ASPECTS OF SYMBIOTIC FIXATION OF NITROGEN

by

Salma Mian

Summary

The thesis relates to the nitrogen-fixing root nodules inhabited by actinomycete-like organisms which occur on a number of non-leguminous species of angiosperms.

Some parts of the thesis describe comparative studies of the nodules of various examples (mostly foreign) of the above plants which were in culture in the glasshouse of Glasgow University Botany Department. The species available were as follows:-

Myrica gale (Britain), M. faya (Canary Islands), M. cordifolia and M. pilulifera (Africa), M. cerifera, M. pensylvanica and M. carolinensis (all U.S.A.), M. javanica (Indonesia), Alnus glutinosa (Britain), A. viridis and A. incana (Europe), and species of Purshia and Dryas (U.S.A.), Coriaria (Europe), Casuarina (Australia) and Hippophaë (Britain).

One such study concerned the structure of the nodules and the appearance of the endophyte, under the light and electron microscope, in species which previously had received little or no such investigation. Close similarities to the nodules of previously-examined related species were found. A second comparative study involved the determination of the number of chromosomes in the meristematic cells of the nodules of six of the species as compared with the number in the dividing cells of the root tips. In each species the numbers

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were found to be identical, contrasting with the tetraploidy in the nodule cells reported for many leguminous species. In a third such study, confined this time to the genera Myrica and Alnus, the extent to which an endophyte associated with a particular host species is able to symbiose with another host species in the same genus was studied by means of cross-inoculation trials. Among Myrica species little evidence of specialisation was obtained, except that the M.gale endophyte failed to set up satisfactory symbiosis with any other species. It did induce the formation of numerous nodules, but they remained of minute size and failed to fix nitrogen. It is concluded that in any classification of these nodule endophytes, the M.gale endophyte should be separate from the rest. The microscopic examination of the ineffective nodules induced by M.gale showed that they lacked vesicles. In Alnus the endophyte of A.glutinosa was found to symbiose satisfactorily with A.viridis plants.

The first of two more specialised studies related to the time of appearance of nitrogenase activity detected by the acetylene-reduction technique in young alder plants which were just beginning to nodulate, and to its relation to endophyte differentiation. Activity was detected two weeks before evidence of fixation was provided by the greening of the leaves, and was found to be closely related to vesicle development by the endophyte, studied by means of the microscopic examination of squash preparations.

In a further study the effect of the level of carbon dioxide in the rooting medium on growth and nitrogen fixation

in Alnus glutinosa and Myrica gale plants was examined by growing plants in water culture with either air or air with 2% carbon dioxide bubbled constantly through the culture solution. Rather variable results were obtained, but the final conclusion was that the extra carbon dioxide has a harmful effect on nitrogen fixation and plant growth. Acetylene-reduction tests on detached nodules of the two species also indicated an inhibiting effect of excess carbon dioxide on nitrogenase activity, possibly due primarily to a reduction in respiration. The latter effect appears to prevent any enhancement of fixation which might otherwise follow the greater activity of carboxylase enzymes in the 'dark' fixation of carbon dioxide in the presence of an increased level of that gas.

ASPECTS OF SYMBIOTIC FIXATION OF NITROGEN (WITH SPECIAL REFERENCE TO NON-LEGUMINOUS ROOT NODULES)

Thesis Presented By

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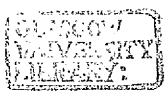

CONTENTS OF THESIS

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The thesis has been prepared substantially in accordance with British Standard 4821: 1972.

PREFACE

If attention is confined to the instances which involve a higher plant, the symbioses with which fixation of nitrogen is definitely associated are as follows:

1. between leguminous plants and the bacterium *Rhizobium*;

2. between non-leguminous angiosperms and actinomycete-like organisms;

3. between species of *Gunnera* and *Nostoc*;

4. between cycads and a blue-green alga variously identified as *Nostoc* or *Anabaena*.

In associations of types 1, 2 and 4 the site of the actual symbiosis is within root nodule structures, though a distinction can be drawn between the intra-cellular situation of the endophyte in the nodules of types 1 and 2, and its inter-cellular situation in type 4. A recently discovered link between types 1 and 2 is provided by the root nodules found by Trinick (1) on a species of *Trema*, a member of the Ulmaceae, and shown by him to be tenanted by a rhizobium.

Excluded from the above list are rhizosphere associations, such as that between the grass *Paspalum notatum* and *Azotobacter paspali* (Dobereiner & Campelo, 2),

where considerable fixation of nitrogen has been shown to occur. It is a matter for argument whether the term symbiosis should be extended to so loose an association. A similar consideration applies to the phyllosphere association, while additionally there is no clear evidence for the occurrence of fixation in that association.

Because of the far greater number of plant species involved, and the width of their distribution, the nitrogen fixation associated with symbioses of types 1 and 2 is obviously, at the present stage of evolution, of far greater importance than in types 3 and 4.

The present thesis is concerned with symbioses of type 2. As compared with the legumes the study of these has been neglected for the valid reason that while the legumes are of great importance agriculturally, the non-legumes bearing root nodules are all of woody nature and are thus unsuited to use in agriculture, and moreover they mostly yield no edible product. However, they have undoubted ecological importance, while also their fixation can be exploited in silviculture, and in any case there is increasing acceptance of the view that a capacity for the fixation of nitrogen is sufficiently rare for every example to merit study. And so in the last 25 years there has been increasing attention paid to non-legume root-nodule plants.

Table 1 lists the plant genera known to include species bearing root nodules inhabited by an actinomycete-like organism, and also shows the distribution of these genera and the number of species so far recorded to bear nodules. In general, all species of these genera that have been examined in the field have proved to be nodulated, though not necessarily under all conditions. The disparity, particularly large in some genera, e.g. Elaeagnus, between the species complement and the number actually recorded to be nodule-bearing, is due to the fact that some species grow only in very remote parts of the world and so far have not been examined for nodulation. It is probable that in due course all species in most of the genera will be found to be nodule-forming. The total number of species indicated in the Table to be nodule-bearing is 159.

Table 2 shows the systematic position of the genera according to one well-known classification. Although some genera are obviously closely-related, others are not, and the overall impression is of heterogeneity, suggesting that these actinomycete-like endophytes have shown a greater faculty for adaptation to very different host plants than have the Rhizobium endophytes.

In order to help in the understanding of the later parts of this thesis, a brief description of these non-legume nodules will now be given. In all examples

Table 1. Genera bearing root nodules inhabited by an actinomycete-like organism.

Genus	Number of species in genus*	Present distribution	Number of species recorded to bear nodules**
Casuarina	45	Australia, tropical Asia, Pacific Islands	24
Myrica	35	Many tropical, sub-tropical and temperate regions	26
Alnus	35	Europe, Siberia, North America, Japan, Andes	32
Dryas	4	Arctic, mountains of north temperate zone	3
Cercocarpus	20	North America	4
Purshia	2	North America	2
Coriaria	15	Mediterranean, Japan, New Zealand, Chile, Mexico	13
Ceanothus	55	North America	31
Discaria	10	South America, New Zealand, Australia	2
Colletia	17	South America	2
Elaeagnus	45	Asia, Europe, North America	16
Hippophaë	3	Asia, Europe	1
Shepherdia	3	North America	2
Arctostaphylos	70	Northwest and Central America, Europe, Asia	1

*According to Willis (3)

**According to Bond (4)

Table 2. Systematic position (according to Engler, 5) of genera listed in Table 1.

Genus	Family	Order
Casuarina	Casuarinaceae	Verticillatae
**Myrica*	Myricaceae	Juglandales
Alnus	Betulaceae	Fagales
Dryas	Rosaceae (tribe Dryadeae)	Rosales
Cercocarpus	Rosaceae (tribe Dryadeae)	Rosales
Purshia	Rosaceae (tribe Dryadeae)	Rosales
Coriaria	Coriariaceae	Sapindales
Ceanothus	Rhamnaceae	Rhamnales
Discaria	Rhamnaceae	Rhamnales
Colletia	Rhamnaceae	Rhamnales
Elaeagnus	Elaeagnaceae	Thymelaeales
Hippophaë	Elaeagnaceae	Thymelaeales
Shepherdia	Elaeagnaceae	Thymelaeales
Arctostaphylos	Ericaceae	Ericales

*Includes *Comptonia*

studied, in response to infection by an appropriate organism the nodules are initiated as simple, swollen structures with a superficial resemblance to legume nodules, but as a result of rapid, repeated branching, corralloid structures conveniently called nodule clusters are soon formed.

Figure 1 shows the nodule clusters in Alnus glutinosa. The clusters, like the plants bearing them, are perennial, and in Alnus and some other genera, over a period of years, from a single infection point, or more commonly by an impaction of the clusters arising from several adjacent infection points, a ball-like nodular mass several cm in diameter often forms.

For the description of the internal structure of the nodules, to begin with Alnus glutinosa will again be used as the type. Figure 2 shows the structure of a lobe of an alder nodule cluster in longsection under the light microscope. Certain resemblances to the structure of a root are obvious. Thus there is an apical meristem behind which, and laterally to it, files of differentiating cells issue. Centrally these form a stele bounded by an endodermis, outside which is a cortex enlarged both owing to hyperplasia and hypertrophy. The endophyte is found in the cortical region, the enlarged infected cells lying among smaller uninfected cells which often contain starch or tannin. Points of differences from a root are that there is no root cap, root hairs are absent, and there is a superficial periderm.

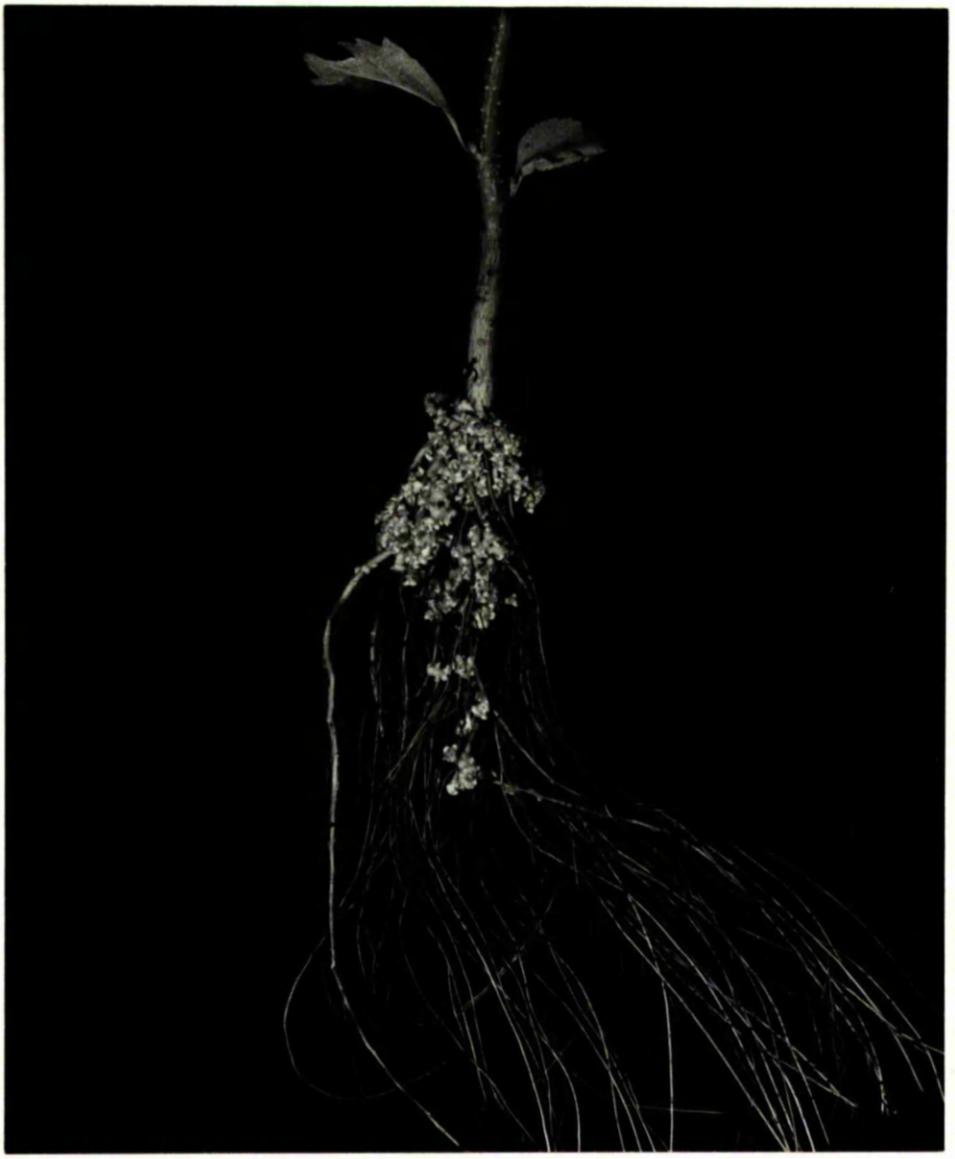

Fig. 1. Part of 4-month-old plant of Alnus glutinosa grown in water culture, showing nodule clusters (x 1).

Photograph kindly supplied by Professor Bond.

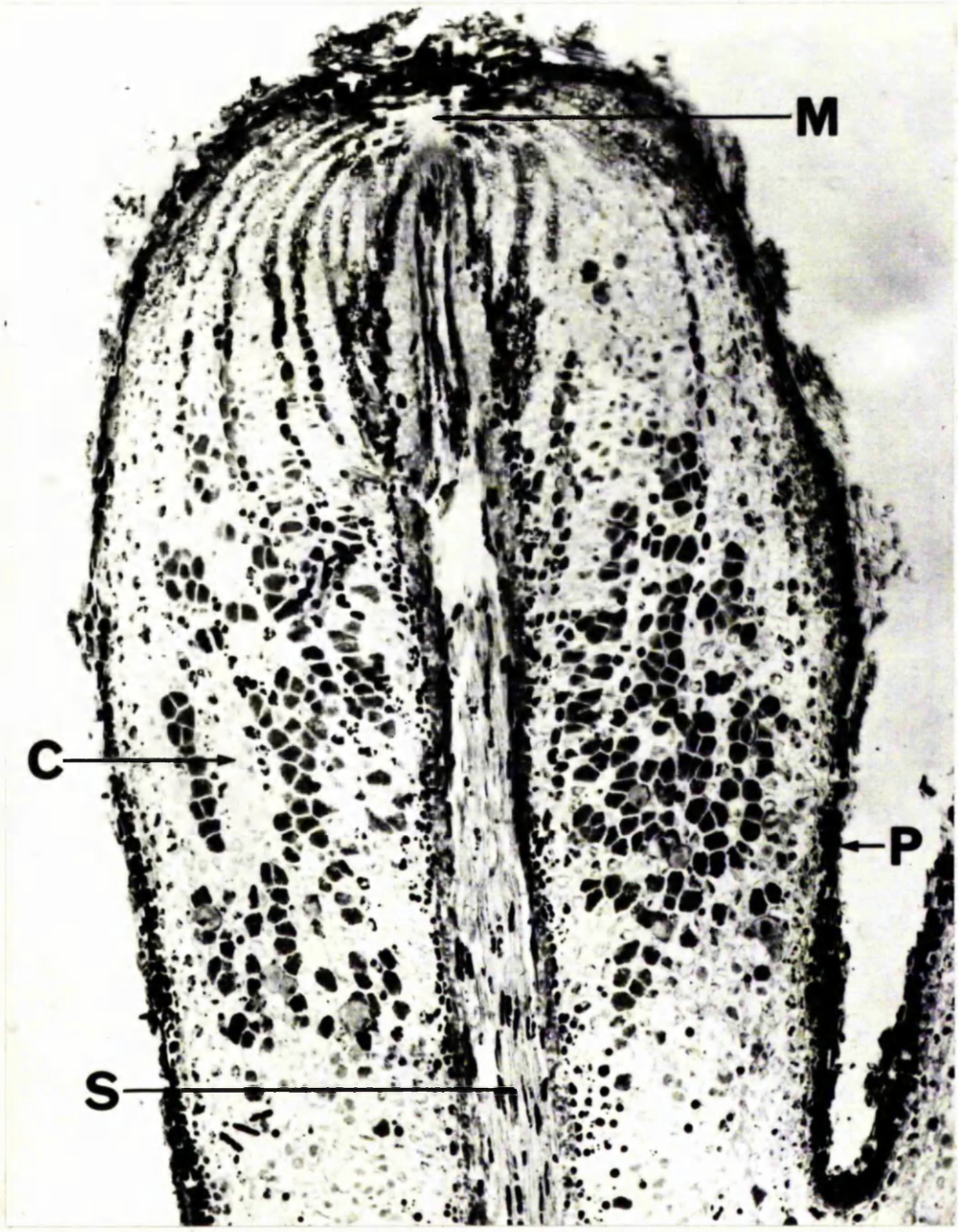

Fig. 2. Radial longsection of nodule lobe of Alnus glutinosa, showing apical meristem (M), central stele (S), periderm (P), and wide cortex (C) with enlarged dark-stained infected cells. The black substance filling cells in various parts of the section is tannin (x 80).

Photograph kindly supplied by Professor Bond.

In some genera, e.g. Myrica (excluding M. gale), the infected cells are confined to a narrow zone in the middle cortex.

The contents of the infected cells in these nodules are very congested and their proper elucidation had to await the arrival of the electron microscope. However, under the light microscope it can be seen that in alder and possibly in all genera - though that has not been properly established - the infected cells are of two types, firstly one in which the endophyte produces numerous so-called vesicles, which usually lie against the cell-wall and in alder and some other genera are spherical in shape, but in other examples (e.g. Myrica) are club-shaped. In the second type of infected cell the endophyte forms very large numbers of minute, polyhedral cells termed bacteroids or granula. These features are shown for alder in Figure 3.

As noted, the use of the electron microscope, for example by Gardner (6), has greatly aided the study of these nodules. It has now been established beyond any doubt that the endophytes are basically finely hyphal in nature, the hyphae being 0.6-1.0 μm in diameter and septate. The vesicles, formed by enlargement of the hyphal tips, are found, when mature, to be usually sub-divided by walls running in various

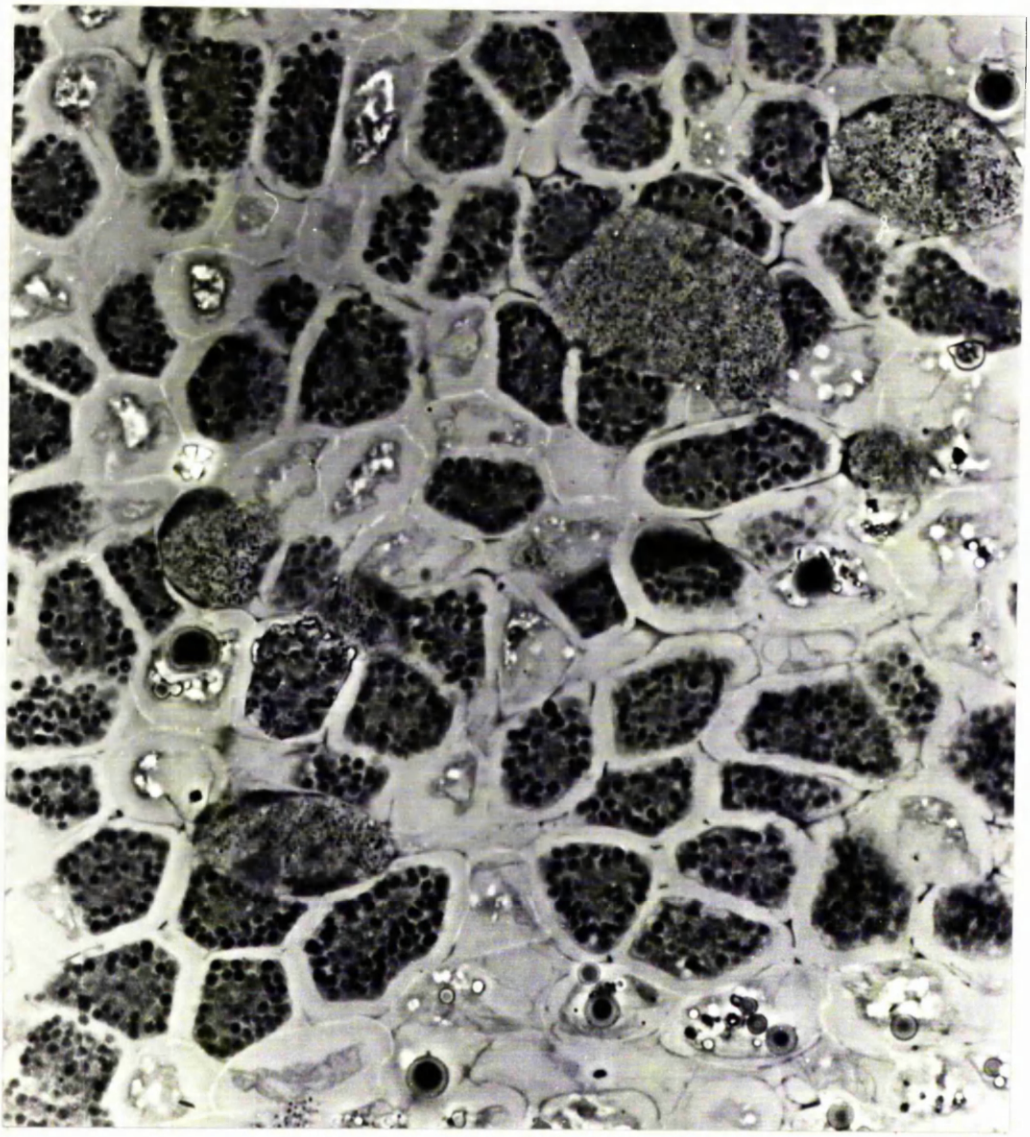

Fig. 3. Area of cortex from transection of nodule of Alnus glutinosa. The infected cells have dark-stained contents. In most of them very dark spherical vesicles are present, but in two relatively large cells in the top right-hand area very numerous, minute bacteroids are present (x 400).

Photograph kindly provided by Dr C.T. Wheeler.

planes*. These features are shown in an electron micrograph (Fig. 4) of an infected cell in Colletia nodule, where the vesicles are spherical as in alder.

When the characteristics of the hyphae seen within these nodules are compared with those of other known organisms, they prove to be most similar to those of actinomycetes, and it is chiefly for this reason that the endophytes are commonly regarded as belonging to that group. The failure so far of the many efforts made to isolate these endophytes into pure culture, denies workers in the field the closer knowledge of the identity of the endophytes that would otherwise be available.

Another characteristic of non-legume nodules is that they are active fixers of elemental nitrogen. By means of growth experiments, ^{15}N and acetylene-reduction tests, the nodules of at least one species (in some cases several) from each of the genera listed in Table 1 except Arctostaphylos have been examined for nitrogen fixation, with positive results. By comparison of glasshouse-grown plants (Bond, 7) showed that fixation per unit nodule weight was as high in non-legumes as in legumes. Measurement of fixation in the field is more

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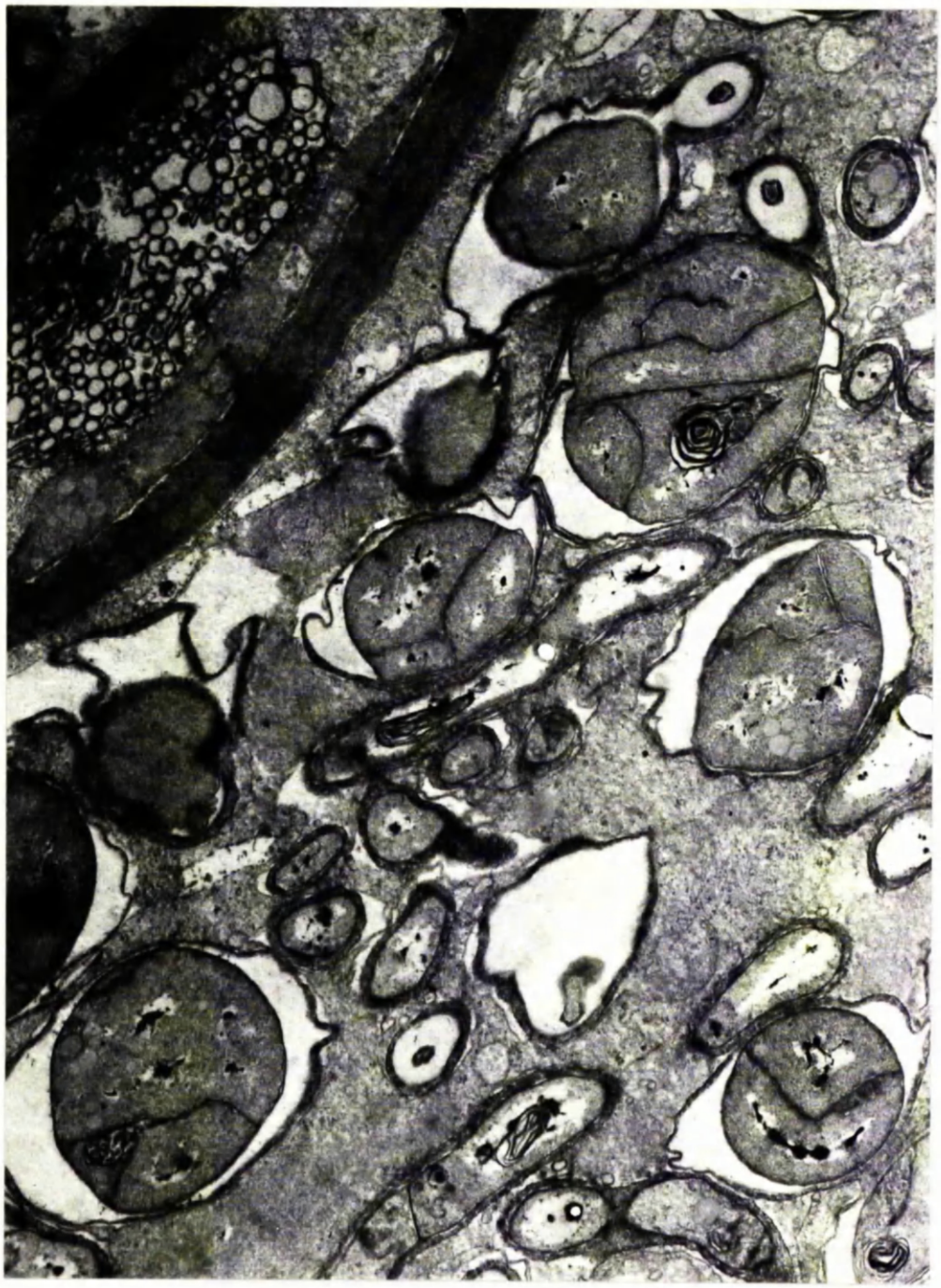
*Although most observers using the light microscope failed to see the sub-divisions within the vesicles, Shibata (9) in a rarely quoted paper saw and illustrated them in Alnus incana vesicles. Schaede (10) also saw indications of sub-divisions in the club-shaped vesicles of Myrica gale.

Fig. 4. Part of an infected cell from a section of a nodule lobe of Colletia paradoxa seen under the electron microscope. Endophytic hyphae cut in various planes are present, also the larger roughly spherical vesicles showing internal septa. The inclusion with coiled structure in the largest vesicle is a plasmalemmasome. Smaller ones are visible in the hyphae (x 17000).

Photograph kindly supplied by Professor Bond.



difficult, but Akkermans (8) after numerous acetylene-reduction assays, concluded that fixation in alder stands in The Netherlands was of the order of 60 kg nitrogen per hectare annually. This is less than half of common estimates for legume crop plants such as clover, but it must be remembered that such crops are grown in a very close stand. Presumably the endophyte is actually responsible for the fixation in non-legume nodules.

Apart from the different nature of the endophyte, the non-legume nodules show other resemblances to those of legumes. Briefly, it may be noted that they both require molybdenum and cobalt for proper functioning and development, while their formation is reduced if substantial amounts of combined nitrogen are available to the host plants. In both types the fixed nitrogen quickly passes within the nodules into the form of amino acids, and it is in such forms that the host plant receives it from the nodules.

When the writer's research programme was being discussed, Professor Bond pointed out that he had a rather unique collection of nodulated species growing in the glasshouse, gathered from many different countries, and that there were opportunities for comparative studies on the material. Three of the Chapters (II, III and IV) of the thesis describe studies of that nature. The remaining Chapters describe more restricted studies

involving only one or two species. The objectives and the purposes of the different Chapters and the relevant literature will be explained and presented in the Introduction to each Chapter.

CHAPTER I

The Onset of Fixation in Alder Plants and its Relation to Differentiation in the Endophyte

CONTENTS

INTRODUCTION

As indicated in the Preface, the endophytes of non-legume nodules show a higher degree of structural differentiation than those of legume nodules. Though essentially hyphal organisms, they also produce vesicles and bacteroids.

The question arises of whether or not the fixation of nitrogen that proceeds within these nodules is contributed to by all of these endophytic structures. The most obvious way of testing this might seem to be to prepare from nodule homogenates, by the use of the centrifuge, separate preparations of these endophytic structures, and then test them for the possession of the nitrogen-fixing property. Akkermans (11) succeeded in obtaining vesicle suspensions from alder nodule homogenates, but failed to detect fixation by them, doubtless because, as found many times in unpublished work by Professor Bond and Dr Wheeler, fixation ceases in non-legume nodules as soon as they are crushed or homogenised. Failing to make any progress by that approach, Akkermans (loc. cit.) immersed alder nodule lobes halved lengthwise in solutions of various tetrazolium salts and subsequently examined sections of the lobes under the microscope. When reducing conditions exist in a cell, dark crystals of formazan are deposited in the cell through an enzymic reduction of the tetrazolium. In Akkermans' tests, such crystals formed in abundance in the vesicles, indicative of the presence of reducing conditions such as are required at a nitrogen-fixing site, but he had difficulty in deciding whether there was any crystal formation

in the very fine hyphae. No reduction was observed in the bacteroids. In any case it can be concluded that their contribution, if any, to nitrogen fixation must be small, since they are formed rather sparsely and, at least in Alnus, are absent from young nodules which are definitely fixing nitrogen.

It seemed that an opportunity for the study of this problem might be provided during the early development of nodulated alder plants. The writer's preliminary observations had shown that in very young nodules only the hyphal form of the endophyte was present. This continued to be true for several days, and only then did vesicle formation start. By sectioning alder roots on which nodule formation was just beginning, Quispel (12) has more recently been able to describe how a hypha - presumably formed by an external spore-like body - penetrates a root hair and continues into the cortex of the root. Then, as also shown earlier by Taubert (13), meristematic divisions commence in the cortex of the mother root in the vicinity of the infection, leading to the formation of a small but visible swelling, which Quispel terms the primary nodule. The nodule proper arises as a result of the initiation of a lateral root primordium in the pericycle in the neighbourhood of the primary nodule. As this grows through the cortex of the mother root, its own cortex becomes infected, and the further development of the structure changes from that of a root to that of a nodule. Quispel states that the endophyte in the primary

nodule remains mostly in the hyphal form for some days. This agrees with the present writer's statement above. Under conditions of water culture, red anthocyanin pigment commonly forms in the primary nodules, with the result that they become readily visible to the naked eye as 'red spots' on the roots. It was thought to be of interest to attempt to find whether there is detectable fixation of nitrogen in primary nodules while their cells contain only the hyphal stage of the endophyte, and whether there is any notable rise in fixation as vesicles develop.

A rough idea of the time of onset of fixation in young alder plants can be gained by the visual inspection and comparison of inoculated and uninoculated plants growing in nitrogen-free culture solution. It usually requires 2-3 weeks from inoculation for nodules to begin to appear, and during that interval a degree of chlorosis appears in the leaves of both types of plants. It is not until at least a further 2 weeks after the first appearance of nodules that the leaves of the inoculated seedlings become visibly greener, showing that fixation has started and its products are reaching the shoots. It is obvious that fixation actually starts some time before these visible signs appear, especially considering that in very young nodules the fixed nitrogen may be wholly or largely retained in the nodules, although in later stages the bulk of the fixed nitrogen is quickly exported to the rest of the plant (Stewart, 14). It would be very difficult to detect by the relatively insensitive Kjeldahl process the

very small increase in the nitrogen content of the plant produced by the earliest fixation. The ^{15}N technique is more sensitive but very time-consuming, and there is no doubt that the acetylene-reduction technique was best-suited to the present purpose, considering its quickness and its great sensitivity - it is commonly held to be 10^3-10^4 times more sensitive than the ^{15}N method, which in turn is held to be 10^3 times more sensitive than the Kjeldahl method (Hardy et al., 15). In addition, the acetylene technique has the very great advantage that the nodules are not destroyed in the completion of the assay - as they are in the other two methods - and are thus available for subsequent cytological study. Thus the planned procedure was to raise a sufficient population of inoculated alder seedlings, and as soon as nodules began to appear to assay samples of the plants daily for nitrogenase activity by the acetylene technique. Subsequently squash preparations of the assayed plants would be examined microscopically and the stage of development of the endophyte noted. The results of the two procedures would be compared. Two such experiments will now be described.

MATERIALS AND METHODS

Plant Culture

In the first experiment, carried out in 1972, the seed of *Alnus glutinosa* (L.) Gaertn. that was used had been obtained from Messrs Vilmorin-Andrieux, Paris; this seed was preferred to locally-collected samples since the individual seeds were on average larger. However, French seed was unavailable for the second experiment, carried out in 1973, and was replaced by Scottish seed which before use was placed on a 2 mm sieve and shaken; only the seed remaining on the sieve was actually used, i.e. the larger individual seeds, this selection being made in order to obtain larger and more uniform seedlings. Seed was sown in trays of Peralite moistened with Crone's culture solution (nitrogen-free formula, see below) at 1/8th normal strength.

