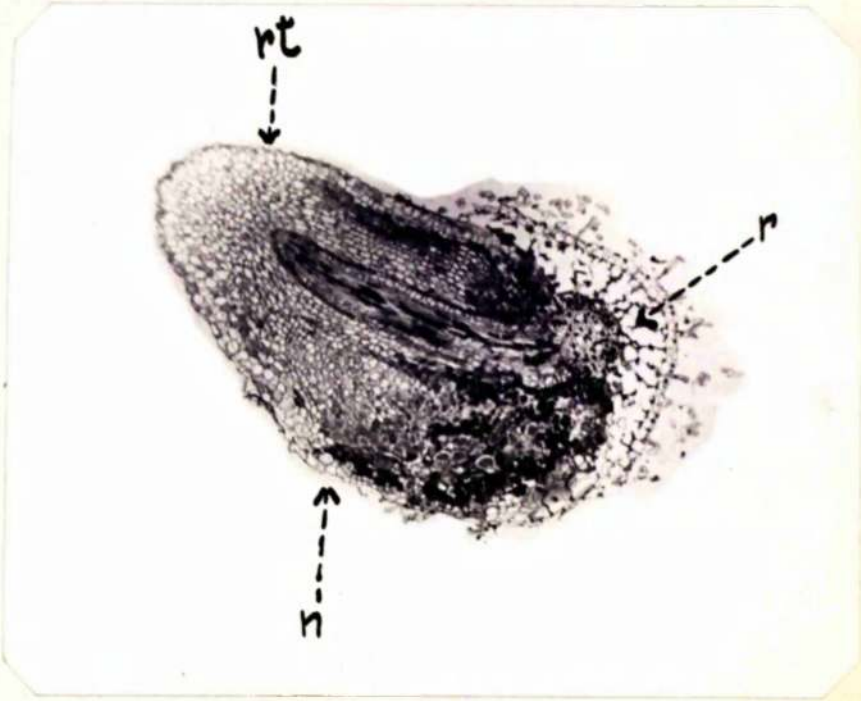


Figure 32.

Photomicrograph of young nodule (n) emerging from the parent root (r). Note that even at this stage the tip of the nodule is being prolonged to form a nodule-root.
4 weeks after inoculation.
Stained Sharman's method. X120.

filled either with tannin or starch grains.

Soon after emergence of the nodule from the parent root a cork cambium is established in the outer cortex and this cuts off 2-3 layers of cork cells to the exterior. As these cells become suberised they presumably isolate the nodule from communication with the external medium be it water, soil or air. Its main communication must then be via the nodule-root which remains unsuberised for the greater part of its length, and also via the main root.

It may be instructive at this point to describe the origin and structure of the leguminous nodule so that it can be compared with the Myrica nodule.

According to Allen and Allen (1950) there are two types of leguminous nodule as regards origin. In the exogenous type (which is by far the more common) infection is limited to the parenchymatous cells of the cortex. Aside from vascular linkages the endodermis and those tissues within the central stele of the root are not directly concerned in their formation. Endogenous legume nodules, on the other hand, comprise tissues which are differentiated entirely from the pericyclic cell proliferations.

The Myrica nodules, as noted above, are endogenous in origin arising from the pericycle.

Investigators are in agreement that the histological patterns of legume nodules from widely different plant species are remarkably similar. Four areas are conspicuous in median longitudinal sections of all nodules. The exterior consists of a spongy layer of loosely packed cortical parenchyma cells generally devoid of prominent contents (suberised cells in Myrica). Within this nodule cortex is contained a peripheral vascular system (central in Myrica) which unites with the primary xylem groups in the root stele. Innermost (pith or medulla) is the infected area , many cells of which are packed with bacteria.(The infected area in Myrica is cortical). Many of the uninfected cells are packed with starch grains. A meristematic zone of compact, small actively dividing non-invaded cells is found between the distal ends of the vascular branches and the outer boundary of the bacteria-filled region. (The meristematic zone in Myrica is terminal and it is difficult to tell whether or not these cells are invaded due to the masking effects of tannin).

To return to the Myrica nodule. Soon after differentiation of the vascular strand the tip of

the nodule, as already noted, is carried forward as a fine nodule-root which has a prominent root-cap (Figure 33). Close examination of the cells of this nodule-root fails to reveal the presence of the endophyte and as already stated it never bears root nodules. Why the hyphae of the endophyte stop short at the apex of the nodule and do not pass into the nodule root is unresolved. This may be related to the absence of sparse coating of root-hairs though no doubt other explanations are possible, e.g as noted later (p 81.)the endophyte may be micro-aerophilic and if these are aerating organs as suggested by Bond(1952) then the concentration of oxygen within them may be too high for the endophyte. Examination of sections of such nodule-roots shows that the general structure is similar to that of the normal root, there being many air-spaces in the cortex which is composed of trabeculae. Nodule roots from field material collected in November were also examined. In them it could be seen that there were abundant air spaces but not all nodule-roots were alike in structure. The majority showed trabeculae passing from the outermost layer of the cortex to the endodermis (Figure 34) closely resembling the young root structure already described. A few

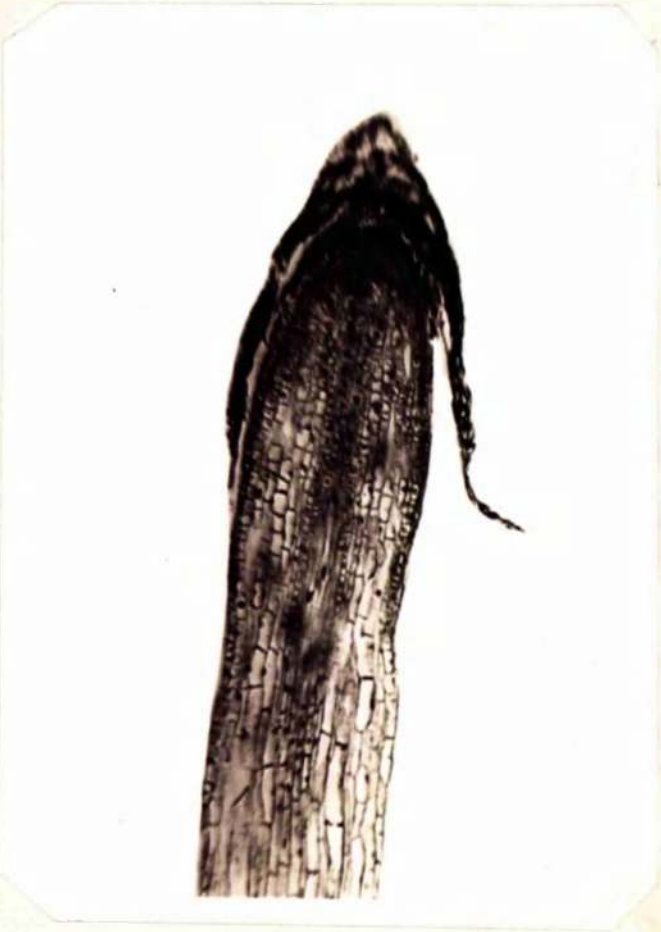


Figure 33.

Photomicrograph of L.S. of root-cap of nodule root of M.gale.

Stained Safranin and Fast Green. X 240.

showed no evidence of trabecular structure. (Figure 35). The reason or reasons for this diversity of structure is not clear but it may be related to the conditions under which the plant is growing. Thus the trabecular type of nodule-root, invariably present in plants growing in water culture, may as a result of some causal mechanism be developed in water-logged soils thereby effecting greater aeration, whilst the other type is developed in drier soils where there is not such an acute oxygen shortage.

Regarding the distribution of corky tissues within the nodule-root, many sections of field nodules with prominent nodule-roots, again collected in November, were cut and stained with Sudan 111. By means of longitudinal sections it could be seen that the nodule was covered, as already described, with a well developed cork layer and that this extended for some short way into the nodule-root. (Figure 36). The origin of the cork layers in the two organs, nodule and nodule-root, is however different. In the case of the nodule it will be remembered the cork layer is formed from a cambium which arises in the outer cortex. The cork layer in the nodule-root on the other hand is formed by the suberisation

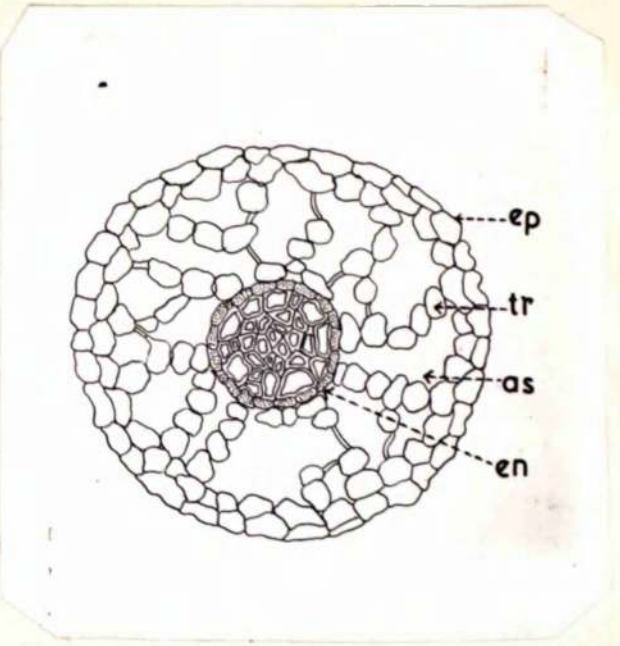


Figure 34.

T.S. Nodule-root. Note epidermis (ep), trabeculae (tr), air-spaces (as) and endodermis (en). Field material. X50.

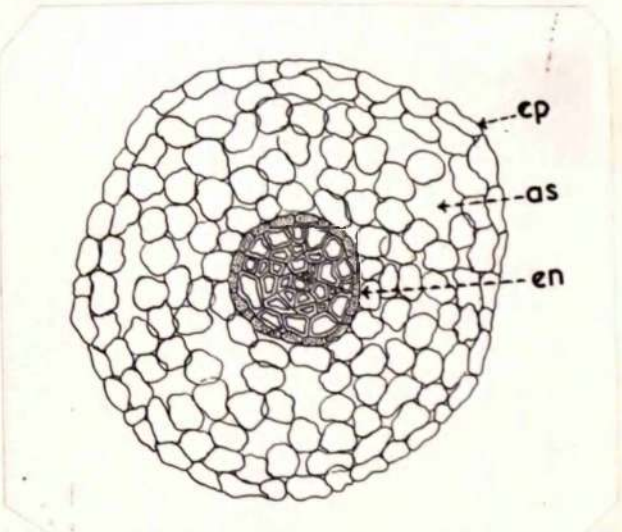


Figure 35.

T.S. Nodule-root. Note that in this there is no trabecular structure such as seen in Figure 34. Field material. X50.

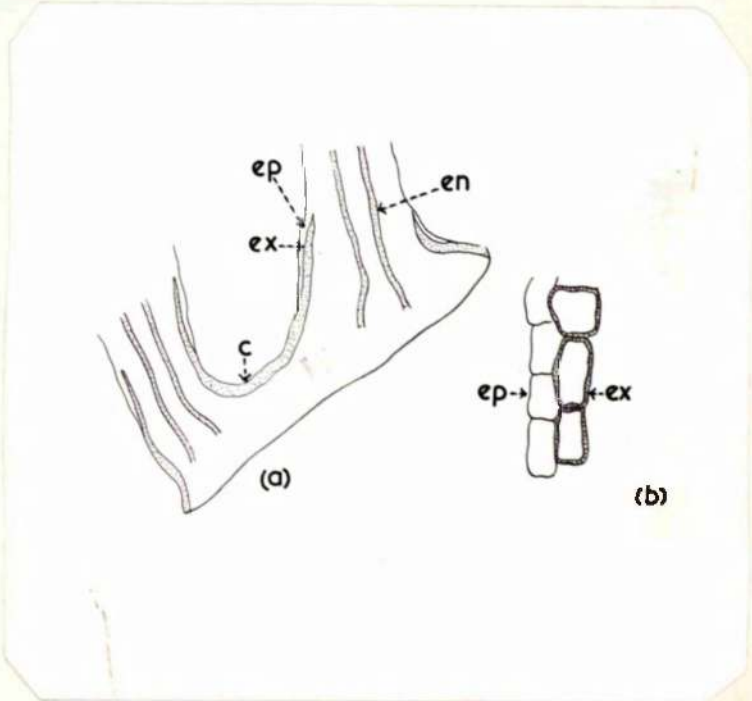


Figure 36.

(a) L.S. apex of field-nodule of M.gale showing distribution of suberised tissues (dotted) X50.

(b) portion of base of nodule-root showing suberised exodermis. X160.

ep - epidermis
ex - exodermis
en - endodermis
} of nodule-root.

c. - cork layer of nodule.

of an already existing exodermis.(Figures 36 , 37). External to this cork layer the epidermis of the nodule-root can clearly be seen.

The endodermis of the nodule-root, consisting of closely fitting brick-shaped cells, is heavily suberised and stains well with Sudan III. It can be clearly seen in both the longitudinal and transverse sections.(Figures 36, 37). Beneath it is the pericycle consisting of large irregularly-shaped cells and internally is the xylem which resembles the xylem of the root in being roughly tetrarch in shape and in having its elements mostly thickened with cellulose with deposits of lignin at the corners. Again the phloem and cambium are not easily distinguished. There has been some doubt as to the morphological nature of these nodule roots. This is of especial interest in view of their abnormal tropic behaviour. Chevalier (1900-02) reported them as having a "pseudo" root cap formed of suberised tissues (presumably derived from the mother nodule) Bottomley (1912) stated that they were devoid of a root cap.

A root cap has been demonstrated by the present author (Figure 33) and though its tissues do become suberised latterly, close examination

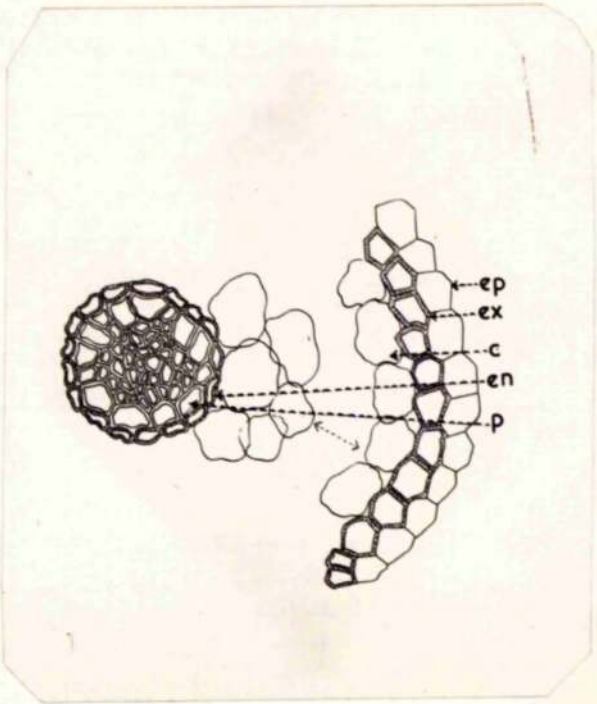


Figure 37.

T.S. of base of nodule-root showing distribution of suberised tissues (dotted)

X160.

ep -epidermis

ex -exodermis

en -endodermis

c - cortex.

p - pericycle.

shows that they are derived from meristematic cells situated near the root tip.

The presence of root caps on the nodule-roots, the fact that they occasionally bear root-hairs, and their central vascular strand, would appear to establish them as true roots.

The nodule may be looked upon as a modified root the development of which has for a time been arrested by the nodule-forming process.

Thus the present author's views regarding the morphological nature of the nodule coincide with those of Chevalier (1900-02) and Hawker and Fraymouth (1951) rather than with those of Dangeard and Trnka (1929); but they are better founded on observation.

SUMMARY OF SECTION III.

1. The water culture technique is utilised for the first time in a study of the development of the root nodules of *Myrica gale*.
2. Observations are made on the germination of *Myrica gale* seeds.
3. The root hairs of inoculated plants are twisted and contorted and this is a manifestation of the organism responsible for nodule formation.
4. It is thought that the organism responsible for nodulation enters the plant, via the root hairs, as bacteria-like bodies which are portions of Actinomyces threads.
5. The external and internal features of nodule formation are described. The nodule arises in the pericycle and in the young condition is generally red due to the presence of anthocyanin.
6. It is at first spherical but gradually becomes pear shaped and from its distal end a nodule-root grows out and grows upwards. The latter seldom bears root hairs but has a true root cap.
7. A central vascular strand connects the nodule to the vascular system of the parent root. This strand continues into the nodule-root. The cortical region of the nodule is hypertrophied.
8. The nodule is enclosed in a cork layer.