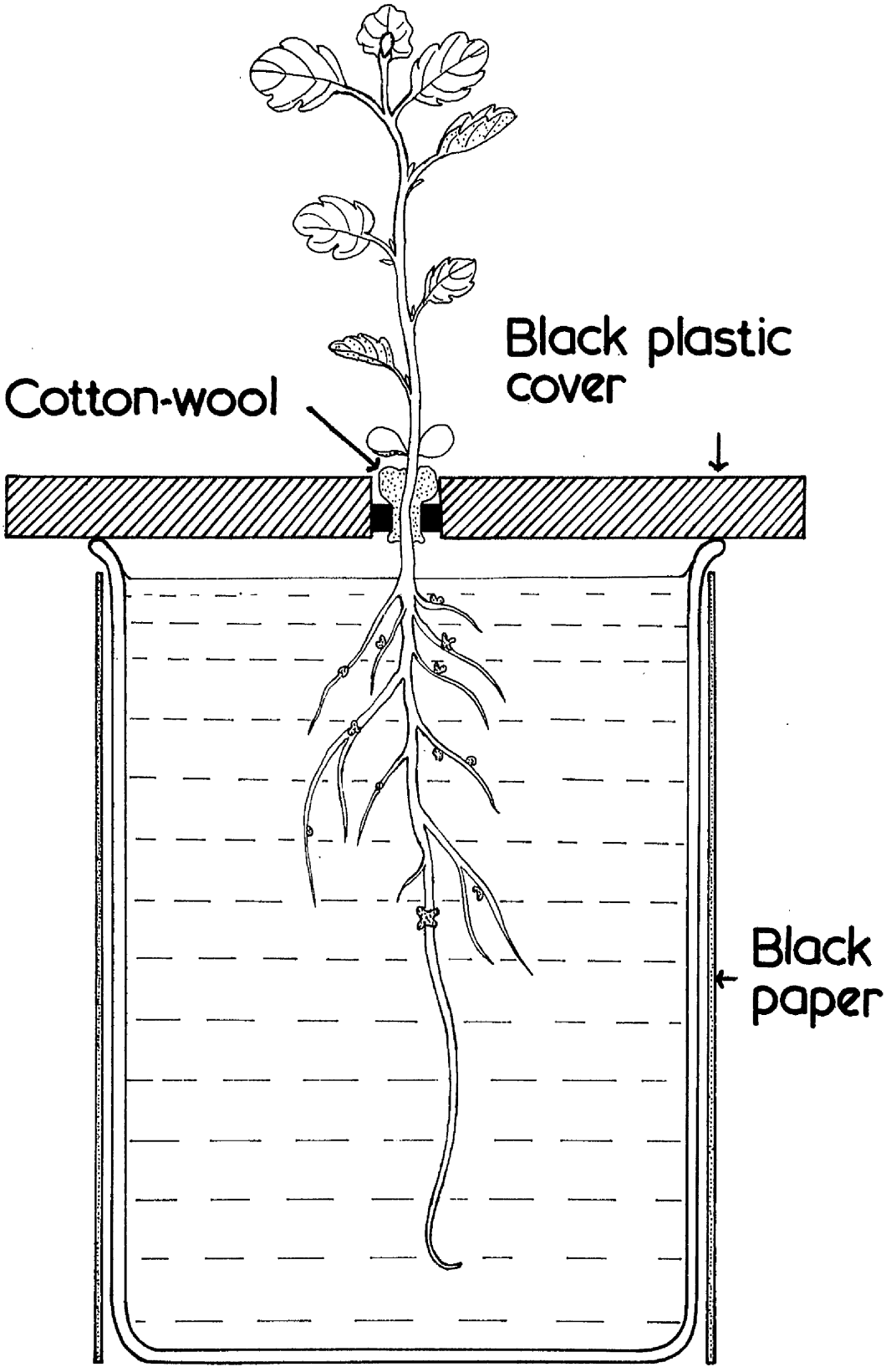
At the two-leaf stage seedlings, selected for uniformity of size, were transferred into water culture in 800 ml beakers (initially heat sterilised) filled with Crone's solution. The recipe for this solution (nitrogen-free version) at full strength is as follows:

KCl	0.75 g	$CaSO_4 \cdot 2H_2O$	0.50 g
$MgSO_4 \cdot 7H_2O$	0.50 g	$Ca_3(PO_4)_2$	0.25 g
$Fe_3(PO_4)_2 \cdot 8H_2O$	0.25 g	Distilled water	1 litre

A small quantity of a concentrate containing all known minor elements (including cobalt) was added to the solution. The

above recipe gives full strength Crone's solution. For the present purpose the solution was prepared at half strength, and the pH was adjusted to 5.8. The beakers were wrapped in black paper and covered by squares of black polyethylene (1 cm thick) bored with 5 holes of diameter 1 cm; the squares were sterilised against the nodule organism by immersion for 10 minutes in methylated spirit followed by copious rinsing in distilled water. A section of rubber pressure tubing of suitable diameter was fixed in each hole, and the root system of a seedling was inserted through the smaller hole thus provided (Fig. 5). This arrangement has been found very advantageous in accommodating seedlings with short hypocotyls, also it allows transfer of seedlings from one beaker to another at quite an advanced stage in growth, and again it makes provision for increase in the thickness of the stem. In order to prevent an extreme nitrogen deficiency arising in the seedlings during the period of nodulation, in 1972 3 mg ammonium sulphate-nitrogen was added per beaker just after transplanting, but in 1973 the addition was limited to 1 mg (supplied in this case 7 days after transplanting) in order to accelerate the development of visual evidence of fixation. Inoculation was effected 2 days after transplanting. An inoculum was prepared by grinding nodules, taken from stock alder plants growing in the greenhouse, in distilled water, and then placing a small quantity of the inoculum on each root system in certain beakers. The seedlings in other beakers were left uninoculated.

Fig. 5. Arrangement for culture of alder plants. Actually there were 5 plants per beaker. The solid black in the central hole in the plastic cover represents the section of rubber pressure tubing (x 1).


Black plastic cover
Cotton-wool
Black paper

In the 1972 experiment transplanting was effected at the end of February, and since the days were still short and natural light intensity low, the natural light was supplemented for 16 hr daily by that from a 500 W compensated mercury vapour lamp suspended 60 cm above the beakers, the light intensity at plant level being approximately 10,000 lux. In the 1973 experiment transplanting was in early May, and in this case the plants received only natural light. In both experiments the position of individual beakers on the greenhouse bench was changed daily in case of inequalities in illumination.

Acetylene Assays

For the examination of nodules of different stages of development for nitrogenase activity, two alternative procedures were considered, as follows. (1) The assays could be made on whole plants taken from the population on suitable, successive occasions. The earliest of such occasions would be that at which nodule development was at the 'red spot' stage. On later occasions each plant would show nodules at varied stages of development. (2) The plants could be allowed to grow until all of them showed nodules at various stages of development. Then, perhaps on a single day, all the nodules would be excised from the plants, either fully or partially (i.e. still attached to 1 cm lengths of root), and samples made up of nodules all of the same approximate sizes. These samples would then be assayed.

As indicated already, procedure (1) was adopted. One reason for this was that complete or partial detachment of such young nodules would almost certainly cause a reduction of nitrogenase activity. Again, to assay so many nodule samples on a single day would have been very arduous. Further, it was of definite interest to find exactly when nitrogenase activity commences in the developing alder plant, and the first procedure is obviously likely to give a more precise answer to this question.

Thus assays were made on whole plants taken from the population at intervals of two days in earlier stages, but at longer intervals at later stages. Figure 6 shows a typical plant as used in the assays. For the assays the plants were enclosed in stout round-bottom glass tubes of length 12 cm and diameter 1.7 cm, of total volume 17.5 ml, and equipped with a side arm bearing a short length of rubber tubing which could be closed by means of a screw clip (Fig. 7). Tubes of this type are used by Dr Wheeler in his assays. In 1972 two nodulated or two non-nodulated plants were placed in each tube, while in 1973 three plants were enclosed together in the earlier assays, reduced to two in the later assays. Since there was considerable plant-to-plant variation within the population in respect of plant size and the rate of nodulation, on each assay occasion a representative sample of plants was selected for assay. The non-nodulated plants were assayed in case the alder plant

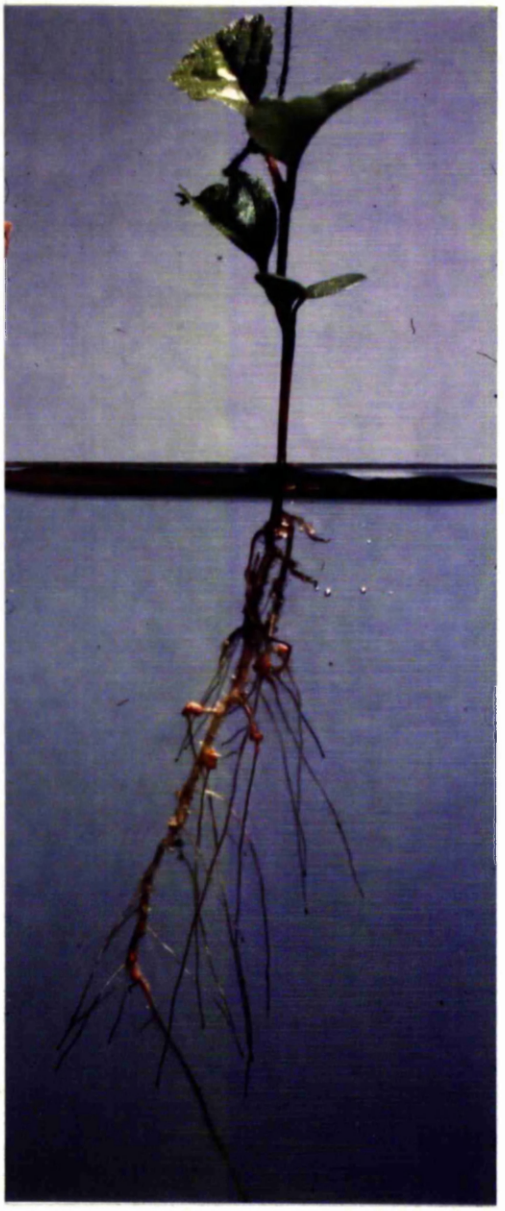


Fig. 6. An alder plant as used in the assays. This particular one is at a fairly advanced stage and has nodules of appreciable size (x 1).

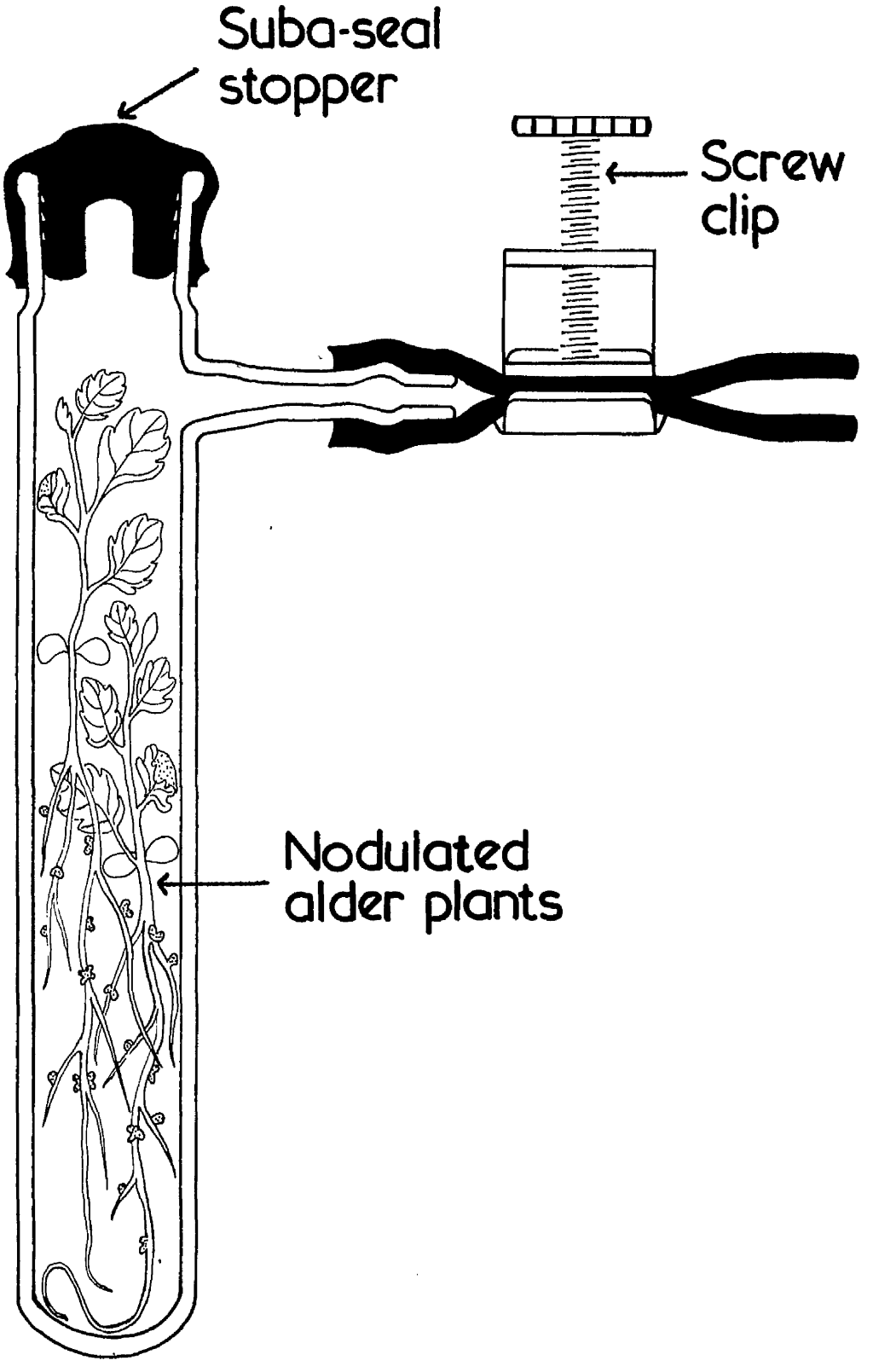


Fig. 7. Method of enclosure of plants for acetylene assays (x 1).

tissues produced ethylene in their normal metabolism. All assays were started at approximately 1400 hr. The root systems of the plants were dried prior to enclosure by pressing them between filter papers in a standard manner. The tubes were then closed by means of 'Suba-seal' stoppers.

The tubes were then attached to a manifold through the side arm, evacuated and subsequently filled to atmospheric pressure with a gas mixture comprising 20% acetylene, 20% oxygen, and 60% argon. The tubes were then sealed, detached from the manifold, and incubated at 25°C - a temperature being known to be near optimal for nitrogenase action in alder nodules - for 1 hr in the 1972 experiment and 2 hr in 1973. Subsequently 2 ml samples of the gaseous contents of the tubes were quickly withdrawn, in each case after filling and emptying the syringe three times following the insertion of the needle through the 'Suba-seal' closure, the object of this procedure being to ensure that the gaseous contents were well mixed up prior to sampling. In 1972 the gas samples were analysed for ethylene on an Aerograph 200 gas chromatograph located in the Chemistry Department, while in 1973 a Pye Unicam Series 104 gas chromatograph Model 4, in the Botany Department was employed. Ethylene contents were obtained by measuring the peak heights on the graphical record provided by the instrument, and then by referring to a calibration curve. In case any ethylene was present as an initial contaminant in the gas mixture, a tube containing the

latter only was included on most assay occasions.

After the plants were withdrawn from the assay tubes a note was taken of the height of the shoot, number of leaves in both the nodulated and the non-nodulated plants. The number of nodules on the nodulated plants was ascertained.

Measurement of Nodule Development

In addition to determining nitrogenase activity on a per plant basis, it was desirable that activity could also be expressed per unit of nodule development. Since the nodules were required for squash preparation it was not possible to determine their dry weight, and the only attribute that could be measured was nodule volume. In 1972 the diameter of each nodule was measured to the nearest 0.5 mm and the volume calculated on the assumption that the nodules were spherical. In 1973, a specific gravity bottle (Fig. 8) was used very successfully to determine the total volume of the nodules (detached) from the plants within each assay tube. No volume measurements were made for nodules in the 'red spot' stage in either year. The procedure was to fill the bottle exactly to the top of the capillary in the stopper with distilled water at room temperature, and then, after carefully drying the exterior of the bottle, to ascertain its weight. Then the nodules, which in all cases had been equilibrated for 30 min in a small petri dish containing a filter paper moistened with a standard amount of water, were weighed and introduced into the specific gravity bottle and the

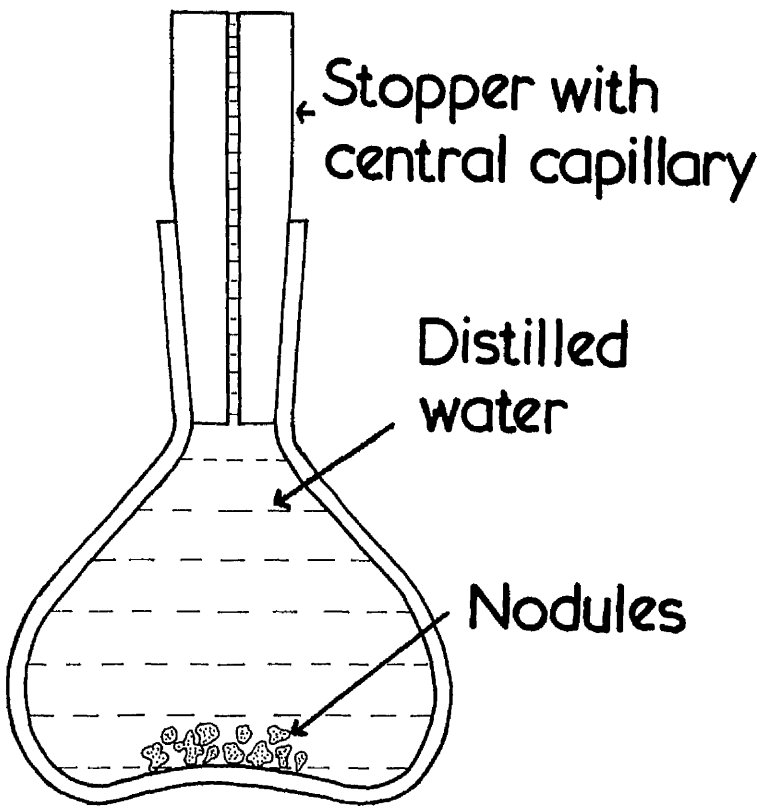


Fig. 8. A specific gravity bottle as used for determination of nodule volume (x $1\frac{1}{2}$).

stopper replaced. The water displaced through the stopper was carefully removed, again leaving the capillary quite filled with water. The bottle was again weighed, and by subtracting the weight of the nodules from that weight, the weight of the bottle plus the residual water was obtained. By subtracting this from the original weight (bottle filled with water), the weight of water displaced by the nodules was found; assuming the specific gravity of water to be exactly unity, the weight of water displaced is equal to the volume of the nodules.

Preliminary tests were made to test the accuracy of the above method. The volume of four glass beads calculated from their weight and a density of 2.8 was 0.205 ml, while by the bottle method the volume was estimated to be 0.201 ml. More convincing (because of greater certainty of the density) was a test with a small quantity of mercury - its volume by calculation (knowing weight and density) was 1.198 ml, and by the specific gravity method 1.192 ml.

Microscopic Study of Nodules

After the volume determination the nodules were fixed in formalin-acetic alcohol, and later squashed individually on slides, stained with cotton blue, and examined under the light microscope after mounting in lactophenol. The stage of development of the endophyte was the particular matter of interest. Photomicrographs were taken under a Zeiss Standard WL microscope equipped with a 24X 36 mm camera.

DATA OBTAINED

1972 Experiment

In Table 3 are presented data for nitrogenase activity in the nodules on successive occasions, together with numbers and details of nodule size. As already mentioned, two plants were placed in each assay tube in order to provide a larger amount of nodule tissue. Since it was obvious that in the inoculated population of plants there was considerable variation in respect of plant size and nodule development (possibly due to difference in size between the original seed), in preparing the assay tubes a relatively small plant was paired with a relatively large one, again with the object of obtaining a detectable amount of ethylene. Actually the assays were started later than intended, for, as the Table shows, an appreciable number of nodules was present on the first occasion of assay; this was mostly due to the situation that some days' notice had to be given in order to have the use of the Chemistry Department gas chromatograph.

As indicated in Table 3, ethylene production attributable to the nodules was obtained by subtracting that found in the tubes containing non-nodulated plants from that found in the tubes with nodulated plants. The ethylene contents found in tubes containing plants of the first type (one tube on each occasion) were 4.94, 4.28, 6.24 and 6.53 respectively. Analysis of the original acetylene used in the assays showed that of the above quantities, 4.14 mμmole per tube was due to contaminant

	1	0.44		13	2	1	10	0	0	1.29	
	2	0.90		13	0	0	13	0	0	2.49	
18	3	0.90	Mean 1.07	9	0	0	7	2	0	2.35	Mean 2.77
	4	0.44		11	3	1	7	0	0	1.44	
	5	2.68		15	0	1	7	7	0	6.29	
	1	0.67		19	2	0	14	3	0	6.21	
	2	0.44		16	2	2	12	0	0	1.91	
22	3	3.82	Mean 3.77	22	1	3	12	6	0	5.49	Mean 5.17
	4	13.72		15	2	0	6	5	2	8.27	
	5	0.22		22	3	3	16	0	0	3.97	
	1	14.90		29	0	2	21	5	1	9.92	
	2	0.72		28	11	3	13	1	0	2.68	
25	3	1.40	Mean 5.48	14	0	0	10	3	1	7.89	Mean 7.89
	4	8.60		30	4	0	19	6	1	10.08	
	5	1.80		26	1	3	16	6	0	8.89	
	1	25.00		47	3	2	34	8	0	13.03	
	2	51.52		37	4	0	22	9	2	17.03	
27	3	72.22	Mean 30.14	49	1	2	36	6	4	22.54	Mean 13.88
	4	1.47		32	3	2	22	5	0	8.27	
	5	0.50		54	21	13	15	5	0	8.52	

*Each tube contained two plants.

†Values obtained by subtracting ethylene production in tubes containing non-nodulated plants from that in tubes with nodulated plants.

ethylene; subtraction of this leaves small balances of ethylene, namely 0.80, 0.14, 2.10 and 2.39 mμmole respectively. This apparently indicates an appreciable production of ethylene in the normal metabolism of the organs of alder plants other than the nodules. However, as will be reported later, the corresponding figures obtained in the 1973 experiment were negligibly small, and it seems probable that the 1972 figures in question arose from errors in assay. For this reason the overall results of the 1972 experiment should be regarded as of preliminary nature.

The observations (Table 3) made at 18 days after inoculation show that in the first four tubes there was apparently a production of ethylene by the nodules, but the amounts are very small and are of doubtful significance for the above reasons. Only in the fifth tube was there clear nitrogenase activity. Scrutiny of the data for nodule size suggests that the presence of nodules of diameter 1 mm or more is necessary before there is definite activity.

In the 22nd day assays there was undoubted nitrogenase activity in two tubes, and here again nodules of diameter 1-1.4 mm were present, while still larger nodules were also present in tube 4 and obviously contributed substantially to the activity. Rather surprisingly the presence of nodules in the 1-1.4 mm range in tube 1 was not productive of a clear positive result.

On the 25th day the plants in four tubes possessed nodules in the larger categories, and showed definite

activity. In the single tube which failed to show a clearly positive result only one nodule in the 1-1.4 mm range was present. At 27 days four tubes gave clearly positive results, with massive production of ethylene in three cases, again accompanied by the presence of many relatively large nodules. Even at this late stage the nodules of the plants in tube 5 failed to show clear activity despite the presence of some nodules in the 1-1.4 mm range; however it will be noted that an unusually high number of very small nodules was also present on the plants in that tube, and it is possible that this constituted a drain on the plant and thus slowed up the activity of the larger nodules.

Thus in this experiment it was found that nitrogenase activity was not clearly detectable until some days after the first appearance of nodules, and that the nodules need to attain a diameter of the order of 1 mm or more before they show definite activity. As shown in Table 3 mean activity of the nodules increases successively with time, especially between the 25th and 27th days, and although these means are based on very variable material, analysis of variance shows that the differences between them are significant at $\underline{P} = 0.05$.

From the data given in Table 3, mμmole ethylene produced per cu.mm of nodule tissue per hour can be calculated. The mean values obtained for the four successive assay occasions were 0.36, 0.49, 0.60 and 1.68. Comments on these figures will be offered later.

Table 4 shows growth data for the plants used in the above assays. A gradual increase in shoot height and number of leaves over the period of the experiment is shown for both nodulated and non-nodulated plants, and a rapid increase in the number of nodules in the inoculated plants. The data also show that by the end of the experimental period there was still no significant mean difference between the two types of plants in respect of shoot height and leaf number. Neither was there any difference to the eye in respect of leaf colour, although as the data in Table 3 indicate, nitrogenase activity had been detectable for some 9 days.

A squash preparation was made from each of the total of 500 nodules present on the assayed plants. The endophyte was found to be entirely in the hyphal form in nodules at the 'red spot' stage. Vesicles, though very sparse in number, were observed in nodules which had emerged slightly from the root. It follows from this that some vesicles were already present in the larger nodules on the plants assayed at 18 days from inoculation (Table 3). As the size of the nodules increased, the number of vesicles also rose, and soon they masked the hyphae completely in the older infected cells. Photographs will be provided later.

1973 Experiment

As noted, the plants utilised here were grown by natural light over the period May to June. Acetylene assays were started as soon as the first signs of nodulation were

Table 4. Mean height, number of leaves and number of nodules for the alder plants used in the acetylene assays in the 1972 experiment. Means are per plant and are based on the 10 nodulated plants and 2 non-nodulated plants used on each occasion.

	Sampling occasion (days from inoculation)	Height of shoot, cm, also range of values on each occasion	Number of leaves	Number of nodules, also range of values on each occasion
Nodulated plants	18	2.2(1.5-3.0)	3.8	6.1(2-10)
	22	2.6(1.6-3.9)	4.3	9.5(5-11)
	25	2.8(2.1-3.7)	4.8	12.6(5-18)
	27	3.2(2.2-4.7)	5.1	21.9(8-23)
Non-nodulated plants	18	2.0(1.4-2.6)	4.0	0
	22	-	-	0
	25	2.8(2.6-3.1)	5.5	0
	27	3.2(2.8-3.6)	5.0	0

detected, and were carried out more frequently than in the previous experiment. Despite the degree of selection for seed size, there was still considerable variation in plant stature and rapidity of nodulation. Ethylene production by the nodules was again obtained by subtracting the ethylene content of tubes containing non-nodulated plants from that of tubes containing nodulated plants. In this experiment there was little evidence of any production of ethylene by non-nodulated plants, since on seven assay occasions the ethylene content of tubes in which such plants had been incubated was exactly the same as that in tubes without any plants, i.e. there had been no increase over the ethylene present at the beginning as a contaminant in the acetylene. On the two remaining assay dates the apparent production of ethylene by the non-nodulated plants was 0.06 and 0.11 mμmole respectively, which are quite negligible amounts.

The assay results are presented in Table 5. Because of the greater frequency of assays, only the mean production of ethylene for the five tubes set up on each assay occasion are shown, but an indication of the maximum variation between replicates is included. It will be seen in the Table that there was no detectable nitrogenase activity at the 9-day stage, and that the same applies to the assays on the 12th day, when again only 'red spots' were present. On the 14th day some slightly emerged nodules were present, although in this experiment the diameters of the nodules were not measured, so that no

Table 5. Nitrogenase activity in the nodules of alder plants of the 1973 experiment on successive sampling occasions, and details of nodule volumes.*

Sampling occasion (days from inoculation)	Mean mμmole ethylene produced by the nodules of two* plants per hr, also range of values on each occasion‡	Mean number of nodules per two plants	Mean total volume of nodules of two plants, cu.mm
9	0	4.8	**
12	0	14.0	**
14	0.08(0.03-0.21)	15.6	0.5
16	0.20(0.09-0.40)	18.2	1.4
19	1.08(0.29-2.20)	21.6	2.4
22	1.60(0.17-4.53)	20.0	3.0
27	2.02(0.52-5.17)	21.0	7.2
34	25.77(3.55-55.43)	25.6	14.8
42	49.64(8.73-104.9)	27.0	36.6

*The data are based on the examination of 10 plants, grouped in pairs in five tubes, on each occasion, except that on the first two occasions there were 3 plants in each tube. In the third column the numbers shown for the first two occasions are adjusted to two plants.

‡Values obtained by subtracting ethylene production in tubes containing non-nodulated plants from that in tubes with nodulated plants.

**The nodules were too little emerged for detachment to be possible.

indication can be given in the Table of spread of nodule size on the plants used. On this 14th day there is a suggestion of a trifling nitrogenase activity, but even in the most active tube ethylene production was only 0.21 mμmole. On the 16th day total volume of nodules per tube was nearly three times greater than on the previous occasion, but nitrogenase activity was still small, attaining only 0.4 mμmole in the most active tube. From the 19th day onwards there is clear evidence of activity, with a very rapid increase between the 27th and 34th days.

Mμmoles ethylene produced per cu.mm of nodule tissue per hr was also calculated for successive assay dates in this experiment. The values obtained for the seven assay occasions are as follows: 0.16, 0.14, 0.42, 0.51, 0.26, 1.55 and 1.27. These are of the same order as the values previously reported for the 1972 experiment, and will be discussed with the latter in the next section.

Table 6 shows growth data for the plants used in the above assays. It will be observed that clear differences in shoot height and leaf number between the two sets of plants only became obvious on the 34th day after inoculation. At that same time the leaves of the nodulated plants became greener. Thus undoubted nitrogenase activity was detected 15 days before visible benefit to the plant from the nodules arose. It is of interest to compare the data in Table 6 with those in Table 4. Comparison of growth data after particular intervals after inoculation shows that the 1972 plants tended to be taller, to have

Table 6. Mean height, number of leaves and number of nodules for the alder plants used in the acetylene assays in the 1973 experiment. Means are per plant and are based on the nodulated plants and non-nodulated plants used on each occasion.*

	Sampling occasion (days from inoculation)	Height of shoot, cm, also range of values on each occasion	Number of leaves	Number of nodules, also range of values on each occasion
Nodulated plants	9	0.13(0.1-0.2)	1.0	2.4(0-12)
	12	0.16(0.1-0.3)	1.5	7.0(3-15)
	14	0.30(0.2-0.6)	2.0	7.8(4-18)
	16	0.50(0.2-0.7)	2.0	9.1(7-12)
	19	0.90(0.6-1.4)	2.5	10.8(8-15)
	22	1.10(0.6-1.4)	3.0	10.0(6-13)
	27	2.10(1.1-2.9)	4.0	10.5(7-13)
	34	3.10(2.3-4.3)	5.0	12.8(8-18)
	42	3.55(3.0-3.9)	6.0	13.5(10-17)
Non-nodulated plants	9	0.10(0.1-0.1)	1.0	0
	12	0.15(0.1-0.2)	1.3	0
	14	0.20(0.2-0.2)	1.5	0
	16	0.25(0.2-0.3)	2.0	0
	19	0.60(0.5-0.7)	2.5	0
	22	1.10(0.5-1.7)	2.5	0
	27	2.05(2.0-2.1)	3.5	0
	34	2.60(2.4-2.8)	4.5	0
	42	2.70(2.2-3.2)	4.0	0

*Ten nodulated plants and 2 non-nodulated plants were used on each occasion, except that on the first two occasions there were 15 nodulated plants and 6 non-nodulated ones.

more leaves and, in the case of inoculated plants, more nodules, while reference to other tables shows that the nodules were also on the whole larger. The light intensity in the 1972 experiment was probably more consistently favourable than in 1973, but most of the growth differences can be attributed to the circumstance that, as noted earlier, a single addition of 3 mg combined nitrogen per beaker was made in 1972, reduced to 1 mg in 1973. The larger addition in the first experiment explains the stronger plant growth, also the more numerous and larger nodules, but at the same time it tends to delay the appearance of visual evidence of fixation in the nodulated plants, this being the reason for the reduction in the amount of combined nitrogen added in the second experiment.

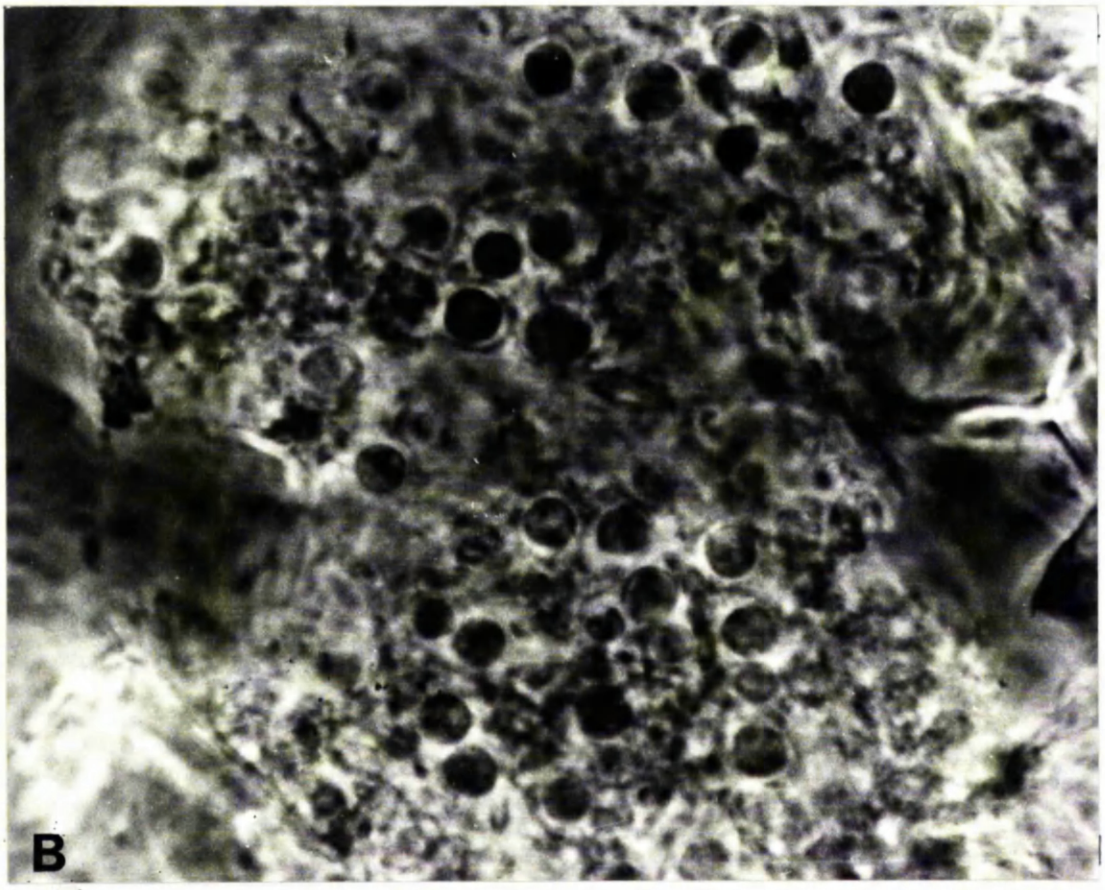
Again in the 1973 experiment, a squash preparation was made from every nodule present on the assayed plants. On the first two assay dates, though masses of hyphae were present within the nodules, no vesicles were found. On the 14th and 16th days all pairs of plants showed sparse vesicles, but in the largest nodules only; the highest number of such nodules was present in the assay tubes which, in Table 5 produced ethylene in amounts of 0.21 mμmole (14th day) and 0.40 mμmole (16th day) respectively. On the remaining assay dates the larger nodules on all plants contained abundant vesicles. Figures 9 (A and B) show squash preparations from two nodules, one in which the endophyte is still in the hyphal stage in the infected

Figs 9 (A & B). Photomicrographs of squash preparations of alder nodules (x 1800).

(A) A very young nodule in which the endophyte is only in the hyphal stage. In the centre are seen the contents of one infected host cell. The spherical structure towards the top of the cell is the host nucleus.

(B) A preparation from a somewhat older nodule in which numerous spherical endophytic vesicles are present together with hyphae.


A


B

cells, while in the other numerous vesicles are also present. It may be noted that the hyphae of an alder endophyte are 0.7-1.0 μm in diameter and the vesicles 4-5 μm in diameter.

DISCUSSION

As stated, the main object of this study was to seek evidence on whether the fixation of nitrogen is associated with any particular morphological part of the alder nodule endophyte, or more specifically whether it is shown by both hyphae and vesicles, or only by the latter.

The data for the 1973 experiment (Table 5) show that on the first two assay dates, when the nodules - all containing hyphae only - were present in each assay tube, no evidence of nitrogenase activity could be detected by the very sensitive acetylene method. Only on later assay dates when vesicles had appeared was nitrogenase activity present. It has already been pointed out in the previous section that on the 14th and 16th days after inoculation only the assay tubes containing a relatively high number of vesicles were observed to produce appreciable quantities of ethylene. This further strengthens the conclusion that it is the vesicles and not the hyphae that possess nitrogenase. Thus the conclusion previously drawn by Akkermans (11- also see Introduction) on the basis of tetrazolium tests that the vesicles are active in fixation is now confirmed by a more direct method. Further, the first positive evidence that the hyphae are not nitrogen-fixing is now presented.

The present state of knowledge only allows us to suggest that it is some feature of the complex structure of the vesicles that makes them suited to the role of nitrogen fixation. It may be noted that in legume

nodules also fixation occurs in enlarged forms - bacteroids - of the entering endophyte.

A smaller point of interest mentioned in the Introduction was the matter of by how many days the appearance of nitrogenase activity in the nodules precedes visual evidence of benefit from the fixation by a nodule-bearing plant growing in a rooting medium almost free of combined nitrogen. For the 1972 experiment it was concluded that the interval was more than nine days, while in 1973 a more precise estimate of 15 days was possible. In explaining this situation, it may be noted that initially the amount of fixation will be extremely small, and that for some days most of the fixed nitrogen may be utilised within the nodules themselves. Only when fixation becomes more extensive may the rest of the plant receive part of the products.

Calculated data indicating nitrogenase activity per unit volume of nodule tissue at different stages in the growth of nodulated alder plants were reported for both experiments in the preceding section. To facilitate further consideration and comparison they are reproduced in Table 7. The values for 1972 tend to rise during the period of observation, especially at the end. There was much variation from plant to plant in the values obtained on a given assay day, but 't' tests show that the increase in activity between the 18th and 27th days is very close to significance at $P = 0.05$. The values for the 1973 experiment suggest a ten-fold increase over the period of observation. Since the nodules from all plants on each

Table 7. Nitrogenase activity per unit volume of nodule tissue during early stages in development of nodulated alders.

Days from inoculation	Mμmole C_2H_4 produced per cu.mm nodule tissue per hr	
	1972	1973
14		0.16
15		
16		0.14
17		
18	0.36	
19		0.42
20		
21		
22	0.49	0.51
23		
24		
25	0.60	
26		
27	1.68	0.26
34		1.55
42		1.27

of the first four assay occasions were bulked prior to volume measurement, no statistical treatment is possible there, but 't' tests made on the data for the last three assay days show that the 34th and 42nd day figures were significantly greater than the 27th day figure. It is reasonable to believe that over the whole period of this experiment, nodule activity per unit volume was really increasing.

One obvious reason for the increasing nitrogenase activity per unit volume of nodule tissue is the more extensive formation of vesicles in the nodules as they develop. Another factor is probably the supply of photosynthates to the nodules from the leaves; as soon as fixed nitrogen begins to reach the leaves their growth, hitherto arrested by the lack of nitrogen, is resumed and must result in increased flow of photosynthates to the nodules.

A graphical treatment of the data obtained in these experiments reveals further points of interest. Figure 10 shows that when nitrogenase activity, as measured by ethylene production by two plants, is plotted against the time lapse after inoculation, curves of exponential type are obtained, as was confirmed by regression analysis. Thus over the period of the observations, the rate of increase in nitrogenase activity becomes greater with the passage of time.

In Figure 11 ethylene production per plant is plotted against nodule volume. The result shows that the

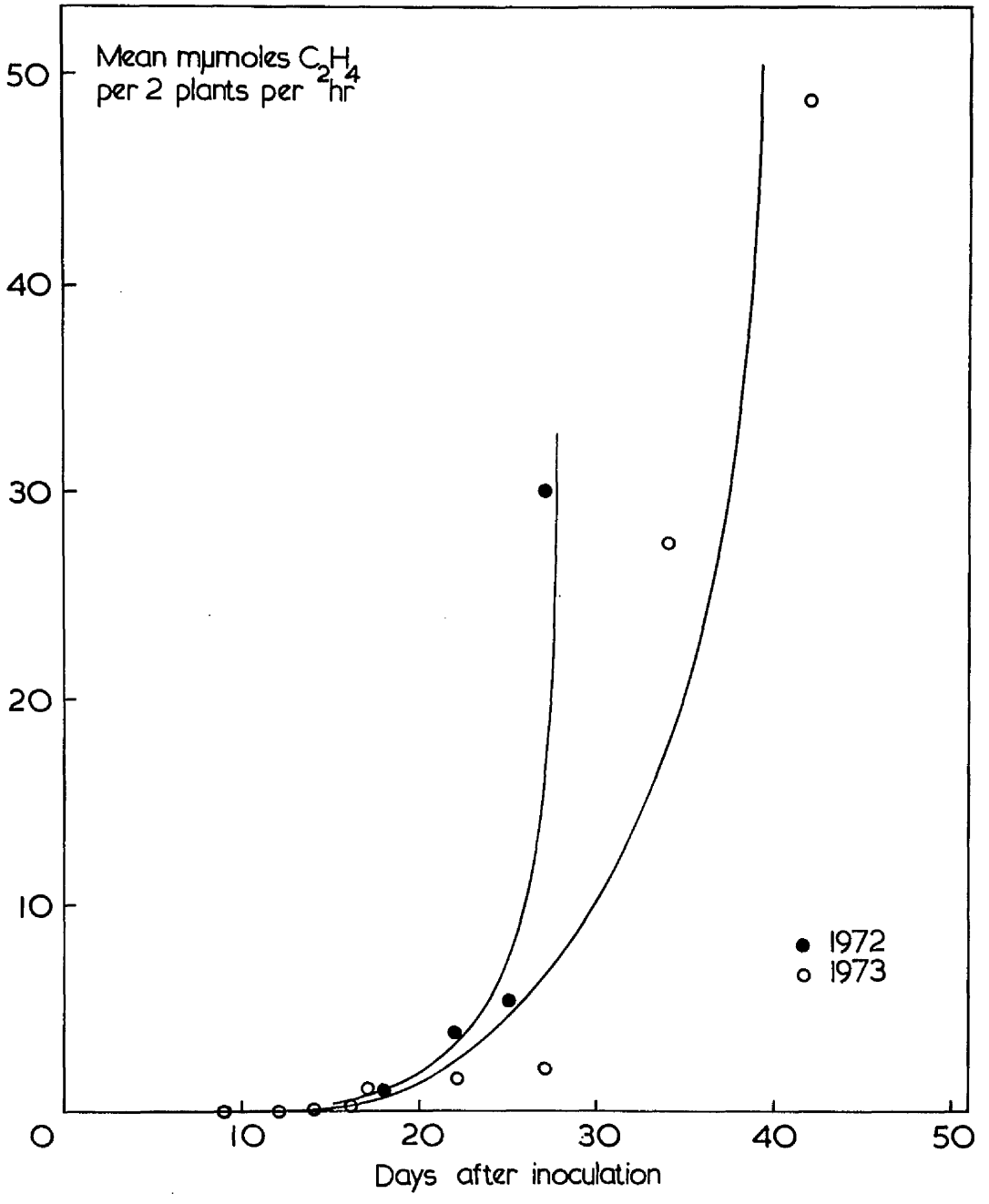


Fig. 10. Graph showing ethylene production by alder plants on successive occasions during nodule development.

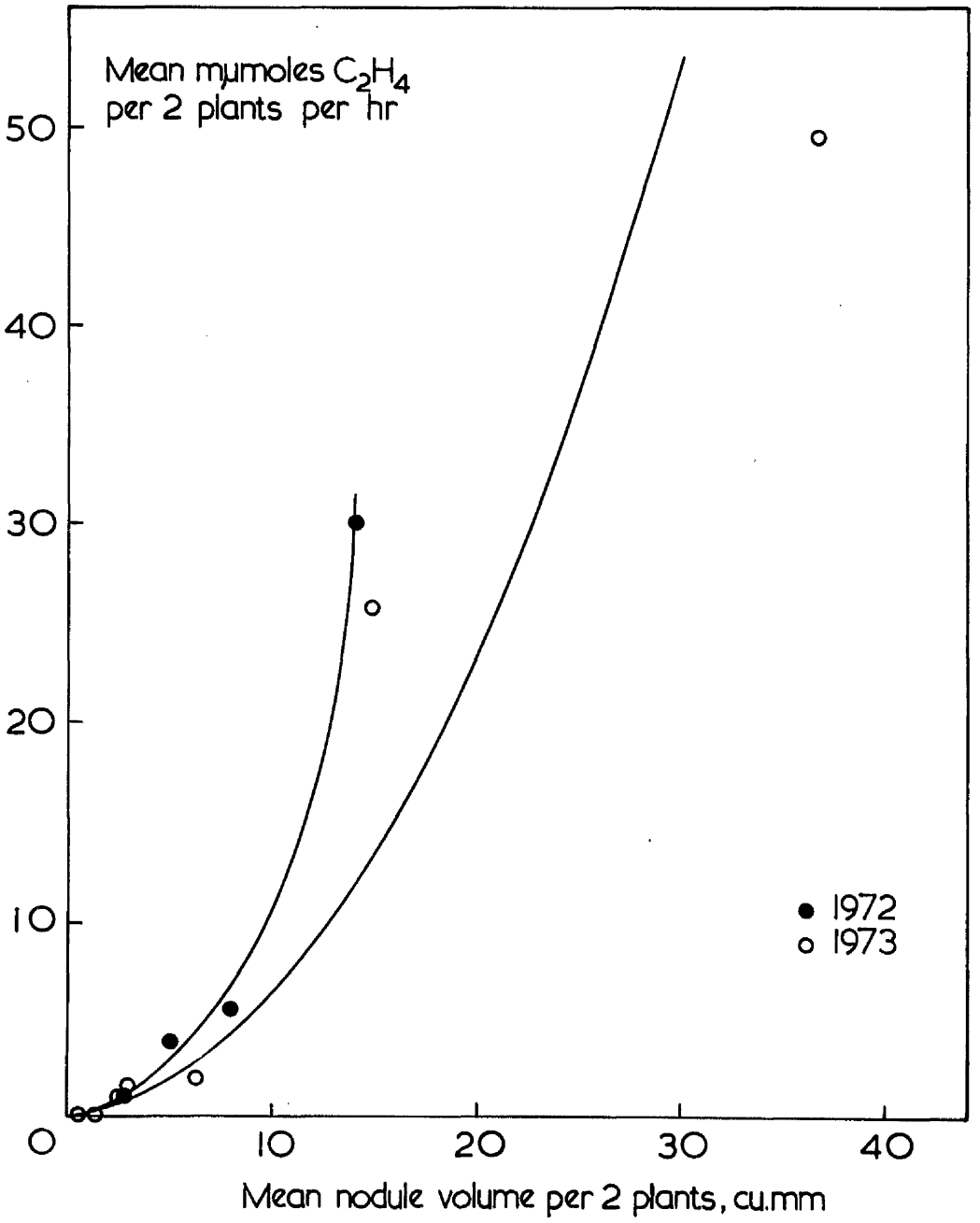


Fig. 11. Graph showing relationship between ethylene production and nodule volume in alder plants.

relationship is not of a straight line type, but is quadratic. This was shown by non-linear regression analysis, in which a power coefficient of 2 was indicated. In keeping with that, when the square root of ethylene production per plant is plotted against nodule volume (Fig. 12), a linear relation is indicated for both experiments.

The previous observations of Stewart (14) in this department show that the rising activity per unit of nodule tissue indicated by the present writer's data is only a temporary phenomenon. His data, based on Kjeldahl analyses, indicated that the nodules of alder plants growing by daylight in a greenhouse fixed a mean of 16.3 mg nitrogen per day per g nodule dry matter between the 42nd and 54th days after inoculation. Over the next ten weeks the value declined slowly, due, Stewart suggested, to the nodules becoming increasingly corky and lignified. Subsequently, in what was now September the value fell sharply to 1.2 mg nitrogen per day per g nodule dry matter. This was attributed to a shortening of days and falling light intensity by Stewart.

In these experiments it has been assumed that the rate of acetylene reduction by a given sample of nodules provides a measure of the rate of nitrogen fixation that the same sample would show under normal conditions. Some check on this is possible from the data of Stewart (14). As noted above he estimated that between the 42nd and 54th days after inoculation, alder nodules fixed a mean of 16.3 mg nitrogen per day per g dry weight of nodule tissue.

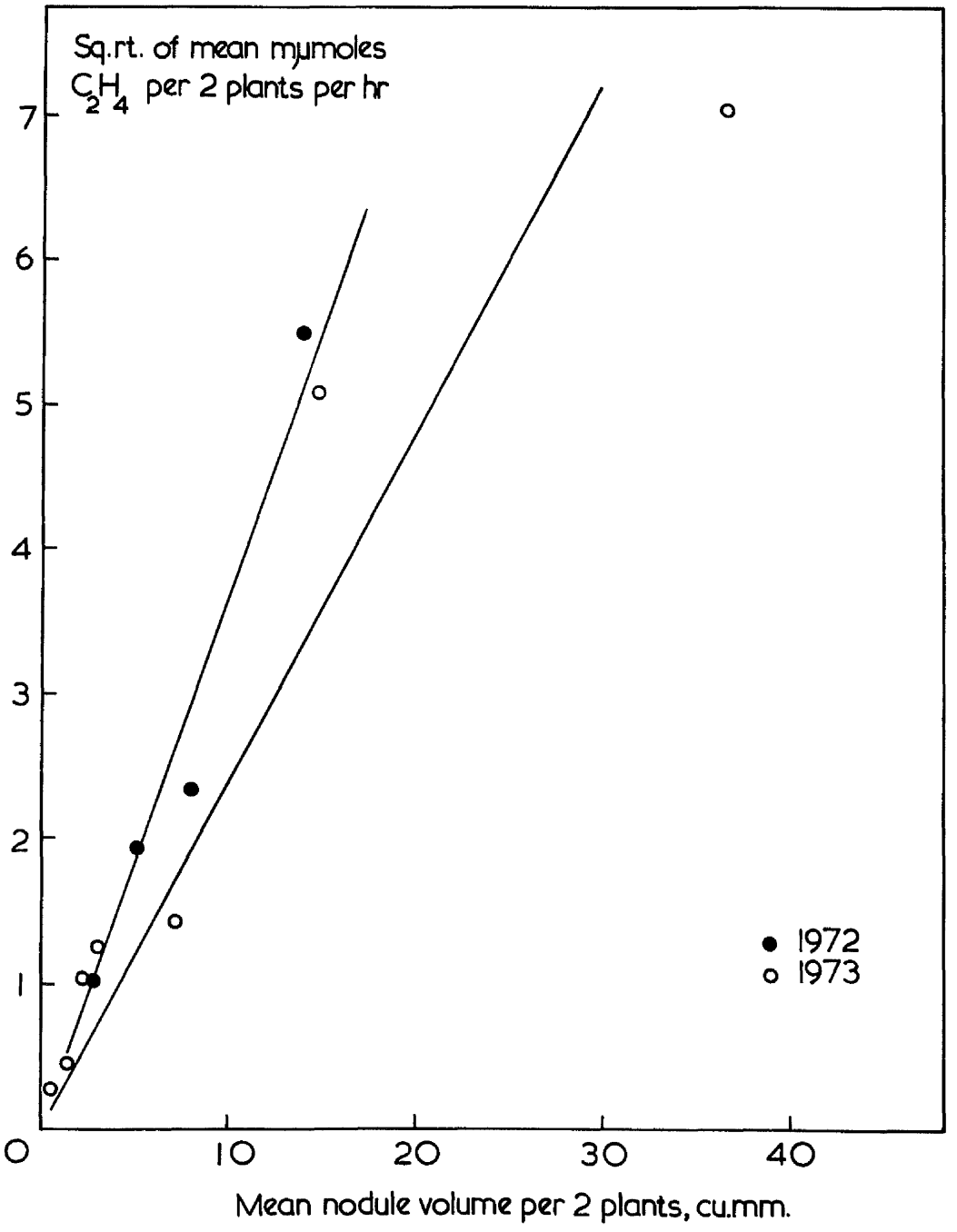


Fig. 12. Graph showing linear relationship between the square root of ethylene produced and nodule volume per plant.

When the ethylene production indicated for the 42nd day in Table 7 above is converted into term of nitrogen fixed, assuming a 3:1 ratio*, a value of 1.7 mg nitrogen per day per g nodule dry matter is indicated. Growth conditions were probably superior in Stewart's experiment, also there is a strong probability that the activity of the nodules at the beginning of his 12-day period was much below the quoted mean. Taking these points into account it is considered that the comparison with Stewart's data supports the belief that the acetylene assays were measuring correctly the nitrogen-fixing capacity.

Finally it may be noted that so far as the writer is aware the acetylene technique has not yet been applied to the study of the onset of nitrogen fixation in young plants of legumes. From the standpoint of endophyte morphology there seems to be a parallel situation in alder and a legume, since in the latter the invading rhizobium at first exists in the developing nodule in the form of cocci and rods, and only at a slightly later stage passes into the enlarged, bacteroid form. The bacteroids are usually regarded as the nitrogen-fixing forms in the legume nodule, but it seems that the acetylene assay could be used to test whether the coccus and rod forms also show nitrogenase activity.

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*The derivation of this ratio will be explained in Chapter V.

SUMMARY

1. The sensitive acetylene assay has been used to trace the early development of nitrogenase activity in the nodules forming on young, recently inoculated alder plants. The assays were made on samples drawn at intervals of a few days from a population of such plants, and were continued for a total period of 33 days. After assay, squash preparations were made from all the nodules and examined microscopically in order to determine the stage of development of the endophyte.

2. No nitrogenase activity could be detected at the earliest stage of nodule development, where the endophyte was entirely in the hyphal condition. Only in somewhat older and larger nodules, in which endophytic vesicles were present, could nitrogenase activity be detected. It is concluded that the vesicles, but not the hyphae, contain nitrogenase and are normally the site of nitrogen fixation.

3. Nitrogenase activity was detected in the nodules some 15 days before visible benefit to the plant from the fixation was shown.

4. Ethylene production by nodules per plant increased in exponential fashion over the period of the experiments. Nitrogenase activity per unit volume of nodule was calculated and showed a steady increase over the period of the experiments, presumably because the number of vesicles and the supply of photosynthates from the leaves were rising.

CHAPTER II

Specialisation of *Myrica* and *Alnus* Nodule Endophytes to Particular Host Species

CONTENTS

INTRODUCTION

This part of the thesis is really concerned with the classification of the non-legume nodule endophytes. It is a natural desire to see what resemblances or differences exist between the endophytes of the different genera and species of host plants in order to find what classification is possible. The same problem exists with the rhizobia. The characteristics available there for classification include the morphology of the organisms in pure culture, such as the type of flagellation, also the rate and type of growth of the organisms on various media and their ability to ferment different carbohydrates. The antigenic properties have also been used. Another available characteristic is the morphology of the bacteroids formed within the legume nodule cells. Overriding importance is, however, given to the symbiotic affinities of a given rhizobium, i.e. the identity of the legume genera or species with which it can exist in symbiosis. On this basis a number of distinct species of rhizobium have been recognised and named according to their host plants, e.g. Rh. meliloti, infecting species of Melilotus and Medicago; Rh. trifolii, infecting species of Trifolium.

The number of characteristics available in the case of the non-legume organisms is smaller owing to the lack of pure cultures of them. The morphology of the endophytes within the nodule cells is still available as a criterion for classification. Thus differences are seen between the endophytes in the nodules of different host genera in respect of width of the hyphae, and of the size, shape and structure of the vesicles formed terminally by certain hyphae. In addition, there are available the results of cross-inoculation

trials, in which information has been sought on the ability of the endophyte within the nodules of a particular host species to set up symbiosis with other host species in the same or a different genus. However, the lack of pure cultures is a considerable handicap in such trials, for the experimenter has to resort to inocula prepared from crushed nodules or from habitat soil. When cross-inoculation is being attempted between two host species both of which are native to a particular region of the world, it is always possible that the nodules of the first species carry the endophyte of the second as a surface contaminant. Surface sterilisation of nodules to be used for inoculum preparation has often been attempted, but the careful study of Quispel (16) showed that it was impossible to kill all the surface contaminants on alder nodules by immersion for reasonable periods in mercuric chloride or other sterilising fluids, since after such treatment a substantial proportion of the nodules, when plated out in a nutrient medium, showed evidence of the continuing presence of common saprophytic micro-organisms, leaving open the possibility that any contaminating nodule endophyte had not been completely killed. This risk is perhaps absent when cross-inoculation is attempted between host species whose world distribution does not overlap.

On the basis of the existing information on the aspects noted above, Becking (17) has recently suggested a classification of the endophytes of non-legume nodules. He proposed that they should be classified in a genus Frankia (a name suggested for historical reasons), this genus being assigned to the Actinomycetales. Nine species of the genus are

created by Becking on the basis of the genus to which the normal host plant belongs. Thus the endophytes of alders are assigned to the species F. alni, those of casuarinas to F. casuarinae, while those associating with species of the genus Myrica are, again for historical reasons, assigned to F. brunchorstii. Becking's proposals represent a useful preliminary classification. Particularly in respect of the genera newly-discovered to be nodule-bearing (Purshia, Dryas, Discaria, Cerocarpus), the scheme is based on assumptions rather than facts. Even in much more studied genera, such as Alnus and Myrica it seems doubtful whether Becking's scheme as it stands is adequate, in view of the existing evidence from cross-inoculation studies.

In order to substantiate the last statement, and to prepare the way for the writer's own studies, a brief review will now be given of cross-inoculation results in the two genera indicated in the title to this Chapter, e.g. Alnus and Myrica.

Roberg (18) reported that he had secured cross-inoculation between four European species of Alnus, namely A. glutinosa, A. incana, A. cordata and A. viridis. He attempted the surface sterilisation of nodules to be used in inoculum preparation by immersing them in 3% hydrogen peroxide for a short time and then in 80% ethyl alcohol followed by flaming. He actually concluded that the nodules of all four species are normally tenanted by one and the same endophyte; however his data do not confirm that the unusual combinations were satisfactory as regards the activity of the nodules in fixing nitrogen. Benecke (19) presented results differing

notably from the above. Benecke's work was done in New Zealand, a country which has no native species of Alnus, though species have been introduced; he records that plants of several Alnus species raised in tree nursery beds nodulated freely without inoculation. Inocula were prepared from nodules taken from plants of A. viridis, A. glutinosa, A. nepalensis and A. sinuata growing in such nursery beds, the last two species being of Asian origin. The nodules were shaken in 0.1% $HgCl_2$ for 10 minutes prior to their use. The effect of applying these inocula to A. viridis plants growing in sand in pots, with some added autoclaved nursery soil, was tested. Satisfactory nodulation was obtained only where the A. viridis inoculum had been applied; occasional nodules formed in other pots but were considered to be due to contamination. Benecke concluded that the endophytes in the nodules of A. glutinosa, A. nepalensis, and A. sinuata are different from that in A. viridis nodules.

Meanwhile Rodriguez-Barrueco (20) had tested the effect of inoculating plants of A. jorullensis (a South American species) from nodules of A. glutinosa, and found that although numerous nodules formed, they all remained small and fixed little nitrogen. In one trial the number of nodules per plant was twelve times as many as in plants of A. jorullensis inoculated with the normal endophyte. Mackintosh & Bond (21) tested seventeen unusual combinations of host plants and endophytes in the genus Alnus, and although nodulation occurred in all combinations, the symbiosis was unsatisfactory in three cases. Geographical distribution and taxonomic affinity of the host species provided a partial explanation of the results. In summary,

there is evidence that the Alnus endophyte exists in a number of forms, each able to symbiose satisfactorily with a restricted number of host species.

Turning to the genus Myrica, Bond (22) reported that the inoculation of plants of M. cerifera with an inoculum prepared from M. gale nodules resulted in the development of very numerous (mean of 235 per plant) nodules of minute size which were scattered over most of the root system and which fixed little or no nitrogen. This situation resembles strongly that of legumes bearing the so-called ineffective nodules. Mackintosh & Bond (21) attempted twelve other unusual combinations of host plant and endophyte in the genus. Inocula prepared from two African species failed to induce any nodule formation in M. gale, and though nodules formed in all other combinations, the symbiosis (on the basis of plant dry weight and nitrogen content) was judged to be satisfactory in one combination only. M. gale inocula applied to an African and a Japanese species induced just the same kind of nodulation as recorded above in M. cerifera plants. It thus appeared that within the genus Myrica there is very considerable specialisation of nodule endophytes towards particular host species.

In the work to be described in this thesis, further cross-inoculations have been attempted in the genera Myrica and Alnus. The work has been facilitated as a result of the world-wide survey of nodulation in non-leguminous plants recently conducted by Professor Bond on behalf of the International Biological Programme (Bond, 4). Some collaborators in the survey sent seed collected from plants

found to be nodulated in the field to the Botany Department, Glasgow University, to facilitate further study of the nodules. A few collaborators sent endophyte sources also (nodules, habitat soil).

Using seed received in the above manner, the response of four species of Myrica to inoculation with the endophytes of other species of Myrica has been studied. The first species to be thus used as a host plant is Myrica javanica Blume which, as its name implies, is native to Java, Indonesia. The occurrence of nodules on this species was first recorded by von Faber (23), but apart from a brief statement by Becking (24) without detail, that nodulated plants growing in pot culture showed evidence of the occurrence of nitrogen fixation, no experimental use has been made of the species. Another species to be grown is Myrica faya Ait., native to the Azores and at least naturalised and possibly native also to parts of Portugal, the Canary Islands and Madeira. The occurrence of nodules in this species was first recorded by Rodriguez-Barrueco in the course of the I.B.P. survey (Bond, 4). In the latter account, Bond reported evidence of vigorous fixation of nitrogen in nodulated plants of M. faya which he had cultured in the greenhouse, when associated with the normal endophyte, and also that preliminary tests had indicated a favourable response of M. faya plants to inoculation from M. cerifera L. (a North American species) but not to inoculation from M. gale L. (Europe and North America).

The third Myrica species to be grown as a host plant is M. caroliniensis Mill., native to the eastern part of

North America, and first recorded by Youngken (25) to be nodule-bearing, though there is still no evidence that the nodules are nitrogen-fixing. The fourth species is M. pensylvanica Loisel. which is of common occurrence on sand dunes and other sites in the north-east of North America. Though the occurrence of nodules was recorded long ago by Chevalier (26), no experimental or other study of the nodules has hitherto been made.

Two species of Alnus have been grown as host species, firstly A. viridis Regel, widely distributed in north temperate regions. Roberg (18) recorded this species to be nodulated and provided evidence that the nodules were nitrogen-fixing. As stated already, there are conflicting reports as to the ability of A. viridis to symbiose with the endophytes of other alders, and it was hoped to clear this situation up in the present study. The second species is A. orientalis Dcne., native to eastern Europe and south-west Asia and first reported by Uemura (27) to be nodule-bearing.

Donor species, i.e. those whose nodules or habitat soils have provided inocula for application to the roots of the above species, are Myrica gale, M. cerifera, M. cordifolia L. and M. pilulifera Rendle (both native to southern Africa) and M. faya. Donor species for Alnus were A. glutinosa (L.) Gaertn. (Europe, Asia Minor) and A. incana (L.) Moench (Europe, North America). Nodulated stock plants of these species were available in the greenhouse, in water culture or Peralite culture, or in some cases in habitat soil. Comparison of this list of species with that

of host species in the preceding paragraphs will reveal that sources of normal endophytes for the host species were mostly unavailable. This was regretted, but it will become clear later that this lack did not seriously handicap the work.

MATERIALS AND METHODS

Source of Seed

Seed of *Myrica javanica* was sent by Mrs Estiti B. Hidajat, Biology Department, Bandung Institute of Technology, Indonesia. Seed of *Myrica faya* and of *Myrica caroliniensis* were sent by Dr C. Rodriguez-Barrueco, Centro de Edafologia y Biologia Aplicada, Salamanca, Spain, and were in both cases collected by him in the botanic gardens at Coimbra University, Portugal. Seed of *Myrica pensylvanica* was provided by Dr D.E. Eveleigh, Department of Biochemistry and Microbiology, Rutgers University, New Brunswick, U.S.A.

Seed of *Alnus viridis* was forwarded by Dr V.J. Radulović, Faculty of Agriculture, Sarayevo, Yugoslavia. Mr A.C. Crundwell kindly collected seed of *Alnus orientalis* in the Mugla Province of Turkey. Seed of *Alnus glutinosa* was collected from the Glasgow district.

Germination and Plant Culture

In *Myrica* species the waxy coating on the seed was rubbed off prior to sowing, and in the case of *M. caroliniensis* and *M. pensylvanica* the seed was given several weeks pre-treatment in moist peat at a temperature of 2°C in a refrigerator, following the recommendation of Barton (28). Chipping of seed was also resorted to, but despite all these measures germination was slow and irregular, so that uniform stands of seedlings for the commencement of a particular experiment could not be obtained, and the experiment had to be done in staggered manner. No pre-

treatment of Alnus seed was considered necessary. No surface sterilisation of seed of either genus was attempted, since experience in the department is that the seed of non-legumes does not carry the nodule endophyte.

Seed was sown in sterilised trays filled with Peralite and moistened with Crone's solution (nitrogen-free formula, see p.27) at 1/8th of its normal strength. Seedlings were transplanted into sterilised 800 ml beakers or glazed earthenware jars of 1- or 2-litre capacity filled with Crone's solution now at 1/4 or 1/3rd of normal concentration. The containers were covered by sterilised black plastic or waxed teak squares. A small addition of combined nitrogen was added to the jars, its amount depending on the extent of the delay that there was to be before inoculation. The plants were always transferred to nitrogen-free solution just prior to inoculation.

Inoculation

As has been explained in the Introduction, since isolates of the endophytes of non-legume nodules are not available, reliance for inoculation purposes has to be placed on preparations of crushed nodules or of habitat soil.

Nodules for the preparation of inocula were usually taken, as already noted, from stock plants associated with their normal endophytes and growing in the greenhouse. In some instances inocula were prepared entirely from nodules, but in others habitat soil - procured from a part of the world where the host species is native - was also used. With the donor species Myrica gale, M. cordifolia and M. pilulifera, inocula were prepared entirely from nodules,

but in M. cerifera and M. faya habitat soil was used in conjunction with nodules. Inocula from donor species of Alnus were prepared from nodules only. Nodules were ground in distilled water in a sterilised mortar, usually in the proportion of 1:20 by weight, a further one part of habitat soil being added to the mortar in the instances mentioned. In general, surface sterilisation of nodules was not attempted since, as noted earlier, there is no known effective but safe method that can be routinely used. Small quantities of the prepared inocula were applied to the root systems of the appropriate plants by means of a small brush or pipette. The plants in some containers were left uninoculated, partly to provide a check against the possibility of cross infection between the plants of different containers.

Subsequent Treatment of Plants

The plants were grown in a greenhouse, with supplementary artificial light in the winter months. Precautions were taken at all stages to avoid contamination between different plant containers when topping-up with water, supplying fresh culture solution, or inspecting the root systems. Forced aeration of the culture solution was provided where appropriate, using air from a 'Reciprotor' pump. Checks were made periodically on the pH of the culture solution in the containers and any drifts corrected; this was most necessary where substantial amounts of combined nitrogen had been supplied.

At harvest the height of the shoot and the dry weights of shoot and root and also of nodules were determined.

Total nitrogen was determined by the Kjeldahl process (see p.181).

The success of the symbiosis between a given host species and an unusual endophyte was assessed by comparison of the plants against other plants of the same host species associated with the normal endophyte where available, or, failing that, against plants supplied with ample combined nitrogen.

DATA OBTAINED

Myrica javanica Blume

Some 200 seeds were sown in February, 1972. The first seedlings appeared after two months, followed by others over the ensuing five weeks, giving a total of twenty-one seedlings.

As they became available, seedlings were transplanted into five one-litre jars. Those in three of the jars received applications of inocula prepared from *M. gale*, *M. cerifera* and *M. cordifolia* respectively. The plants in the fourth jar were left uninoculated, as were also those in the fifth jar, and to the latter a liberal addition of ammonium nitrate was made from time to time during the growth period, since no source of the normal endophyte of *M. javanica* was available.

Although nodules eventually appeared in all three inoculated jars, this did not occur until 4-6 months from the time of original inoculation, which is much slower than in any other *Myrica* species previously raised in the greenhouse, even with unusual endophytes. This is possibly because *M. javanica* is a tropical species for which conditions in a temperate greenhouse are quite sub-optimal. During the interval prior to the appearance of nodules at least one re-inoculation was made, in case the original inoculum had for some reason been inactive. The plants in the uninoculated jars remained nodule-free.

During the 1972-3 winter supplementary light was provided for this evergreen species, and also in that period forced

aeration was commenced, since the roots had shown a reluctance to grow into the depth of the jars.

The nodules forming on the inoculated plants were of the usual Myrica type, equipped with nodule-roots. The nodules induced by the M. gale inoculum took the form of very small clusters scattered over a much greater part of the root system than in the case of the nodules induced by the two other inocula.

During the period prior to nodule development, all the plants except those supplied with combined nitrogen showed leaf chlorosis and a slowing up of shoot growth, but after nodules appeared these features gradually disappeared in most of the plants inoculated from M. cerifera and M. cordifolia, but persisted in plants inoculated from M. gale. These and other features are shown in Figure 13, a photograph taken shortly before harvest. The vigorous growth of the non-nodulated plants supplied with combined nitrogen will be noted, also that some plants inoculated from M. cerifera and M. cordifolia had grown well, though others in the same jars were weaker - this being associated with a paucity of nodules. It will be seen that the plants associated with the M. gale endophyte were all very poor in growth, and similar as regards shoot growth to the nodule-free plants in nitrogen-free solution.

Harvest was made approximately eight months from the original date of inoculation, and data for plant height and dry weight are shown in Table 8. They confirm the strong

Fig. 13. Plants of Myrica javanica after eight months' growth from date of inoculation. Jars left to right: uninoculated plants; inoculated from M. cerifera; inoculated from M. cordifolia; inoculated from M. gale; uninoculated with combined nitrogen supplied (x 1/4).