9. From its morphological and anatomical features it is concluded that the nodule is a modified lateral root.

IV. THE MATURE NODULE.

A. EXTERNAL FEATURES.

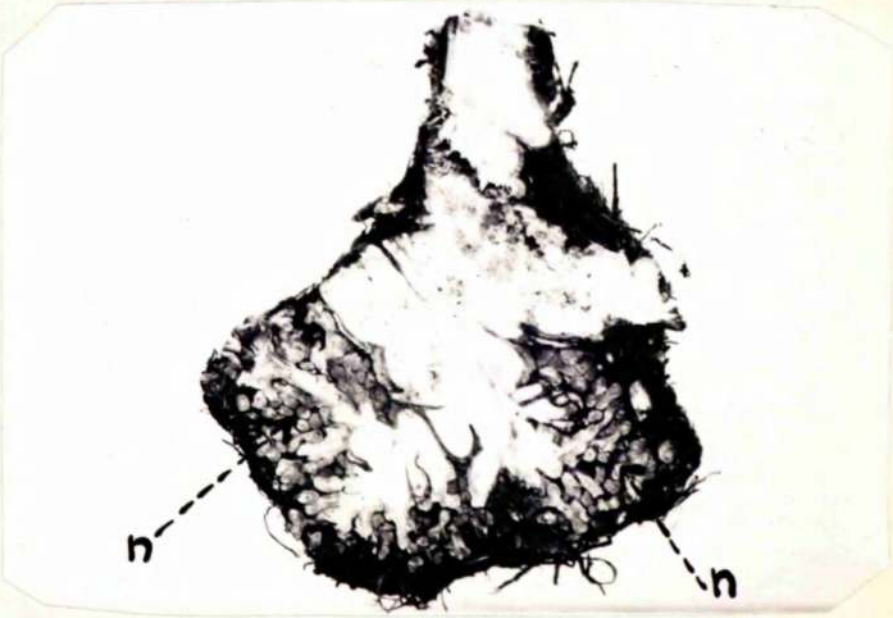
Introduction.

Many authors have described the external features of the mature nodule of Myrica namely, Brunchorst (1886-87) Chevalier (1900-02) Arzberger (1910) Bottomley (1912) Youngken (1919) Arcularius (1928). Consequently there is not much that is original in this Section. But the material has been re-examined and the description is included here for the sake of completeness.

Observations.

The original nodule terminates as already described in a nodule-root. Near the junction of the nodule-root and the nodule, new meristems (usually three in number) are initiated and these give rise to new nodule lobes each of which terminates in a nodule-root. The process continues so that eventually a close cluster of nodule lobes is formed which in the field attains the size of a hazel-nut. Dr. Bond, of this Department, has shown to the present author a cluster as big as a golf ball from a three year old plant growing in water culture. (Fig 38) bed. (Figure 38). When grown in water culture the nodules may be completely concealed by the upwardly growing nodule-roots. (Figures 19, 39-42).

(a)



(b)

Figure 38.

(a) Nodule mass (n) (plus supporting roots) at the top of the root system of a three year old Myrica gale plant in water culture

X5/7

(b) same specimen sawn in half X6/7

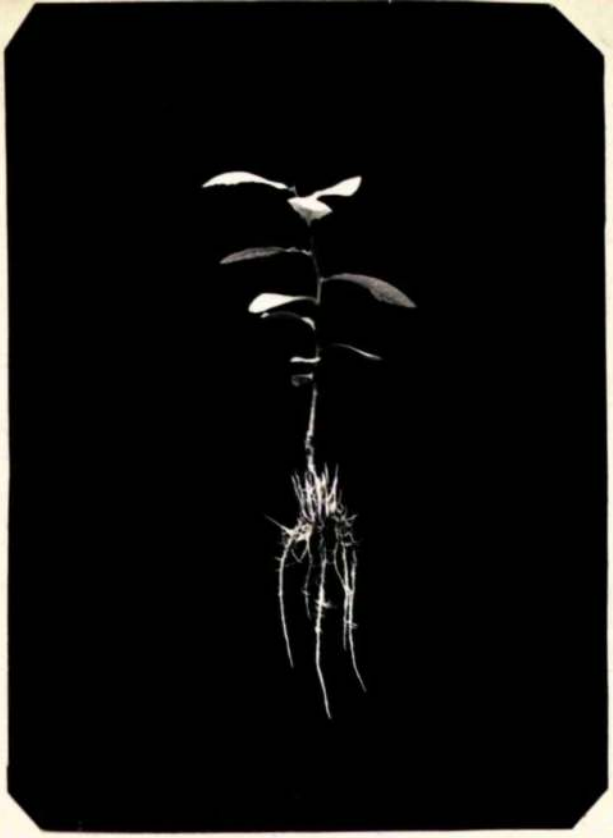


Figure 39.

1st year water culture plant. Note upwardly growing nodule roots. 27. 9. 50. X½.

Figure 40.

2nd year water culture plant. Note upwardly growing nodule roots. 18. 8. 49. X1/10.

Figure 41.

2nd year plant from water culture. Note that the nodules are confined to the upper part of the root.
Note also the nodule-roots. 19/5/51 X½

Figure 42.

2nd year plant from water culture. Note that the nodules are distributed over the root system.

Note also the nodule-roots. 2/7/52 X 1/3.

The appearance of field nodules depends on the time of year that they are collected. Thus those collected in June show the presence of prominent white nodule-roots which may be two inches long.(Figure 43). During the winter however the nodule-roots wither and when seen in March-May may consist of wispy brown threads. (Frontispiece).

Figure 43.

Field nodules collected and photographed 27. 6. 51.

Note prominent nodule-roots. Nat. size.

IV. THE MATURE NODULE (CONTINUED).

B. INTERNAL FEATURES.

(I) GENERAL ANATOMY.

Introduction.

The internal structure of the nodule has also been the subject of investigation by many authors, namely, Brunchorst (1886-87), Chevalier (1900-02), Arzberger (1910), Bottomley (1912), Shibata and Tahara (1917), Youngken (1919), Arcularius (1928), Dangeard and Trnka (1929) and Schaede (1938-39). Consequently the main features of its anatomy are well established. But as we shall see in a later Section IV B(2) there is considerable disagreement as to the cytological details particularly those relating to the form of the endophyte.

Observations.

There is no essential difference in the anatomy of nodules collected from plants growing in the field and nodules from plants growing in water culture in the greenhouse. Both types have been examined. In transverse section of a nodule the following tissues may be noted:-(Figure 44)

(1) an outermost covering consisting of 2-4 layers of narrow elongated suberised cells. This layer (as already noted) effectively shuts off the nodule from the exterior.

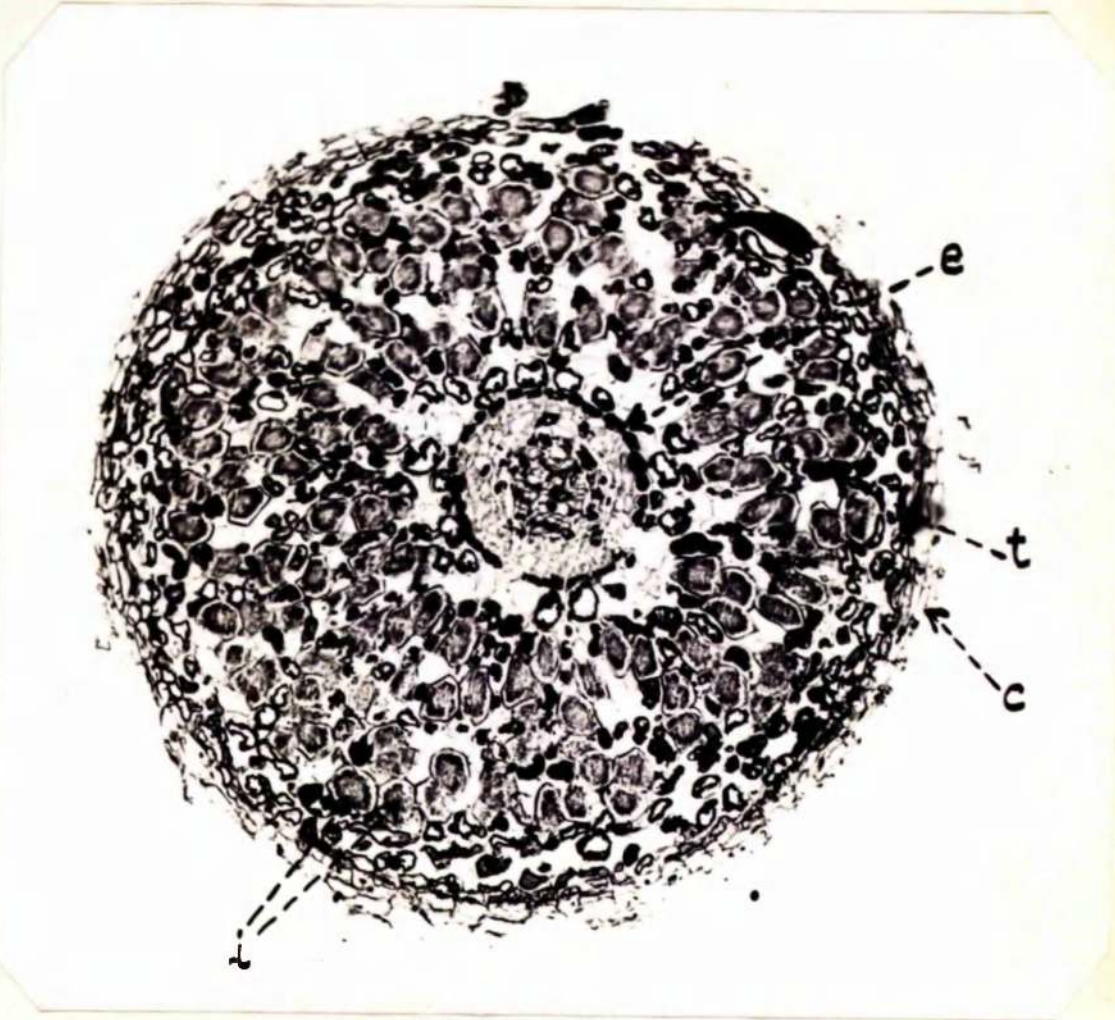


Figure 44.

Photomicrograph of T.S. of nodule. Note cork layer (c) well marked endodermis (e) infected cells (i) tannin cells (t). Stained Safranin and Fast Green. X95.

These cells are filled with a substance which Chevalier (1900-02) by means of extensive chemical investigation has shown to be gummy lignin or wound gum.

(2) a fairly wide hypertrophied cortex in which we may make out the following types of cells:-

(a) Large cells containing the endophyte. These cells are 2-3 times larger than the uninfected cells. The walls of these cells are suberised according to Chevalier (1900-02). Shibata and Tahara (1917) and Schaede (1838-39) state that they are lignified. Investigations by the present author have shown that both lignin and suberin are present on the cell wall. The presence of both lignin and suberin is not surprising. Butler and Jones (1949) state that lignification is one of the commonest modifications of cell membranes in the path of an invading mycelium; invasion is followed commonly by suberisation or wound cork. They remark that the defence mechanism is far from being invariably successful.

(b) Normal uninfected cells. These often contain starch grains. In them the

cytoplasm and the nucleus is clearly seen. Some of the uninfected cells contain tannin in densely staining masses. In these it is difficult to detect the presence of cytoplasm and nucleus.

(c) endodermis - in the mature nodule this is in the secondary condition. Each cell, except for an occasional passage cell, is suberised and lignified and densely filled with tannin.

(3) The central cylinder consisting of :-

(a) the pericycle lying within the endodermis consists of some 3-4 layers of irregularly shaped living cells.

(b) The phloem is poorly developed and not easily distinguished.

(c) The xylem is prominent, tetrarch in structure. The thickening is mainly of cellulose with deposits of lignin at the corners. This feature has already been noted for root and nodule-root and may be related to the wet conditions under which Myrica gale is usually found growing.

(d) Cambium cannot be easily distinguished. It seems likely, however, that some form of secondary thickening must be brought

into play where the nodule joins the parent root for the original vascular tissue would seem to be inadequate for supplying the needs of nodules the size of golf balls.

A transverse section cut near the base of the nodule may appear trilobed,(Figure 45) due to the branching, as will the central cylinder which sends one branch to each lobe. In longitudinal section the lobing and re-lobing can be clearly seen with the meristematic apices showing dense tannin-filled cells. (Figure 46).

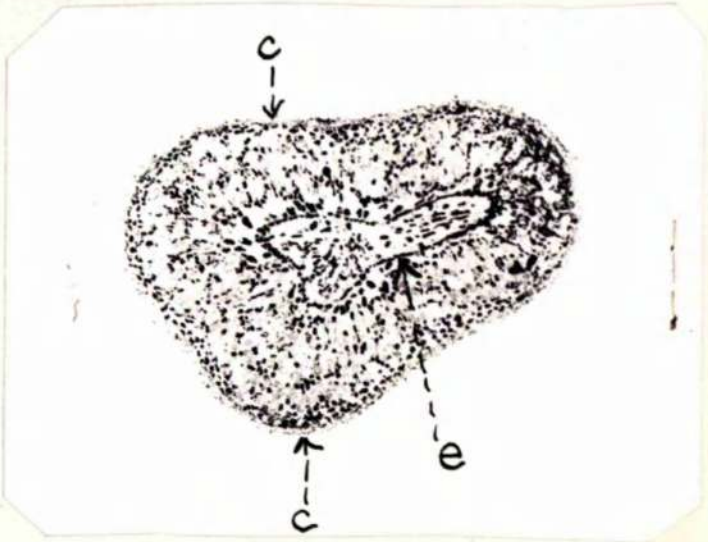


Figure 45.

Photomicrograph of T.S. of three-lobed nodule. Note cork layer (c) and well marked endodermis (e)
The dark masses are tannin-filled cells.
Stained Haematoxylin and Orange G. X35.

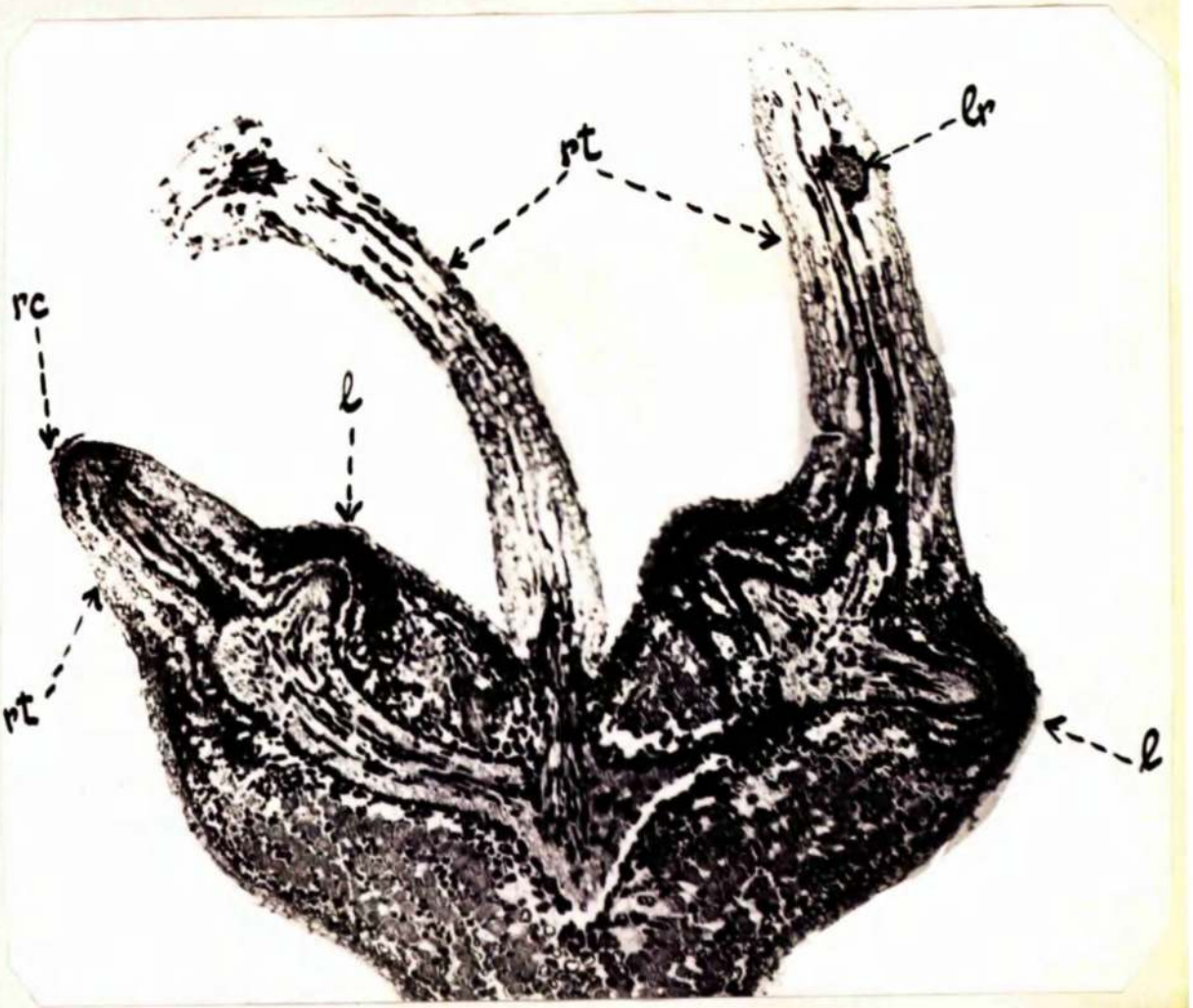


Figure 46.