Table 8. Mean growth and dry weight data for *Myrica javanica* plants after eight months from inoculation.

Treatment*	No. of plants harvested	Shoot height, cm	Dry weight per plant, g		Nodule dry weight as % of total dry weight
			Nodules	Whole plant	
Inoculated from *M. gale*	3	5.1	0.023	0.217	10.6
Inoculated from *M. cerifera*	2	13.1	0.051	1.141	4.5
Inoculated from *M. cordifolia*	1	13.5	0.105	1.441	7.3
Non-inoculated plants	4	5.1	0.000	0.109	-
Non-inoculated plants supplied with ample combined N	2	21.2	0.000	3.294	-

*All plants were grown in nitrogen-free solution unless otherwise stated.

growth of plants inoculated from M. cerifera and M. cordifolia - the poorly nodulated plants mentioned above were excluded from the harvest - and the weak growth of the plants inoculated from M. gale. It is notable that in the latter plants the nodules accounted for over 10% of the dry weight of the whole plant, an unusually high figure, despite which they were of little value to the plants.

Nitrogen data obtained by Kjeldahl analysis of the harvested plants are shown in Table 9. The data for total nitrogen per plant show that the plants bearing nodules induced by M. cerifera and M. cordifolia endophytes achieved a considerable accumulation of nitrogen which showed some approach to that of the plants amply supplied with combined nitrogen. Under the conditions of the experiment this accumulation must have been the result of fixation in the nodules; the actual amount fixed is calculated by subtracting the nitrogen content of the non-nodulated plants grown in nitrogen-free solution from that of the nodulated plants. When this is done for the plants inoculated from M. gale, a trifling amount of fixation (0.8 mg) is indicated. The great contrast in nodule activity in fixation in the different combinations is clearly revealed in the right-hand column of Table 9.

Thus it may be concluded that the endophytes of M. cerifera and M. cordifolia nodules are capable of setting up effective symbioses with plants of M. javanica, while that of M. gale nodules is not.

Table 9. Mean nitrogen data for *Myrica javanica* plants after eight months from inoculation*.

Treatment	Per cent N in whole plant dry matter	Total N per plant, mg	Mg N fixed per g dry weight of nodules formed
Inoculated from *M. gale*	0.956	2.1	35
Inoculated from *M. cerifera*	1.391	15.9	286
Inoculated from *M. cordifolia*	1.735	25.0	226
Non-inoculated plants	1.154	1.3	-
Non-inoculated plants supplied with ample combined N	1.142	37.6	-

*The numbers of plants analysed were the same as those harvested (Table 8). Corresponding plants were bulked together prior to analysis.

Myrica faya Ait.

The author took over some young, uninoculated plants left over from Professor Bond's preliminary experiments mentioned in the Introduction. In addition, she made sowings herself. The first seedlings emerged 3-4 months from sowing, followed by others at irregular intervals, the total germination being about 30%. The plants were set-up in 2-litre jars, several in each jar. Since *M. faya* is again an evergreen species, supplementary light was provided for the plants during the winter months. Forced aeration of the culture solution was also provided.

A first experiment was made in 1972, in which a comparison was made of the effects of inoculation of the *M. faya* plants with the normal endophyte, and with the endophytes of *M. gale*, *M. cerifera* and *M. cordifolia*.

Nodules appeared in all four host plant-endophyte combinations. They came most quickly (after four weeks) where the *M. gale* endophyte had been applied, after five weeks with the normal endophyte, but not until about ten weeks where the endophytes of *M. cerifera* and *M. cordifolia* were involved. As with *M. javanica*, except for those induced by the *M. gale* endophyte the nodules were of the typical *Myrica* type, with prominent upward-growing nodule-roots. Figure 14 shows part of the root system of a *M. faya* plant associated with its normal endophyte, and it will be noted that the nodule clusters - whose position is indicated by the white nodule-roots - are mainly confined to two sites on the root system. On the other hand (again

Fig. 14. Part of root system of Myrica faya plant associated with its own endophyte. The white nodule-roots indicate that the nodules are mostly confined to two sites on the root system (x $1\frac{1}{2}$).

Photograph kindly supplied by Professor Bond.

as in M. javanica), the nodule clusters induced by the M. gale endophyte were scattered over a large part of the root system and were all minute in size, consisting mostly of only 1-2 lobes, still equipped with nodule-roots. These features are shown in Figure 15.

In all combinations except that involving the M. gale endophyte, the appearance of nodules was followed by an obvious relief of the symptoms of nitrogen deficiency which the plants had developed prior to nodulation, namely leaf chlorosis and reduced shoot growth. These symptoms, however, persisted in the plants associated with the M. gale endophyte, as shown in Figure 16.

Unfortunately, though the three uninoculated control plants included in the experiment remained free of nodules for four months after the commencement, they subsequently formed a small number of nodule clusters, and in due course a greening of their leaves showed that benefit was being derived from the nodules. Obviously by accidental contamination an effective endophyte - possibly the normal M. faya endophyte - had gained access to the jar. Contamination between jars rarely happens with the practices followed in the greenhouse; thus Mackintosh & Bond (21) were able to record that in their study only one out of 160 control plants became nodulated. Further reference to this contamination in the present experiment is made below. The plants in question were discarded.

The nodulated plants were harvested after 6-8 months' growth. Figure 17 shows the plants shortly before harvest.

Fig. 15. Root system of *Myrica faya* plant associated with *M. gale* endophyte, 1972 experiment. Numerous minute nodule clusters scattered over the root system are seen. As a result of the disturbance caused by the photograph, the original upward orientation of the nodule-roots was upset (x 2/3).

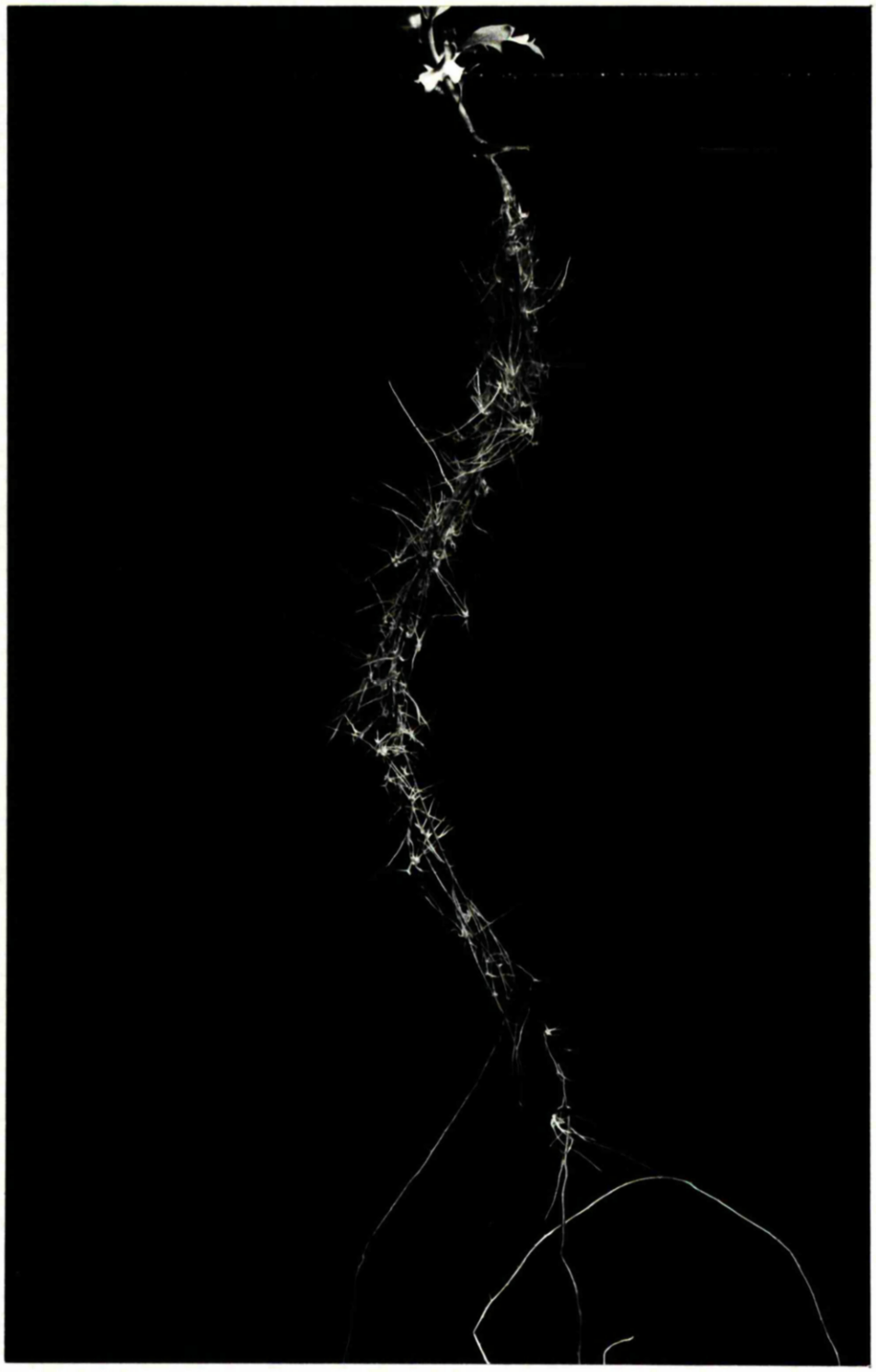


Fig. 16. Plants of Myrica fava after five months' growth in nitrogen-free solution. Left: plants inoculated from M. gale. Right: plants inoculated with the normal endophyte (x 1/3).

Fig. 17. Plants of Myrica faya just prior to harvest, 1972 experiment. Endophytes used for inoculation, left to right: normal endophyte; M. gale endophyte; M. cerifera endophyte; M. cordifolia endophyte (x 1/5).

The poor growth of the plants associated with the M. gale endophyte will again be noted, also the healthy growth of most of the plants associated with the normal endophyte or with those of M. cerifera or M. cordifolia. The smaller stature of the plants associated with the two latter endophytes, compared with those in the left-hand jar, is at least partly due to the later development of nodules plus - in the case of the plants inoculated from M. cordifolia - the shorter growth period.

Harvest data for height and dry weight are shown in Table 10. They confirm the impression of the relative vigour of plant growth in the various combinations given by Figure 17. The relatively high proportion of the total plant dry weight accounted for by the nodules in the plants associated with the M. gale endophyte is again noteworthy. Counts showed that at harvest the mean number of nodule clusters on these plants was 170 per plant. Although in the plants associated with other endophytes the nodule clusters were too compacted together to be reliably counted, there is no doubt that the number was much less than 170.

Nitrogen data are provided in Table 11. The per cent figures show that the tissues of the plants inoculated from M. gale were very low in nitrogen. Calculation of nitrogen fixed cannot be made in the normal way, owing to the contamination of the control plants noted already, but an approximate guide can be obtained by using the figure (1.2 mg) for the control plants in the 1973 experiment (see later). On that basis it appears that a trifling amount of nitrogen (1.4 mg) was fixed in the nodules induced

Table 10. Mean growth and dry weight data for Myrica faya plants after several months' growth in N-free culture, 1972 experiment.

Treatment	No. of months of growth	No. of plants harvested	Shoot height, cm	Dry weight per plant, g		Nodule dry weight as % of total dry weight
				Nodules	Whole plant	
Inoculated from M. faya	8	3	31.8	0.179	4.219	4.2
Inoculated from M. gale	8	5	8.2	0.073	0.415	17.6
Inoculated from M. cerifera	8	3	20.5	0.087	1.903	4.6
Inoculated from M. cordifolia	6	3	14.8	0.072	1.370	5.3

Table 11. Mean nitrogen data for *Myrica faya* plants after several months' growth in culture, 1972 experiment.*

Treatment	Per cent N in whole plant dry matter	Total N per plant, mg	Mg N fixed per g dry weight of nodules formed
Inoculated from *M. faya*	1.394	58.8	322
Inoculated from *M. gale*	0.626	2.6	19
Inoculated from *M. cerifera*	2.002	38.1	424
Inoculated from *M. cordifolia*	1.832	25.1	332

*The numbers of plants analysed were the same as those harvested (Table 10). Corresponding plants were bulked together prior to analysis.

by the M. gale organism, and substantial amounts fixed in the other combinations.

In 1973 a further experiment was set up. In part it was a repeat of the previous experiment - repetition was necessary since the contamination of the control plants in 1972 made the evidence for successful symbiosis between M. faya plants and the M. cerifera and M. cordifolia endophytes slightly suspect. In 1973 those two combinations were set up again, and, in addition, some plants were inoculated from M. pilulifera. This time the control plants remained nodule-free. Plants associated with the M. cerifera or M. cordifolia endophytes again grew well, as did also those associated with the M. pilulifera organism. These statements are confirmed by the Tables 12a and b, and by Figure 18.

These two experiments show that M. faya plants are able to set up effective symbiosis with the endophytes of M. cerifera, M. cordifolia and M. pilulifera, but not with the M. gale endophyte.

Myrica caroliniensis Mill.

Although some 400 seeds of this species were sown, only three healthy seedlings were obtained. Two of these were inoculated from M. gale nodules. Nodules duly appeared on the plants after two months and resembled those induced by an M. gale inoculum on the two preceding species, i.e. they were numerous, minute, and scattered over the root system. Again they conveyed very little benefit to the plants, which remained in an obvious state of nitrogen

Inoculated from *M. cordifolia*	5	1	11.0	0.125	1.253	10.0
Inoculated from *M. pilulifera*	5	5	9.7	0.065	0.710	9.2
Non-inoculated plants	6	10	4.2	0.000	0.198	-

Table 12B. Mean nitrogen data for *Myrica faya* plants after several months' growth in culture*, 1973 experiment.

Treatment	Per cent N in whole plant dry matter	Total N per plant, mg	Mg N fixed per g dry weight of nodules formed
Inoculated from *M. cerifera*	1.195	26.6	195
Inoculated from *M. cordifolia*	2.051	25.7	196
Inoculated from *M. pilulifera*	1.816	12.9	180
Non-inoculated plants	0.602	1.2	-

*The numbers of plants analysed were the same as those harvested (Table 12A). Corresponding plants were bulked together prior to analysis.

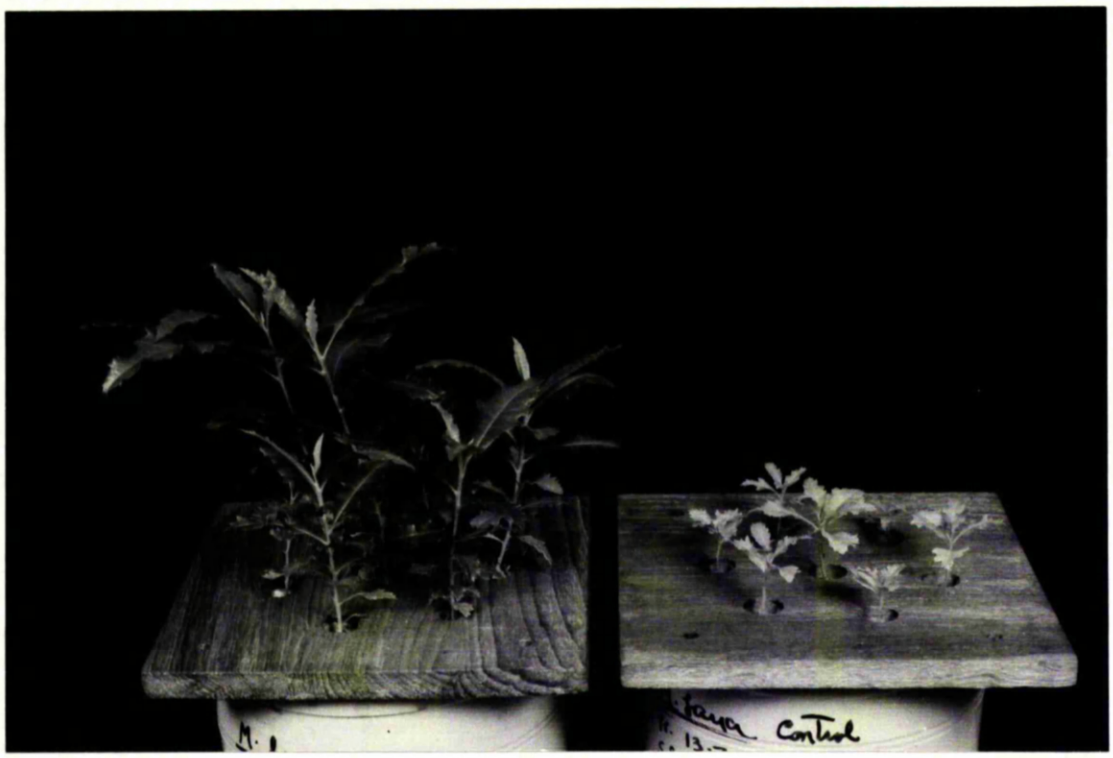


Fig. 18. Plants of Myrica faya after five months' growth in nitrogen-free solution, 1973 experiment. Left: plants inoculated from M. pilulifera. Right: uninoculated, non-nodulated control plants (x 1/3).

deficiency for the whole of the eight months' growth period allowed. This was confirmed by the harvest data, which showed a mean dry weight per plant of only 0.67 g, a per cent nitrogen content of 0.817, and an absolute nitrogen content of 4.1 mg per plant. Though the lack of control plants prevents calculation of the nitrogen fixed, this could not have been much more than 1 mg. At harvest a mean of 115 nodule clusters was found to be present on the plants.

The third seedling was inoculated from M. pilulifera. Nodules began to appear after a delay of three months, and in contrast to those induced by M. gale the clusters were mostly concentrated at three sites of the root system (Fig. 19). Subsequently the plant grew strongly. It has not been harvested, but it is very clear that the host plant has established a satisfactory symbiosis with this particular unusual endophyte. Figure 20 shows a recent photograph of the plant.

Myrica pensylvanica Loisel.

Out of 250 seeds sown only five seedlings were obtained. Four of these were inoculated from M. gale. Nodules appeared after ten weeks, but as in the previous host species they conveyed no obvious benefit to the plants, as is indicated in Figure 21 where the appearance of these plants is contrasted with a non-nodulated plant supplied with combined nitrogen. No analyses were made on these plants, and the ineffective nodules induced by M. gale were used to test whether an inoculum prepared from them could induce nodulation in M. gale plants (see Chapter III).

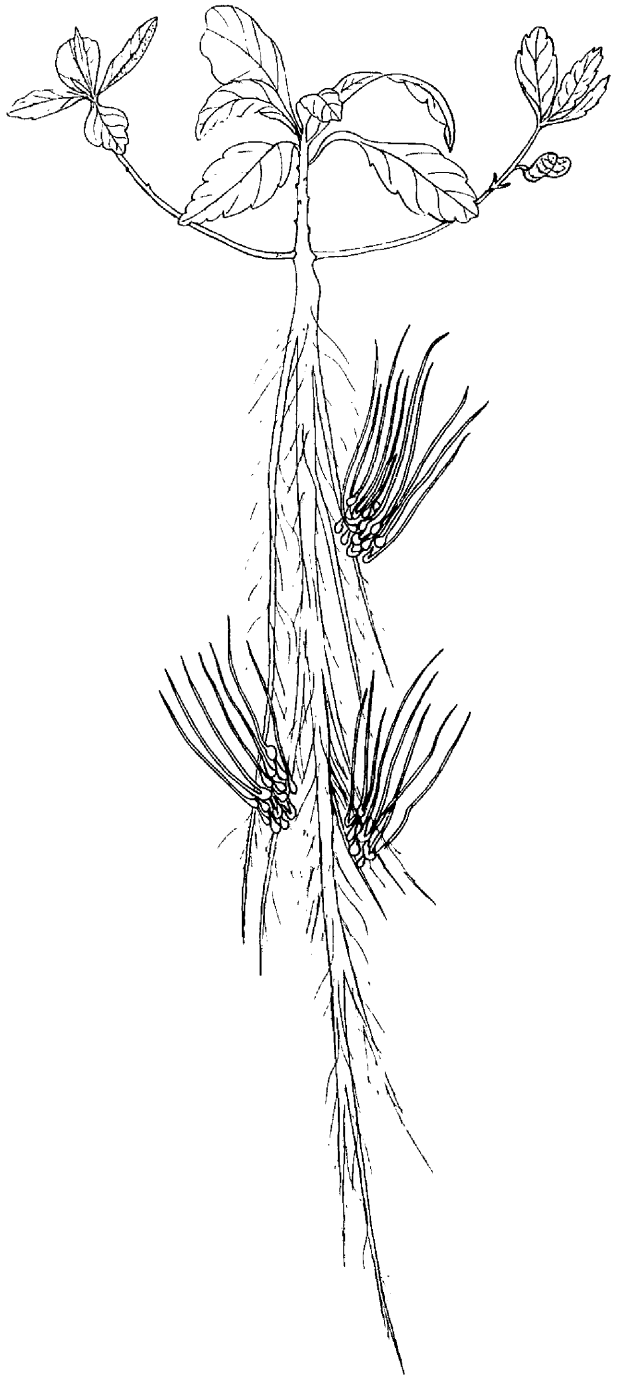


Fig. 19. Sketch of plant of Myrica caroliniensis inoculated from M. pilulifera, after eight months' growth. The nodules are concentrated at three sites (x 1/2).

Fig. 20. Plant of Myrica caroliniensis inoculated from M. pilulifera, after twelve months' growth in nitrogen-free solution (x 1/4).

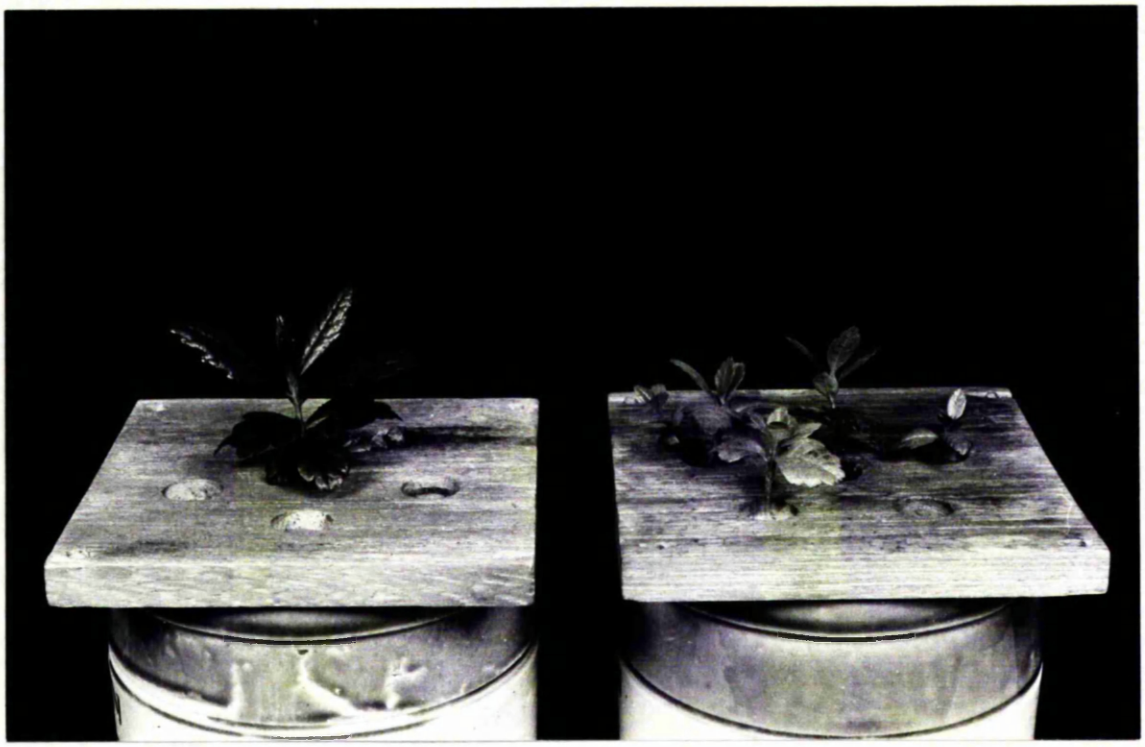


Fig. 21. Plants of Myrica pensylvanica. Left: uninoculated plant supplied with combined N grown for two months. Right: plants inoculated from M. gale grown for five months (x 1/3).

It was, however, recorded that the mean number of nodules on the four plants concerned was 92 per plant. Figure 22 shows the scattered distribution of the nodules.

Alnus viridis Regel.

As indicated in the Introduction, the main object here was to try to resolve contrary statements in the literature on the ability of A. viridis to set up satisfactory symbiosis with the endophytes of other European species of Alnus, a minor objective being to test another statement in the literature (Benecke, 29) that A. viridis plants do not thrive on nitrate nitrogen.

Germination was good and rapid. Seedlings were set out in water culture in 800 ml beakers, five per beaker. Inocula were prepared from the nodules of stock plants of A. glutinosa and A. incana. Two types of inocula were prepared from the nodules of each of those species. In preparing the first type the nodules were not surface-sterilised, but for the second type the nodules were shaken for 4 min in a solution of 0.1% $HgCl_2$ in 0.05N HCl and then rinsed in many changes of distilled water. This was done bearing in mind that the original A. incana nodules from which the stock plants had been inoculated had come from Norway, where both A. incana and A. glutinosa grow, making it possible for nodules of the former to be surface-contaminated with the endophyte of the latter species. Three beakers, with a total of 15 plants, were set up for each of the above four treatments. In addition, in order to check whether the A. glutinosa

Fig. 22. Sketch of Myrica pensylvanica plant five months after inoculation from M. gale. The more prominent roots are nodule-roots, with the minute nodules at their bases (x 1/2).

inocula (both types) were normally infective for their usual host, beakers of A. glutinosa plants were included and were treated with the same A. glutinosa inocula as were applied to the A. viridis plants; owing to a lack of A. incana seed similar checks could not be made on the inocula prepared from nodules of that species. Uninoculated control plants of A. viridis (four beakers with a total of twenty plants) were included. Finally three beakers of A. viridis plants were supplied with 12 mg sodium nitrate-nitrogen per litre of culture colution prior to inoculation, further quantities being added during the growth period.

The plants were grown during the winter months, with supplementary electric illumination. The position of the individual beakers was changed frequently by a definite system in order to equalise the lighting.

After three weeks from inoculation all inoculated plants were well-nodulated, except for those treated with the A. incana inoculum prepared from surface-sterilised nodules, where nodules did not appear until four weeks from inoculation and in some plants were still absent at harvest. The uninoculated control plants were all nodule-free at this stage and remained so to the end of the growth period. As regards the A. glutinosa plants treated with the inocula prepared from A. glutinosa nodules, the good nodulation shown by all these plants confirmed that the inocula (from both unsterilised and sterilised nodules) were normal in activity, and these plants were discarded part way through the main experiment.

Differences in top growth appeared fairly soon after nodulation and persisted through the growth period. They are shown in Figure 23 for the plants in nitrogen-free solution, the photographs being taken shortly before harvest. All the nodulated plants are of healthy appearance and much superior to the control plants, except for those inoculated from surface-sterilised A. incana nodules.

Harvest was carried out eleven weeks after inoculation - rather earlier than planned, owing to the energy crisis in January, 1974. Data are shown in Table 13. They indicate that the application of each type of A. glutinosa inoculum resulted in satisfactory nodulation of A. viridis plants - the value of 4.5 for the nodule dry weight as a per cent of whole plant dry weight is a normal figure for Alnus species. Also the per cent nitrogen in the tissues is satisfactory, as is the actual fixation (calculated as usual by subtracting the mean nitrogen content of the control plants from that of the nodulated ones). Although owing to the unavailability of A. viridis field nodules, no A.viridis plants infected with their normal endophyte could be included in the experiment, it is difficult to suppose that had such plants been included, their growth would have been much better than that of the plants inoculated from A. glutinosa nodules.

Table 13 also shows that equally good plants were obtained where the A. incana inoculum prepared from unsterilised nodules was applied, but not where surface-sterilised nodules had been used - it will be seen that all

Fig. 23. Plants of Alnus viridis in nitrogen-free solution after eleven weeks from inoculation. No. 16: uninoculated, non-nodulated control plants; no. 1: inoculated from A. glutinosa nodules; no. 5: as no. 1 but nodules surface-sterilised; no. 7: inoculated from A. incana nodules; no. 12: as no. 7 but nodules surface-sterilised (x 1/5).

Table 13. Harvest data for *Alnus viridis* plants grown in nitrogen-free solution and inoculated in various ways, after eleven weeks from inoculation.*

Source of inoculum	Mean shoot height cm	Mean dry weight per plant, mg		Mean nodule weight as % of whole plant weight	% N in whole plant dry matter‡	Total N per plant mg
		Nodules	Whole plant			
A. glutinosa unsterilised nodules	5.4	4.4	98.8	4.5	1.302	1.3
A. glutinosa surface-sterilised nodules	5.1	4.3	94.8	4.5	1.522	1.4
A. incana unsterilised nodules	6.5	5.2	119.6	4.3	1.636	2.0
A. incana surface-sterilised nodules	3.3	1.4	53.9	2.6	1.171	0.6
No inoculum applied (controls)	2.2	0.0	40.0	-	0.814	0.3

*The number of plants in each treatment was 15 except in the last group in which case there were 20 plants.

‡Dry matter of all plants in each group bulked together prior to Kjeldahl analysis.

the growth data are inferior. This was chiefly due to the late appearance of nodules on these particular plants. The reason for the different results for the two A. incana inocula cannot be decided from the data available, chiefly because,as noted, no A. incana plants were available for checking the activity of the two inocula. It is possible that A. incana nodules are more permeable to mercuric chloride than those of A. glutinosa, perhaps because of the thinner periderm that they have, with consequent injury to the endophyte, but another possibility is that as noted earlier the A. incana nodules may have been superficially contaminated with the A. glutinosa organism, and this may have been responsible for the good results obtained with the unsterilised nodules, the poor results with the sterilised nodules being perhaps due to the elimination of some of the contaminating organisms.

As noted already, some further A. viridis plants were given nitrate-nitrogen at the time of inoculation. These plants grew much more strongly than any of the others. Figure 24 compares their appearance shortly before the harvest date with that of similarly inoculated plants not given nitrate. At harvest the mean growth and nitrogen data for these plants were as follows: shoot height 13 cm, dry weight of nodules 6.7 mg and of whole plant 543.3 mg, nodule dry weight as per cent of whole plant dry weight 1.2, nitrogen content 10.2 mg per plant. It will be observed that these plants formed a greater absolute weight of nodules than any of the plants in nitrogen-free solution, though the weight relative to that of the whole

Fig. 24. Plants of Alnus viridis after eleven weeks' growth, all inoculated from A. glutinosa nodules. No. 1: nitrogen-free solution; no. 19: nitrate-nitrogen supplied (x 1/3).

plant was reduced. Thus both plant and nodule growth were favoured by addition of nitrate-nitrogen, especially that of the plant.

Alnus orientalis Dcne.

A brief account of work with this species will be included, although the results obtained are not all clear-cut.

The forty seeds available were sown in a tray of Peralite which was divided across the centre by an impervious plastic partition. After sowing, a suspension of crushed nodules from A. orientalis brought by Mr. Crundwell from Turkey, was applied to one half of the tray. In due course two seedlings were obtained from the inoculated part and one from the other, and were set up in water culture.

After 17 days from transplanting, no nodules had, in fact, appeared on the two plants where they were expected, and considering that the inoculum had been in contact with the seedling roots in the tray for about two weeks prior to transplanting, it was concluded that the inoculum had been inactive, perhaps because the nodules had been off the tree for more than a week before use. An inoculum prepared from A. glutinosa was nowapplied to the two plants. Apparently in response to this, nodules appeared after 13 days, which is the normal interval for alder species in the greenhouse. After a further interval these nodulated plants began to grow strongly, whereas the

uninoculated and still nodule-free plant made little further growth. Figure 25 shows the plants after three months from the inoculation from A. glutinosa. Obviously good fixation was proceeding within the nodules.

Unfortunately, at a slightly later stage a few nodules appeared on the control plant, and it is not clear why this happened. It could have been due to contamination from some other culture, but alternatively it might signify that the A. orientalis inoculum applied to the seed tray was, in fact, active, that a trace of it gained access to the uninoculated part, and further, that the nodules which appeared on the inoculated plants were, despite all appearances, due to the A. orientalis endophyte.

There is no doubt that the A. orientalis plants at any rate formed nodules very active in fixation, though whether the nodules contained the normal endophyte or that of A. glutinosa is uncertain.

The nodulated plants continued to grow very strongly. At harvest, after 16 weeks from inoculation, the mean height of the two plants was 54 cm and their mean nitrogen content was slightly over 300 mg, indicating extensive fixation.

Fig. 25. Plants of Alnus orientalis in nitrogen-free solution, on the left: nodulated; on the right: non-nodulated. After three months from inoculation (x 1/3).

DISCUSSION

Before discussing the cross-inoculation aspect, it may be noted that some of the data obtained in this work have significance in another connection. As already pointed out in some instances, in the past there has been little or no evidence on whether the nodules normally present on plants of Myrica javanica, M. caroliniensis, M. pensylvanica and Alnus orientalis under field conditions are nitrogen-fixing. Since the normal endophytes of these species (with the possible exception of A. orientalis) were not available to the writer, she was unable to obtain direct evidence on this matter. However, except for the third one, it has been shown that these species, in response to inoculation with certain unusual endophytes, form nodules which fix nitrogen vigorously. In previous comparisons where the normal enodphytes have been available, Professor Bond has never found nodules containing an unusual endophyte to be more efficient in fixing nitrogen than those inhabited by the normal endophyte. Myrica faya provides an example of this in the present work. Thus the present work provides evidence, though indirect, that the normal nodules of M. javanica, M. caroliniensis and Alnus orientalis are nitrogen-fixing. The present results with M. faya confirm the report of Bond (4) to the effect that in combination with its own endophyte nitrogen is fixed strongly.

In the course of the attempted cross-inoculations made in the present work, ten unusual combinations were attempted in the genus Myrica. All gave satisfactory

symbiosis except those involving the M. gale endophyte. The latter induced the formation of nodules ineffective in nitrogen fixation on all the host species employed, namely M. javanica, M. faya, M. caroliniensis and M. pensylvanica, just as Mackintosh & Bond (21) previously recorded with M. cerifera, M. cordifolia and M. californica as host plants. In another part of the thesis a microscopic study of these ineffective nodules will be reported. It was undertaken to find whether the structural development of the endophyte provided any clue on the reason for the lack of fixation. In any event it seems that some physiological factor in the cells of the unusual host prevents the occurrence of fixation. That there is a degree of taxonomic and hence of physiological apartness between M. gale plants and those of other species of the genus is indicated by the fact that some botanists have placed M. gale in a separate genus on account of catkin features. Thus Chevalier (26) called it Gale palustris. In addition, M. gale differs from all other Myrica species that have been grown in Glasgow in being deciduous, a further difference being that in M. gale nodules the endophyte occupies a much larger part of the cortex than in the nodules of other species. The M. gale endophyte appears to be adapted to the distinctive physiology of its normal host, and cannot symbiose properly with others.

The remaining six unusual combinations attempted by the author in the genus Myrica gave satisfactory symbiosis, except that nodule development was often quite slow, though the lack of normal enodphytes prevented direct comparison except in the case of M. faya. There

nodule induction by M. cerifera and M. cordifolia inocula took about twice as long as when the normal endophyte was applied. These delays perhaps indicate a degree of resistance by the host plant to unusual endophytes. The degree of success in these six combinations was greater than that found by Mackintosh & Bond (21), and it is rather surprising that good combination should have been shown between the endophyte of an African species and an American host plant, and between an American endophyte and a Javanese host. Indeed on the basis of the present study it might be suspected that apart from that of M. gale, the endophytes of all Myrica species are quite similar, both morphologically and symbiotically, but taking into account the further nine combinations tested by Mackintosh & Bond (loc. cit.) it is clear that differences do exist.

The inclusion by Becking (see Introduction) of the endophytes of all species of Myrica under the one specific name Frankia brunchorstii, though possibly justifiable on the grounds of endophyte morphology, is not justifiable when symbiotic affinities are considered, particularly as regards the M. gale endophyte which clearly differs from the others and should be classed separately, at least as a sub-species.

Of the more limited data for the genus Alnus, those in which A. viridis was used as host plant are of chief interest. The present writer's finding that the combination of the A. viridis plant with the A. glutinosa

endophyte gave good results is in agreement with that of Roberg (18), and adds to it by better evidence of satisfactory fixation. The present findings for the A. viridis plant - A. incana endophyte combination also agree with those of Roberg if it is assumed that the less satisfactory results obtained when the inoculum was prepared from surface-sterilised nodules were due to injury to the endophyte within the nodules.

As already mentioned, Benecke (19) reported that in his trials an inoculum prepared from A. glutinosa failed to induce nodulation in A. viridis plants, contrary to the findings in the preceding paragraph. Two possible explanations of this situation can be mentioned. One is that the long period (10 min) of immersion of the nodules in mercuric chloride which Benecke employed, resulted in injury to the endophyte in the case of the A. glutinosa nodules. The other is more involved and stems from the situation that no alder species is native to New Zealand; the various species which have been introduced have presumably been raised from seed in the country, and the original source of the endophytes within the nodules which the introduced plants bear is uncertain. Their occasional presence as surface contaminants of seed sown is one possibility. It is possible that the endophyte within A. glutinosa nodules in New Zealand is not identical with that within European nodules of that species.

The very restricted cross-inoculation tests in the genus Alnus now reported do not conflict with Becking's

assignment of all alder endophytes to Frankia alni. However, the evidence reviewed in the Introduction indicates that there are differences in respect of symbiotic affinities.

SUMMARY

1. The response of plants of four species of Myrica, native to Java, the Azores, and North America (two species) respectively, to inoculation with the endophytes of five other species of Myrica, native to Africa, North America and Europe respectively, has been studied. In all instances, though inoculation with the M. gale (Europe, North America) endophyte resulted in nodulation, the nodules were ineffective in fixing nitrogen, and were also distinctive by being minute size and widely scattered over the root system. Otherwise inoculation with unusual endophytes resulted in the formation of nodules which were normally active in fixation, and so made possible a good plant growth in a nitrogen-free rooting medium. It is concluded that in its symbiotic properties the M. gale endophyte is clearly different from the endophytes of other Myrica species, and should be classified separately.

2. In the genus Alnus cross-inoculation tests showed that the A. glutinosa endophyte gave good results when inoculated into a second European species of alder, A. viridis. The same is probably true of the A. incana endophyte, though the results here were less conclusive. It was also shown, contrary to a previous report, that A. viridis plants grow strongly on nitrate-nitrogen.

3. For two of the *Myrica* species grown as host plants in this study and one of *Alnus* (*A. orientalis*) there was previously little or no evidence on whether the nodules normally present on the plants in the field are nitrogen-fixing. The present study provides strong though indirect evidence that they are.

CHAPTER III

Nodule Structure and Endophyte Morphology in Some Non-Legumes

CONTENTS

INTRODUCTION

There is an urgent need for the study of nodule and endophyte morphology in additional non-legumes, in order that the full range of variation in these aspects may become known. Nearly all previous workers in this field have concentrated on the nodules of species native to their region, while the nodules of species growing in regions where no biologists existed or none were interested in root nodules have remained uninvestigated.

In the present study structural examination has been made of the nodules of additional species of Myrica, including the ineffective nodules obtained in the greenhouse by the application of an unusual endophyte (see Chapter II). Also, studies have been made of the nodules of species of Dryas, Purshia and Coriaria.

In the genus Myrica previous study by the light or the electron microscope has referred almost exclusively to M. gale, M. rubra and M. cerifera. For reasons of brevity individual citations to the rather numerous authors involved will not be made, and only a generalised account will be given of the facts established for those species. As already noted in the Preface and Chapter II, while in M. gale the infected cells are scattered through most of the cortex, as in Alnus, in M. rubra and M. cerifera, and also (as briefly noted by various authors) in M. adenophora, M. cordifolia and M. pilulifera they are confined to a narrow layer in the middle cortex. The endophyte becomes established first in the central region of the host cell,

and in most cells subsequently produces radiating hyphae whose terminating portions enlarge to form club-shaped vesicles. The recent work of Gardner (6) showed definitely that in M. gale and M. cerifera these vesicles are internally sub-divided, as in Alnus glutinosa. The production of bacteroids by the endophyte has also been recorded in Myrica species.

The structure of the nodules incapable of nitrogen fixation, obtained in this department by the inoculation of other Myrica species with the M. gale endophyte, was briefly examined by Mackintosh (30) in sections from wax-embedded nodules taken from M. cerifera. She found that the endophytic hyphae were sparsely developed within the host cells, and no vesicle formation was to be found.

The occurrence of fleshy root nodules in the genus Dryas was first recorded by Lawrence (31) in respect of plants of D. drummondii growing at Glacier Bay, Alaska. Nodules have since been found on that and other species of Dryas at sites in North America, but so far they have not been found in any other country. The only previous microscopic study of the nodules is that made by Quispel included in the paper by Lawrence, Schoenike, Quispel & Bond (32). He found that nodules to be of typical non-legume type, the infected cells containing the usual fine hyphae and vesicles, oval or spherical. Some evidence of bacteroids was obtained. Quispel used only the light microscope, so that he could only observe the grosser structural features.

The occurrence of nodules on the two species that comprise the genus Purshia was first recorded by Wagle & Vlamis (33). They are woody shrubs, commonly known as bitterbrush, covering vast areas in the western U.S.A. and providing an important source of browsing for animals. Wagle & Vlamis (loc. cit.) merely state that the nodules contain a 'fungus' which a colleague had tentatively identified as an actinomycete. No report on the structure of these nodules and on the detailed appearance of the endophyte is known to the writer.

Coriaria, as noted in the Preface, comprises 15 shrubby species and provides an often-quoted example of extreme discontinuous distribution, with some species in New Zealand, others in South America, one in southern Europe, and others in Japan and adjacent countries. The presence of nodules in the genus was first noted in respect of Japanese species, by Shibata & Tahara (34), and the feature has subsequently been observed for most of the species that grow elsewhere. Shibata & Tahara (loc. cit.) also provided a good account of the structure of the nodules and the appearance of the endophyte in C. japonica nodules, and so revealed remarkable differences from other non-legume nodules. The stele does not lie centrally in the nodule lobe, but rather to one side, and the endophyte is confined to the side of the nodule on which the cortex is deeper, features which will be more readily understood from the present writer's illustrations to be provided later. Further in contrast with the infected cells of other non-legumes, these in Coriaria japonica have

conspicuous vacuoles. The endophyte, which they considered to be an actinomycete-type with hyphae about 0.4 μm in diameter, proliferates in the cytoplasm and forms club-shaped vesicles which, most remarkably of all, are all orientated towards the vacuole, rather than to the cell-wall. Allen, Silvester & Kalin (35) reported similar features in the New Zealand C. arborea.

MATERIALS AND METHODS

The material of effective and ineffective nodules of Myrica faya Ait., M. javanica Blume and M. caroliniensis Mill. was taken from plants grown in connection with Chapter II of this thesis. The material of nodules of Purshia tridentata (Pursh) DC. and of Coriaria myrtifolia L. was taken from plants which Professor Bond was growing in the greenhouse. Nodules of Dryas drummondii Richardson had been fixed earlier in formalin-acetic-alcohol by Professor Bond from living plants from the field received from Glacier Bay, Alaska.

Hand-sections were cut from fresh material and stained with cotton blue. For microtome sectioning small pieces of nodule material (0.5 mm) were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.0) at 4°C, usually for overnight, after which the material was rinsed in several changes of the buffer alone. Further fixation treatment was given by immersion for 2-4 hr at room temperature in the same buffer now containing 1% osmic acid. After washing in distilled water the material was dehydrated in an alcohol series, and embedded in either Araldite or Epon 812 overnight at 60°C. Blocks were sectioned cold with an LKB Ultratome, using a glass knife. Sections 2 μm thick were cut for light microscope study, and 0.05 μm thick for electron microscope study.

Sections for the light microscope were in some cases stained with toluidine blue, washed in distilled water and mounted in Canada balsam; in others the sections were mounted unstained in the Zeiss L25 medium for phase contrast

microscopy.

For the electron microscope study sections which had been collected on a copper grid were dried on filter paper and stained first with 2% aqueous uranyl acetate for 1 hr and, after washing, with Reynold's lead citrate for 15 min. The sections were observed under an AEI EM6B type electron microscope equipped with a plate camera.

OBSERVATIONS MADE

Effective Nodules of *Myrica* Species

By effective is meant nodules normally active in nitrogen fixation, i.e. those induced by the normal endophyte or by the endophytes of certain other *Myrica* species, as shown in Chapter II.

Figure 26 shows the general structure of a lobe of a nodule of *M. faya* containing the normal endophyte. It will be noted that the endophyte is confined to a layer 1-2 cells deep, situated a little beyond the middle point in the cortex, so that in this respect *M. faya* nodules resemble those of all other *Myrica* species that have been examined (see Introduction) except *M. gale*.

Figure 27 shows part of a section of a nodule as portrayed in Figure 26, now at a higher magnification. The rich contents of the infected cells include endophytic hyphae and club-shaped vesicles arranged radially in the cell as shown, for example, by Fletcher (36) for *M. gale* nodules.

Figure 28 presents a view under the electron microscope through a part of an infected cell, again from a *M. faya* nodule containing the normal endophyte. The endophytic structures are seen in transection, the wider ones being vesicles with a diameter of about 1.5 μm and showing sub-divisions by inner walls, some of which are still incomplete. The narrower structures are hyphae, of diameter about 0.9 μm.

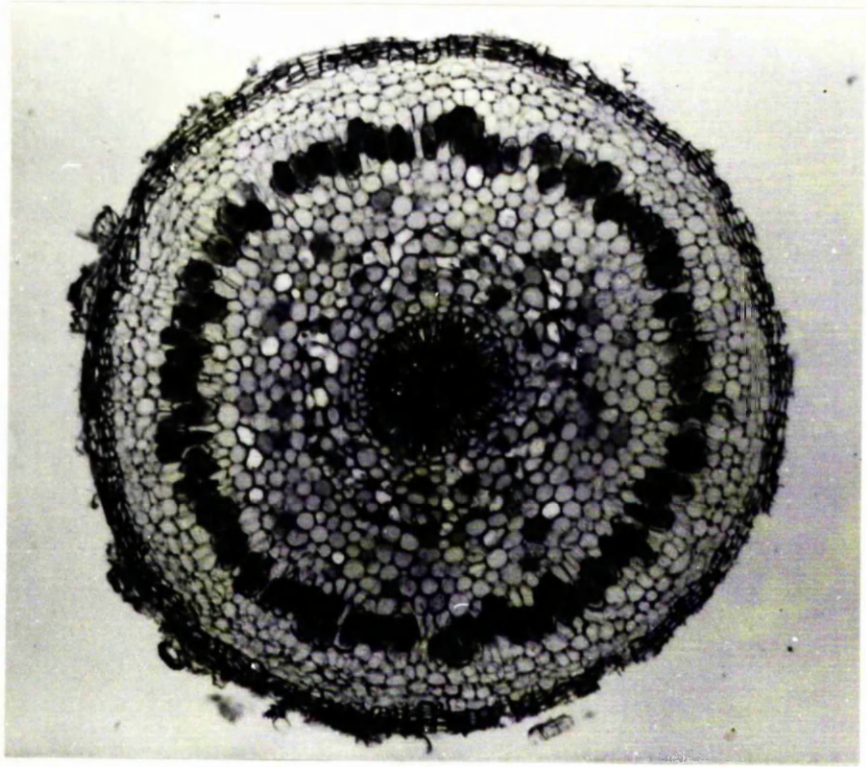


Fig. 26. Hand transection of a Myrica faya nodule lobe infected with its normal endophyte. Within the outer periderm is a deep cortex in which the dark-stained infected cells form a ring 1-2 cells thick. The inner cortex is collenchymatous (x 80).

Fig. 27. Ultra-thin section of part of a Myrica faya nodule lobe infected with its normal endophyte. Within the infected cells are to be seen hyphae and club-shaped vesicles which tend to be arranged radially in the cell. Starch grains are present in adjacent uninfected cells (x 2600).

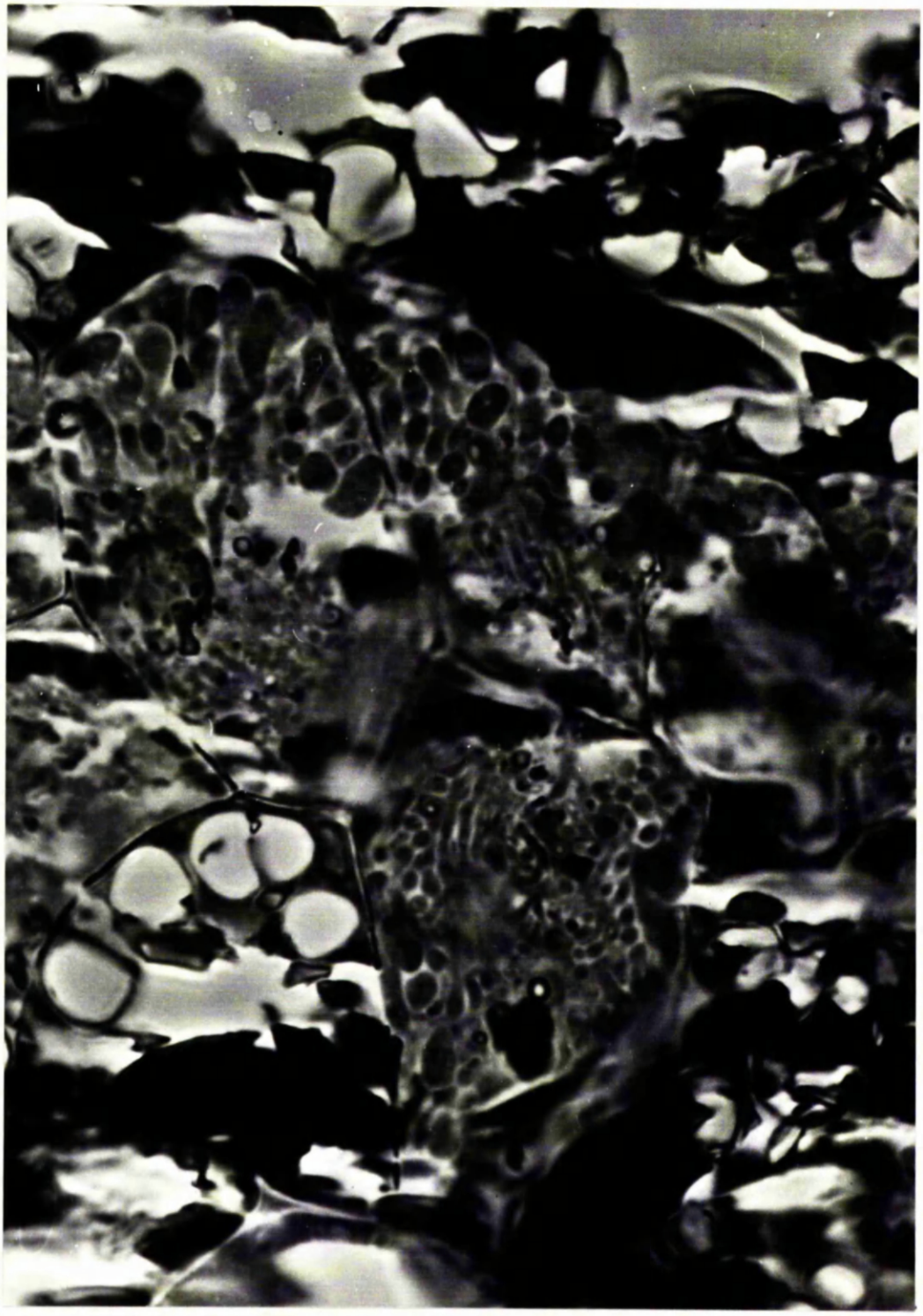


Fig. 28. Electron micrograph through an infected cell of M. faya nodule lobe infected with its normal endophyte, showing hyphae (narrower) and vesicles cut transversely. Internal subdividing walls are present in some vesicles (x 22,000).

Figure 29 shows a few infected cells from a nodule induced to form on M. faya by a M. cordifolia inoculum. The structures seen are obviously very similar to those in Figure 27 (above).

Ineffective Nodules in Myrica Species

This part refers to the numerous, minute and scattered nodules which fix little or no nitrogen, induced to form on several Myrica species by inoculation with the M. gale endophyte.

Figure 30 shows part of a transection through such a nodule taken from a M. faya plant. Although the distribution of the infected cells is the same as in the effective nodule, the endophytic development within the cells is quite different. The radiating club-shaped vesicles are absent, while the hyphae show frequent cross-walls and are mostly confined to the central region of the cell. The infected host cells are narrower than in the effective nodule, another difference being that starch grains are present actually in the infected cells.

Figure 31 shows a similar section of an ineffective nodule taken from a M. javanica plant. Here the hyphae more nearly fill the cells than in Figure 30 and the host cells are larger, but again the hyphae show numerous internal septa and appear stunted, while no vesicles are to be seen. Sections of ineffective nodules from M. caroliniensis showed similar features to those in Figures 30 and 31.

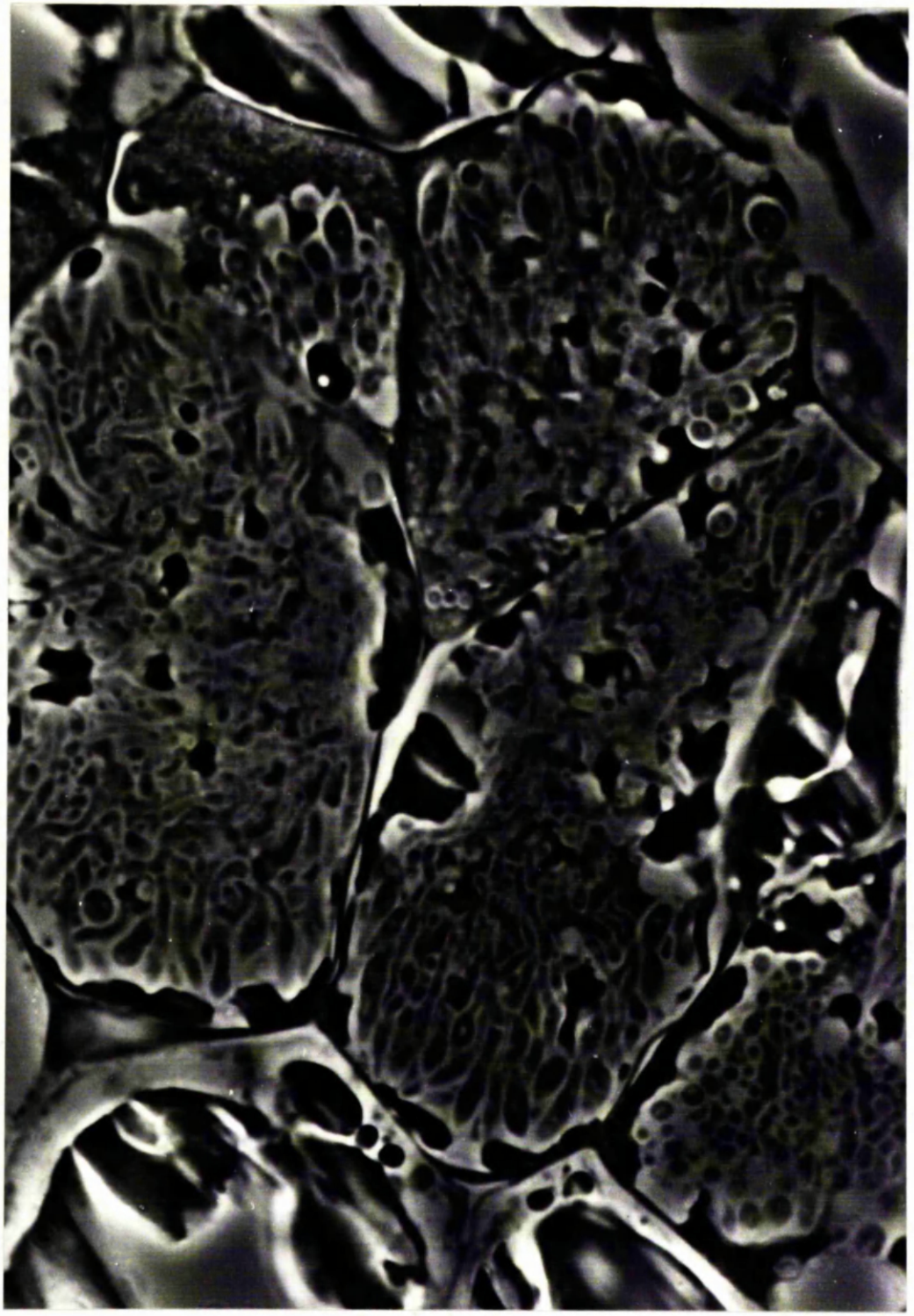
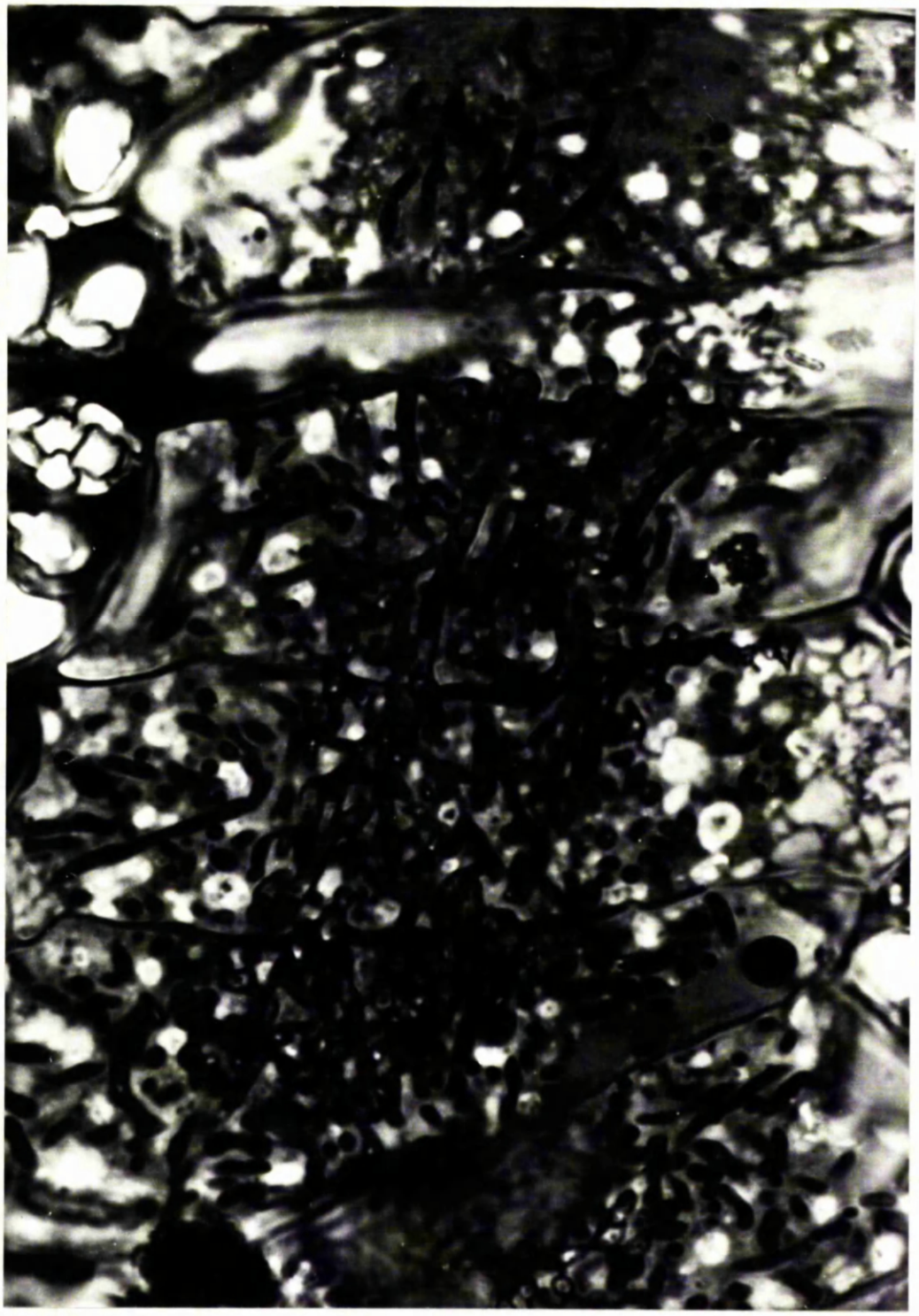


Fig. 29. Similar section to that shown in Figure 27 except that here the endophyte is that from M. cordifolia nodules. The features seen are the same as in Figure 27 (x 2600).

Fig. 30. Ultra-thin section through lobe of Myrica faya nodule infected with the M. gale endophyte, i.e. an ineffective nodule. Several infected cells and small parts of adjacent uninfected cells are included. In the former, hyphae are prominent, but no vesicles are present (x 2600).



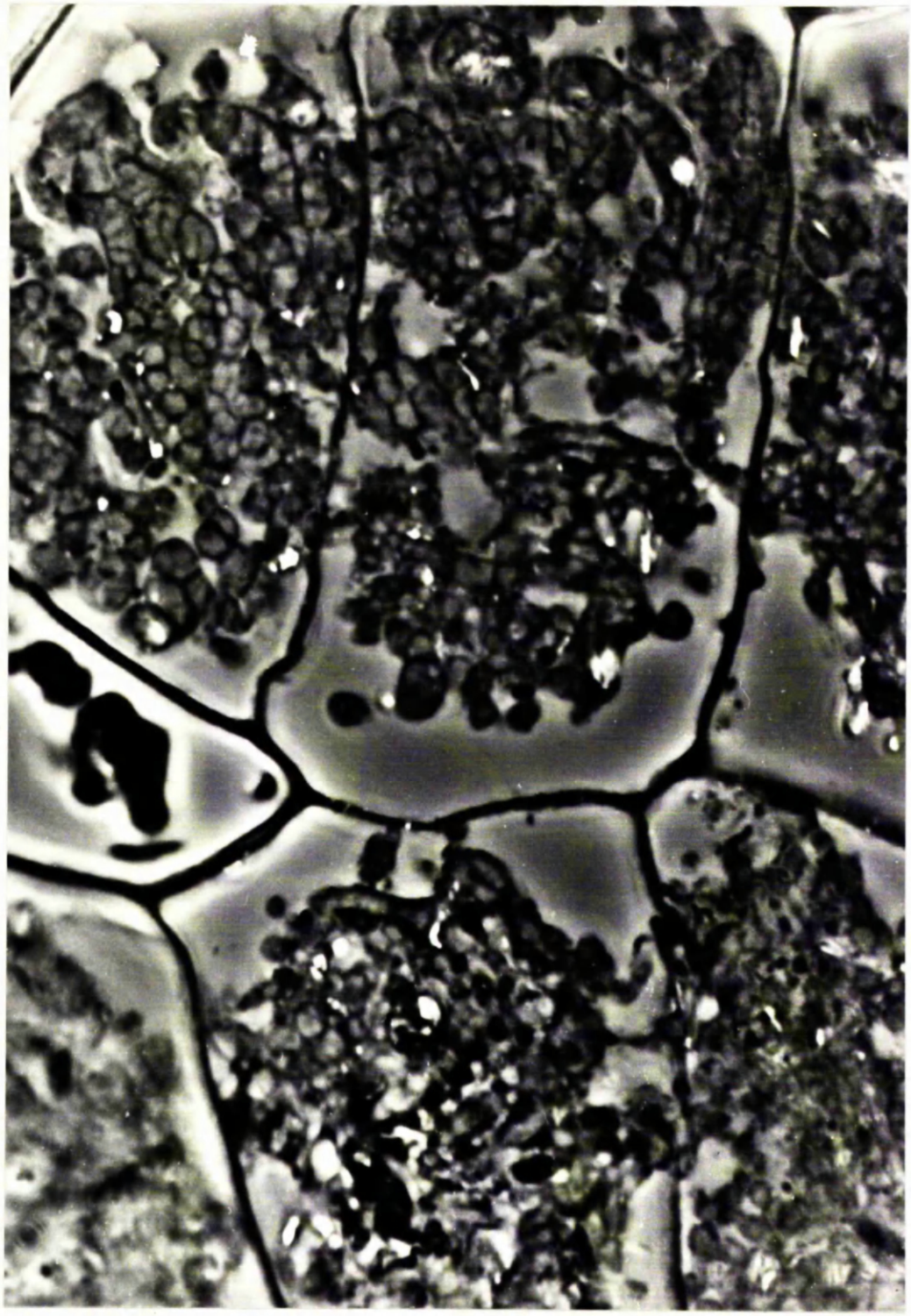


Fig. 31. Ultra-thin section similar to that in Figure 30 except this ineffective nodule is from the M. javanica plant. Again the hyphae are highly septate, while vesicles are absent (x 2600).

Nodules of Dryas drummondii

Figure 32 shows a transection of a nodule lobe. The appearance agrees with that described by Quispel (see Introduction) in that the infected cells are very much hypertrophied and occur in the outer half of the cortex. The uninfected cells are seen to be starch-laden, while the periderm is much thinner than in most other non-legume nodules.

In Figure 33 is shown part of an infected cell, hyphae and vesicles being visible, while Figure 34 shows the dense mass of hyphae from a single burst host cell in a squash preparation. Vesicles are visible, especially round the periphery.

Figures 35 and 36 are photographs taken under the electron microscope, showing endophytic structure; as noted earlier, the nodules used had not been specially fixed for E.M. study, and the clumping of the endophytic contents is to be attributed to that. In Figure 35 are seen hyphae in lengthwise and transverse view. They are of diameter 0.3-0.5 μm and show no cross-walls in this particular picture. Figure 36 shows vesicles cut mostly lengthwise; one includes part of the bearing hypha, while the vesicles are pear-shaped, their length 3-5 μm, diameter 2-3 μm.

Nodules of Purshia tridentata

Figure 37 shows a transection , and the appearance is extremely similar to that in Figure 32 above and shows the

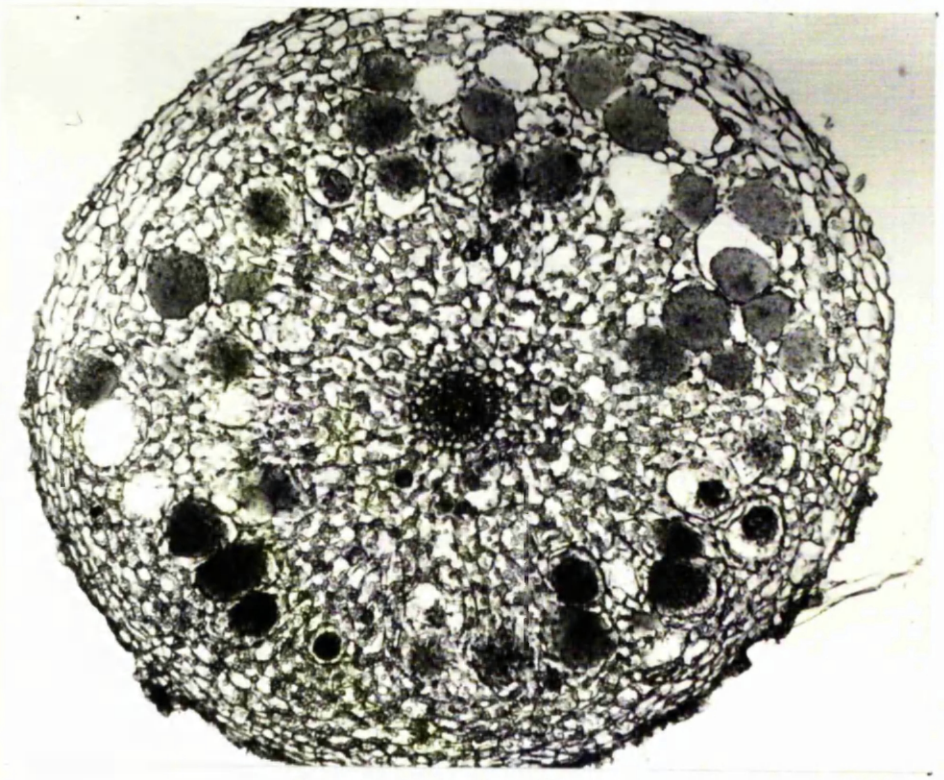


Fig. 32. Hand section of a Dryas drummondii nodule lobe. The highly hypertrophied infected cells with dark-stained contents lie in the outer cortex. The inner cortex is starch-laden. Periderm is very thin (x 80).

Fig. 33. Ultra-thin section of Dryas drummondii nodule lobe, showing part of a large infected cell. Hyphae and vesicles are seen (x 2600).