Photomicrograph of L.S. of lobed nodule showing new lobes forming (l) and nodule-roots (rt). Note that one of the nodule-roots shows presence of a root-cap (rc) and that another shows formation of lateral root (lr).

Stained Safranin and Fast Green.

Top of solution is to the left of the Figure.

× 70.

(2). CYTOLOGICAL STUDY.

Introduction.

It will be shown in a later Section (V) that the present author has been unable to isolate the endophyte. Lacking then a pure culture, the basis of identification must rest primarily upon Cytological evidence. It seems to the present author that the identification must not be carried too far on this basis and a tentative attempt is made in this Section to determine whether the endophyte be bacterium, actinomycete, myxomycete or fungus, without however attempting to assign it to a particular genus or species. Brunchorst (1886-87) who was the first to note and describe the root nodules of M. gale, believed from a cytological examination that the endophyte was the same fungus as he had previously described (Brunchorst (1886-88)) as occurring in the nodules of Alnus, namely Frankia subtilis. He was in some doubt as to Frankia's systematic position, since the sporangial characters resembled the Mucorineae or Saprolegniales but the septate hyphae of Frankia counted against this. Moeller (1890) examined the nodules and though agreeing that the endophyte belonged to the genus Frankia thought that it differed sufficiently from Frankia subtilis on account of its short hyphae and club

shaped sporangia to be assigned to a new species which he named Frankia brunchorstii. He described both F.subtilis and F.brunchorstii as being aseptate or unicellular and assigned them to the Hyphomycetes. Chevalier (1900-02) in an excellent monograph on the Myricaceae dealt at some length with the nodules occurring on various Myrica species particularly M.gale. He accepted the endophyte as Frankia brunchorstii but promised another memoir on its organisation and biology. (This memoir has not been published.) The various cells occurring in the nodule were examined in some detail: particularly the cells in which Frankia occurs, and the changes effected in these latter cells, e.g. disappearance of starch, hypertrophy and final disappearance of the nucleus; suberisation of the walls of the cells. Shibata (1902) differed from Brunchorst and Moeller on the nature of the endophyte. He thought that it was an actinomycete, showing threads which broke up into bacteroid-like particles. Harshberger (1903) examined the nodules of Myrica cerifera and described their external and internal form. He used dried museum material which he first boiled and then treated with 35% alcohol and from this material he cut sections. From an examination of these sections he considered that the genus Frankia

should be placed among the Oomycetes close to the genera Pythium and Peronospora principally because of the coenocytic hyphae; but in the absence of oogonia and zoosporidia Harshberger remarked that the relationship cannot be insisted upon. It seems to the present author that observations on such material can have little value. Arzberger (1910) worked principally with Myrica cerifera but also looked at M. gale and M. asplenifolia. Regarding the identity of the organism he stated that it is difficult to place systematically on the basis of nodule cytology but thought Shibata (1902) "probably correct in placing it with Actinomyces". He noted the presence of club-like structures at the periphery of the actinomycete rosette. They were also noted by Peklo (1910) who dealt mainly with the isolation of the endophyte (see next Section) but also dealt with the host-endophyte relationship. He (Peklo) described, in some detail, the digestion of the endophyte to form a structureless clump. Bottomley (1912) considered that the "infected cells" of the nodule of Myrica gale are filled with bacteria, not fungi or actinomycetes as thought by previous workers. He stated that the organism responsible for nodulation was a bacterium close to Bacillus radicicola. He did find true

fungal hyphae ramifying through the cells of the basal zone of the nodules and sometimes filling the cells in old nodules, and thought that these, interpreted by him as due to a secondary infection, were wrongly considered by earlier workers to be the cause of the nodules. Shibata and Tahara (1917) criticised Bottomley's findings for Myrica. They state that the "bacteria" seen in the nodules were probably artefacts due to faulty micro-technique. They remarked that it is scarcely possible that Bottomley's material should have differed so much from that of all the other authors. They did not commit themselves to an identification. They figure huge club-like bodies occurring within the infected cells, at the ends of the hyphae of the endophyte. Youngken (1919) dealt with the morphology, taxonomy and distribution of the Myricaceae in the Eastern United States. He examined the nodules of a number of Myrica species including M.gale and described their structure and histology. The responsible organism is, according to him, an Actinomycete which he named Actinomyces myricarum; also it is a parasite and passes via the pitted vessels of the main roots to the stem, being conveyed by the transpiration stream to the flowers, bracts and fruits. He noted the club structures seen by

previous authors and agreed with Chevalier that the nucleus of the infected cell becomes hypertrophied and perishes. Arcularius (1928) dealt with the anatomy and histology of the nodules of Myrica gale and the plant-endophyte relationship. According to his findings the endophyte is eventually digested by the host cell, much as is believed to happen in Neottia. He also figured structures which he thought to be symbiotic bacteria which he likened to the bacteroids of legumes in addition to the normal endophyte. Regarding the latter, i.e. the normal endophyte, he is in some doubt as to its systematic position. He observed that the host nucleus shows changes of form and is said to be cleaved. He was unable to see any clubs in nodule sections. Dangeard and Irnka (1929) from cytological examination thought that the endophyte of Myrica gale is a filamentous bacterium which can break up into pieces of variable length. It has certain points of resemblance to Rhizobium but the filaments justify a distinct genus for which they propose the name Rhizobacterium and the species is Rhizobacterium myricae. They say that this should not be confused with a true mycelium which often develops abundantly in the intercellular spaces of the root cortex. The relationship between plant and bacterium is interpreted as one of symbiosis. They

state that the nucleus of the infected cell conserves its vitality to the end without notable changes in volume or in structure. Schaede (1938-39) dealt principally with the life history of the endophyte within the nodules of Myrica gale. The report by Bottomley of the presence of bacteria in the nodules is noted and agreement expressed with Shibata and Tahara's view that Bottomley was in error. Schaede noted thick (2-2.5 μ) and thin (less than 1 μ) hyphae within the nodule but was unable to determine whether one developed from the other or whether there were two organisms present. Digestion of the hyphae is noted and described. On some rare occasions bacteroid formation has been seen. He considered the endophyte to be an actinomycete. Regarding club-formation he stated that it is infrequent. He looks upon the huge clubs described and figured by Shibata and Tahara as monstrosities. He observed that the nucleus of the host cell enlarges sometimes to the point of bursting. His paper is accompanied by excellent illustrations. Hawker and Fraymouth (1951) examined the nodules of species of Elaeagnus, Hippophäe, Alnus and Myrica. They concluded that in all of these the endophyte is a member of the Plasmodiophorales but whereas those of Elaeagnus, Hippophäe and Alnus could well be

included in the genus Plasmodiophora that of Myrica must go into a separate genus. They go so far as to say that the endophyte in this plant "is in many ways an approach to the actinomycetes" but still insist that when modern fixatives are used, there are no cross walls and no "obvious containing walls" while in fresh material the strands are even less like hyphae. Presumably they imply that with older and less perfect fixatives, apparent containing walls might arise as artefacts. They noted that the width and branching of the strands are very variable. They describe and figure club-shaped bodies which they regard as sporangia which break up into packets soon after they are formed. The particles have been observed by these authors to move with a dancing movement. They are tadpole shaped with the posterior end pointed.

Thus we have many conflicting views and interpretations; some believing that the causative organism is a bacterium, others that it is an actinomycete, others that it is a fungus and still others that it is one of the plasmodiophorales. Some authors believe that there may be more than one organism present. It is the opinion of the present author that it may be possible to reconcile many of these divergent views.

Observations and Discussion.

The wall of the infected cell is at first composed of cellulose but the cell increases in size with the advent of the endophyte and lignification takes place early. The infected cell may, as noted, be 2-3 times the size of the uninfected cell, and becomes filled with a dense network of very fine mycelium, the hyphae being less than 1μ in diameter. (Figures 47, 48). Indeed it is extremely difficult to see individual hyphae at this stage. The "ray arrangement" of the typical actinomycete is not always observed but may be seen in a number of cells. Cell cytoplasm is not easily detected in the infected cells but must still be present since the nucleus can be clearly seen. As noted above, there are conflicting views on the fate of the nucleus. The observations of the present author are that the nucleus may double in size, become lobed and show a prominent nucleolus. (Figure 48). The nucleus persists for a long time and may even be seen in the cells in which the hyphae are empty and swollen. Eventually, however, it disintegrates, becoming indistinguishable from the mass of collapsed hyphae. These observations are generally in accord with the findings of the above authors other than Dangeard and Trnka. It is difficult to

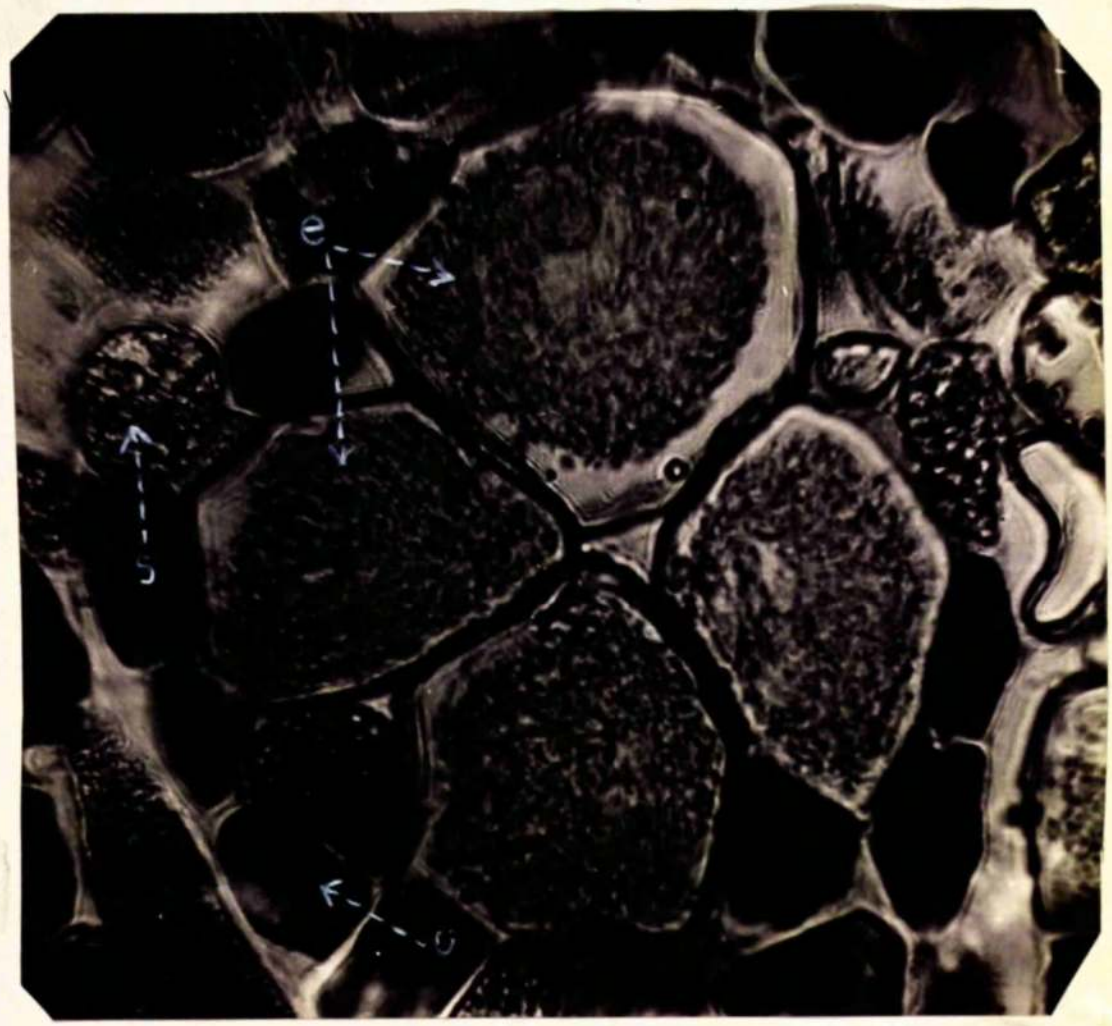


Figure 47.

Photomicrograph of nodule cells of M.gale. Note enlarged cells filled with the endophyte (e) and small uninfected cells some of which are filled with tannin (u) and some with starch grains (s).

Stained Safranin and Fast Green. X1800.

Figure 48.

Photomicrograph of nodule cells of M.gale. Note that the infected cells contain masses of the endophyte in which it is difficult to distinguish the individual hyphae. Note lobed nucleus at (n).

Stained Safranin and Fast Green. X1800.

see how their findings can differ so much from the others unless by an error of technique or observation. Schaede (1938-39) noted that occasionally very small starch grains can be detected in the infected cells and states that the disappearance of starch is significant for the physiological relation of host and endophyte. The findings of the present author are that the infected cells may at first contain, as do many of the uninfected cells, starch grains, but these are digested , presumably by the action of the endophyte, although possibly by the action of the host cell in an attempt to meet the food demands of the endophyte. A few starch grains may be found in cells in which there are only a few strands of hyphae but as the hyphae fill the cell so the starch grains disappear.

Starch grains may also be seen in the bacteroidal cells of the leguminous nodule. Fréd Baldwin and McCoy (1932) note that as the nodule degenerates starch begins to disappear. From this it has been inferred that the starch serves as a source of energy for nitrogen fixation. But they report Beijerinck as stating that the bacteria are unable to attack starch. Unfortunately the physiology of the Myrica endophyte cannot be studied until the organism is isolated in pure culture.

Single hyphae or strands of hyphae can be seen passing from cell to cell (Figures 49, 50). No, or very occasional, septa can be observed in these hyphae but in some, bacteria-like bodies lying end to end may be seen (Figure 51). These may represent some of the hyphae in course of segmentation as noted by Shibata (1902) leading to the formation of the bacteria which have been observed (see earlier Section) in and around the root hairs. It is here of interest to note that Butler and Jones (1949) observe that, according to Lutman et al, the spores abstricted in segments from Actinomyces scabies may look more like segments of the filament than organised spores. This segmentation may have led to Dangeard and Trnka (1929) considering that the endophyte is a "filamentous bacterium" which may break up into pieces. These bodies were also observed by Shibata and Tahara (1917) and Schaede (1938-39). As the cell grows older it appears that the contents of the hyphae are digested, whether by the action of the host cell or because of a shortage of food after exhausting the host cell is not clear. The former appears to be the more likely since it is often possible to find the host cell nucleus still present after the hyphae have lost their contents.

Figure 49.

Photomicrograph of nodule cells of M.gale. Note swollen hyphae passing from cell to cell.

Stained Sharman's method. X1800.

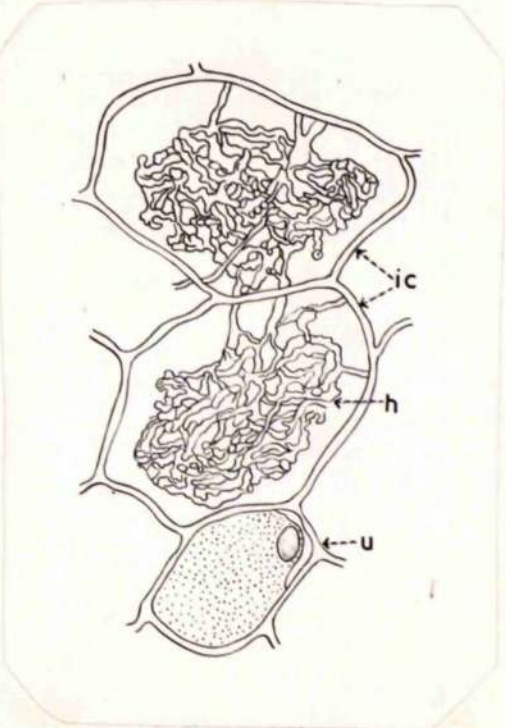


Figure 50.