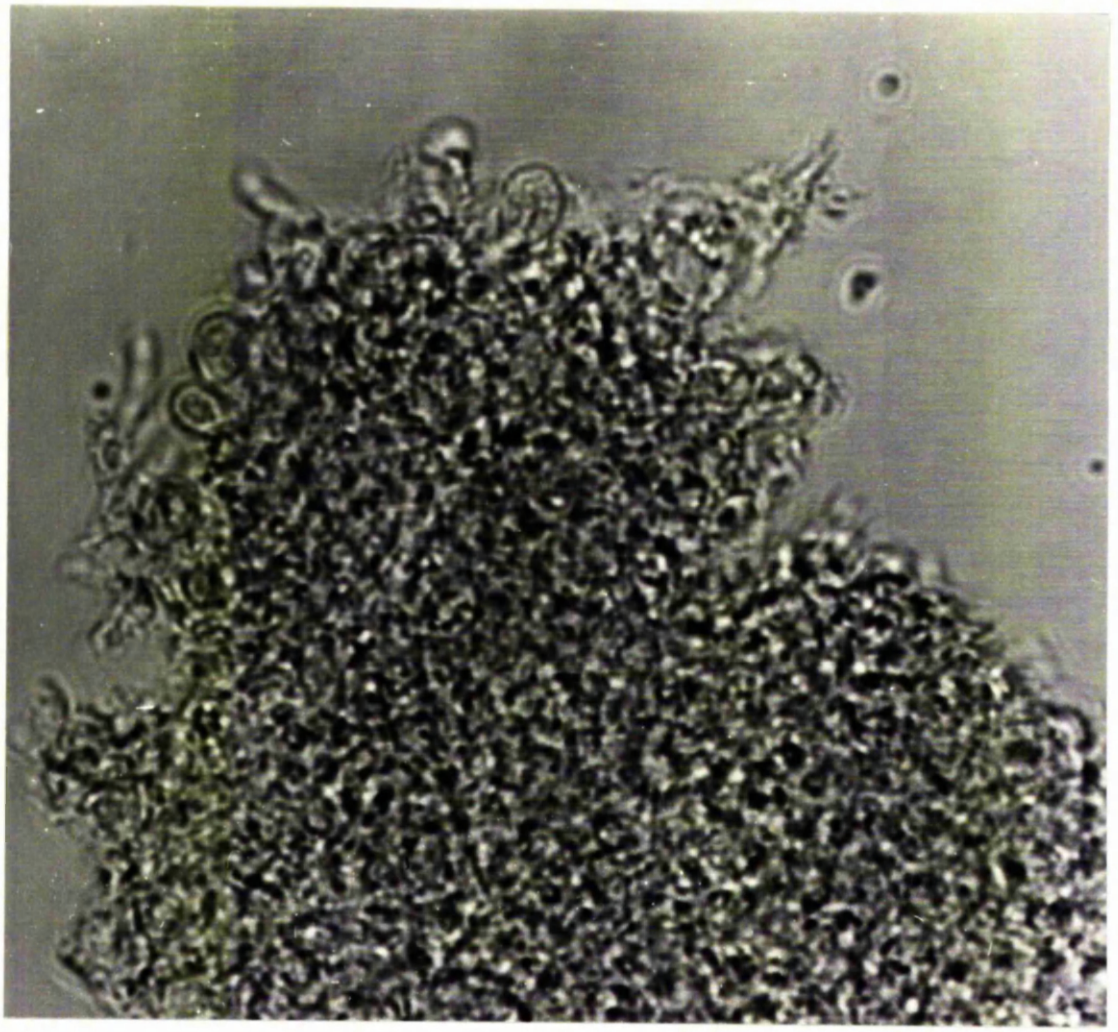


Fig. 34. Squash preparation from Dryas drummondii nodule lobe showing the dense mass of hyphae extruded from a single burst cell, with some vesicles visible round the periphery (x 2600).

Fig. 35. Electron micrograph of part of an infected cell from Dryas drummondii nodule showing hyphae in lengthwise and transverse view (x 33,000).

Fig. 36. Electron micrograph of part of an infected cell from Dryas drummondii nodule showing the pear-shaped vesicles, mostly cut lengthwise. Part of the bearing hypha is included in one case (22,000).

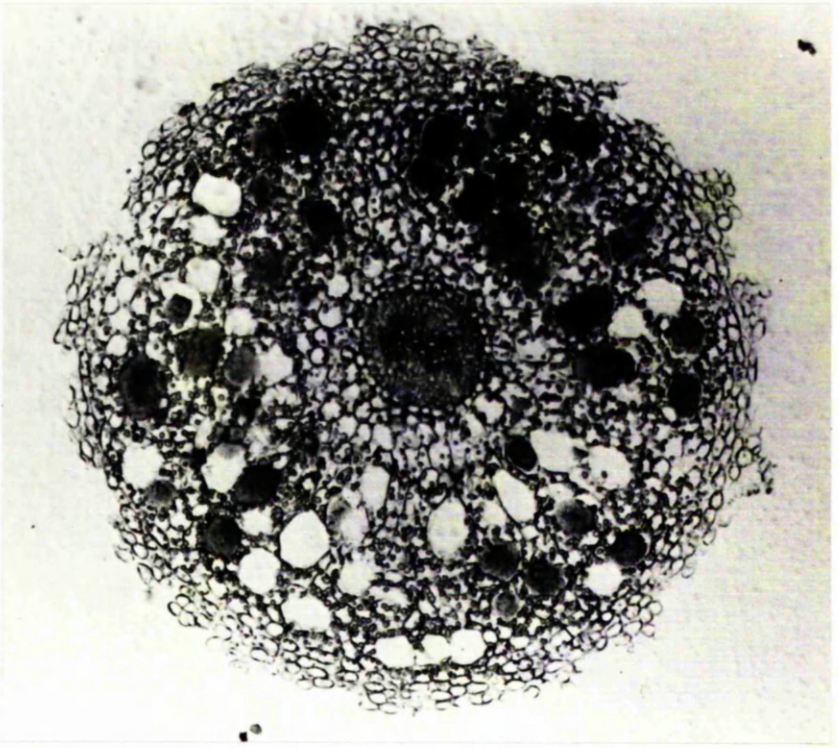


Fig. 37. Hand transection of a Purshia tridentata nodule lobe, showing numerous very large infected cells in the cortex and the lack of a clear periderm (x 80).

same distribution of the infected cells and the lack of a clear periderm. In the preparation of material of these nodules for microtoming much difficulty was encountered in securing proper infiltration of the fixative into the tissues, and the fixation was not good. Figure 38 shows an infected cell, obviously considerably shrunken (cf. Fig. 37), but showing vesicles (diameter about 3.0 μm) and traces of hyphae. Cells filled with bacteroids or granula were also observed, one being shown in Figure 39, while in Figure 40 a few of the bacteroids are seen under the electron microscope; they are about 1 μm in length and 0.5 μm in width and show translucent enclosures which according to Gardner (6) are typical of bacteroids from other non-legume nodules and may be food reserves.

Nodules of Coriaria myrtifolia

The transection shown at two magnifications in Figures 41 (A and B) indicates that as in the previously-described nodules of other species in the genus (see Introduction) in C. myrtifolia also the stele is situated excentrically, with the infected tissue forming a kidney-shaped area in which - as noted by Shibata & Tahara (34) in C. japonica - practically every cell is infected, unlike the situation in all other non-legume genera. The stele lies in starch-filled tissue. An inner layer of the thick periderm detaches itself and approaches the endodermis, thus sealing off the infected area. Also visible in the figures is the vacuolated nature of the infected cells.

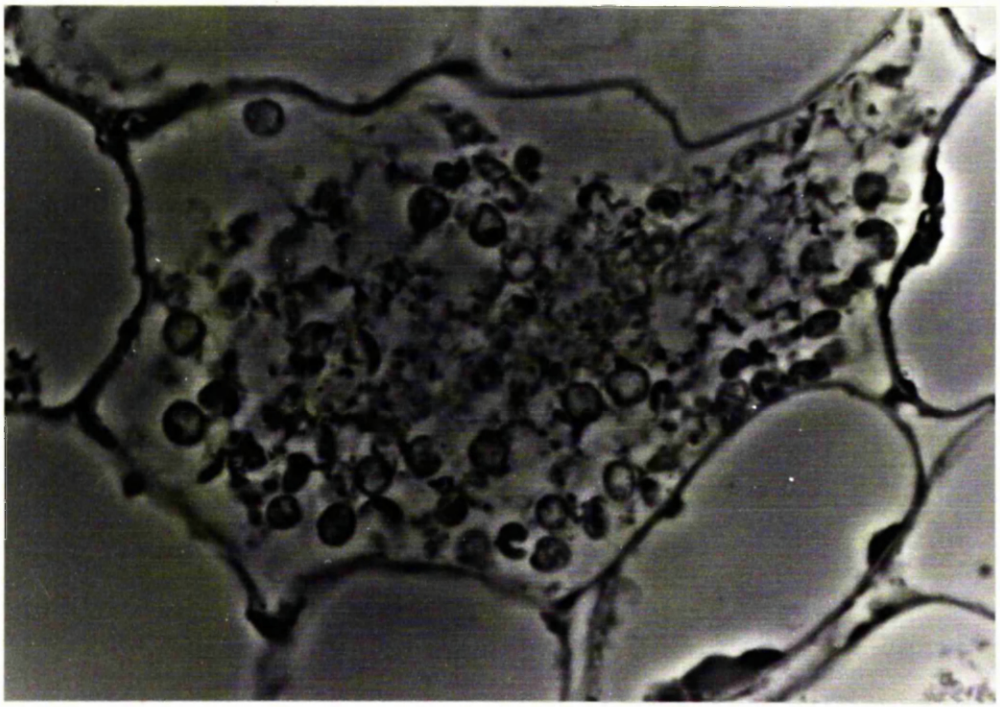


Fig. 38. Ultra-thin transection of a Purshia tridentata nodule lobe, showing an infected cell with hyphae and spherical vesicles (x 1800).

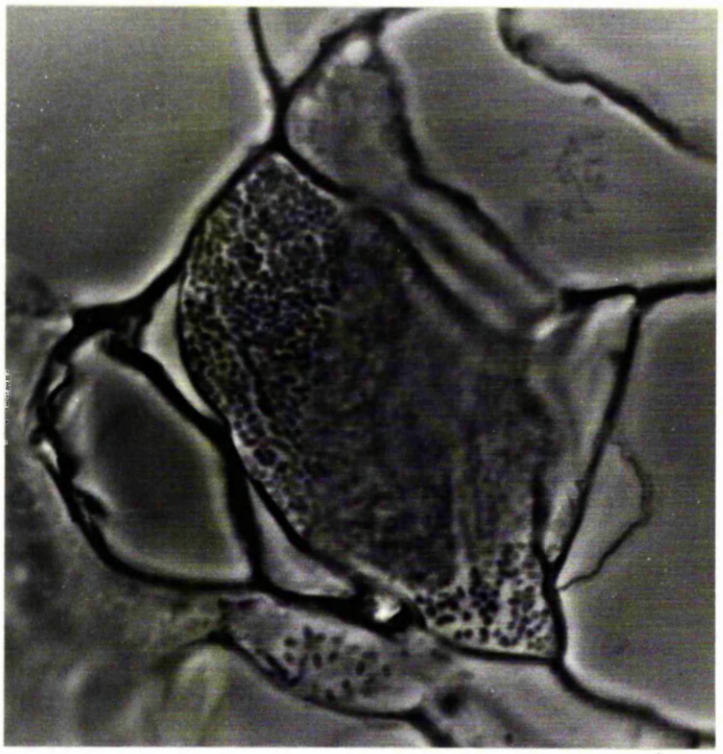
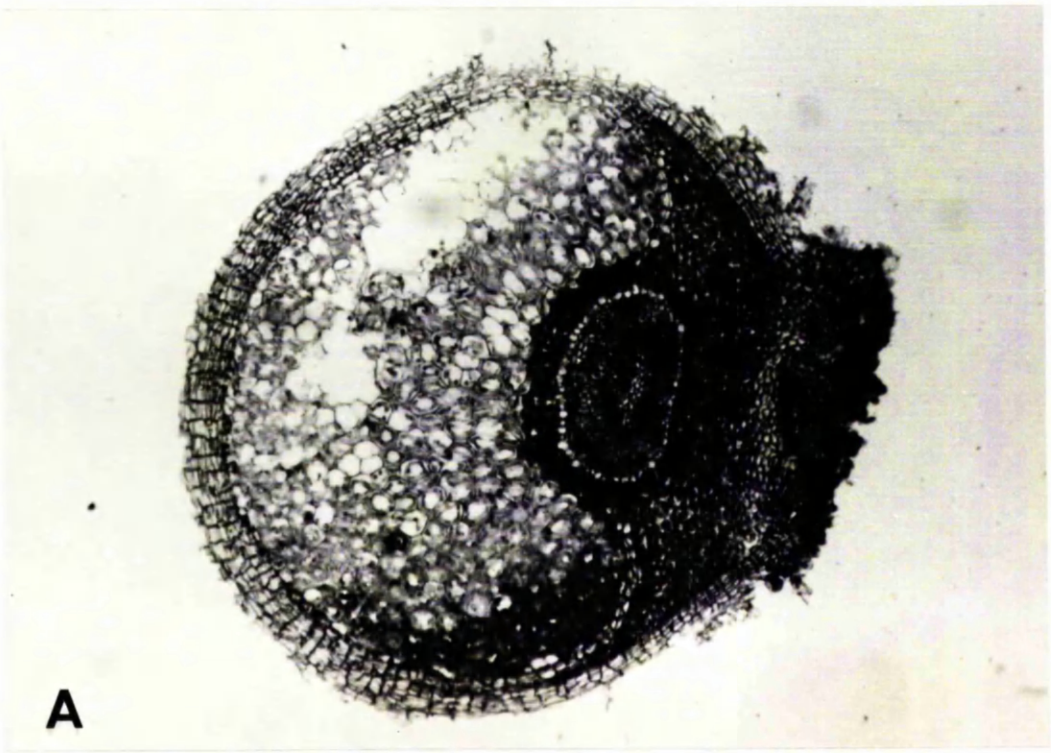
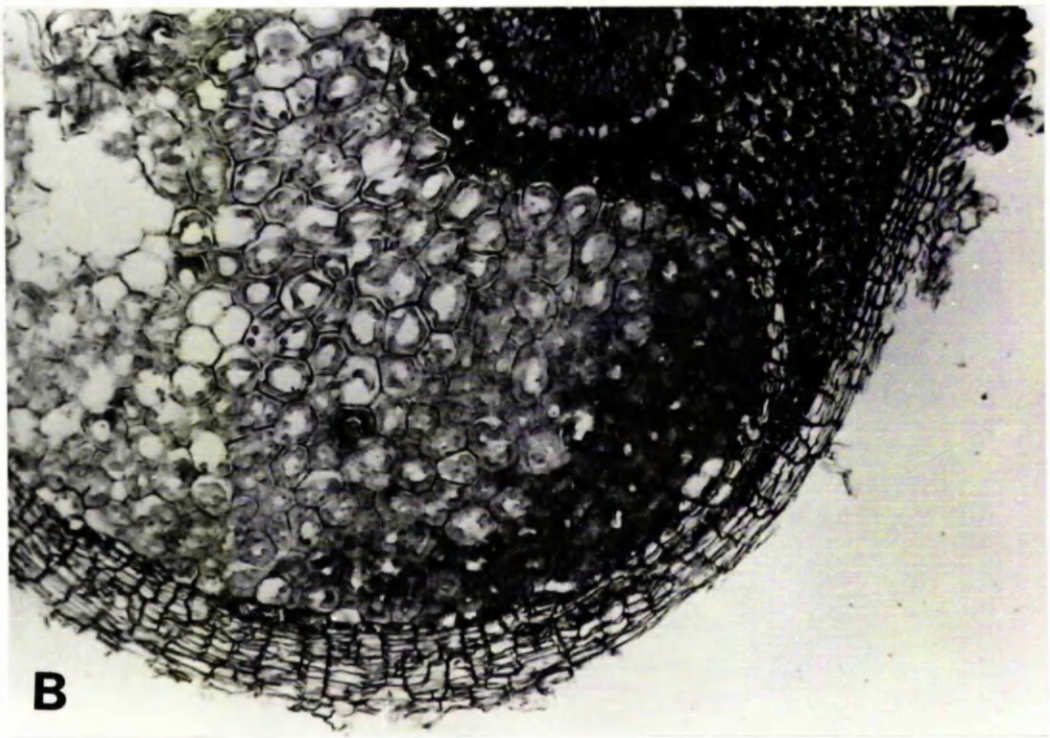


Fig. 39. Cell filled with bacteroids from ultra-thin transection of Purshia tridentata nodules (x 1800).

Fig. 40. Electron micrograph showing bacteroids in an infected cell of Purshia tridentata nodule (x 30,000).

Figs 41 (A & B). Hand transection of Coriaria myrtifolia nodule lobe, showing excentric stele, kidney-shaped area of vacuolate infected cells, and deep periderm the inner layer of which connects up with the endodermis (A x40, B x80).


A


B

Unfortunately unsatisfactory fixation was experienced in this genus also, and the limited supply of nodules prevented any experimenting with alternative fixatives. The sections obtained were unsuited to photography, but in Figure 42 a diagrammatic drawing of an infected cell under high power is provided, and shows how - as noted by Shibata & Tahara (34) for C. japonica - the original base for hyphal development is against the host cell wall, and that the club-shaped vesicles eventually formed are arranged in a close-packed layer directed away from the cell wall and towards the vacuolar membrane. In the preparations available there was no indication that the vesicles are sub-divided by internal walls. Shibata & Tahara (loc. cit.), also on the basis of light microscope examination show them as free of sub-divisions.

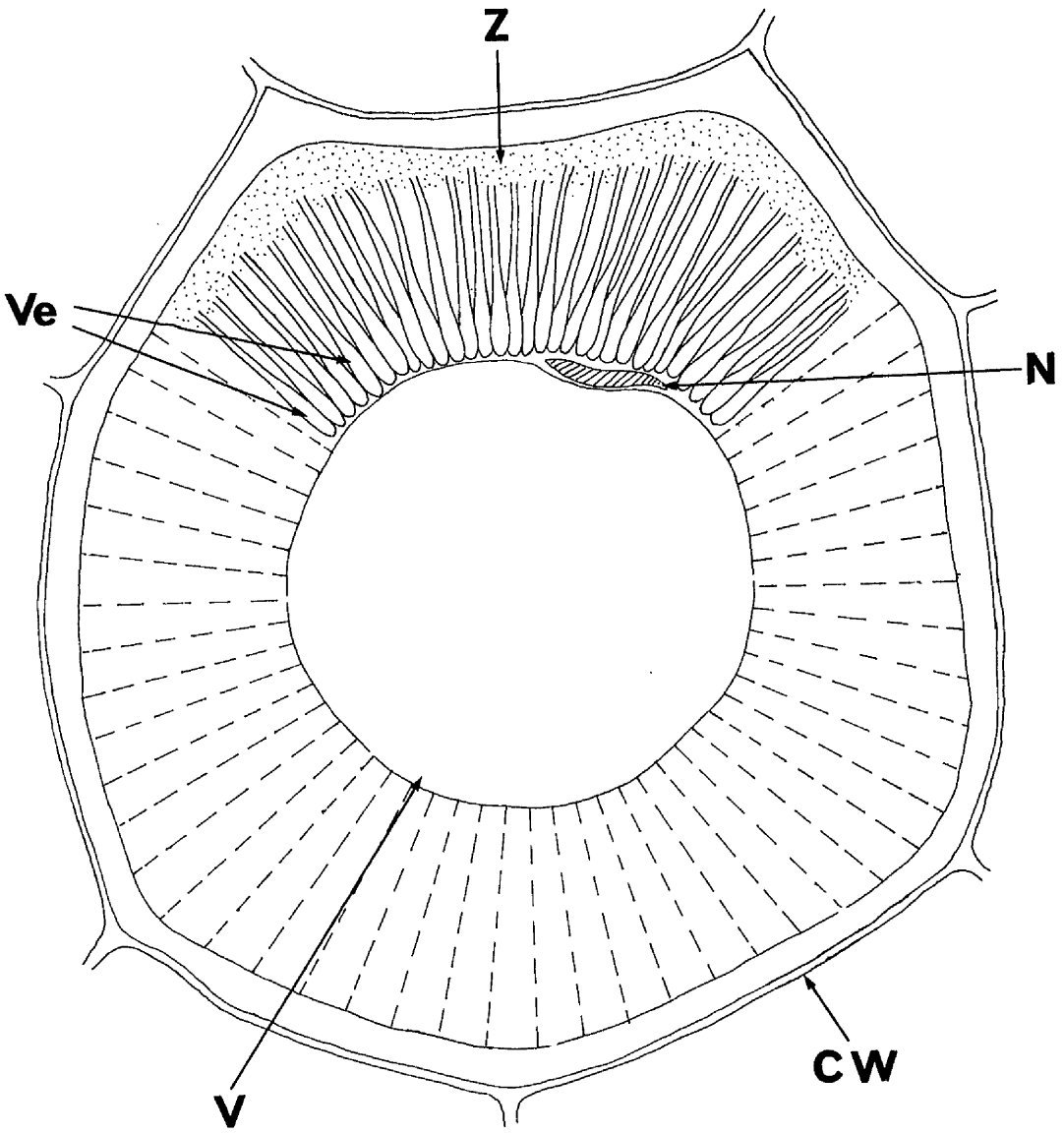


Fig. 42. Diagram of median section through infected cell of Coriaria myrtifolia, showing zone with dense hyphal growth (Z), club-shaped vesicles formed by enlargement of inward directed hyphae (Ve), cell nucleus (N), central vacuole (V), and host cell wall (CW) (x 2,080).

DISCUSSION

The distribution of the infected cells in nodules induced to form on Myrica faya by the normal endophyte or by unusual endophytes is shown to be of the M. rubra type, i.e. the endophyte is confined to a narrow central layer in the cortex. It is also shown that the vesicles which form in effective nodules of M. faya are internally sub-divided, thus adding a third species to the two already shown by Gardner (6) - see also Introduction - to have this feature. Bacteroids were not seen in M. faya sections.

The chief finding emerging from the structural study of the ineffective nodules induced to form on several other Myrica species by the M. gale endophyte, is the failure to detect any production of vesicles by the endophyte in its unusual environment. If it is assumed that the endophytic hyphae in Myrica are normally nitrogen-fixing, but not when they lie in unusual host cells, then the failure to produce vesicles might be simply due to extreme nitrogen deficiency. It would be more in keeping with the conclusions in Chapter I for alder to believe that in Myrica also the vesicles are the site of fixation, and that it is their failure to develop in the ineffective nodules that is responsible for the lack of fixation.

Another property of non-legume nodules which has been thought to be associated with a particular morphological part of the endophyte is their frequent ability, in the form of a crushed nodule inoculum, to induce nodulations in fresh seedlings of the appropriate host species. There

is some evidence, based mostly on tests with Alnus glutinosa, that the bacteroids or granula are the infective bodies which are active in nodule inocula, and which under natural conditions survive the eventual decay of the nodule and maintain infectiveness in the soil. The evidence of this in alder is by no means conclusive. Thus Akkermans & van Dijk (8) found that nodule inocula prepared from particular nodules which were devoid of bacteroids - one strain of the alder endophyte produces such nodules - still had infective power, though that of inocula made from nodules containing bacteroids was 100 - 1,000 times greater.

In Myrica gale the occurrence of bacteroids or granula was seen by Shibata & Tahara (34) and again by Schaede (10), while Gardner observed them in M. cerifera. Schaede noted that in Myrica gale again some nodules were devoid of bacteroids, but no tests have been made on the infectivity of such nodules. As noted, the present writer saw no bacteroids in any of the Myrica nodules sectioned, though no intensive search was made for them. It was, however, thought to be of interest to find whether the nodules - ineffective in fixation - induced to form on other Myrica species by the M. gale endophyte had any ability, in the form of the usual crushed-nodule inoculum, to infect the original host plant, i.e. M. gale. This question arose in the writer's discussion with Dr Wheeler. A back cross-inoculation was carried out, using ineffective nodules from M. pensylvanica. The nodules were shaken for 1 min in 0.1% $HgCl_2$ in 0.05N HCl before being crushed in distilled water in the proportion of 1 g to 100 ml water.

Fifteen young plants of M. gale in water culture were inoculated with this preparation, but bore no nodules when finally inspected after 11 weeks. Other test plants of M. gale, treated with an inoculum made from nodules taken from stock plants of M. gale and shaken in the mercuric chloride for the normal time of 4 min, nodulated normally. However, it cannot be finally concluded that the ineffective nodules were also non-infective, since even the shortened sterilisation time which they received may have killed the endophyte, because of the minute size of the nodules and their feebly developed periderm. On the other hand, if a still briefer sterilisation had been given, and nodulation of test plants had been secured, this could have been attributed to the presence of normal infective bodies of the M. gale endophyte present as a surface contaminant, and surviving from the original inoculation of the M. pensylvanica root systems. Thus it is only a possibility that the ineffective nodules are also non-infective; if they are, the reason could be either that some particular infective structure such as the bacteroids is not present, or that the general weakness of the endophyte as a result of extreme nitrogen starvation was responsible.

The account now given of the structure of Dryas drummondii nodules confirms and adds further detail to the one previous account, including that the vesicles are not internally divided. The observations now presented on Purshia nodules are the first to be reported, and though less complete than had been hoped they show that

the endophyte is finely hyphal in nature and bears spherical vesicles of the usual dimensions for non-legume endophytes, while bacteroids are also produced. In their general structure the nodules of Dryas and Purshia are, as noted, extremely similar in having particularly large infected cells and a thin periderm. As indicated in the Preface (Table 2) those two genera are placed by Engler in the tribe Dryadeae of the Rosaceae. The third member of that tribe known to bear nodules is Cercocarpus, and the brief account of the structure of its nodules provided by Hoeppel & Wollum (37), on the basis of a light microscope study, shows that it also isvery similar to that in the two other genera.

The observations now presented on the nodules of Coriaria myrtifolia show that the very unusual features previously described for C. japonica and C. arborea are also present in C. myrtifolia. It is a pity that so little attention has been paid to the nodules in this genus, the main reason for this being that most species grow in regions remote from the main centres of nodule studies. It is of some interest that the general structure of the rhizobial root nodules recently found on a species of the non-legume Trema (Trinick, 1, see also Preface to this thesis) resembles that of Coriaria nodules.

SUMMARY

1. By means of hand sections, ultra-thin sections examined under the light microscope, and observations under the electron microscope, the structure of various types of non-legume nodules and of their endophytes has been studied. The types studied had received little or no previous examination.

2. The general structure of the normal *Myrica faya* nodules is shown to resemble that previously described for the nodules of other *Myrica* species, except *M. gale*. It is shown that the club-shaped vesicles are internally sub-divided.

3. The examination of the abnormal nodules, ineffective in nitrogen fixation, induced to form on several other species by the *M. gale* endophyte, shows that the endophytic hyphae are of unusual appearance and arrangement, and that no vesicles are present. It is possible that their absence is the reason for the lack of nitrogen fixation. Some evidence is provided that these nodules also lack infective power.

4. The account given of the structure of *Dryas drummondii* nodules confirms and adds to the one previous account, including that the vesicles are not internally sub-divided.

5. The study now made for the first time of *Purshia* nodules shows that they contain a finely-hyphal endophyte which produces vesicles and bacteroids.

6. The examination of Coriaria myrtifolia nodules shows that they resemble the previously described nodules of two other Coriaria species, and thus display the same deviations from the standard non-legume nodule type.

CHAPTER IV

A Study of Ploidy in the Meristems of Some Non-Leguminous Root Nodules

CONTENTS

INTRODUCTION

In leguminous root nodules, as long ago as 1908 Fred (38) reported that in many species there were indications of an unusual cytological situation in that the mitotic figures in the nodule meristem were much larger than in the root tip in the same species, and that more chromosomes seemed to be present, though he was unable to make actual counts. Little further attention was paid to the matter until thirty years later when Wipf & Cooper (39) and Wipf (40) reported that in a wide range of legumes used in American agriculture, sections through the nodule meristem revealed that the dividing cells were mostly tetraploid, as compared with the diploid root tips. This implies that the mature tissues of the legume nodule are also composed of tetraploid cells. The authors pointed out that there were no aberrant features in mitosis in the meristematic cells, such as absence of a spindle, formation of multipolar spindles, or irregularities in chromosome numbers, which do occur in pathological tumerous growths in plants and animals. In a further study Wipf & Cooper (41), in seeking to explain the tetraploidy in the nodules, reported that occasional tetraploid cells were present in the cortex of the roots of several legumes, unexposed to rhizobia. When rhizobial infection threads came into the vicinity of such tetraploid cells, the latter were stimulated into meristematic divisions, thus initiating the nodules. The tetraploidy in the nodules, it was concluded, was not induced by Rhizobium, but was due to pre-existing

tetraploid cells in the roots. Wipf & Cooper pointed out that tetraploid cells had been observed in the roots of many plants, not only legumes.

Subsequently several workers confirmed the tetraploid nature of the meristematic cells in many legume nodules, but Tatuno & Kodama (42) and Kodama (43, 44) in various legumes not examined by previous workers, such as broad bean, soya bean, and ground nut, found only diploid cells in the nodule meristems. Thus a confusing situation exists as regards legume nodules. Tetraploidy does not appear to be necessary for successful symbiosis. Moreover, even in those species where tetraploidy exists in the nodules, it has been questioned whether the number of tetraploid cells in the mother roots is adequate to explain the number of nodules formed.

Kodama (45, 46) was the first to examine the cytological situation in non-legume nodules. In squash preparations of the nodules of the Japanese species Alnus pendula, A. hirsuta and A. sieboldiana he found 28, 56 and 112 chromosomes respectively in the dividing cells, which are the diploid numbers for these species.

In the study now to be described, comparisons of chromosome numbers in nodules and root tips of some further non-legumes have been made.

MATERIALS AND METHODS

The species studied were Alnus glutinosa, Myrica gale, Myrica faya, Myrica cerifera, Hippophaë rhamnoides L. and Casuarina cunninghamiana Miq. Nodules and root tips were taken from plants of these species growing in the greenhouse in water culture or in Peralite, in both cases without combined nitrogen.

As compared with serial sectioning, the squash technique was preferred because of its rapidity and the larger number of mitotic figures which a single preparation provides. A range of fixatives and stains was tried before the two following techniques were selected, one for use for certain species and the other for the remainder.

Technique A

This was based on the procedure of Dyer (47). The nodule tips and root tips were fixed in modified Carnoy's fluid (ethanol, acetic acid, chloroform, formalin in proportions 10:2:2:1) for 5 min and then macerated in 1N HCl at 60°C, the length of the maceration varying from 5 to 15 min with different material. The specimens were then stained in lacto-propionic orcein (2 g orcein dissolved in 50 ml each of lactic and propionic acids) for 2 min, mounted on a slide in 50% lactic acid and squashed under a coverslip, with warming if necessary. The coverslip was then sealed with nail varnish. With nodules the outer cork was teased away before squashing.

Technique B

In a few instances the above technique failed to clear the cytoplasm sufficiently, and for these a different procedure was adopted, based on the technique of Snow (48), in which the material was fixed in acetic-alcohol (acetic acid, 95% ethanol in proportions 1:3) for 24 hr and then washed thrice in 70% ethanol at 1 hr intervals. After draining on absorbent paper the specimens were placed in a liberal quantity of hydrochloric acid-carmine (4 g carmine dissolved in 50 ml distilled water and 1 ml conc. HCl by boiling gently, then cooling and adding 95 ml of 85% ethanol and filtering) for 24 hr, and subsequently washed in 70% ethanol in which reagent they were also stored if required. Microscopic preparations were made by macerating specimens in 45% acetic acid at 60°C for 5 min, then mounting on a slide in the same fluid, squashing and sealing as before.

For each of the species over 100 root and nodule specimens were squashed. The slides were studied by phase contrast under a Zeiss Standard WL microscope with attachment for a 35 mm camera. Photographs were taken under oil immersion, with a green filter to increase contrast between the chromosomes and the cytoplasm.

OBSERVATIONS MADE

Since, by choice, no pre-treatment with colchicine or 8-hydroxyquinoline had been given prior to the fixation of the material the spreading of the chromosomes was not very good. Counting presented no great difficulty, but often the chromosomes were not sufficiently in one plane for good photographs to be obtainable.

The numbers of chromosomes found in root tips and nodule tips are shown in Table 14. The numbers recorded were verified in at least 10 preparations in each case except in Casuarina where, at the time when observation was made, mitosis was occurring rather infrequently, so that only about five mitotic figures with a countable number of chromosomes were seen. Darlington & Wylie (49) had previously reported the diploid chromosome complements for the first, second, fourth and fifth species listed in the Table, and Barlow (50) for the sixth species. The present counts agree with these published figures. No previous count on the 2n figure for Myrica faya has been traced. It will also be seen in the Table that the number of chromosomes found in the dividing cells of the nodules agreed with that in the root tips, and is thus the diploid number. No tetraploid cells were detected, neither were any aberrant features such as aneuploidy.

In Figures 43-48 photomicrographs showing the chromosome complement in root tip and nodule are provided for each of the species investigated, together with drawings which are basically tracings from the photomicrographs presented, but which also take into account the appearance

Table 14. Numbers of chromosomes found in dividing cells in root and nodule apices.