Nodule cells of *M.gale*. Note infected cells (ic) containing partially digested swollen hyphae which pass from cell to cell. Note also (u) uninfected cell. X800.

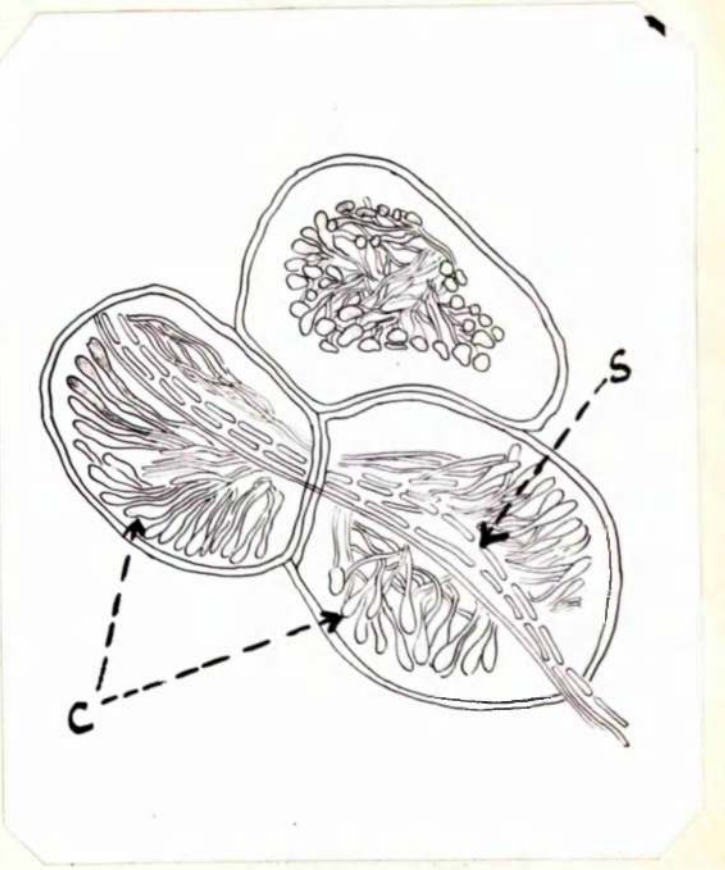


Figure 51.

Nodule cells of M.gale showing "club formation"(c). The clubs in the two lower cells are seen in median section: those in upper cell in transverse section. Note also strands of segmented hyphae (s) passing from cell to cell. X1400.

These hyphae can be seen as empty branching threads some 2--2.5 μ in diameter. (Figures 52, 53, 54, 55). The empty branching threads are distorted and twisted this being probably a manifestation of age. It is at this stage that the nucleus of the host cell begins to disintegrate. The diameter of these hyphae has probably led many authors to state that they are fungal but it is the conviction of the present author that they are derived from the fine (less than 1 μ) hyphae which are of actinomycetal dimensions. Schaede (1938-1939) noticed both these thin and thick hyphae but could not decide whether the thick developed from the thin hyphae or whether they are new growths. He noted that the fine hyphae are at first present along side the stout ones but later disappear. He did note hyphae at the base of the nodule whose contents had been digested. It has been the experience of the present author that the thick hyphae are never found in young nodules. In them the thin hyphae are found exclusively. In older nodules thin and thick hyphae may be found and when present the thick hyphae are always found at the base, i.e. the older part of the nodule. Furthermore starch is sometimes found in cells with fine hyphae but never in cells with thick hyphae.

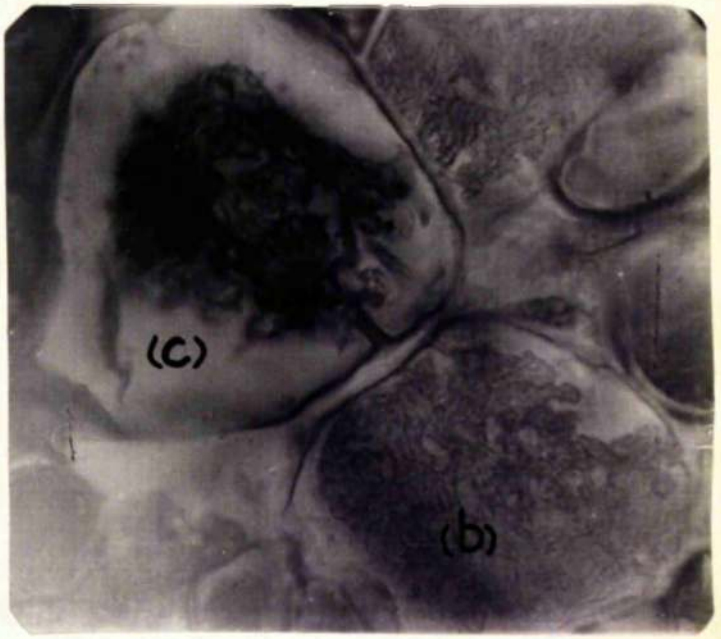


Figure 52.

Photomicrograph of nodule cells of M.gale.
Note (b) Infected cell in which are strands of partially digested swollen hyphae.
(c) Infected cell in which the hyphae are clumping due to digestion.
Stained Sharman's method. X1800.

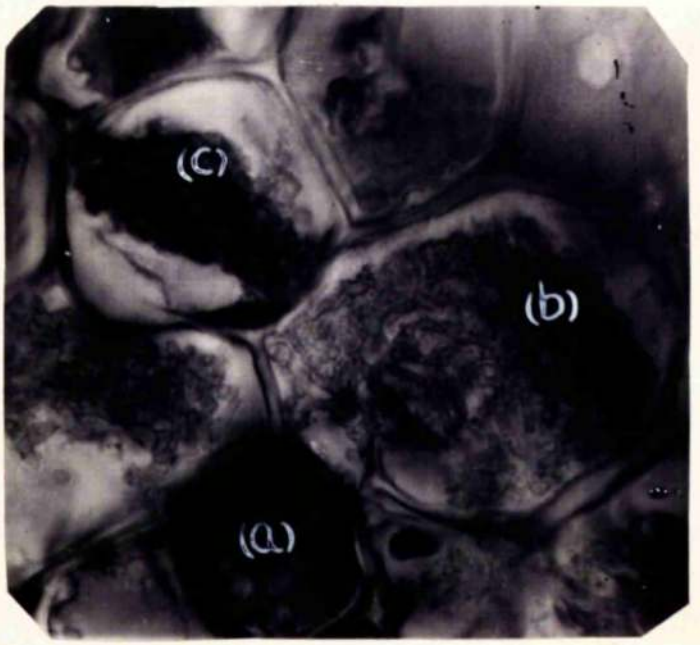


Figure 53.

Photomicrograph of nodule cells of M.gale. More advanced stage of digestion than shown in Figure 52. Note (a) uninfected cell filled with tannin. (b) infected cell in which are strands of partially digested swollen hyphae. (c) infected cell in which the hyphae are almost wholly digested and form a dark clump. Stained Sharman's method. X1800.

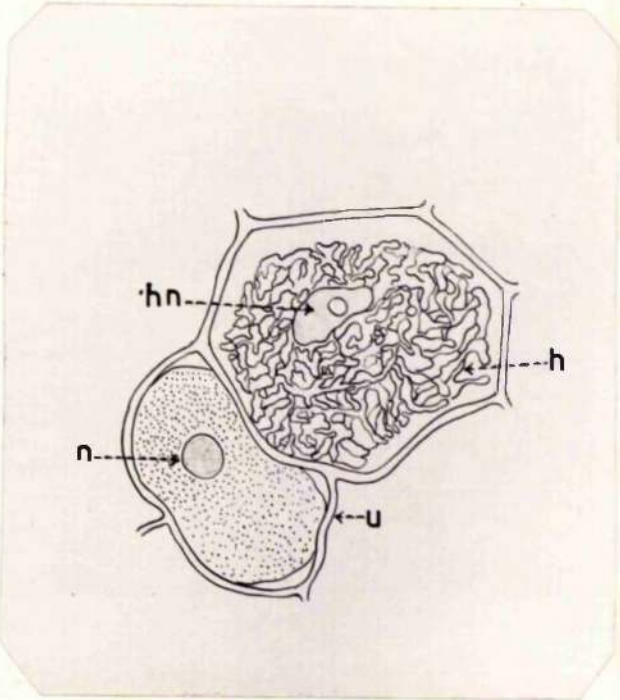


Figure 54.

Nodule cells of M.gale. Note partially digested hyphae within infected cell(h): lobed hypertrophied nucleus with prominent nucleolus (hn); uninfected cell (u) with nucleus (n). X800.

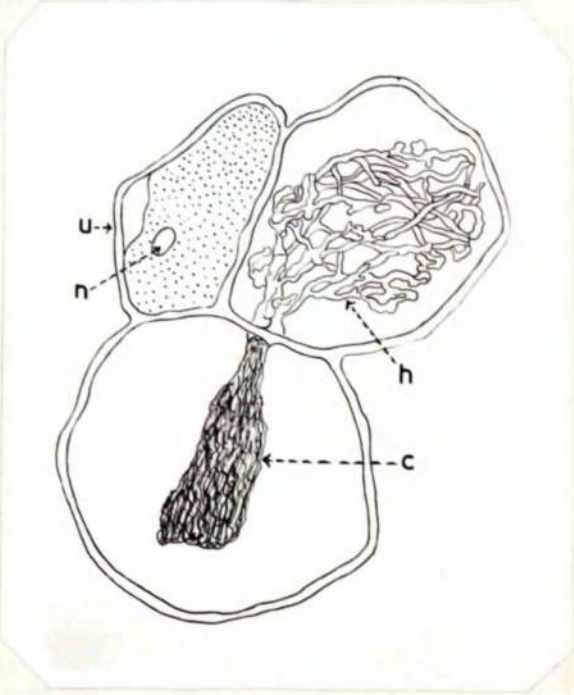


Figure 55.

Nodule cells of M.gale. Note infected cell with partially digested hyphae (h): clump of digested hyphae within infected cell(c); ininfected cell (u) with nucleus (n).

X800.

These facts would seem to indicate that the thin hyphae precede the thick hyphae in time. Whether the former give rise to the latter is conjectural, but if it is not so then there must be two organisms capable of causing nodule formation in Myrica gale since both types of hyphae cause enlargement of the cells in which they are found. Furthermore the thick hyphae are never found in the nodule in the living state since they i.e. the hyphae are always empty. Regarding Hawker and Fraymouth's findings (see Introduction) for Myrica, the virtual absence of cross walls need not count against the organism being an actinomycete. Henrici (1948) states that although there is some difference of opinion concerning the occurrence of septa in the mycelium of actinomycetes, most investigators deny the existence of septa. The present author has examined fresh material of M.gale nodules both from water culture material and from the field and has observed what are undoubtedly actinomycete hyphae within them (Figure 56). Even in this fresh condition they have obvious containing walls. These walls cannot then be dismissed as artefacts due to faulty fixation.

Regarding the variability in width and branching of the strands observed by Hawker and

Figure 56.

Hand section of field nodule of M.gale showing actinomycete hyphae (h) passing from cell to cell. X1800.

Fraymouth this is not an uncommon feature of endotrophic fungi and bacteria e.g. it is observed in the hyphae of the fungus causing endotrophic mycorrhiza of orchids and there is great variability in the width of the rhizobia within any one legume nodule.

The club-like endings observed by Hawker and Fraymouth and others have been noted by the present author (Figure 57) but he has been unable to make out any details of their internal structure. Their very infrequent appearance would appear to count against them being reproductive structures and it seems much more likely that the swellings are due to the action of the host cell upon the endophyte.

In view of the above evidence, so far as can be deduced from nodule cytology, it is considered that the organism responsible for nodulation in Myrica gale is an actinomycete.

It seems reasonable then to assume that there is only one organism and that the thin hyphae give rise to thick empty hyphae as they age. This swelling of organisms as they age is readily observable in the bacteroids of Rhizobium and it is considered that a similar process is taking place here.

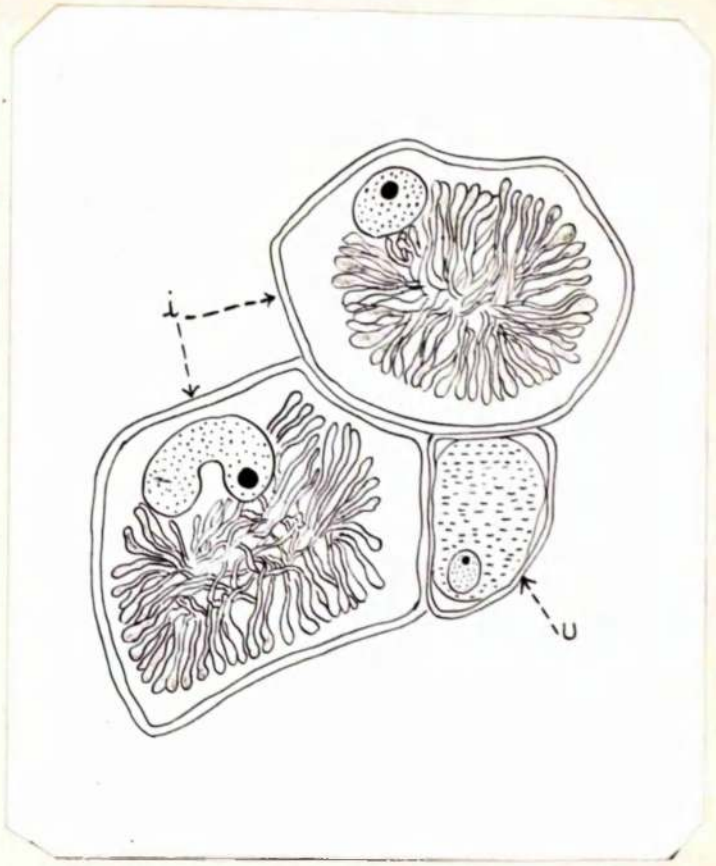


Figure 57.

Nodule cells of M.gale showing "ray arrangement" of actinomycete endophyte, within infected cells (i). Also club formation and lobed nucleus with prominent nucleolus.

Note also (u) uninfected cell. X1400.

SUMMARY OF SECTION IV.

1. An examination is made of the mature nodule of *Myrica gale*. Both the external and internal features of field and water culture material are described.
2. On the basis of cytological evidence it is concluded that although thin and thick hyphae are observed there is only one organism present in the nodule and that it is an actinomycete.
3. It is proposed that the thin hyphae as they age give rise to the thick.
4. Stages in digestion of the contents of the hyphae are described and figured.

V. THE ATTEMPTED ISOLATION OF THE ENDOPHYTE.

Introduction.

A number of attempts have been made by previous authors to isolate, in pure culture, the organism responsible for the formation of nodules on the roots of *Myrica*.

The first attempt was that by Peklo (1910) who claimed to have isolated the organism on a beerwort medium as a rich slimy zoogloeal thread. He did not state whether this was an Actinomycete. He reported that he was also successful using a mineral solution to which was added asparagine and mannitol. The nodule material was first washed, then the nodule pieces rotated in a bunsen flame until they began to burn, placed in the medium and crushed by means of a sterile glass rod. *But owing to lack of seedlings Peklo performed no re-inoculation tests with his isolates.* Bottomley (1912), using maltose agar medium, isolated an organism similar to *B. radicicola*. The nodules were sterilised by means of acid mercuric chloride. His re-inoculation experiments appear to be very unsatisfactory since he used six young plants two of which possessed nodules when planted. Of the remaining four two showed active growth when watered with a "liquid culture of *Myrica* nodule

organism " but it is not stated in the body of the paper whether this was accompanied by nodule formation, though there is a statement to this effect in the summary, Youngken (1919) claimed to have isolated the organism which causes nodulation of Myrica cerifera and " probably most, if not all, of these lesions on other plants of the Myricaceae". Tubercle clusters were washed thoroughly with clean water and transferred into a test tube with 1:1000 corrosive sublimate for 20 seconds. They were then, by means of sterile forceps, put into a tube of sterile distilled water and cut up into small pieces with a sterile scalpel. The fragments were transferred to five tubes of sterile slant agar, and stored in a dark closet for several weeks. All five cultures revealed Actinomyces rosettes, non-septate thin filaments and rods of different sizes as well as coccus forms, all of which stained well by Gram's method. According to Youngken the coccus forms are probably for the most part, products of the degeneration of the above filament. The Actinomyces rosettes were found in the depths of the agar " thus showing the anaerobic nature of the organism".

Five seedlings of M.cerifera were removed from the soil and their roots washed in water,

dipped quickly into 1:1000 corrosive sublimate and then washed in sterile distilled water. Small portions of the Actinomyces culture were pricked into the roots of four seedlings. One negative control was pricked with a sterile needle. Each seedling was then planted in a pot with sterile sand, placed in the greenhouse and daily watered with sterile Knop's solution. After nine weeks the plants were examined for tubercles. According to Youngken these were found in "a primitive state" at the points of inoculation on all but two including the control. Thin hand sections revealed the presence of Actinomyces in the same condition as observed in cells of tubercles of M.cerifera. He named the isolate Actinomyces myricarum but it is noteworthy that there is no reference to re-isolation from the inoculated roots. Arcularius (1928) made unsuccessful attempts to isolate the organism on "Biomalx" agar. Hawker and Fraymouth (1951) state "Neither we ourselves nor Miss S. Mount (unpublished) have been able to cultivate these organisms (those responsible for nodulation of Myrica, Alnus, Hippophäe and Elaeagnus) by methods which sufficed for the isolation of the clover bacterium. This is additional evidence that they are not identical or even similar". It is not stated whether they attempted other methods

Methods and Results.

The present author has made many attempts to isolate the endophyte of *Myrica gale* but none has proved to be successful. They are as follows:-

1st Attempt - 26/1/50.

The nodules were taken from plants growing in water culture solutions in the greenhouse and were sterilised by various methods :-

1) By flaming. This was the method described by Peklo (1910). The nodule pieces were first washed in water and then rotated in the bunsen flame until they singed.

2) By Bottomley's method. Nodule pieces submerged in the following fluid (conc. HCl 2.5 gms. $HgCl_2$ 1.0 gm. distilled water 500 cc) for two minutes , then washed frequently in sterile distilled water .

3) By Youngken's method. Nodule pieces shaken in 0.1% $HgCl_2$ for thirty seconds then washed frequently in sterile distilled water.

4) By using hydrogen peroxide. Nodule pieces shaken for fifteen minutes in 15% H_2O_2 and then washed frequently with sterile distilled water. Nutrient agar pH 7.0 was used as the medium and eight Petri plates of this medium were poured. 2 plates were inoculated with crushed nodules

sterilised by flaming; 2 inoculated with crushed nodules sterilised by Bottomley's method; 2 inoculated with crushed nodules sterilised by Youngken's method; and 2 inoculated with crushed nodules sterilised by H_2O_2. The plates were incubated at 24°C.

Results. 72 hours after inoculation all eight plates showed growth of a Gram -ve bacterium. By 12/2/50 i.e. some 17 days after plating, six colonies of an actinomycete had appeared on one of the plates . Subcultures were taken of both the bacterium and the actinomycete. Two jars (i.e. 14 plants) of young Myrica gale plants growing in Crone's solution were inoculated with the bacterium and two jars of plants with the actinomycete. Neither set produced any nodules although the root hairs of the plants inoculated with the actinomycete showed contortions. Control plants inoculated with crushed nodules produced nodules.

2nd Attempt - 4/4/50.

Twenty nodules (from plants growing in water culture in N-free Crone's solution in the greenhouse) were surface sterilised with acid mercuric chloride (Bottomley's fluid) for two minutes followed by six washes of sterile distilled water over a period of 30 minutes. A further twenty were

surface sterilised by shaking with 0.1% $HgCl_2$ for 30 seconds (Youngken's method) and washed with several changes of sterile distilled water. The forty nodules were then transferred to a sterile Petri dish. Each nodule was picked up singly with sterile forceps and cut in half with a sterile scalpel. A little of the tissue was scooped out of the centre of each nodule with the tip of a sterile scalpel and the tissue macerated on the inside edge of a tube of nutrient agar pH 7.0 kept liquid at 40°C in a water bath. Each tube was rotated so that the tissue was well mixed through the agar. The agar was then poured into Petri dishes, allowed to set and incubated at 24°C. Thus forty plates were set up.

Results.

None of the above plates developed an actinomycete though they were kept till 6/6/50 i.e. 8 weeks. A few remained completely sterile. The majority were overgrown by various fungi and bacteria e.g. Penicillium sp. Aspergillus sp. Rhizopus, Mucor and various yeasts; Bacillus subtilis, Bact. prodigiosum, Staphylococcus sp. Micrococcus sp.

3rd Attempt - 27/5/50.

Plotho (1941) claimed to have isolated the endophyte of Alnus on glycerine agar. He sterilised

the nodules merely by cleaning thoroughly in soapy water, rinsing many times in distilled water then flaming. This method (with slight modifications) was adopted for Myrica gale. The nodules which were obtained from plants growing in the field were shaken for 10-15 minutes in 1% soap solution, then washed several times in sterile distilled water, crushed and plated on glycerine agar (2% glycerine; 1.2% bacteriological peptone (Plotho used de Witte); 0.8% Lab. Lemco (Plotho used Liebig meat extract) 0.2% NaCl). The plates were incubated at 24°C.

Results.

Two actinomycetes were isolated from these plates. One (yellowish in colour) on 5/6/50. Another,(white wrinkled) on 20/6/50. Inoculation experiments were carried out with both isolations. Twenty five plants growing in N-free Crone's were inoculated with the first isolate and twenty with the second. Two months later there was no sign of nodulation. Control plants inoculated with crushed nodules showed nodulation at this time.

4th Attempt - 2/6/50.

The same medium as above was employed i.e. glycerine agar. The field nodules were washed with 1% soap, as above. Washed with sterile distilled water.

Each nodule was cut in half with a sterile scalpel and the centre portion scooped out and plated. Seven plates were set up in this way and incubated at 24°C.

Results.

By 9/6/50 a white wrinkled actinomycete (similar to that isolated in 3rd Attempt) appeared on one of the plates. Twenty five plants growing in N-free Crone's were inoculated with this isolation but no nodules developed. (Controls with crushed nodules were positive).

5th Attempt - 29/6/50.

It was thought that the organism might require anaerobic conditions for growth, so the following apparatus was set up. 6 Petri dishes of glycerine agar were inoculated with crushed greenhouse nodules sterilised by Bottomley's method. The dishes were placed on a plate and a jar with a two-way stopper placed on top. The jar was sealed to the plate by means of vaseline. A continuous stream of CO_2 from a cylinder was allowed to pass through the jar displacing the air in the jar. The CO_2 was allowed to pass in for some six hours initially and then for one hour in each of the subsequent days, thus maintaining anaerobic conditions. The plates were incubated at room

temperature.

Results.

No actinomycetes appeared on the plates.

6th Attempt - 29/6/50.

Parallel with the above, six plates of glycerine agar were inoculated and incubated aerobically at 24°C.

Results.

No actinomycetes appeared on the plates.

7th Attempt - 28/7/50.

Peklo (1910) reported that he used the following medium with good results for the isolation of the endophyte of Alnus.

Distilled water	200 cc.
K_2HPO_4	1 gm.
KH_2PO_4	1 gm.
$MgSO_4$	0.6 gm.
NaCl	0.2 gm.
$CaCl_2$	0.1 gm.

The above portion well shaken ; not filtered, then:

Above mineral solution	100 cc.
Distilled Water	400 cc.
Asparagine	2 gms.
Mannitol	10 gms.
$FeCl_3$	trace.
Agar	.75%.

This medium is clear and colourless.

It was thought that it would be worth trying this out with nodules of Myrica. Plates and slopes were prepared using 2% agar. Greenhouse nodules were sterilised by singeing in the bunsen flame (as

recommended by Peklo). They were then crushed, using a sterile glass rod, in a sterile Petri dish with a little sterile distilled water. A loopful of the inoculum was spread on to each of 12 Petri dishes and 11 slopes of the above agar, and incubated at 24°C.

Results.

One month after inoculation only one plate out of the 12 showed the presence of an actinomycete. All others were contaminated with various organisms. Of the slopes, 10 remained sterile; one was contamin - ated with a fungus. Inoculation experiments were carried out with the actinomycete. Four jars (i.e. 28 plants) of Myrica gale were set up in N-free Crone's solution and inoculated but all failed to develop nodules.

8th Attempt - 25/9/50.

Peklo (1910) reported the isolation of the Alnus endophyte using the following medium:-

Beerwort	500 cc.
Distilled water	450 cc.
K_2HPO_4	8 gms.
K_2CO_3	6 gms.

This medium was used for attempted isolation of the Myrica endophyte. The pH before autoclaving was 9.12. It was distributed in 130 cc. amounts in conical

flasks and sterilised by autoclaving at 15 lbs. pressure for 15 minutes .Greenhouse nodules were sterilised by singeing, crushed with sterile water in a sterile Petri dish, inoculated into flasks which were incubated at 24°C.

Results.

All the flasks remained completely sterile. 7/11/50. This was thought to be due to the high pH and so the following was attempted.

9th Attempt - 10/10/50.

The following medium was made up:-

Beerwort .....500 cc.
Distilled water .....450 cc.
K_2HPO_4 ..... 1.5 gms.
K_2CO_3 ..... 1.5 gms.

This gave a medium of pH 7.6. It was tubed in 10 cc. amounts and autoclaved 15 lbs. / 15 minutes. The tubes were inoculated with singed, crushed greenhouse nodules. 16/10/50. Ten tubes were set up.The above medium plus 1.5% agar was also made up and subcultures made from each of the tubes on to the agar distributed in Petri dishes.

Results.

One plate remained sterile, two showed the presence of a fungus with extremely fine hyphae, four the presence of an ascosporic yeast and six a

zoogloeal mass of a motile rod -shaped bacterium. Inoculation tests were performed on Myrica gale plants growing in N-free Crone's with all three isolates (1) fungus (2) yeast (3) bacterium, but there was no nodulation. (12 plants were tested with each isolation).

10th Attempt - 20/2/51.

Jensen (1928) reported the isolation of an actinomycete (Actinomyces acidophilus) from the soil on an acid medium. The organism was never found growing on neutral or alkaline media such as are commonly employed for the isolation of actinomycetes. The organism appeared to live only in acid media, a property hitherto unknown to the actinomycetes. Jensen's medium was utilised for the attempted isolation of the Myrica organism.

Dextrose	20 gms.
Asparagine	2.0 gms.
KH_2PO_4	2.0 gms.
$MgSO_4$	0.5 gms.
NaCl	0.5 gms.
$FeCl_3$	0.1 gms.
Agar	25 gms.
Distilled water	1000 cc.

Two lots were made up, one batch pH 4.12; the other pH 5.12 (by pH meter).

Two methods of sterilisation of nodules were employed:-

(1) Shaken up for 2 minutes in Bottomley's fluid, followed by frequent washing in sterile distilled water. The nodules were crushed in sterile distilled water in a sterile Petri dish.

(2) Shaken for 30 seconds in 0.2% $HgCl_2$ followed by frequent washing in sterile distilled water. The nodules were crushed in sterile distilled water in a sterile Petri dish.

Inoculated plates of agar were incubated at one of two temperatures 24°C or 30°C.

To sum up:- Plates set up 20/2/51.

(a) Nodules sterilised by method (1) plated on Jensen's medium pH 4.12 - 6 plates.

(b) Nodules sterilised by method (1) plated on Jensen's medium pH 5.12 - 6 plates.

(c) Nodules sterilised by method (2) plated on Jensen's medium pH 4.12 - 6 plates.

(d) Nodules sterilised by method (2) plated on Jensen's medium pH 5.12 - 6 plates.

All of the above 24 plates were incubated at 24°C. In addition a duplicate series of the above were set up - again 6 plates of each - and labelled (e), (f), (g) and (h). These were incubated at 30°C.

Results.

No actinomycetes were present on any of the plates when they were examined 28/2/51. Plates (a) showed

most of the nodule pieces surrounded by a greenish coloured fungus. Subcultures were taken of this organism. One of the plates from(b) also showed the presence of this fungus.

3 plates of (c) showed the presence of a finely filamentous fungus, white in colour. This fungus was also present on 3 of the plates of (d); 2 of the plates of (e); 5 of the plates of (f); 3 of the plates of (h).

Inoculation experiments with both of these isolations failed to induce nodulation on Myrica gale plants growing in water culture.

Discussion.

As noted in the introduction to this Section Peklo (1910) performed no re-inoculation tests with his isolate. Without such evidence of re-inoculation it is surely impossible to accept proof of identification of the supposed isolate. Until some worker is able to demonstrate nodulation of Myrica gale plants using a pure culture of isolated organism then he cannot claim to have isolated the causative organism. Furthermore, attempts by the present author to repeat Peklo's isolation methods have failed. Attempts were made using the beerwort medium described by him and sterilising the nodules by singeing as recommended.

Peklo states that the nodule pieces should be rotated in the bunsen flame until they glow. This was thought by the present author to be rather drastic and so the pieces were merely passed through the bunsen flame a few times before being crushed in the medium. Even with this modified treatment the beerwort medium remained completely sterile six weeks after inoculation. This perhaps was not so very surprising since the original pH of the medium was 9.12.

Reduction of the pH to 7.6 of a fresh batch of medium and re-inoculation resulted in the growth, among other organisms, of a zoogloeal mass of bacteria which may have corresponded to Peklo's isolate. But inoculation of Myrica gale plants with this zoogloeal mass failed to effect nodulation.

No one has substantiated Bottomley's claim to have isolated the endophyte. As noted in the introduction to this Section it is not sure whether or not nodules were produced after inoculation with the bacterium he isolated. If nodulation did occur then it should not necessarily be ascribed to the presence of the culture organism, since, earlier, some of the uninoculated controls also developed nodules and had to be

discarded. Indeed Bottomley's isolate may be regarded as a contaminant and not as the endophyte for the following reasons:-

(a) The inadequacy of his re-inoculation experiments.

(b) The failure of any workers to repeat his isolation results.

(c) Cytological examination of the nodule indicates that the responsible organism is not bacterial in nature.

(d) Inoculation of Myrica gale plants with various strains of Rhizobium has failed to effect nodulation. (See later Section (VI) on cross-inoculation experiments). This does not of course exclude the possibility of the organism within the Myrica nodules being a specialised strain of Rhizobium which is not cross-inoculable with other strains.

As noted in the introduction Youngken (1919) also claimed to have isolated the endophyte and the presence of Actinomyces threads in cells of the roots pricked with his isolate appears to have satisfied him as to its nature; but the claim will not bear critical examination, and his results cannot be accepted. Many attempts have been made by the present author to repeat Youngken's findings using Myrica gale and sterilising the nodules by

the method recommended by him, namely, shaking up for 20 seconds in 1:1000 corrosive sublimate followed by repeated washings in sterile distilled water. A variety of agar media have been used and although actinomycetes have been isolated on some of them, none of the isolates have produced nodules when applied to the roots of young Myrica gale plants in water culture.

Youngken's work may be discounted then because of his failure to perform satisfactory re-inoculation tests with Actinomyces myricarum.

Finally, Arcularius (1928) and Hawker and Fraymouth (1951) have reported unsuccessful attempts to isolate the endophyte of Myrica gale nodules.

Thus six workers, including the present author, have reported attempts to isolate the endophyte of Myrica nodules and no doubt there are many unreported attempts by other workers. All have been unsuccessful for all isolations fail at the critical test of satisfactory re-inoculation

The failure to isolate the causative organism from the nodules of Myrica gale is disappointing but not wholly unexpected. Indeed, despite the outstanding example of the root nodules of the Leguminosae from which the causative organism can

be relatively easily isolated, nodular organisms and mycorrhizal forming fungi have remained difficult and often impossible to isolate e.g. the organisms responsible for nodulation of the roots of Alnus, Hippophäe and Elaeagnus have not yet been grown apart from the host plant. The ease of isolation of Rhizobium from the nodules of the Leguminosae should be regarded as the exception rather than the rule. Regarding mycorrhizae in general Kelley (1950) states that " isolating the fungus directly from the mycotrophic organ has proved, in most cases, impracticable". As noted Hawker and Fraymouth (1951) consider that the endophyte is a member of the Plasmodiophorales. If so then this would explain the failure of many authors to isolate it. But in the previous Section (p48.) of this thesis it is deduced from cytological examination that the endophyte is a member of the actinomycetes. Is then the relationship to be regarded as being of an obligate nature? The presence of nodules, and consequently of the organism is not necessary for the healthy growth of the Myrica gale plant. Supplied with an inorganic source of nitrogen, such as $NaNO_3$ or NH_4NO_3, uninoculated and non-nodulated plants of Myrica gale will grow excellently and produce

viable seed. Clearly then, the presence of the organism is not necessary for the growth of the plant; but the question remains - can the organism survive without the presence of the plant? The failure, so far, to culture the organism on any of a wide range of artifical media (nutrient agar, glycerine agar, mineral salt agar, beerwort liquid and agar, Jensen's agar) using a wide range of pHs (4.12 - 9.12) and varied methods of surface sterilising the nodules (by fluming, by H_2O_2 , by $HgCl_2$, by HCl + $HgCl_2$, by washing in soapy water) would indicate that the organism is, at least not easily cultured. But it should not be assumed from this that it cannot be cultured. It may be that the organism is very exacting in its food requirements and we have not yet hit upon the composition of the correct medium; or perhaps the pH tolerance is narrow though this would not appear to be so from Bond's (1951) finding that nodulation can occur over a fairly wide range 3.3 - 6.3 (the latter being the highest pH tested by Bond). But it should be remembered that this merely indicates that the organism can survive for a time in media of these pHs not that it can multiply in them, for it will be shown in a later Section (VIIA) that there is no correlation between the pH of the medium and

the pH of the sap of roots of Myrica gale growing in the medium. Despite the fact that the external pHs range between 4.1 and 6.9 the pH of the roots remain constant between 5.0 and 5.3. The organism then needs to survive for only a short spell in the adverse pHs before entering the plant where a suitable pH is present regardless of the pH existing in the external solution.