Species	Chromosome complement found	
	In root tip	In nodule apex
Alnus glutinosa	28	28
Myrica gale	48	48
Myrica faya	16	16
Myrica cerifera	16	16
Hippophaë rhamnoides	24	24
Casuarina cunninghamiana	18	18

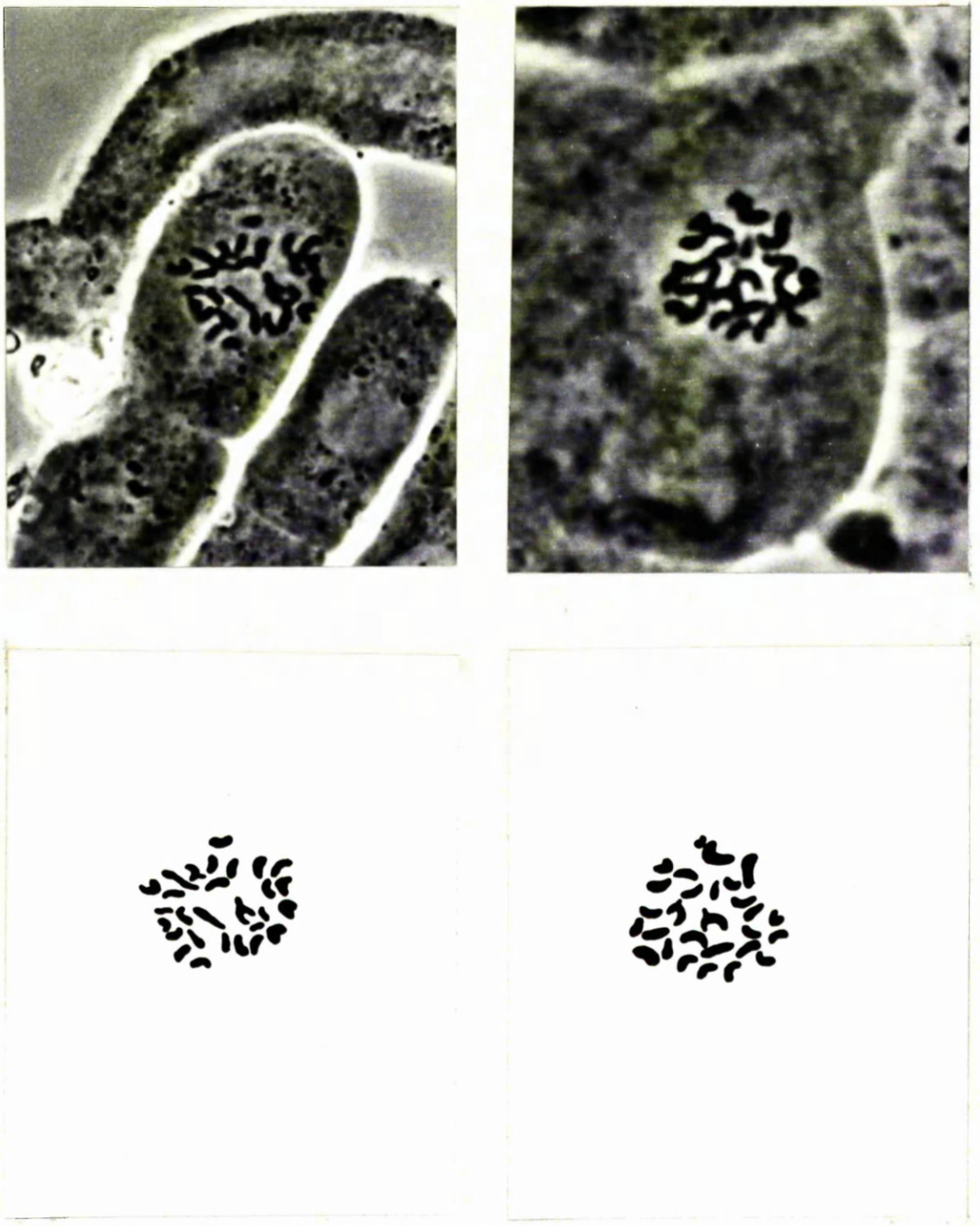


Fig. 43. Above: photomicrograph showing chromosomes in dividing cells in root tip (left) and nodule (right) of Alnus glutinosa (x 3400).

Below: tracings made of the above photographs, root again on left. The number of chromosomes is 28 in both organs.

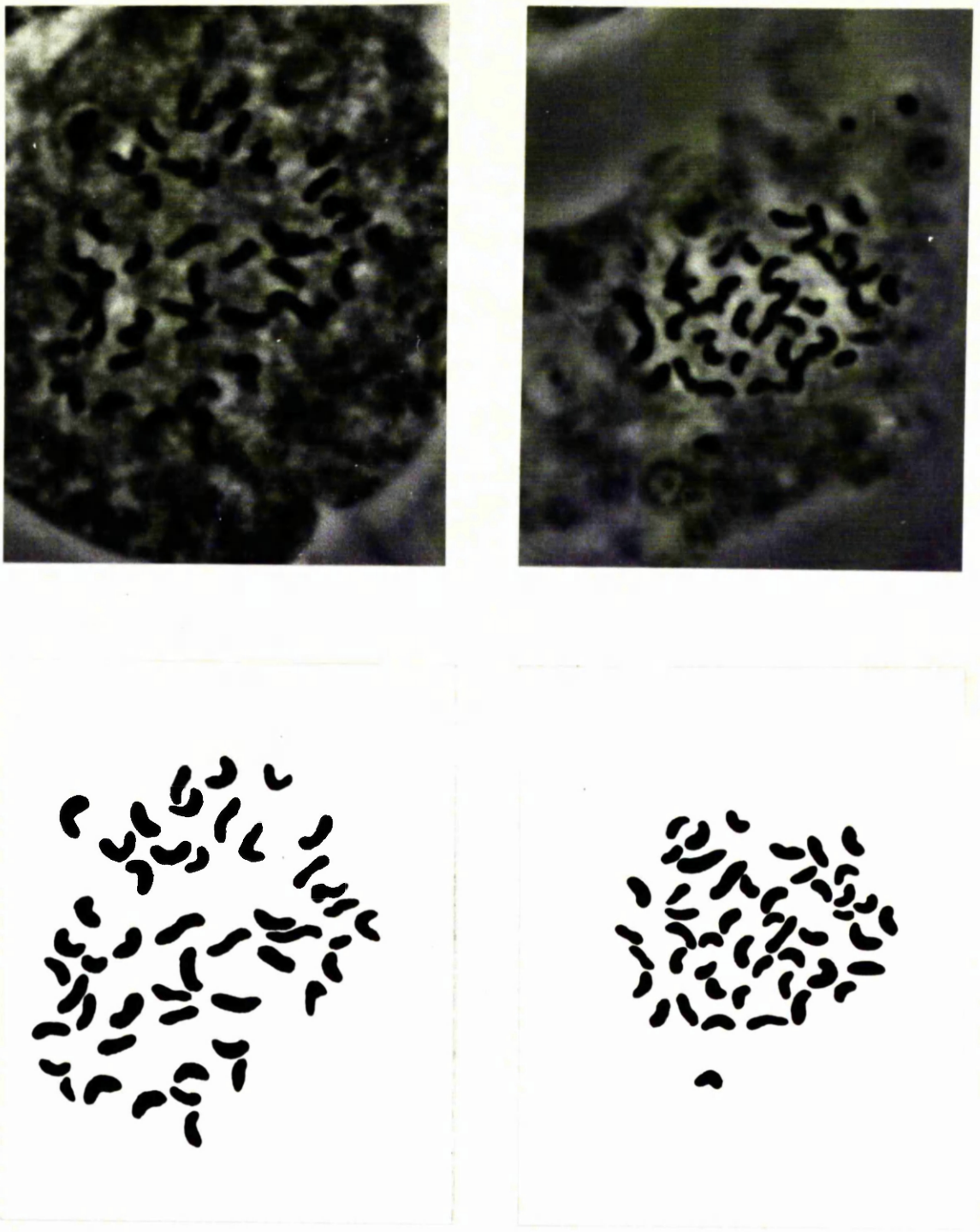


Fig. 44. Above: photomicrographs showing chromosomes in dividing cells in root tip (left) and nodule (right) of Myrica gale (x 3400).

Below: tracings made of the above photographs, root again on left. The number of chromosomes is 48 in both organs.

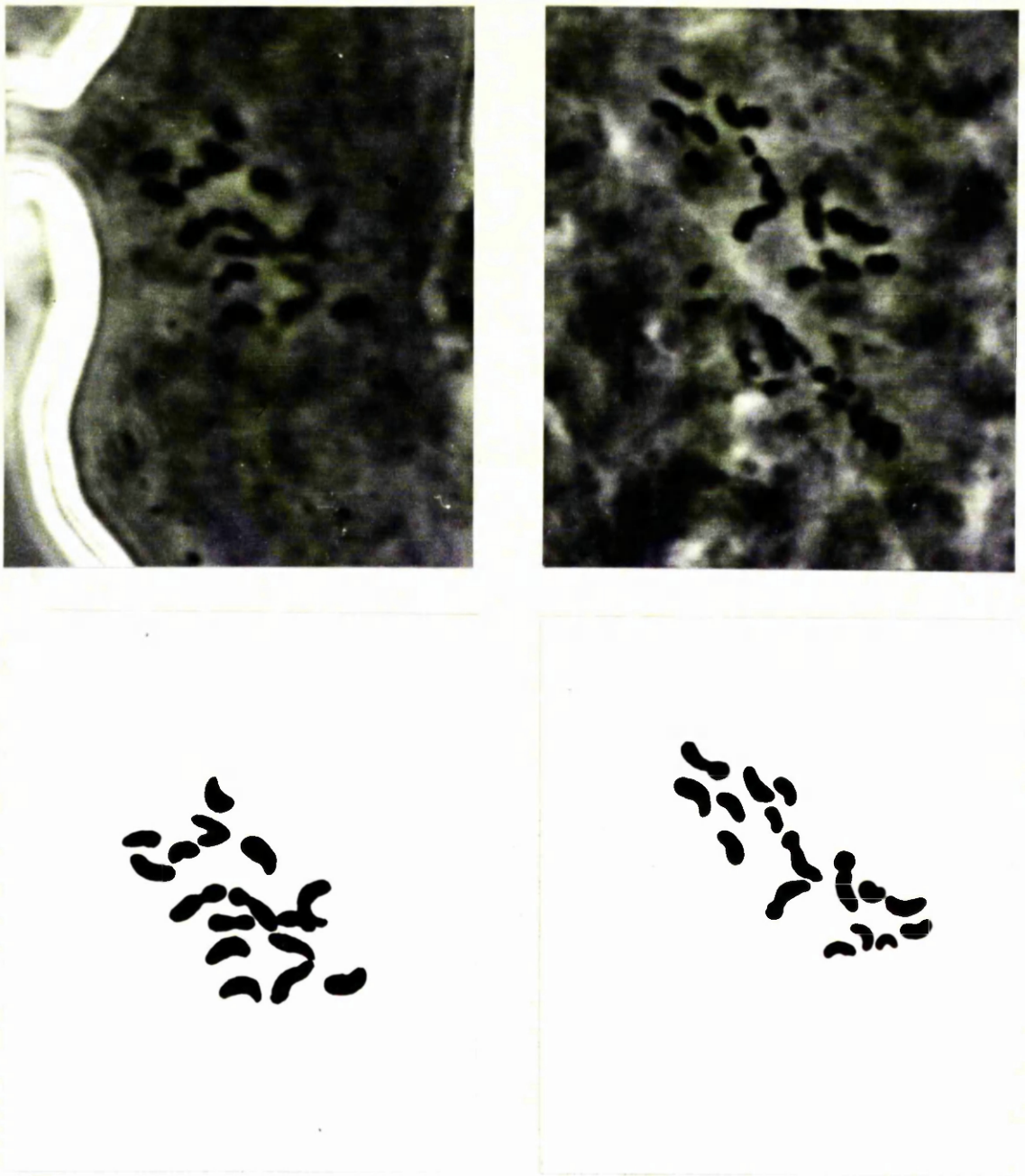


Fig. 45. Above: photomicrographs showing chromosomes in dividing cells in root tip (left) and nodule (right) of Myrica faya (x 3400). In the nodule preparation the chromosomes in two cells are included, those of the upper one being obviously clearer.

Below: tracings made of the above photographs, root again on left. The number of chromosomes is 16 in both organs.

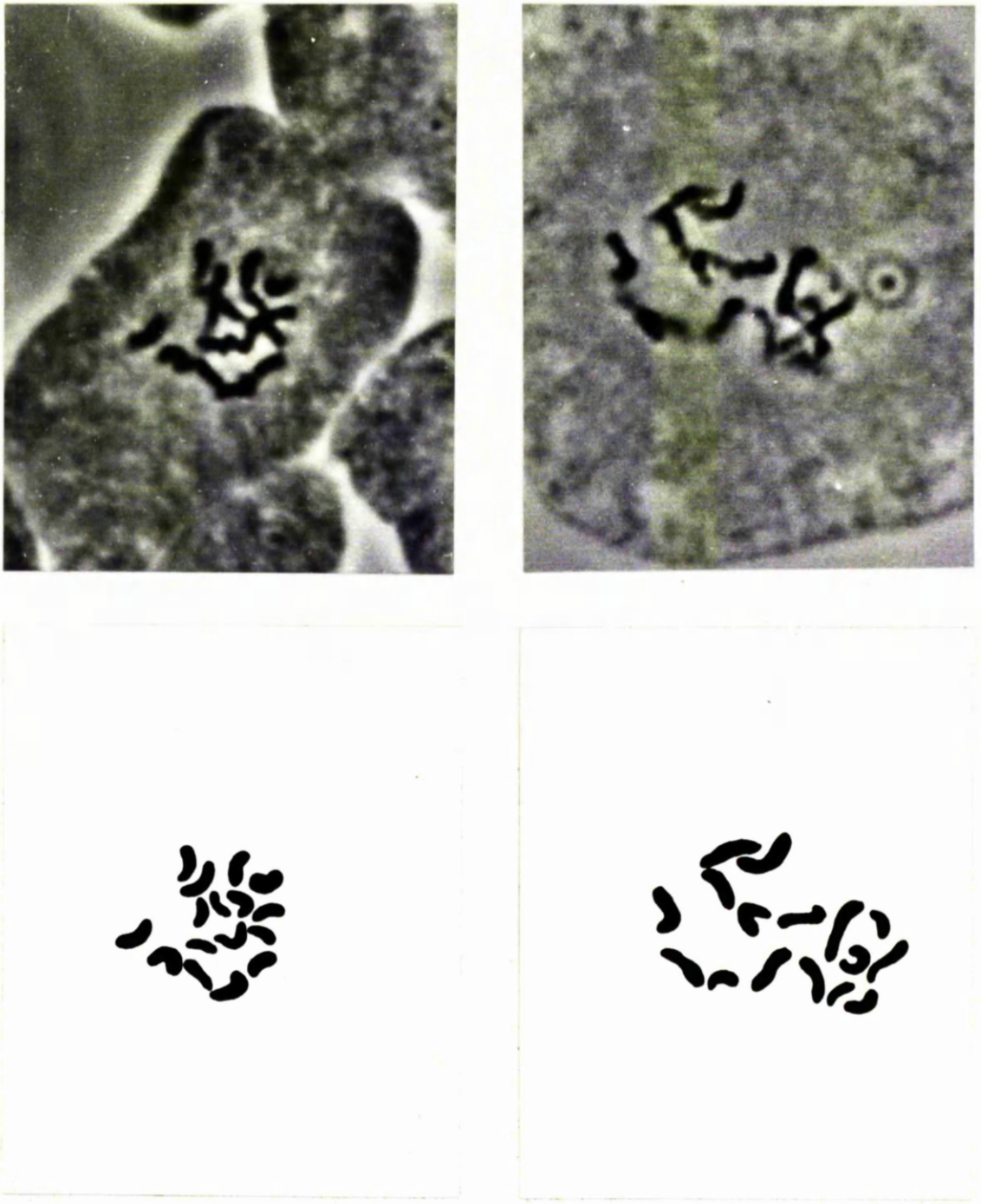


Fig. 46. Above: photomicrographs showing chromosomes in dividing cells in root tip (left) and nodule (right) of Myrica cerifera (x 3400).

Below: tracings made of the above photographs, root again on left. The number of chromosomes is 16 in both organs.

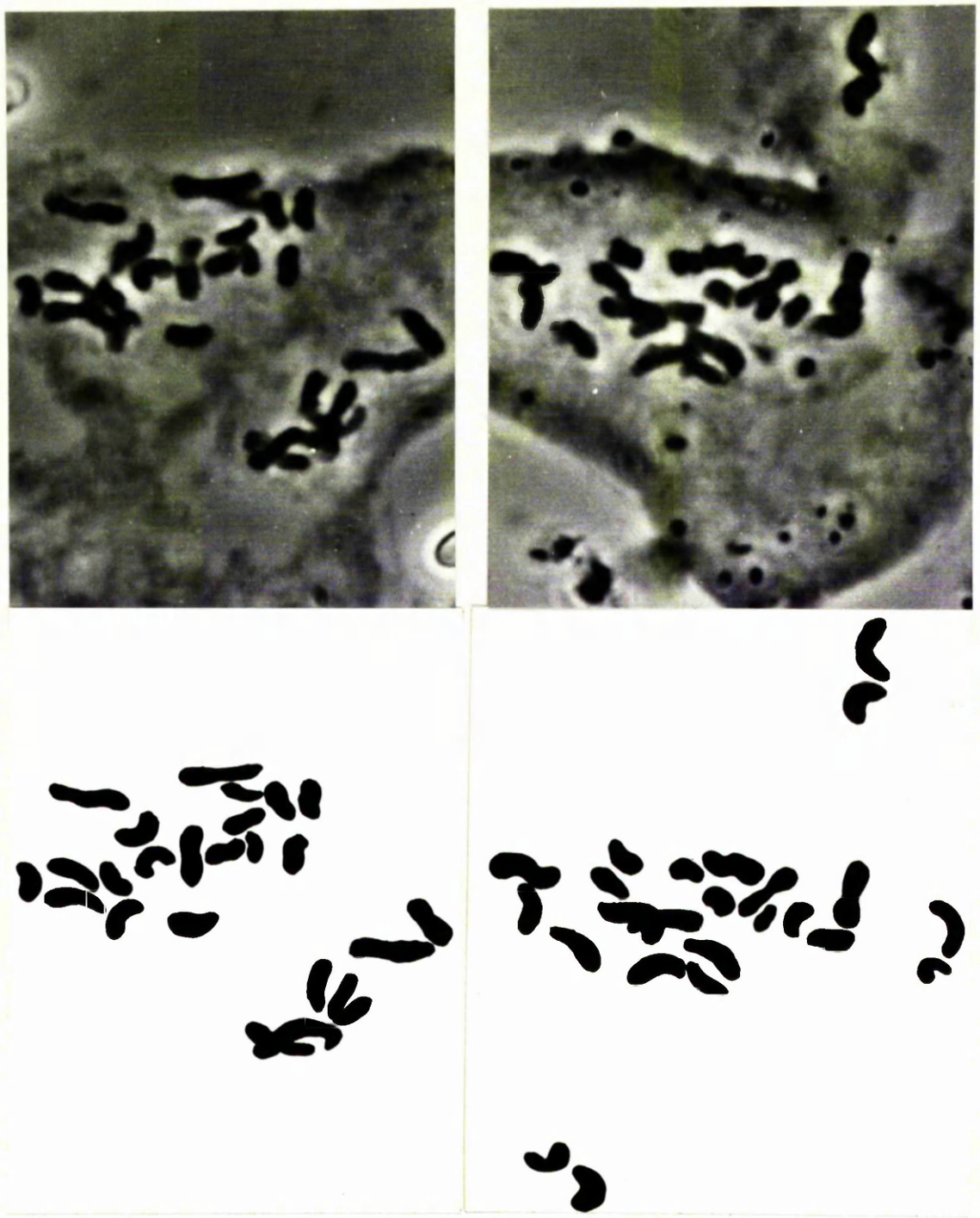


Fig. 47. Above: photomicrographs showing chromosomes in dividing cells in root tip (left) and nodule (right) of Hippophaë rhamnoides (x 3400).

Below: tracings made of the above photographs, root again on left. The number of chromosomes is 24 in both organs.

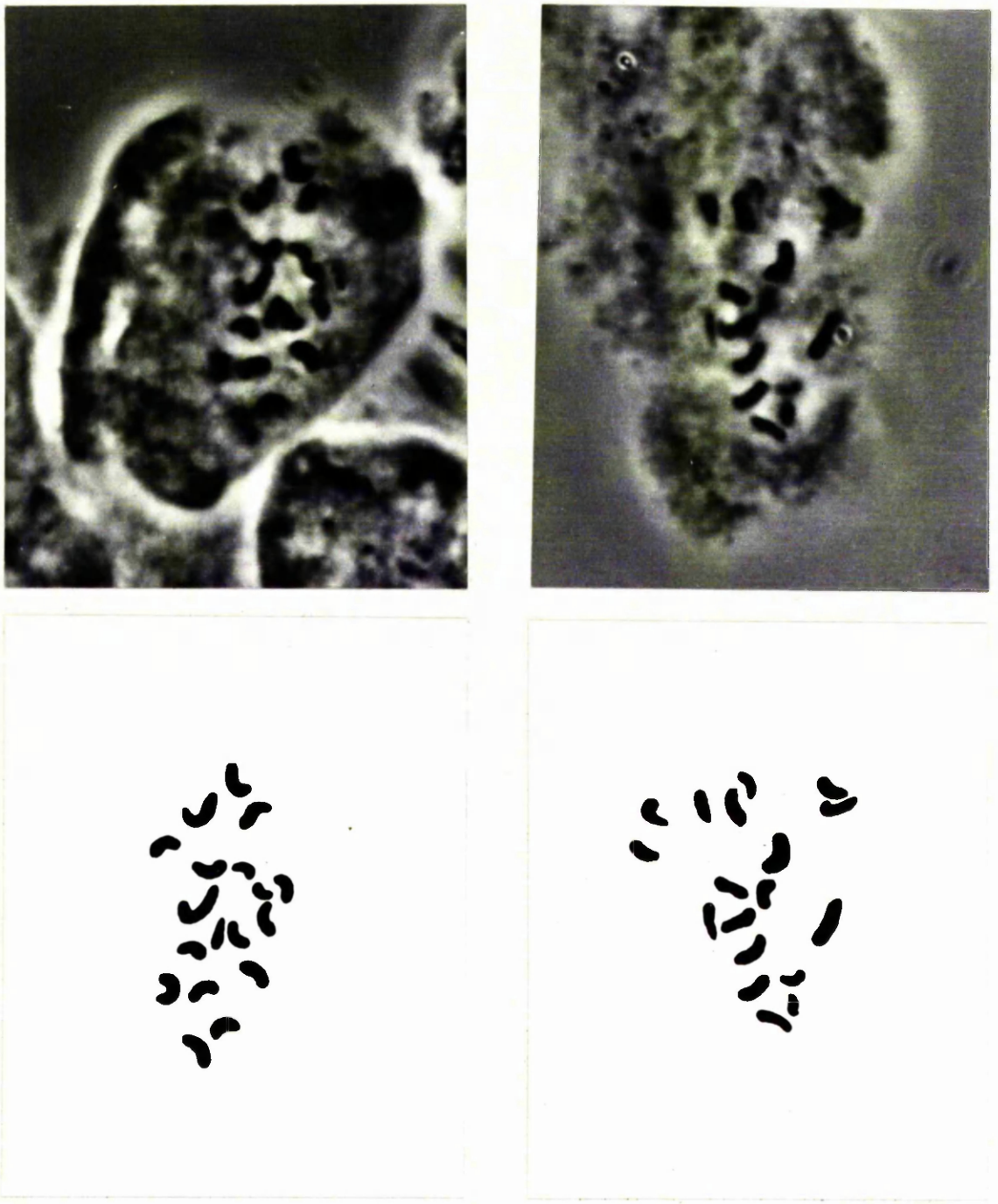


Fig. 48. Above: photomicrographs showing chromosomes in dividing cells in root tip (left) and nodule (right) of Casuarina cunninghamiana (x 3400).

Below: tracings made of the above photographs, root again on left. The number of chromosomes is 18 in both organs.

of the same chromosomes at slightly different foci, thus facilitating counting.

DISCUSSION

From the observations now reported it is clear that tetraploidy is not present in the cells of the nodule meristem in the six species studied. The nodule cells show the same number of chromosomes as the cells of the root. These findings extend those reported by Kodama (45 & 46, see Introduction) to an additional species of Alnus, to three species of Myrica, and to species of Hippophaë and Casuarina.

As indicated in the Introduction, although it has been claimed that in many legumes the nodules arise from tetraploid cells already present in the mother root, in other legumes this does not seem to be true. In the nine non-legume species so far examined there is no evidence of tetraploidy in the nodule meristems.

In the present study tetraploidy was sought for only in the nodule meristem cells, i.e. the only cells in which the chromosomes can be seen. By other techniques evidence of tetraploidy can be obtained in respect of resting nuclei. Thus in some species the chromocentres remain visible and are countable. By this means Kodama (51) obtained evidence that in the legume Astragalus sinicus, contrary to the findings of Wipf & Cooper (41) in other legumes, the cells of the mother root cortex are all diploid, but that penetration by the rhizobial infection thread induces endomitosis, a process by which the chromosomes divide independently of cell-division, giving a tetraploid condition. A second technique involves microphotometric measurement of the DNA content of nuclei after

Feulgen staining. Kodama (44) used this technique also and obtained evidence that in the legume Vigna catiang - a kind of cow-pea - although the nodule meristem cells are diploid, the infected cells in the mature nodule are mostly tetraploid, following endomitosis apparently induced by the presence of rhizobia. It is possible that the application of these additional techniques to non-legume nodules would reveal the occurrence of similar endoduplication of the chromosomes in the mature, much hypertrophied infected cells. It is obvious that the present study completes only the first stage in the investigation of ploidy of such root nodules.

SUMMARY

1. The chromosome complements of dividing cells of root tips and nodule apices have been determined in *Alnus glutinosa*, *Myrica gale*, *M. faya*, *M. cerifera*, *Hippophaë rhamnoides* and *Casuarina cunninghamiana* by the use of the squash technique.

2. In all six species the number of chromosomes found in the nodule meristem cells was the same as in the root tip cells. These results agree with those obtained by a previous worker for three Japanese species of *Alnus*, but disagree with those obtained for a number of leguminous species in which the nodule meristem is composed of tetraploid cells.

3. These findings, however, do not preclude the occurrence of tetraploidy in the mature, much hypertrophied, infected cells of non-legume nodules, which could arise by endomitosis, but could only be detected by techniques other than that used in the present work.

CHAPTER V

The Effect of the Level of Carbon Dioxide in the Rooting Medium on Growth and Nitrogen Fixation in Alder and Bog Myrtle

CONTENTS

INTRODUCTION

Since it is an end product of the respiratory processes of host plant and endophyte, carbon dioxide is constantly present in the dissolved condition in the cells of nodulated root systems, and in the gaseous form in the air spaces of the tissues, from whence it diffuses into the air spaces of the soil at a rate dependent on the gradient of partial pressure of the substance. In the wet, badly aerated soils for which both alder and bog myrtle show a preference, it is certain that relatively high levels of carbon dioxide are often present in the soil. As will be shown below, there are metabolic reasons for suspecting that a possible reason for the preference of these plants for such soils is that they re-cycle some of the carbon dioxide in a manner which favours nitrogen fixation.

Interest in this department in this question was aroused partly by the work of Mulder & van Veen (52) with several leguminous species, which were grown in a greenhouse in water culture constantly aerated with either CO_2-free air or with air containing 4% CO_2. The authors recognised that in the latter cultures the enriched air issuing from the culture vessels might stimulate photosynthesis in those particular plants, and since they wished to study only the effect of CO_2 on the root system they took the precaution of releasing the issuing gas well above the plant level. Inoculation with appropriate rhizobia was effected 1-2 weeks after the seedlings had been set up in water culture, and to sustain growth over this early period some combined nitrogen was provided.

In an experiment with red clover, Mulder & van Veen (loc. cit.) reported that the plants supplied with 4% CO_2 developed more nodules, showed an 80% increase in top growth, and fixed 50% more nitrogen, though the method of calculation of the latter is not clear since no information is given on the extent of uptake of the combined nitrogen supplied initially. In a further experiment with red clover evidence was obtained suggesting that the CO_2 benefit was chiefly exerted during the early phase of growth; analysis of the nodules revealed considerably higher amounts of keto-acids than in nodules grown without supplied CO_2. However, in a third experiment with red clover no clear effect of the CO_2 treatment could be found.

In a similar experiment with peas, the weight of nodules formed per plant was somewhat greater (20%) where CO_2 was supplied, and though owing to incomplete data fixation per plant cannot be calculated exactly, it may have been enhanced by about 50% in the presence of CO_2. Nodulation and fixation in French Bean were also found to be enhanced by CO_2.

Mulder & van Veen refer to the possibility that the organic acids which, as noted, they found to be more abundant when CO_2 was supplied had arisen by the occurrence of 'dark' fixation of CO_2, and they suggested that these resulting acids might favour nitrogen fixation, though they did not say how this could be. It is now known that the ammonia formed by nitrogenase action in legume nodules

is taken up by organic acids such as keto-glutaric to form amino acids, and it is possible that at times the supply of these organic acids from normal sources does not match up to the capacity of the nitrogenase to produce ammonia, and that an extra supply provided by 'dark' fixation promotes fixation. Also it has been suggested that in plants in general the significance of 'dark' fixation lies in its supplementation of the Kreb's cycle acids. By thus favouring respiration and ATP production, in nitrogen-fixing plants fixation could again be promoted.

Actually there does not appear to be any published evidence demonstrating directly that legume nodules are capable of 'dark' fixation, but in a personal communication to Professor Bond, Dr H.J. Evans (Oregon State University) has indicated that he has clear evidence of the presence of a CO_2-fixing system in soya bean nodules.

Bergersen (53) sought to confirm Mulder & van Veen's findings by means of short term ^{15}N tests on detached soya bean nodules. In his very brief account he records that nitrogen fixation was, in fact, enhanced in the presence of added CO_2 (amount not indicated) when levels of oxygen up to 0.3 atm. were provided. At higher oxygen levels the addition of CO_2 had no effect on fixation, presumably, he thought, because the enhanced respiration was nowproviding all the CO_2 needed by the nodules. Bergersen's statements imply that at the normal level of oxygen, CO_2 had a promoting effect on fixation, which is

in agreement with Mulder & van Veen.

Turning now to the non-leguminous plants with root nodules, the work of Leaf, Gardner & Bond (54, 55) on alder and bog myrtle, using ^{15}N, showed that here also the nitrogen fixed in the nodules passes quickly into the form of amino acids, presumably by the uptake of the ammonia by organic acids, so that there is again the possibility that an augmentation of the supply of the latter through 'dark' fixation would hasten nitrogen fixation. In the particular instance of alder there is an additional reason why the CO_2 supply might be important. The amino acid citrulline occurs in all parts of the alder plant, including the nodules, and the studies of Leaf, Gardner & Bond (54) showed that a substantial part of the newly-fixed nitrogen in the nodules is in the form of citrulline. The acid has the following formula:-

$$NH_2 - CO - NH - (CH_2)_3 - CHNH_2 - COOH$$

In animal liver tissue, at least, the acid is known to arise from ornithine, the formula of which is as follows:-

$$NH_2 - (CH_2)_3 - CHNH_2 - COOH$$

The citrulline synthesis involves the initial formation of carbamoyl phosphate ($NH_2 - COO \sim \textcircled{P}$) by reaction between ammonia, CO_2 and ATP. This substance then reacts with ornithine to give citrulline (Baldwin, 56). Obviously this reaction involves a special kind of 'dark' fixation of CO_2. Gardner & Leaf (57) showed by the use of $^{14}CO_2$

that the nodulated root system of alder does readily fix CO_2, presumably in citrulline synthesis, though their data do not exclude fixation by other reactions.

Additional information on this matter is provided in unpublished work by Dr C.T. Wheeler (personal communication), in which detached nodules and root portions of alder and bog myrtle were surface sterilised by immersion in calcium hypochlorite solution and then separately exposed to air with added $^{14}CO_2$. The samples were then dried, ground, and extracted with ethanol, the extract being then separated into 'acidic' (predominantly organic acids), 'basic' (mainly amino acids) and 'neutral' (principally carbohydrates) fractions by an ion exchange method. The radioactivities of the original alcoholic extract and of the fractions were determined, with results as shown in Tables 15 and 16. In both species the original ethanol extracts of the nodules showed substantial radioactivity, indicative of an appreciable incorporation of the labelled carbon into nodule constituents. The data for the fractions show that after 3 hr exposure, in both species rather more than half of the total radioactivity of the nodules was on average in the 'basic' fraction, i.e. in amino acids. A substantial part of the radioactivity was, however, contained in the 'acidic' (organic acid) fraction. The radioactivity of the ethanol extracts of the roots was considerably lower than that of the nodules, especially in the case of alder, while another notable difference was that in roots there was on average much more radioactivity in the 'acidic'

Table 15. Experiment with nodules and roots of *Alnus glutinosa*. 0.2 mCi and 4 g plant material per 250 ml flask. Exposure period 3 hr at 25°C.

Fraction	C.p.m. per g fresh weight of tissue extracted			
	Nodules		Roots	
	Sample A	Sample B	Sample A	Sample B
Original ethanol extract	32,750	30,000	4,320	8,350
Acidic	7,009	6,426	2,572	5,631
Basic	18,045	16,400	1,950	1,109
Neutral	2,750	2,720	216	43

Table 16. Experiment with nodules and roots of *Myrica gale*. 0.2 mCi and 5 g plant material per 250 ml flask. Exposure period 3 hr at 25°C.

Fraction	C.p.m. per g fresh weight of tissue extracted		
	Nodules		
	Sample A	Sample B	Roots
Original ethanol extract	42,009	30,920	20,579
Acidic	14,420	10,080	11,940
Basic	21,340	17,345	3,531
Neutral	1,080	2,589	1,480

than in the 'basic' fraction. An acceptable explanation would be that organic acids are produced as a result of CO_2 fixation, and that in nodules these are rapidly converted into amino acids by reaction with ammonia produced in nitrogen fixation; in roots, of course, no nitrogen fixation occurs. The synthesis of citrulline in the alder nodules could account for part of the radioactivity in the 'basic' fraction.

Walker & Brown (58) studied the effect of CO_2 concentration on the activity of phosphoenolpyruvic carboxylase, an enzyme which appears to be commonly present in plants and whose action produces oxal-acetic acid. They used the partially purified enzyme extracted from Kalanchoë leaves. The saturation level of CO_2 was in the region of 0.5%. As CO_2 was further increased the enzyme was not much affected until a level of 3% was attained. After that there was some inhibition, but the enzyme was still considerably active in the presence of 10% CO_2. Biale & Young (59), working with whole lemon and orange fruits, considered that the activity of the above enzyme was still rising when the external CO_2 level had reached 10%, and that in this way the enhanced respiration rate shown at that level of CO_2 was to be explained.

In the experiments now to be described, a comparison has been made between the growth of nodulated plants of alder and bog myrtle in culture solution kept continuously in equilibrium with either normal air or air containing 2% CO_2. The latter level was chosen in the light of (a) the

results of Mulder & van Veen (loc. cit.) with legumes, (b) the levels of CO_2 that are likely to prevail in the soil at the sites where these plants occur in the field, and (c) the findings by other workers (see preceding paragraph) on the effect of CO_2 concentration on the commonest of the 'dark' fixation enzymes. Water culture was employed because it greatly facilitates the control of the rooting medium as regards CO_2 content, pH and ion supply, and, in addition, permits ready inspection of the root system. [Unlike Mulder & van Veen's procedure, the present experiments were started with plants which were already nodulated and fixing nitrogen; this was done partly to allow the selection of a fairly uniformly nodulated set of plants for use in the experiments, while again very small plants are liable to be damaged while being set up in sealed cultures. Thus the experiments were not designed to reveal any CO_2 effects on the early stages of growth. The plants were grown in culture solution free of combined nitrogen, and so were entirely dependent on the nodules for nitrogen compounds.]

Supplementary evidence has been sought by means of short-term tests on the effect of CO_2 on nitrogen fixation in detached nodules, using the ^{15}N and also the acetylene technique. It was, however, appreciated that any benefit to fixation from the formation of organic acids by 'dark' fixation would not necessarily be revealed by the latter technique.

MATERIALS AND METHODS

Preliminary Culture of Plants for Growth Experiments

All plants used in these experiments were grown during the period April to October in a glasshouse lit by natural light. In the daytime the glasshouse temperature was usually in the range 20-27°C and slightly lower overnight.