Possibly, also, the organism is microaerophilic. The present author has made one attempt to isolate it under micro-aerophilic conditions without success but many more attempts must be made before the possibility is discarded. Myrica gale is usually found growing in wet boggy situations where one could reasonably assume that there was an oxygen shortage. Further we have already noted that a cork layer is formed round the nodule at a fairly early stage of development. This too would tend to cut down the amount of oxygen available to the organism within the nodule. We have already noted (p 36.) that the organism is never found in the nodule-roots emerging from the nodule tips. We have also already noted that Bond (1952) considers that the nodule-roots are aerating organs, and it may well be that the organism is prevented from migrating to them

because of the relatively high concentration of oxygen present there. All of the above remarks point to the possibility of the organism being at least partially anaerobic or micro-aerophilic.

If the organism is an obligate symbiont, how does it get from plant to plant? If spores were formed, then infection could be established by the spores becoming attached to the roots and germinating there so that the organism would not have to live saprophytically at all. Cytological examination by the present author has failed to reveal the presence of spores but Hawker and Fraymouth(1951) have observed particles formed from the breakdown of the previously noted peripheral clubs (p 54.). These may be spores but the present author has been unable to detect discrete contents within the clubs (see p 60.).

There remains however the possibility that the bacterial pieces immediately go into a spore form when they are shed into the soil from the nodules and do not germinate again until they are within the rhizosphere of a Myrica plant. Germination would result in the formation of the bacteria noted in a previous Section, which penetrate the root hairs and initiate nodulation. Examination of the supernatant fluid from crushed nodules has

failed to reveal the presence of such spores though this does not discount the possibility of their occurring in nature.

Most of the foregoing is conjectural and must necessarily be so until either the organism is isolated in pure culture or sufficient workers have reported failure to do so and it may then be classified as an obligate symbiont.

SUMMARY OF SECTION V.

(a) Attempts are described to isolate the organism responsible for nodulation of *Myrica gale*. All of these have proved to be unsuccessful.

(b) An examination is made of the results of previous authors' attempts at isolation and it is concluded, despite claims to the contrary, that the organism has not yet been isolated since no satisfactory re-inoculation tests have been performed.

(c) A discussion follows on the possible reasons for failure and the evidence examined for the possibility of the organism being an obligate symbiont.

VI. CROSS-INOCULATION EXPERIMENTS.

Introduction.

There appear to have been no cross-inoculation tests carried out between Myrica and other nodule-forming plants such as Alnus, and members of the Leguminosae, to determine whether the various endophytes are cross inoculable or not. Roberg (1933-34) has performed cross-inoculation experiments between Alnus and the other nodule formers, Elaeagnus and Hippophäe. His conclusions were that Elaeagnus and Hippophäe are nodulated by the same organism but that Alnus contains a different organism. It is of interest to note that Elaeagnus and Hippophäe both belong to the Elaeagnaceae family while Alnus and Myrica according to Clapham, Tutin and Warberg (1952) are in the Betulaceae and Myricaceae respectively. Bentham and Hooker (1924) however remark that some authors would unite Alnus and Myrica together with several other genera (e.g. Corylus, Fagus) in the Amentaceae or Catkin family.

Methods and Results.

These experiments were carried out on Myrica gale and Alnus glutinosa plants growing in N-free Crone's solution. They were obtained by the germination of seed in peat as already described for

Myrica gale except that no pretreatment was necessary for Alnus seed, and after approximately two months were transferred either to sterilised 2-litre jars or 1-litre beakers each containing the Crone's. In order to obtain an inoculum of Myrica endophyte the method used was that described on p 10. A similar method using Alnus nodules yielded an inoculum of Alnus endophyte. Cultures of Rhizobium were used consisting of a mixed suspension of the following strains:- Pea 317, Pea Nx, Clover F, Lucerne AH. All were active nodule-formers on their appropriate hosts. Each of the strains was grown on a slope of yeast-mannitol agar for one week at 24°C and then the growth was suspended in sterile distilled water. The four suspensions so obtained were then mixed together and an equal volume of the total added to the requisite jars. Inoculation using the Myrica and Alnus inoculum was as described for Myrica in an earlier Section, namely applying a loaded camel hair brush of inoculum to the roots of requisite plants and then adding a little of the inoculum to each jar.

EXPERIMENT I.

Effect of Rhizobium on Myrica gale.

Two jars i.e 14 plants of Myrica gale growing in

Crone's solution adjusted to pH 5.4 (suitable for nodulation in Myrica gale) were inoculated with a suspension of Rhizobium on 13/5/50. There was no evidence of nodulation by the time the experiment was discontinued on 14/7/50.

EXPERIMENT 2.

Effect of Alnus inoculum on Myrica gale.

Two jars i.e. 14 plants of Myrica gale growing in Crone's solution adjusted to pH 5.4 were inoculated with an Alnus inoculum on 13/5/50. The experiment was discontinued on 14/7/50. There was no evidence of nodulation.

EXPERIMENT 3.

Effect of Myrica gale inoculum on Alnus glutinosa.

20 plants of Alnus glutinosa, growing in Crone's solution pH 6.3 (Ferguson (unpublished)) has shown that optimum pH for nodulation of Alnus lies between 6 and 7) were inoculated with a Myrica gale inoculum on 31/5/51 and further inoculation of the same plants was repeated on 2/6/51 and again on 23/6/51. The plants were inspected for nodules on several occasions and finally on 4/9/51. There was no evidence of nodulation.

It may be noted that no controls were used in the above experiments because similar inocula used on many previous occasions had always

induced nodulation, when applied to the correct host plant e.g. Myrica inoculum to Myrica gale plants. In the experiments which follow the Myrica inoculum was obtained from nodules of plants which had been reared from seed in the greenhouse. It was not known for certain whether such an inoculum would induce nodulation on Myrica gale plants. Consequently controls had to be set up.

EXPERIMENT 4.

Group A. 30 plants of Alnus glutinosa, growing in Crone's solution pH 6.3.

Group B. 30 plants of Myrica gale growing in Crone's solution pH 5.4.

Group C. 20 plants of Alnus glutinosa growing in Crone's solution pH 6.3.

Plants in Groups A and B were inoculated on 29/7/51 with an inoculum of Myrica gale from the green - house. Group C was left uninoculated.

By 4/9/51 fifteen plants from Group B had formed nodules and were thriving; also three others of Group B had formed nodules but had later died. These results show that the inoculum was active. No nodules had formed on the plants in Group A nor in Group C.

The results of these cross-inoculation experiments may be tabulated as follows :-

Plant inoculated.

Source of Inoculum		Myrica gale	Alnus glutinosa
Rhizobium Pea 317 Pea HX Clover F Lucerne AH	mixed	no nodulation	not done
Alnus glutinosa.		no nodulation	nodulation
Myrica gale.		nodulation	no nodulation

From these results it may be concluded that the same organism is not responsible for nodulation in Myrica gale and Alnus glutinosa, or at least there is a difference in the strain of the two organisms, despite the existence of taxonomic affinities between the two host plants. The fact that the rhizobia utilised did not nodulate Myrica gale does not mean that there is no Rhizobium capable of doing so. There are many which were not tested and it is always a possibility that if a Rhizobium is responsible then it is one which can nodulate Myrica and no other plant.

VII. EFFECT OF SOME EXTERNAL FACTORS OF NODULATION.

A. THE EFFECT OF pH ON NODULATION IN MYRICA GALE COMPARED WITH THAT IN ALNUS AND TRIFOLIUM.

Introduction.

In this Section of the thesis a comparison will be made of the effect of the pH of the rooting medium on nodule formation in two non-legumes (*Myrica gale* and *Alnus glutinosa*) with that in a typical legume namely Red Clover - (*Trifolium pratense*). The value of such a comparison lies in the light that may be thrown on the nature of the various endophytes, particularly as regards their pH requirements.

For this purpose the data obtained by Bond (1951) on *Myrica gale* and unpublished data on *Alnus glutinosa* kindly communicated to the author by Mr T.P.Ferguson of the Botany Department, Glasgow University will be utilised. In order to provide a direct comparison the author has obtained data for the pH effect on Red Clover grown, as were the two non-legumes, in water culture. Previous authors have studied the pH effect in Red Clover grown in other rooting media e.g. Bryan (1923), Virtanen (1927) and Jensen (1943); Bryan using sand culture, Virtanen sand culture and Jensen agar culture. The results of

all three authors are in general agreement. Bryan noted that there is poor nodulation at pH 4.0 and none at pH 3.0 or below; Virtanen that nodules can be produced at pH 4.6; and Jensen that although there is slight nodulation at pH 4.4 - 4.5 there is none at pH 4.3 or below.

In addition to the above investigations, a comparison is made of the pHs of root tissue, nodule tissue and the medium in which the plants are growing, of both Myrica and Alnus in order to determine whether or not there is a correlation between external and internal pH. Such an investigation will obviously yield important data relating to the effect of pH on nodulation. Jensen (1943) has obtained data of this kind for various species of Trifolium. He grew T.repens, T.glomeratum and T. subterraneum in soil in which the pH ranged from 5.0 - 7.5. The root tissue pH of such plants ranged from 5.5 - 6.0 only, and the nodule tissue from 5.5 - 6.3.

Similar experiments were carried out with T.subterraneum growing in sand ranging from pH 5.1 - 6.5. The nodule tissue pH ranged from 5.96-6.08 only. No figures are given for root tissue pH. Finally T.repens plants were grown in agar at pH 5.3 - 5.5. The nodule tissue pH was 5.7.

Again no figures are given for root tissue pH.

Methods.

In the investigations carried out by Bond (1951) on Myrica gale and by Ferguson (unpublished) on Alnus glutinosa, the plants were first germinated from seed in peat and transplanted at an early stage (2-4 foliage leaves showing) to Crone's N-free solution in 2-litre jars. The pH was adjusted as required by suitable addition of sulphuric acid or sodium hydroxide. There were 7 plants per jar.

Essentially the same methods were employed by the present author for the Trifolium pratense investigation. The seeds were germinated in washed sand in glass troughs, being watered from time to time with Crone's N-free solution. They were transplanted when the first foliage leaves were showing to Crone's N-free solution + the minor-elements A-Z solution in 2-litre jars.

There were 7 plants per jar. 1 mgm. combined-N (as a solution of NH_4NO_3) was added per litre of solution to prevent the plants dying off in the early stages. Inoculation was carried out using Clover strain 49 of Rhizobium. The pHs of the various solutions were adjusted by adding N/1 H_2SO_4 or N/1 NaOH as required.

The following jars were set up:-

Jars 1 - 5; pH adjusted to 3.3
Jars 6 -10; pH adjusted to 4.2
Jars 11 -15; pH adjusted to 5.4
Jars 16 -20; pH adjusted to 6.3
Jars 21 -25; pH adjusted to 7.0

All the plants in the above jars were inoculated with Rhizobium.

In view of the results of previous authors, particularly Bryan (1923) it was thought that there would be a dying-off of the plants at the three lower pHs namely 3.3, 4.2 and 5.4 and in order to determine whether this dying-off was due to the harmful effect of the low pH on the plant, or to a lack of nitrogen due to failure to nodulate, duplicate jars were set up for these pHs containing uninoculated plants growing in Crone's solution + NH_4NO_3. Initially 20 mgms. combined-N / litre was added and then 20 mgms. per litre every alternate day until a total concentration of 140 mgms. combined-N / litre was achieved. Jars were set up as follows:-

Jar 1A pH adjusted to 3.3
Jar 6A pH adjusted to 4.2
Jar 11A pH adjusted to 5.4
} All of these jars contain 140 mgms. combined-N / litre.

Transplanting was carried out 8/2/52 and the plants inoculated the same day. They were then placed

under a battery of fluorescent tubes in a cool greenhouse as described by Low (1948). The intensity of the artificial light alone at plant level (12 cms. below the fluorescent tubes) was approximately 300 ft.-candles, the light being supplied for sixteen hours each day.

The pHs of all jars were checked on making up the solutions, again after distributing the solutions into jars and again after transplanting. Subsequently the pHs were checked every three days and adjusted if necessary. The experiment was terminated 14/3/52.

The methods employed for the determination of tissue pHs of Myrica and Alnus were essentially the same as those described by Jensen (1943) for Trifolium except that the plants were grown in water culture solutions and the pHs adjusted every day or two as required. The roots were carefully washed two or three times with distilled water, all nodules picked off and the root material weighed and cut into small pieces with scissors and crushed in a mortar by means of a glass pestle with ten times its weight of distilled water. The nodules were treated in a similar manner. In both cases the expressed fluid was used for pH determination which was

carried out by means of an electric pH meter. Jensen did not dilute his root material with water, but he diluted the nodules with their own weight of distilled water. He observed that the addition of small amounts of dilute acid or alkali showed that the nodule tissue had high buffering capacity so that little change in pH is likely to result from dilution with water. The present author's findings are in agreement with those of Jensen. Thus the pH remained quite constant whether the root (or nodule) tissue was diluted once, five times, ten times or twenty times with distilled water.

Results.

Observations on Trifolium pratense.

Many of the plants in solution at pH 3.3 were dying off by 21/2/52 (i.e. some 13 days after setting up the experiment) and all were dead by 12/3/52 although those supplied with combined nitrogen lasted longer than the inoculated plants. There was no evidence of nodulation.

When examined on 21/2/52 all plants in solution at pH 4.2 were healthy. By 26/2/52 the leaves of the plants growing in N-free solution were becoming chlorotic although the plants supplied with combined nitrogen, at the same pH, were

growing strongly and continued to do so till the experiment was terminated on 14/3/52. On this date the inoculated plants were dead or dying. There was no evidence of nodulation.

At pHs 5.4, 6.3 and 7.0 the plants grew strongly and there was very good nodule-formation.

The plants, supplied with combined nitrogen, growing at pH 5.4 were bigger and stronger than the corresponding nodulated plants at the same pH.

Many of the above observations are illustrated in Figures 59-62.

The main findings for the experiment are summarised in Table 1. (p 97).

TABLE 1.

MEAN DATA OBTAINED AT HARVEST OF TRIFOLIUM PRATENSE GROWING AT DIFFERENT pH LEVELS.

TREATMENT.	NUMBER OF PLANTS SET UP.	PLANTS SURVIVING AND HARVESTED.	AVERAGE NUMBER OF NODULES PER PLANT.	AVERAGE DRY WEIGHT OF NODULES PER PLANT. (Mg)	AVERAGE DRY WEIGHT OF PLANT. (Mg)	PLANTS NODULATING
pH 4.2 Inoculated	35	13	0	-	16	0
pH 4.2 Uninoculated + combined N*	7	5	-	-	123	-
pH 5.4 Inoculated	35	32	48	1.7	42	32
pH 5.4 Uninoculated + combined N*	7	7	-	-	139	-
pH 6.3 Inoculated	35	33	65	2.1	45	33
pH 7.0 Inoculated	35	31	96	2.7	49	31

*Combined N added as NH_4NO_3. 140 mgms combined N / litre of solution.

TABLE 2.

REACTION OF ROOT NODULES AND ROOTS OF MYRICA GALE COMPARED WITH REACTION OF THE MEDIUM.

pH of solution.	pH of roots.	pH of nodules.
4.2	5.0	5.82
5.4	5.32	5.50
6.3	5.18	5.42
7.0	5.13	5.54

One sample of roots and nodules was tested at each of the above solution pHs.

TABLE 3.

REACTION OF ROOT NODULES AND ROOTS OF ALNUS GLUTINOSA COMPARED WITH REACTION OF THE MEDIUM.

pH of solution.	pH of roots.	pH of nodules.
4.2	5.02	5.92
5.4	4.93	5.8
6.3	5.21	6.0
7.0	5.15	6.05

Each result for roots and nodules is an average of two tests.

Discussion.

From Table 1. it can readily be observed that in this clover experiment no nodulation occurred at pH 3.3 nor at pH 4.2

At pH 3.3 all of the plants died off without making any appreciable growth even when supplied with 140 mgms. nitrate N / litre. Death in this case was not then due to a nitrogen shortage but because the pH had an adverse effect on the Clover plants. At pH 4.2 on the other hand the plants supplied with 140 mgms. nitrate N / litre grew excellently. The pH was therefore suitable and the death of the plants lacking added nitrate N could be directly ascribed to the failure to nodulate and the failure to nodulate could in a like manner be ascribed to the failure of the Rhizobium organism to survive this pH of 4.2. Good nodulation occurred at pHs 5.4, 6.3 and 7.0 and the majority of plants at these pHs thrived. Thus the organism is at least able to survive these pHs. The upper limit has not been determined but the lower lies somewhere between pH 4.2 and 5.4.

The findings then are in accord with those of Bryan (1923), Virtanen (1927) and Jensen (1943).

A comparison of pH effects can now be made in respect of Trifolium pratense, Myrica gale

and Alnus glutinosa, all the plants having been grown under similar conditions in water culture.

Bond (1951) found that some nodulation can occur on Myrica gale growing in water culture at a pH of 3.3. Good nodulation occurred at pH 4.2, 5.4 and 6.3. The best growth of plants and the greatest dry weight of nodules was at pH 5.4. Ferguson (unpublished) found that there was no nodulation on Alnus glutinosa growing in water culture at pH 3.3 but that there was nodulation at pH 4.2, 5.4, 6.3 and 7.0. The best growth of plants and the greatest dry weight of nodules was at pH 5.4.

These findings and those of the present author for Trifolium pratense are best expressed in the form of a graph which is attached (Figure 58).

To summarise :- The endophyte of Trifolium pratense cannot survive a pH of 4.2 or lower. The endophyte of Alnus glutinosa can survive a pH of 4.2 but not of 3.3 or lower. The endophyte of Myrica gale can survive a pH of 3.3.

The findings confirm the Cross Inoculation Experiment findings that the same organism is not responsible for nodulation in Myrica gale and Alnus glutinosa. It should of course be noted

that these findings do not mean that the various endophytes can multiply and grow at the pHs at which they can effect nodulation. It merely means that they can survive these pHs (in some cases perhaps only for a short time). Once inside the plant the endophyte must be able to multiply but as noted earlier Jensen (1943) has shown for the species of Trifolium that there is no direct correlation between external and internal pHs. The present author has shown that the same holds good for Myrica gale (Table 2) and Alnus glutinosa (Table 3). Although the reaction of the solutions in which the plants were growing ranged from 4.2-7.0 , the pH of the root tissues and nodule tissue of Myrica gale remained fairly constant at 5.0 - 5.3 and 5.42 - 5.82 respectively.(See Table 2 p98).

Similarly with Alnus glutinosa the pH of the root tissue was 4.93 - 5.21 and that of the nodule tissue 5.8 - 6.05. (See Table 3 p 99).

This explains Bond's (1951) finding for Myrica gale that "at pH 3.3 many plants failed to form nodules and soon died but one of the plants which did form nodules at this latter pH grew fairly strongly" and also, as noted, that Jensen (1943) found that Rhizobium was killed within one to three days in a well buffered

sucrose-yeast extract at pH 7.6 - 8.7 although good nodulation can be obtained on inoculated plants at this latter pH.

The organisms in these cases were able to survive the adverse pHs long enough to penetrate the root. Once within the root of the host plant they found a more suitable pH and proceeded to multiply with consequent nodule-production.

Regarding the identity of the organisms responsible for nodulation in Myrica gale and Alnus glutinosa this experiment does not reveal a great deal e.g. any of the organisms, bacterium, actinomycete, fungus or myxomycete (though probably not Rhizobium) could survive the demonstrated pHs at which nodulation occurred.

From Jensen's (see Introduction) findings it can be assumed that Rhizobium trifolii multiplies rapidly at pH 5.5 - 6.0 since this is the internal pH of the root inwhich nodules are formed. Similarly the present author's findings for Myrica gale and Alnus glutinosa indicate that the endophytes can multiply rapidly at 5.0 - 5.3 and 4.9 - 5.2 respectively. This does not imply that these are the optimum pHs for growth. Bacteria and actinomycetes in general prefer a neutral pH for growth. Many

exceptions to this are known among the bacteria and as noted earlier in this thesis, Jensen (1928) demonstrated a group of actinomycetes - Actinomyces acidophilus-which grew readily on an agar medium of pH 3.8 - 4.0. The findings of the experiment do not therefore count against the possibility of the organism responsible for nodulation of Myrica gale being an actinomycete, as the present author has concluded from cytological investigation.

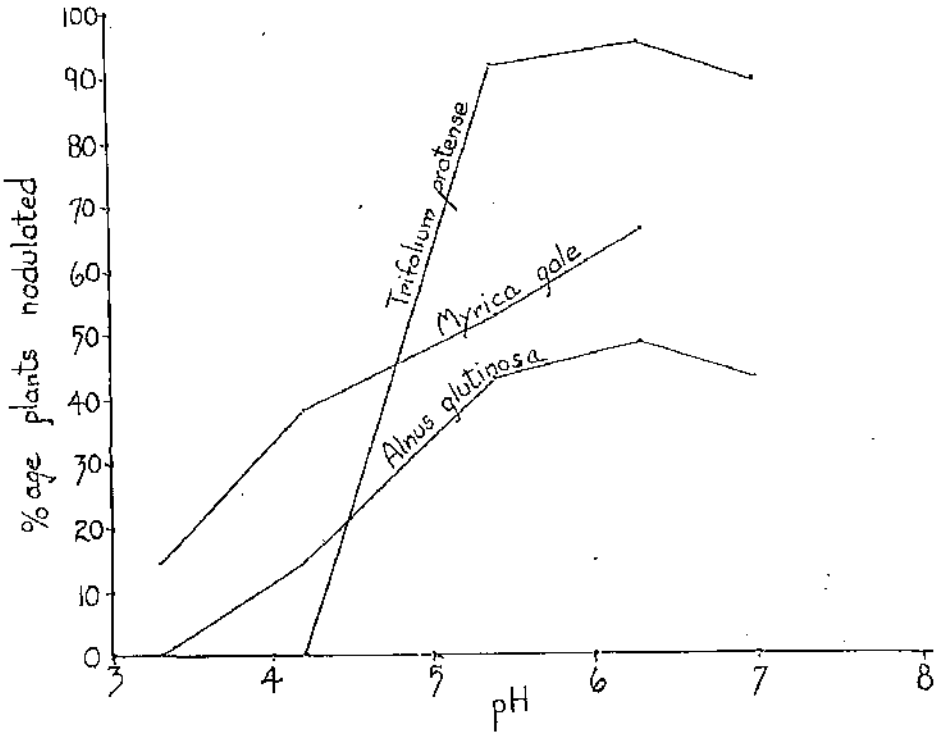


Figure 50.

Graph showing the relationship between ph of culture solution and nodulation of Trifolium pratense, Myrica gale and Alnus glutinosa.

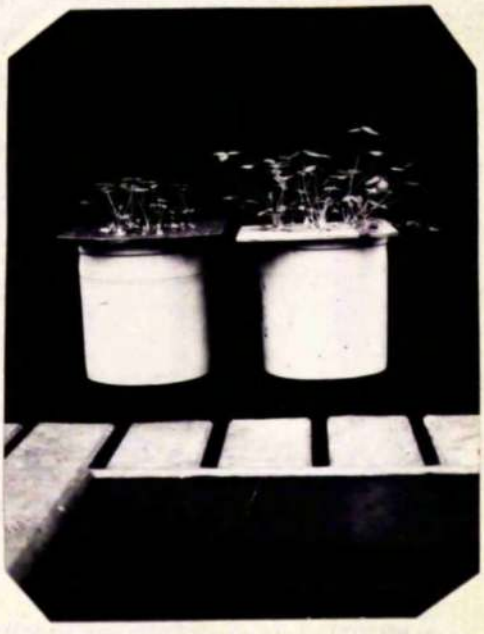


Figure 59. Figure 60.

Jars of plants of Trifolium pratense grown in water culture.

Figure 59 at pH 4.2. Jar on left contains inoculated plants grown in N free solution. No nodules formed.

Jar on right contains uninoculated plants with 140 mgms. combined N/litre added to the solution.

Figure 60 at pH 5.4. Jar on left contains inoculated plants grown in N free solution. Nodules were formed.

Jar on right contains uninoculated plants with 140 mgms. combined N/litre added to the solution.

All plants 7 weeks old. X1/10th

Figure 61.

Plants of Trifolium pratense grown in water culture at ph 4.2. Plants 7 weeks old.
The two plants on the left were inoculated and grown in N-free solution.
No nodules formed.
The two plants on the right were uninoculated, with 140 mgms combined N per litre added to the solution. X1/5th.

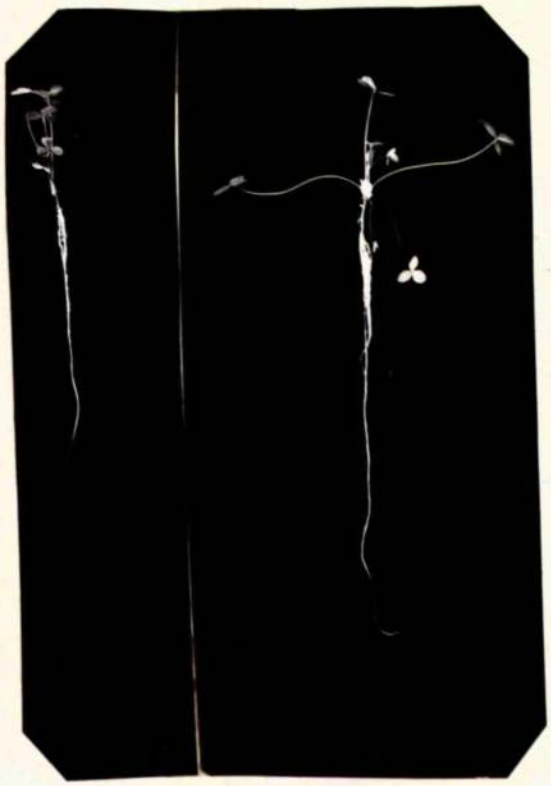


Figure 62.

Plants of *Trifolium pratense* grown in water culture at pH 5.4. Plants 7 weeks old. Plant on left was inoculated and grown in N-free solution. Nodules were formed. Plant on the right was uninoculated, with 140 mgms. combined N per litre added to the solution.

XI/5th.

SUMMARY OF SECTION VII A.

(1) The effect of pH on nodulation of *Trifolium pratense* is investigated using the water culture technique.

(2) The results are compared with the findings of other authors for *Myrica gale* and *Alnus glutinosa*.

(3) It is shown that the endophytes of the above three plants have different pH tolerances.

(4) It is demonstrated for *Myrica* and *Alnus* that the reaction of root and nodule tissues does not vary with the pH of the medium in which the plants are growing.

(5) The findings confirm the Cross Inoculation Experiment findings that the organisms responsible for nodulation of *Myrica gale* and *Alnus glutinosa* are different organisms.

(6) Also that those organisms are different from that of legumes in respect of acid tolerance.

VII EFFECT OF SOME EXTERNAL FEATURES ON NODULATION (continued)

B. THE EFFECT OF COMBINED NITROGEN ON NODULATION IN MYRICA GALE, WITH COMPARATIVE DATA FOR TRIFOLIUM PRATENSE.

Introduction.

A well known feature of the legume-Rhizobium symbiosis is that the extent of nodule formation (including both number and size of nodules) is at its maximum when the plant is grown in a medium free or almost free of available combined nitrogen. Nodule formation is lessened when appreciable amounts of nitrate or ammonium nitrogen are present. As an instance of this, data obtained by Thornton and Nicol (1936) are shown in Table 4. (see p. 107).

The data show that the addition of the nitrate reduced both the number and the size of nodules, the size of nodule being reduced before a reduction in number was apparent. The higher levels of nitrate employed in this experiment are of course far in excess of those normally supplied in sand or water culture of plants. Thus Crone's solution (full formula) contains 140 mgms. nitrogen per litre.

Thornton and Nicol state that -"Repressive effects with increasing doses of nitrate showed

TABLE 4.

EFFECT OF NITRATE-NITROGEN ON NODULATION IN LUCERNE.

(DATA OF THORNTON AND NICOL)

Mg.NITRATE-N SUPPLIED *	NUMBER OF NODULES PER PLANT	MEAN LENGTH OF NODULES (mm.)	DRY WT OF TOPS (gms/plant)	DRY WT OF ROOTS (gms/plant)
0	50	2.2	0.46	0.29
163	51	1.4	0.52	0.34
326	33	1.0	0.53	0.20
651	20	0.7	0.62	0.15
977	7	0.6	0.50	0.12
1628	7	0.6	0.49	0.22

* All added at start to the sand. Each pot held about 2 litres of culture solution.

themselves most clearly in reduced numbers of nodules and in reduced nodule length. With other indices of growth, e.g. dry matter, the effects were either substantially independent of the amount of nitrate added or else tended to have a maximum at about the levels of 1-4 gms. of $NaNO_3$ per pot (163-651 mgms.N); when there was a maximum it was seldom pronounced. The mean root weights showed with increasing doses of nitrate a decline that is just statistically significant."

In a recent paper, Virtanen (1951) has shown that in an unnamed legume, nodulation and nitrogen fixation are much less inhibited by ammonium N than by nitrate N. This, it may be noted, is at variance with the general literature.

There has been much discussion as to the explanation of the reduction in nodulation of legumes in the presence of combined nitrogen. Reviews of the literature on the subject are presented by Fred, Baldwin and McCoy (1932) and by Wilson (1940). An early suggestion (by Hiltner) was that the greater vigour of a young leguminous plant supplied with combined nitrogen from the commencement of growth is associated with a degree of immunity to invasion by the nodule bacteria. In other words the legume is able to "fight off"

the infection. A different view taken by other authors was that combined nitrogen exercised, in the rooting medium, a harmful effect on the nodule bacteria and reduced their infective powers; but in this connection it may be noted that experiment has shown that the cultivation of the organism on media containing nitrate at levels inhibitory to nodulation, results at least in no persisting lowering of infectiveness. There is, however, some evidence which seems to suggest that a relatively low concentration of nitrate in the cell sap of the legume restricts the growth of the organism there.

Yet another explanation draws attention to the probable effect of an ample supply of combined nitrogen in reducing the amount of carbohydrate available within the plant for the development of the bacteria and the nodules. Rapid synthesis of protein by the plant protoplasm will reduce the carbohydrate level and, it is suggested, thus hinder nodule development. In extension of this proposal, Wilson (1940) has argued that the critical factor is the carbohydrate-nitrogen ratio prevailing within the legume tissues. In general a high ratio favours nodulation, a low ratio depresses nodulation.

Essentially it appears to the author this is merely another way of describing the observed phenomena.

Thornton and Rudorf (1936), in a microscopical study of nodule development in the presence of nitrate, found no clear evidence of a shortage of carbohydrate and in fact noted an excessive thickening of cell-walls.

The investigations of Thornton (1936) show that the effect of combined nitrogen begins to operate at a very early stage in the infection process, since he found that in the presence of nitrate the curling and deformation of the root hairs (a normal prelude to infection) is diminished. These root hair effects have subsequently been shown to be due to a production of indole acetic acid by the bacteria (p.18 of thesis), and it thus appears that the effect of the nitrate must either be to reduce the secretion of this substance or to reduce the reaction of the root hairs towards it.

In view of the many similarities that exist between non-legume root nodule plants and legumes - for example, the occurrence of nitrogen fixation and the general state of symbiosis existent between host plant and endophyte - it will be of interest to determine whether a further resemblance exists

in respect of the effect of combined nitrogen on nodulation. No previous investigation of this question has been reported for any non-legume. Roberg (1933-34) after finding that uninoculated plants of Alnus, Hippophäe and Elaeagnus grew satisfactorily if supplied with combined nitrogen, stated that under such conditions the plants "do not need to form nodules" but he did not put the matter to actual test. The present writer has studied the effect of combined nitrogen on nodulation in Myrica gale growing in water culture and has also carried out a similar experiment with red clover (Trifolium pratense) in order to provide comparable data. Previous authors have reported on the effect of combined nitrogen on nodulation in red clover, but not (except for a very early investigation by De Vries (1877)) in respect of plants grown in water culture.

Methods.

Myrica gale.

The procedure for water culture was similar to that already described (p. 9), 2-litre glazed earthenware jars being used with six or seven plants per jar. The pH of the Crone's solution (nitrogen-free formula) was adjusted to 5.0 before use, and the pH of each jar was frequently tested

during the course of the experiments and corrected where necessary by suitable addition of acid or alkali to restore the pH to 5.0. Combined nitrogen, in the form of sodium nitrate or ammonium sulphate, was added to certain of the jars immediately after transplanting and prior to inoculation. Four levels of combined nitrogen were arranged, namely, 0, 35, 70 and 140 mg. nitrogen per litre of culture solution. The highest of these (140 mg. per litre) is that provided in the form of potassium nitrate by the "full" Crone's formula and may be assumed to provide fully for the requirements of normal plants. Inoculation was effected by the method previously described, i.e. by means of crushed nodule inoculum.

Red clover (Trifolium pratense).

After being surface-sterilised by means of alcohol and 0.1 per cent. mercuric chloride, red clover seed was sown in autoclaved sand, contained in glass troughs, and moistened with Crone's solution. At the stage when the first foliage leaf had formed, the clover plants were transplanted into water culture, Crone's solution (nitrogen-free formula) adjusted to pH 6.3 being used. In this case the effect of nitrate-nitrogen only was investigated, since in general the effects of

ammonium and nitrate salts are very similar, despite Virtanen's (see Introduction) finding for an unnamed legume. Appropriate amounts of sodium nitrate were added after transplanting to give the same levels as were employed for Myrica gale, except that an extra level (17.5 mg. nitrogen per litre) was introduced. The plants were then inoculated with an effective strain of clover Rhizobium (Rothamsted No.49) by adding to each jar an equal amount of a suspension of this organism in sterile water.

This experiment with clover was carried out in winter time, the plants being placed under the batteries of fluorescent lights previously described (p. 94). The pH was frequently tested and corrected to 6.3 as necessary.

Results.

Myrica gale.

An experiment relating to the effect of nitrate-nitrogen on nodulation was set up in water culture on 21/8/51 and terminated on 11/10/51. Observations made on the latter date are presented in Table 5. (see p.114).

In addition it was noted that the strongest plants were those receiving 35 mg. nitrogen per litre of culture solution, these showing a combined

TABLE 5.

EFFECT OF NITRATE-NITROGEN ON NODULATION OF MYRICA GALE.

Mgms. NO_3-N* ADDED PER LITRE OF CULTURE SOLUTION	NUMBER OF PLANTS SET UP	NUMBER OF PLANTS NODULATED	AVERAGE NUMBER OF NODULES PER PLANT
0	28	25	5
35	28	25	9
70	28	22	3
140	28	16	6

*Supplied as sodium nitrate.

shoot height and root length of 10 - 11 cm. compared with 6 - 7 cm. in respect of the plants at the three other levels. The 35 mg. plants also showed the best development of nodules and nodule-roots. Statistical analysis confirms that the number of nodules in these plants was significantly greater than in the other series. The higher levels of nitrate seem to have exerted a toxic effect on the plants. Thus those receiving 70 mg. nitrogen per litre showed inferior growth to those at the 35 mg. level, and at the highest level twelve plants died off at an early stage in the experiment.

The conclusions to be drawn from this experiment are (a) a moderate addition of nitrate-nitrogen (35 mg. per litre) leads to increased growth of the plant, indicating that the nitrate was being utilised by the plant, and this was accompanied by more numerous and better developed nodules than in the absence of combined N; (b) with concentrations of nitrate greater than 35 mgms. per litre the extent of nodulation was still as great as in the absence of nitrate, but the growth of the plants was not indicative of much absorption of nitrate. Taken by themselves the results obtained at the 70 and 140 mg. levels merely show that the presence of large amounts of combined

nitrogen in the external solution (as distinct from in the plant cells) does not interfere with the process of infection.

An experiment to investigate the effect of ammonium nitrogen on nodulation in Myrica gale was set up on 30/6/52 and terminated on 4/8/52. Observations made on the latter date are indicated in Table 6.

All the plants receiving combined nitrogen grew better than those in nitrogen-free solution, the mean combined shoot height and root length on the former being 12 cm. as compared with 6 cm. in the N-free plant. This difference became manifest at an early stage in the experiment, prior to the development of nodules, and was thus clearly due to the absorption and utilisation of the ammonium nitrogen. There was no significant difference between the growth of plants at the three levels of nitrogen, suggesting that the lowest level satisfied the requirements of the plants.

The presence of the ammonium nitrogen had no clear effect on the number of plants nodulating or on the average number of nodules per plant. It was observed (data not shown in table) that in presence of the combined nitrogen the nodules and nodule roots were considerably larger by the time

TABLE 6.

EFFECT OF AMMONIUM-NITROGEN ON NODULATION OF MYRICA GALE.

Mg. NH_4-N* ADDED PER LITRE OF CULTURE SOLUTION	NUMBER OF PLANTS SET UP	NUMBER OF PLANTS NODULATED	AVERAGE NUMBER OF NODULES PER PLANT	AVERAGE COMBINED SHOOT HEIGHT AND ROOT LENGTH
0	24	20 x	12	6 cms.
35	24	17	14	12 cms.
70	24	17	14	12 cms.
140	24	20	11	12 cms.

*Supplied as ammonium sulphate.

xPlants dying off did so at an early stage in the experiment before nodulation took place.

of harvest than in the plants dependent on nodule nitrogen alone.

Figure 63, a photograph taken shortly before terminating the experiment (five weeks after inoculation) illustrates many of the above statements. Some of the plants growing in the presence of 140 mgms. ammonium nitrogen were allowed to continue growing for a further six weeks, during which time the ammonium nitrogen solution was renewed. There was no interference with the process of nodule formation.(see Figure 64).

Red Clover (Trifolium pratense).

In an experiment to assess the effect of nitrate-nitrogen on nodulation of red clover, young plants were set up in water culture on 11/12/51 and inoculated the following day. Growth was allowed to continue until 8/1/52 when harvesting was commenced. Data then obtained are presented in Table 7.

It is clear that the presence of only 17.5 mg. nitrogen per litre of culture solution had a notable reducing effect on the number and size of nodules. The dry weight of the plants was appreciably greater with 35 or 70 mg. nitrogen supplied than with 17.5 mg. or no nitrogen as is to be expected in a short-term experiment where

TABLE 7.

EFFECT OF NITRATE-NITROGEN ON NODULATION IN RED CLOVER.

Mg.NITRATE-N ADDED PER LITRE OF CULTURE SOLUTION +	NUMBER OF PLANTS SET UP	NUMBER OF PLANTS NODULATED	AVERAGE NUMBER NODULES PER PLANT	AVERAGE No. OF "LARGE" NODULES * PER PLANT	AVERAGE DRY WT. OF NODULES PER PLANT (mg.)	AVERAGE DRY WT. OF PLANT (mg.)
0	28	22	85	12	1.8	40
17.5	28	23	42	1	0.7	36
35	28	27	37	1	0.9	60
70	28	25	30	1	0.8	55
140	28	27	32	0	0.6	43

+ Supplied as sodium nitrate.

* Of length exceeding 2 mm.

Figure 63.

Myrica gale plants from experiment showing effect of ammonium nitrogen on nodulation.

L to R

1) without combined nitrogen.

2) 35 mgms. ammonium N / litre of solution.

3) 70 mgms. ammonium N / litre of solution.

4) 140 mgms. ammonium N / litre of solution.

Note nodule formation in all plants.

Five weeks after inoculation. X½

Figure 64.

Later stage in growth of *Myrica gale* plants in water culture + 140 mgms. ammonium N / litre. Note continuation of nodule formation.

11 weeks after inoculation. X3/10.

the plants with little or no nitrogen were still feeling the effect of nitrogen shortage. If the experiment had been allowed to continue for a longer period, no doubt the nitrogen fixed by the nodules would have compensated these plants for the absence or shortage of nitrogen in the solution and they would have caught up with the plants growing in the 35 or 70 mg. nitrogen. The dropping of the dry weight at the 140 mg. nitrogen level may have been due to a slight toxic effect of this concentration on the plants.

Discussion.

The experiment with red clover served its purpose by confirming that under the particular conditions of water culture employed, the characteristic effect of combined nitrogen on nodulation in legumes could be demonstrated.

The results with *Myrica gale* were quite different. Here the enhanced development of the young plant consequent on the presence of available combined nitrogen from the time of transplanting, was accompanied by the formation of at least as many nodules as in the absence of combined nitrogen, and the nodules increased in size much more rapidly. There thus appears to be no interference with the infection process, and after that stage

the nodules share in the improved nutrition of the plant.

The present writer's experiments were not intended to yield information on whether or not the nodules forming on Myrica gale in the presence of combined nitrogen maintained their normal function of nitrogen fixation. This would be a matter for a separate investigation. It is quite possible that the stronger development of nodules was accompanied by enhanced fixation. There is no clear evidence that in legumes the reduction of fixation of nitrogen observed in the presence of combined nitrogen exceeds the extent of reduction in nodulation.

The data for Myrica gale show that the growth of the plants supplied with ammonium nitrogen greatly exceeded that of the plants dependent on nodule nitrogen alone. This seems at first surprising in view of the experience of Bond (1951) that plants of the latter type grew as strongly as non-nodulated plants receiving nitrate-nitrogen. It must, however, be noted that in the present writer's experiment the growth period in water culture was only 5 weeks, whereas in Bond's case it was 6 months. Bond (private communication) noted that at first the nitrate plants grew more

strongly, and only at a later stage did the nodulated plants catch up.

The suggestions that have been made by previous authors concerning the effect of combined nitrogen in legumes were reviewed in the Introduction. It was noted that there was no clear idea as to the sequence of events. It appears to the writer that the presence of increased amounts of combined nitrogen in the legume tissues must result in (a) some diminution of the growth-promoting stimulus which the bacteria apply firstly to the tissues of the mother root and later to the nodule tissues, or (b) a redistribution of food substances so that less becomes available to the developing nodules, i.e. there is a differential adjustment of "sink" values, comparable to that which appears to occur, for example, in many plants, quite apart from legumes, whereby in the presence of an abundance of combined nitrogen, top growth is favoured at the expense of root growth. It appears in the legumes, however, that the reduction in nodule growth far outstrips that in the roots, so that a differential effect as between nodules and roots must be assumed. This latter is essentially the explanation favoured by several previous workers

but at present it does not seem possible to choose finally between (a) and (b). Possibly both may apply.

The view of Thornton (1952) about the classical "symbiosis" between leguminous plant legume nodule bacteria is that the "symbiosis" is in effect a controlled parasitism. Thornton and co-workers have demonstrated the existence of several forms of relationships between the leguminous host-plant and the bacterial endophyte of its nodules. In one of these, the strain of nodule bacterium is virtually parasitic; it forms large numbers of small nodules which singly are almost ineffective in nitrogen-fixation. In other forms which may be called more customary or "typical", the bacteria tend to form relatively large nodules, each effective in fixing nitrogen provided combined nitrogen is not present in amounts greater than what can be taken up in the ordinary way by the roots.

It is perhaps not unnecessary to say that the fundamental modes of nitrogen-nutrition in leguminous plants (nodulated or not) are no different from those of any other plant nourished solely by its roots; but many species of leguminous plants, and also the carnivorous

plants, can benefit from a source of nitrogen additional to what is absorbed by the roots. If excess combined nitrogen is present, bacterial nitrogen-fixation is interfered with: either because nodule-formation is impeded ab initio. or because nodules already formed either tend to cease to grow and to develop new bacterial tissue, or tend to become senescent owing to an onset of pronounced parasitism. Still another manifestation appears when the vascular strands do not develop (an extreme but illustrative phenomenon which is probably not encountered in nature), or when the supply of soluble carbohydrate from the host plant to the nodule and its endophytic bacteria is otherwise cut off, as by darkening a plant already bearing normal efficient nodules. The last-mentioned instance, indeed, suggests that even in "normally" well-nodulated legumes the bacteria may be symbiotic by day and parasitic by night.

The lesson of the last two instances is that parasitism by "normal" legume nodule bacteria within the host plant is averted only by sufficiently abundant supply of soluble carbohydrate: failing which, the bacteria attack the insoluble carbohydrates and pectinous and allied

substances of the cell-walls of the nodule. Combined with the observations about the effects of excessive combined-nitrogen supply upon the activities of nodule bacteria within the legume nodules, it appears that the kind of activity manifested by bacteria within the legume nodules must be influenced by the ratio of soluble carbohydrate to combined nitrogen present in chemically simple forms easily soluble in water.

Compare now the results obtained with Myrica gale and with clover. The essential difference seems to be that while in both cases the development of the plant itself is stimulated by the presence of combined nitrogen in the rooting medium, in Myrica gale the nodules both in respect of number and size share in this improved nutrition, whereas in clover they do not.

Consideration has been given to the possibility that this difference in result does not indicate any fundamental divergence in behaviour, but was due to difference in experimental treatment. Thus on account of the slow development of the Myrica seedlings it was necessary to allow a period of two months before the seedlings were of suitable size for transplanting into water culture. During this period

the young plants were growing in horticultural peat of low available nitrogen content. Thus by the time of inoculation a relatively high carbohydrate-nitrogen ratio (as compared with that in the clover) was probably prevailing in the plants. This assumption is supported by the tendency to anthocyanin formation noted in shoots and roots. This high carbohydrate-nitrogen ratio even in the presence of combined nitrogen might persist long enough for the earlier stages in nodulation to be accomplished as successfully as in the nitrogen-free solution. It would however seem likely that fairly soon the ratio would fall as absorption of the combined nitrogen proceeded, and, if events were running parallel to those in legumes, the subsequent development of the nodules would be retarded. Since there was no sign of this (see Figure 64) it is concluded that the effect of combined nitrogen on nodulation is fundamentally different in the two plants. The nodule appears to be more of an integral part of the plant, physiologically in Myrica than in the legume.

Some leguminous species (e.g. Judas tree (Cercis siliquastrum)) are known not to bear nodules, and may thus be relics of a stage of evolution before other legumes had acquired the

ability to exist in partnership with micro-organisms in nodules on roots or leaves.

From the standpoint of the economy of the plant, the Myrica - actinomycete symbiosis seems to be a less highly evolved system than the legume -Rhizobium association (where that exists). The view may be taken that, as far as present knowledge goes, the microbial partner in Myrica nodules is more nearly a true symbiont than are the bacteria in legume nodules: there is evidence that the legume-rhizobium association is fundamentally parasitic and there is no evidence or suggestion that the Myrica - actinomycete association is parasitic. On the contrary, the evidence of the present work that the effects of the Myrica - actinomycete association persist unchanged whether only little combined nitrogen is present as probably occurs in the natural Myrica habitat or whether combined nitrogen is made abundant, strengthens the view that the Myrica - actinomycete association is a true symbiosis.

In so far as the manifestation of "symbiosis" between legume and root-nodule bacteria is controlled by the plant, or is an expression of the reaction of plant + bacteria towards an

excessive supply of combined nitrogen in the root surroundings (that is to say, leaving out of account the existence of strains of root-nodule bacteria which are intrinsically virtual parasites) the legume may be said to possess mechanisms which, in the presence of sufficient combined nitrogen, suitably reduce the expenditure of plant materials for the process of symbiotic nitrogen-fixation when nitrogen-fixation is wholly or partly superfluous. Myrica, in association with its nodular actinomycete, does not exhibit any such mechanism; at least none has been detected to detract from a conclusion that the Myrica-actinomycete association is a true symbiosis within the nodules of Myrica gale.

SUMMARY OF SECTION VII B.

1. The effect of combined nitrogen on nodulation of *Myrica gale* is compared with its effect on a typical legume - *Trifolium pratense* - both species being grown in water culture.
2. It is shown that whereas a small quantity of combined nitrogen (17.5 mgms. N/litre) markedly depresses nodulation (both as regards size and number of nodules) in *Trifolium*, combined nitrogen (up to 140 mgms. N/litre) does not affect the number of nodules found in *Myrica*. Furthermore, the nodules of *Myrica* plants receiving nitrogen increase in size much more rapidly than those developing in N-free solution.
3. It is concluded that the effect of combined N on nodulation is fundamentally different in the two plants.
4. It is suggested that the *Myrica* -actinomycete symbiosis is a less highly evolved system than the legume-*Rhizobium* association since in the former there is a superfluous expenditure of raw materials in the formation of nodules in the presence of combined nitrogen.

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