The seed of *Alnus glutinosa* (L.) Gaertn. that was used had been obtained from Messrs Vilmorin-Andrieux, Paris; although in the past locally collected seed was used, it was noted recently that the French seeds were larger and gave stronger seedlings. The seed of *Myrica gale* L. had been collected locally, and before sowing was given the low temperature treatment previously shown to promote germination. Seed of both species was sown in trays of Peralite moistened with dilute Crone's solution (nitrogen-free formula, see below).

When the seedlings had formed two leaves they were transplanted into glass troughs 12 cm in depth and 30 cm in diameter, covered by black Perspex tops with holes for 30 seedlings. The side of the trough was covered in black paper. The trough was filled with Crone's nitrogen-free culture solution. The recipe for this solution was given in Chapter I. That recipe gives full strength Crone's solution. For the present purpose the solution in the troughs was at half strength for alder and at a quarter for bog myrtle. The pH of the solution, naturally 6.2, was lowered to 5.5 for alder and 5.0 for bog myrtle. To sustain the seedlings before nodules appeared, sufficient

ammonium sulphate was added to the troughs to provide 0.5 mg nitrogen per seedling.

Inoculation of seedlings was effected two days after setting-up in the troughs by placing on each root system, by means of a small brush, approximately 0.25 ml of an inoculum prepared by grinding 5 g nodules, taken from appropriate stock plants in the greenhouse, in 100 ml distilled water. As usual, nodules began to appear on alder plants two weeks after inoculation, and after a slightly longer interval in bog myrtle. The young plants were left in the troughs for a further month, by which time appreciable fixation had occurred in the nodules. The plants were then transferred to the final containers now to be described.

Setting-up the Growth Experiments

The plants for use in growth experiments were selected for uniformity of size from the excess of plants in the troughs.

In the first experiment the plants (alder) were set up in individual test tubes of 180 ml capacity filled with culture colution. Each tube was fitted with a three-holed rubber stopper, the largest hole being partly closed by a rubber inset with a smaller hole through which the root system of the plant was introduced. A second hole in the stopper carried the gas inlet tube, the lower end of which was attached to an earthenware aerator of the type used for aquaria. The third hole was closed by a small rubber stopper bearing the gas outlet tube;

in the case of cultures through which normal air was being bubbled, this outlet tube consisted of a short capillary tube with a right-angle bend, but with cultures through which CO_2-enriched air was passing, the gas outlet consisted of an upright tube extended in height from time to time to keep its upper end above the tops of the plants. These features are shown in Figure 49. The stopper bearing the outlet tube could easily be removed to provide access to the interior of the culture tube for maintenance purposes (see later). The tubes were wrapped with black paper and placed in racks, as will be shown in a later photograph.

Because (as will appear later) the growth of alders in the above tubes was not completely satisfactory, possibly owing to the restricted rooting space, in subsequent growth experiments the plants were set up in glass jars of capacity 2½ litre. Each jar was closed by a waxed cork (cut so that the lenticels ran transversely), bored with holes for three plants and for gas inlet and outlet tubes, the latter being of the same type as before. These features are shown in Figures 50 and 51. The jars were wrapped in black paper. By using these jars the rooting space per plant was increased from 180 ml to 830 ml.

Crone's culture solution was used for plant culture in tubes or jars. A property of this solution is that the constituent salts do not entirely dissolve on first mixing, and it is a valuable feature that the undissolved part forms a reserve of nutrients which gradually dissolves

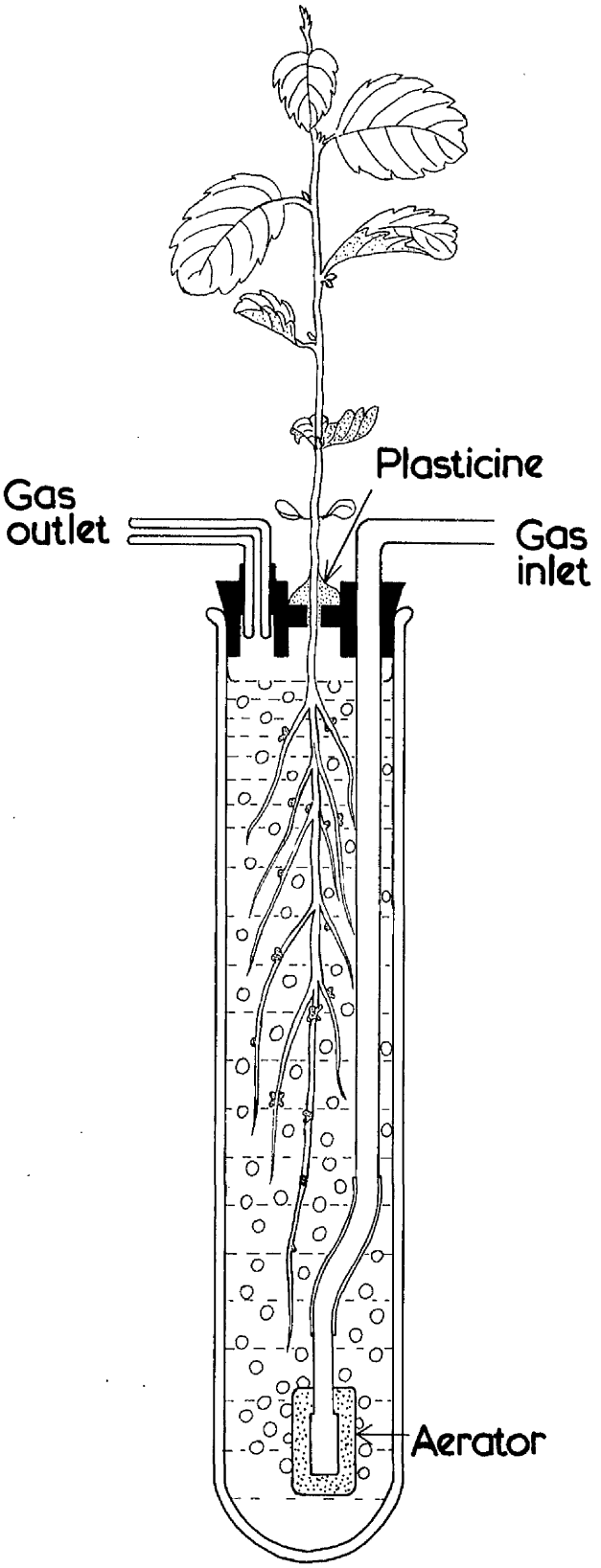


Fig. 49. Apparatus used for tube culture of alder (x 3/4). The particular tube shown is as used for the air series.

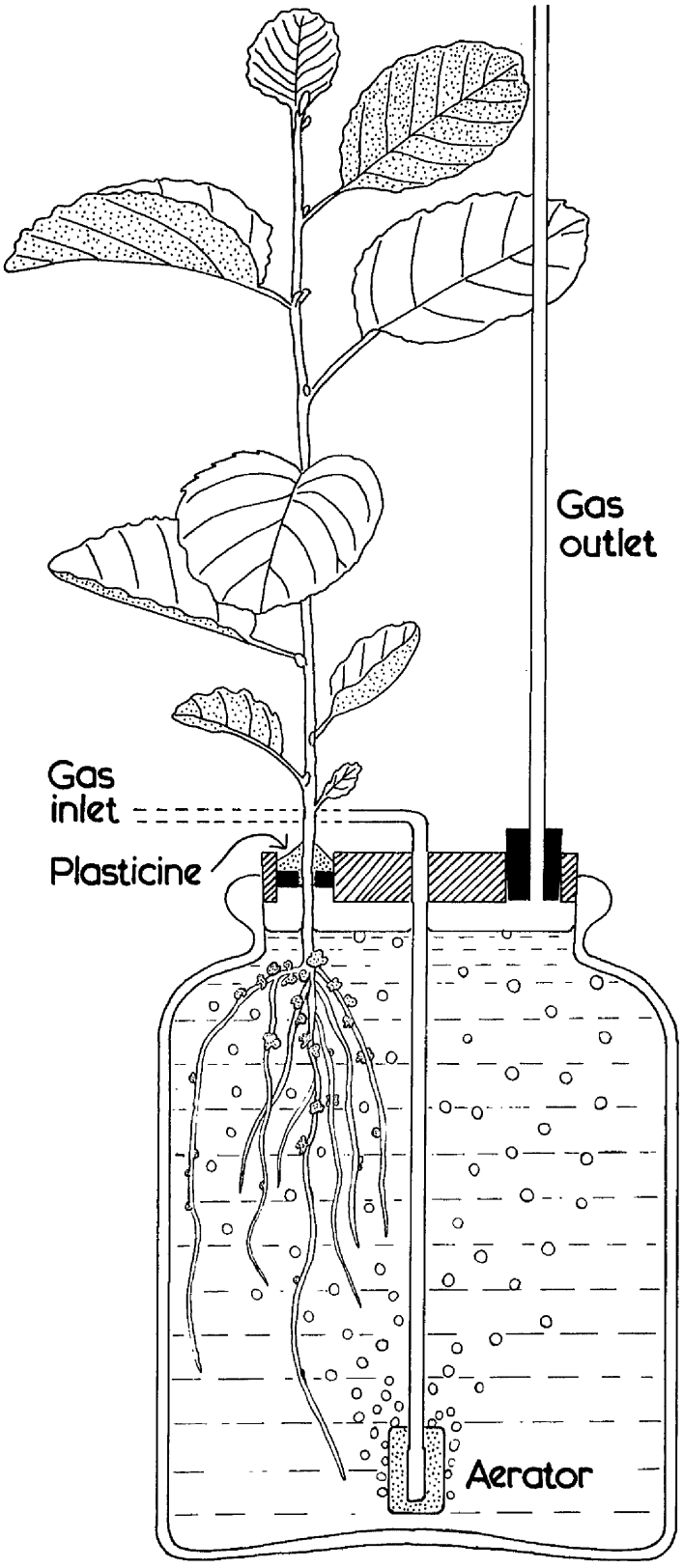


Fig. 50. Apparatus used for jar culture of alder and bog myrtle (x $\frac{1}{2}$). The particular jar shown is as used for the air + 2% CO_2 series.

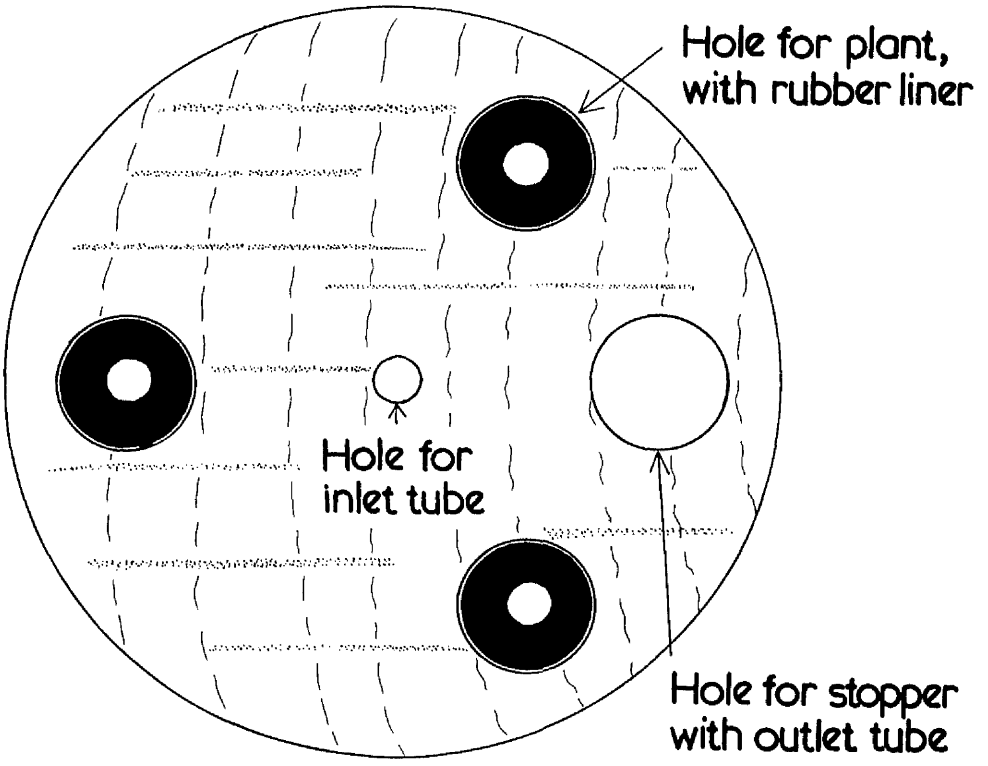


Fig. 51. Plan of cork closure for jar cultures (x 1).

as ion uptake by the plants proceeds. However, in experiments involving forced gas-flow through the solution, the undissolved salts become deposited over the roots and nodules to a possibly harmful degree. In the present experiments the prepared culture medium, after the usual vigorous shaking, was allowed to settle overnight in a suitably-fitted aspirator bottle. Only the clear supernatant solution was supplied to the plant cultures. To compensate for the absence of the undissolved residue the solution in the plant tubes or jars was changed relatively frequently (see below). The strength of the culture solution was increased as the plants grew larger.

After being set up in jars or tubes the plants were allowed a 'settling-down' period of two weeks during which normal air was bubbled through all the containers. The plants were then sealed into the stopper or cork by applying 'plasticine' to the hypocotyl where it passed through the closure. The principal reason for this sealing was to ensure that when CO_2-enriched air was being bubbled through cultures, the issuing gas would all emerge through the appointed outlet tube, above the top of the plants, so as to exclude the possibility that these particular plants would be benefitted in their growth by an increased rate of photosynthesis following the release of CO_2-enriched air at leaf level.

After sealing was complete gas flow was commenced. The adequacy of the sealing was checked by submerging set-up tubes or jars, with the gas-flow operating, in a container filled with water. A lack of any bubbling from

the region of the stopper or cork indicated that the sealing was satisfactory.

In order to provide data on the size, weight and nitrogen content of the plants at the commencement of gas flow, 6-10 plants which had received the same treatment as those to be used in an actual experiment were now harvested (see below for methods).

Gases Supplied to Root Systems in Growth Experiments

The experiments involved two treatments. In one treatment, greenhouse air, compressed by a 'Reciprotor' pump (oil-free) was bubbled constantly through the culture solution in the plant container. In the other treatment a mixture of 98% air and 2% CO_2 was bubbled through the containers, this mixture being obtained in cylinders from The British Oxygen Co. (Special Gases) Ltd. Certificates of analysis provided by that Company in respect of the 1972 delivery stated that the actual CO_2 content was in the range 2.0-2.1%. Samples from the 1973 delivery were analysed by Professor Bond in an Orsat analyser with the writer's assistance, and were duly found to contain 2% CO_2.

The actual rates of flow through the tubes or jars in which the rate had been adjusted to what was considered to be a suitable one, were ascertained on several occasions by leading the emerging air or gas into a 2-litre measuring cylinder filled with water and inverted in a trough of water. By means of a stopwatch the time taken for the accumulation of 2 litres of gas (measured at atmospheric

pressure) was noted. The conclusion was that approximately 500 ml of air or gas was passing through the plant container per hr in the case of tube culture, and 2-3 times as much in jar cultures.

Day-to-Day Maintenance of Growth Experiments

Each day the adequacy of the sealing of the containers was tested by applying a drop of soapy water to the end of the gas-outlet tube and inspecting for bubble formation. Experience had shown that the presence of a leak in the seal resulted in a lack of bubble formation in this test. The rates of gas flow were adjusted daily.

The pH of the culture solution was tested on most days by withdrawing a 5 ml sample from the plant container and establishing the pH colorimetrically. Previous experience had shown that the pH of Crone's solution in which plants are growing shows a gradual fall owing to differential uptake of ions. Also it was to be expected that bubbling the air-CO_2 mixture through the culture solution would lower the pH. Tests with tubes and jars containing culture solution (without plants) showed that the pH remained at the original value of 6.3 when normal air was bubbled through for 24 hr, but when air with 2% CO_2 was passed the pH fell to a value of 5.2 in about 6 hr and then remained steady at that value. It was concluded that the solution was then in equilibrium with the air-CO_2 mixture. Calculations from equations given by Garrels & Christ (60) indicate that pure water in equilibrium with 2% CO_2 should have a pH of 4.8. The

somewhat higher pH obtained here can be attributed to the buffering power of the culture solution. To avoid a persisting difference of pH between the two series of plants, the samples withdrawn as above were titrated with N/100 NaOH to pH of 6.2, and a calculated amount of similar NaOH was added to the culture tubes or jars to restore them to a pH of 6.2.

The culture solution was topped up with distilled water daily, and was completely changed once weekly. The culture tube or jar was emptied by inserting a siphon tube through the hole left by the removal of the gas outlet tube, and fresh solution introduced by means of a small funnel.

The individual positions of the cultures on the greenhouse bench were changed weekly in case of inequality of lighting etc.

In order to reduce the likelihood of a build-up of CO_2 in the greenhouse, free ventilation was provided day and night. Also in the daytime a fan was kept going above the plants in order to hasten the dispersal of CO_2 from their vicinity.

Harvesting of Growth Experiments

At both preliminary and final harvests shoot heights and in some cases leaf areas were measured, the latter by the paper tracing method, with precautions to use paper of uniform thickness equilibrated with atmospheric moisture prior to weighing. Dry weights of shoot, root and nodules of the plants were then separately established by

drying to constant weight in an oven at 95°C. The per cent total nitrogen content of the dry matter was established by subjecting weighed samples of the material (after grinding in a Christy & Norris hammer mill) to the Kjeldahl process as modified by Ranker, described in Official Methods of Analysis of A.O.A.C. (61). The dry matter of all parts of the plant was combined for this purpose. Sometimes each plant was analysed separately, but in some experiments, owing to a temporary lack of laboratory facilities, all the material of one treatment had to be bulked to reduce the number of analyses. In most instances duplicate or triplicate analyses were made on each sample of dry matter. Close agreement was obtained in the results for per cent nitrogen content, as illustrated by the following examples:

Sample A	3.117% 3.111%	Sample B	2.700% 2.671%
Sample C	1.892% 1.899% 1.889%	Sample D	2.411% 2.376% 2.368%

Total nitrogen per plant was then calculated by reference to the dry weight data. Since the plants had grown in a culture solution free of combined nitrogen, the increase in the nitrogen content of the plants during the period of gas flow is all attributable to fixation.

^{15}N Assays of Fixation in Detached Nodules

In addition to the above growth experiments, it was thought that short-term measurement of fixation (here by ^{15}N) in detached nodules exposed to different levels of

CO_2 along with the ^{15}N might yield significant results, although it was realised that it would be impossible to reproduce at all closely the precise conditions experienced by the nodules in the above growth experiments.

First the procedure adopted in the ^{15}N measurements will be described, and afterwards comments will be made on some of its features.

The construction of the containers in which nodules were exposed to the gas mixtures is shown in Figure 52. A strong, round-bottomed glass tube of capacity 40 ml was fitted with a stopper bearing a stopcock. From a hook inserted into the stopper was hung a cylindrical metal basket perforated on its round side and base by close-set holes of 2 mm diameter. The nodule sample was placed in this basket. In one set of tubes 5 ml of 'Indicarb' soda-lime (Hopkins & Williams, 8-14 mesh) was placed at the base. Soda-lime is an intimate mixture of sodium hydroxide and slaked lime, in this particular case with an indicator added to show when the CO_2-absorbing capacity is exhausted. It was anticipated that the soda-lime would absorb any trace of CO_2 present in the original gas mixture, and also the CO_2 evolved during the respiration of the nodule sample. In the other set of tubes, 5 ml of glass beads replaced the soda-lime.

The preparation of the gas mixtures for introduction into the above tubes was done as follows. Gaseous nitrogen containing 95 atom per cent of ^{15}N was obtained

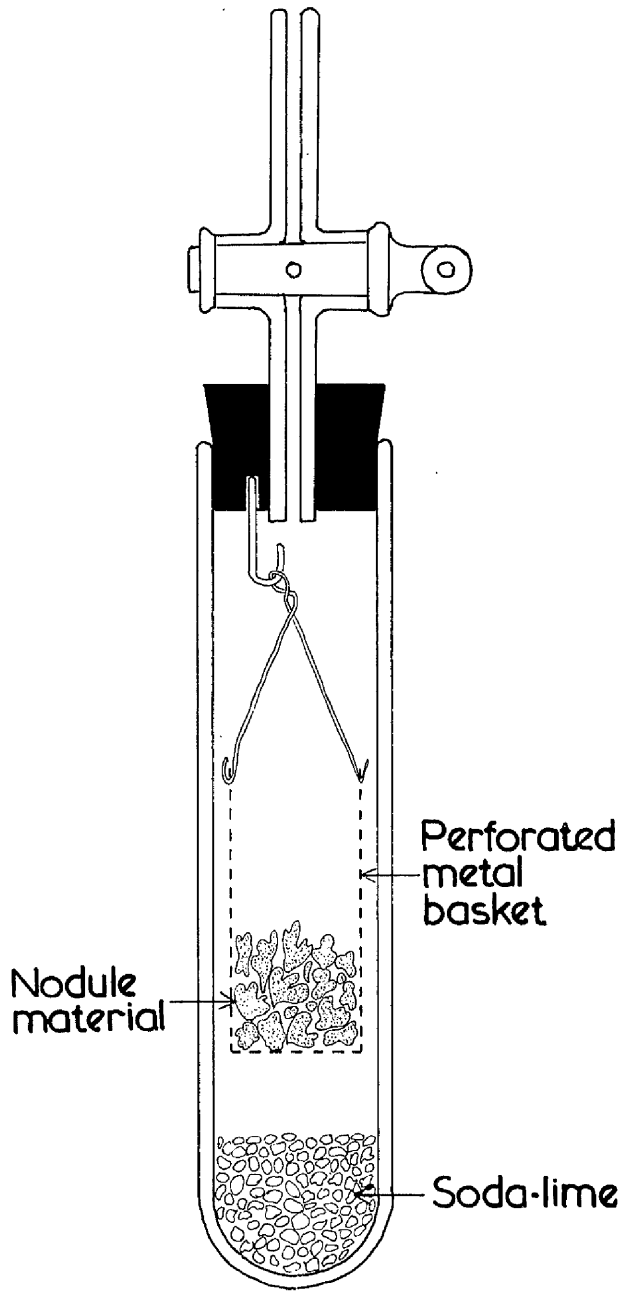


Fig. 52. Incubation tube used for the ^{15}N and acetylene assays (x 1). In other tubes glass beads replaced the soda-lime.

by reaction between a special stock of ammonium nitrate (enriched to the above extent in the ammonium radical) and sodium hypobromite in a nitrometer tube. This nitrogen was subsequently diluted with ordinary nitrogen to obtain an enrichment of 34.7 atom per cent. By use of a manifold, a gas mixture consisting of 30% by volume of this nitrogen, 20% oxygen, and 50% argon was prepared, for use in the tubes containing soda-lime. A second mixture for use in tubes containing glass beads comprised the above proportions of nitrogen and oxygen, plus 2% of CO_2 and 48% of argon.

A warm sunny day was selected for the actual experiment, so that fixatory activity in the nodules might be as high as possible. In the early afternoon a bulk sample of 12 g fresh weight of alder nodules was quickly gathered from stock alder plants growing in water culture. The nodules were blotted, thoroughly mixed, and 1.25 g fresh weight placed in the basket of each of the eight tubes to be used. The tubes were evacuated and re-filled to atmospheric pressure with the appropriate gas mixture. The interval between the start of nodule picking and the completion of the gassing was 35 min.

The tubes were incubated at 24°C for 2½ hr. The nodule samples were then removed from the tubes and subjected to the Kjeldahl process. After titration the distillates were re-acidified and evaporated down to a volume of about 7 ml. The nodule nitrogen now in the form of ammonium chloride in these distillates was assayed for ^{15}N abundance in the mass spectrometer. These

analyses were kindly carried out by Professor W.D.P. Stewart at Dundee University.

In order to ascertain whether 5 ml of soda-lime was capable of absorbing the amounts of CO_2 likely to be evolved in the respiration of the nodules (see below), tubes as shown in Figure 52 were charged with air-CO_2 containing in one instance 5% of the latter constituent and in another 50%. The tubes (in duplicate for each mixture) were closed and incubated at 24°C. Inspection after 4 hr showed that in all the tubes only a few granules of soda-lime on the surface had turned yellow-brown, indicating exhaustion. Striking evidence of the avidity of soda-lime for CO_2 was obtained when tubes were being charged with air plus 50% CO_2 in these tests - the readings on the manometer attached to the manifold indicated that the CO_2 was being absorbed immediately it entered the tubes.

In order to know in advance what changes in the composition of the gas were likely to occur in the gas mixture during the incubation period, measurements of the respiration of detached alder nodules were made in August, 1973, prior to ^{15}N tests. The Pettenkofer constant-flow method was employed. A relatively large sample (3 g fresh weight) of nodules from stock plants in water culture was enclosed in a glass tube measuring 2 x 22 cm, the latter being then immersed in a water bath at 24°C. A constant current of air previously freed of CO_2 was passed through the tube and thence into a Pettenkofer absorption tube containing 50 ml of N/20 baryta. One hour

was allowed for the attainment of a steady state, and subsequently CO_2 production was measured (by titration of the baryta with N/20 HCl) over three successive one-hour periods. These measurements were made on two different days, with closely similar results. CO_2 production was initially 1.2 ml (at S.T.P.) per g fresh weight per hour, falling slightly to 1.0 ml by the third hour.

Calculations from these data indicated that the sample of nodules in each of the ^{15}N tubes would produce some 3 ml of CO_2 during the period of incubation. In the case of the tubes incorporating soda-lime this CO_2 would, it was anticipated, be taken up by that reagent. Since it is known (MacConnell, 62) that alder nodules have a respiratory quotient close to unity, there would be an uptake of some 3 ml of oxygen during the incubation. Thus in these tubes the pressure of the gas would have fallen to about 0.9 atm. It was considered whether this could affect the fixation of nitrogen, since the latter gas will now be in slightly higher proportion than it was initially, or than it would have been at the end of incubation in the tubes without soda-lime. However, although fixation by alder nodules is affected by changes in the proportion of nitrogen at lower levels, where 30% is provided initially small changes either way are most unlikely to affect the fixation.

In tubes lacking soda-lime there should be no change in the gas pressure during incubation. The accumulation of respiratory CO_2 will be expected to raise the proportion of that gas from the initial 2% level to about 9% finally.

The initial level of 2% was provided to ensure the presence of some CO_2 at the time when the nodules were likely to be most active in fixation.

Past experience has shown many times that detached alder nodules are less active in fixation than detached nodules of other genera, such as Casuarina or Hippophaë. The provision of the relatively high proportion of 30% nitrogen, with quite high label, would, it was hoped, somewhat enhance the fixation.

Indirect Measurement of Fixation in Detached Nodules by the Acetylene-Reduction Method

It is generally held that the reduction of acetylene to ethylene by biological material is indicative of the presence of nitrogenase, and that with given material the rate of acetylene reduction under particular environmental conditions provides a measure of what nitrogen fixation would be under similar conditions. For technical reasons the method is much more sensitive than the ^{15}N method.

For acetylene-reduction assays nodules were enclosed in tubes similar to those used for the ^{15}N tests. Here again the nodules were quickly collected from stock plants growing in water culture in the greenhouse, and 1 g fresh weight placed in each tube. Tubes containing also soda-lime were charged at atmospheric pressure with a gas mixture consisting of 20% acetylene, 20% oxygen and 60% argon. Tubes containing glass beads in lieu of soda-lime were charged with a mixture which differed only from

the above in that 2% CO_2 was substituted for the same proportion of argon. Also included were tubes without nodules, for control purposes.

The charged tubes were incubated for 1 hr at 24°C, and then 1 ml samples of the gaseous contents of the tubes were weekly withdrawn, in each case after filling and emptying the syringe three times following insertion of the needle through the rubber stopper of the tube, the object of this procedure being to ensure that the gaseous contents were well mixed up prior to sampling.

The gas samples were analysed for ethylene on a Pye Unicam Series 104 Gas Chromatograph, Model 4. Ethylene contents were obtained by measuring the peak heights on the graphical record provided by the instrument and then by referring to a calibration curve. The nodule samples were oven-dried, and ethylene production was expressed as mole per hr per g nodule dry matter.

DATA OBTAINED

Growth Experiments

An experiment with alders in tube culture was carried out from 23 May-21 June, 1972, with one series of plants receiving normal air and the other air with 2% CO_2.

Numerous additional nodules formed on the plants of both series soon after the start of gas flow, and since red anthocyanin pigmentation was intense - undoubtedly because an appreciable amount of light penetrated into the tubes - they presented a very striking appearance, as shown in Figure 53 (A and B).

Plant growth was moderately satisfactory, and there was no visible difference between the two series within the four weeks of gas flow. Table 17 shows the increments in growth and N content occurring during the period, and Figure 54 (A and B) show typical plants. It will be noted from the Table that no significant differences are shown in the data.

A further experiment, again with alder but now in jars, was conducted over the gas-flow period of 24 July-6 September, 1972 (6 weeks). After a month of growth the visual impression was that the plants receiving 2% CO_2 were growing somewhat more strongly than those in the air series, and this difference persisted until harvest. Growth and nitrogen data are provided in Table 18, while Figure 55 shows typical plants. Even allowing for the longer growing period in this experiment, it is clear that jar culture produced larger plants overall. It will also

Fig. 53A. Part of root system of an alder plant in tube culture showing numerous young nodules containing red anthocyanin pigment (x 1).

Fig. 53B. A small portion of the root system shown in Fig. A, now at higher magnification (x 5).

Table 17. Mean growth and nitrogen data for alder plants grown in tube culture 23 May-21 June, 1972*.

Gas-flow	No. of plants	Shoot height cm	Dry wt per plant, g		%N in whole plant dry matter	Total N per plant mg
			Nodules	Whole plant		
Normal air	13	9.8	0.052	0.936	2.403	22.5
Air with 2% CO_2	13	10.1	0.055	0.849	2.484	21.1
Result of 't' test, $P = 0.05$		N.S.	N.S.	N.S.	Not+ available	Not+ available

*Except in respect of % N, the values above indicate the increases in the various attributes shown during the period of gas-flow. They were obtained by deducting the values recorded at the preliminary harvest of comparable plants at the commencement of gas-flow from those obtained at the final harvest. Mean preliminary harvest data (based on 8 plants) in this experiment were as follows:-

Shoot height 6.3 cm; nodule dry wt 0.005 g; whole plant dry wt 0.135 g; total N per plant 3.4 mg.

+Dry matter of all plants in each series bulked prior to Kjeldahl analysis.

Fig. 54 (A and B). Typical plants at harvest of experiment with alder, 23 May-21 June, 1972 (x 1/5).

Table 18. Mean growth and nitrogen data for alder plants in jar culture 24 July-6 September, 1972*

Gas-flow	No. of plants	Shoot height cm	Dry wt per plant, g		Leaf area per plant cm^2	%N in whole plant dry matter	Total N per plant mg
			Nodules	whole plant			
Normal air	15	18.6	0.116	1.872	346	2.546	47.7
Air with 2% CO_2	15	27.6	0.135	2.461	453	2.634	64.8
Result of 't' test							
$\underline{P} = 0.05$		Sig.	N.S.	Sig.	Sig.	N.S.	Sig.
$\underline{P} = 0.01$		Sig.		Sig.	N.S.		N.S.

*Except in respect of leaf area and %N, the values above indicate the increases in the various attributes shown during the period of gas-flow. They were obtained by deducting the values recorded at the preliminary harvest of comparable plants at the commencement of gas-flow from those recorded at the final harvest. Mean preliminary harvest data (based on 9 plants) in this experiment were as follows:-

Shoot height 7.0 cm; nodule dry wt 0.005 g; whole plant dry wt 0.106 g; total N per plant 1.9 mg.

Fig. 55. Typical alder plants at end of experiment of 24 July-6 September, 1972. Left; plants grown with air + 2% CO_2 bubbled through the culture solution. Right; plants with normal air bubbled through the culture solution.

be noted from the Table that the plus-CO_2 plants showed significant superiority (at $\underline{P} = 0.05$) in respect of increases in height, dry weight per whole plant, leaf area and total nitrogen (= nitrogen fixed) per plant. The differences in the first two characteristics were also significant at $\underline{P} = 0.01$. Since there was no significant superiority in nodule dry weight on the part of the plus-CO_2 plants, it appears that the nodules were more effective in fixation. Calculation shows that, in fact, the formation of 1 g nodule dry matter was on average attended by the fixation of 495 mg nitrogen in these plants, but of only 423 mg in the air plants; the 't' test shows the difference between these means to be highly significant.

It was decided to seek, during the 1973 season, confirmation of the beneficial result of 2% CO_2 on alder suggested by the experiment just described, and also to extend the study to bog myrtle.

A repeat experiment with alder was made over a gas-flow period 27 June-21 August, 1973, following the same procedure as in the previous experiment. At first the plus-CO_2 plants grew more strongly than the air plants, but during about the fifth week the plus-CO_2 plants began to show a noticeable yellowness in the leaves, which appeared to indicate some slowing of fixation. These symptoms persisted, with the inevitable result that these plants fell behind those receiving air, which were mostly growing strongly.

Data obtained at harvest are shown in Table 19. Due to the amount of plant-to-plant variation, the differences between the means in respect of height and whole-plant dry weights fail to attain significance, but at least it can be said that the values tend to be lower for the plus-CO_2 plants. The largest differences between means are in respect of %N and total N, and although an actual statistical test cannot be made, in view of the magnitude of the differences between means it cannot be doubted that they are real. Considering the relatively low %N in the plus-CO_2 plants it seems certain that their growth had latterly been limited by an inadequate supply of N from the nodules. Calculation as before shows that in those plants the formation of 1 g nodule dry matter was accompanied by the fixation of 228 mg N, while for the air plants the figure was 502.

In view of the unexpected result of the above experiment, a further experiment was conducted with alder, the period of gas-flow being 13 August to 8 October, 1973. The plants were again grown in jars. After only one week the leaves of the plus-CO_2 plants had developed a slight chlorosis, while those of the air plants were a normal green. After a little further time this difference in leaf colour intensified, while a retarded elongation of the plus-CO_2 shoots also became obvious. After two weeks the jars which had so far received 2% CO_2 were transferred to air-flow for one week resulting in a distinct improvement in the growth of the plants. The restoration

Table 19. Mean growth and nitrogen data for alder plants in jar culture 27 June-21 August, 1973*

Gas-flow	No. of plants	Shoot height cm	Dry wt per plant, g		%N in whole plant dry matter	Total N per plant mg
			Nodules	Whole plant		
Normal air	15	20.5	0.167	3.328	2.490	82.9
Air with 2% CO_2	15	15.3	0.229	2.809	1.893	53.2
Result of 't' test						
$\underline{P} = 0.05$		N.S.	Sig.	N.S.	Not+ available	Not+ available
$\underline{P} = 0.01$			N.S.			

*Except in respect of %N, the values above indicate the increases in the various attributes shown during the period of gas-flow. They were obtained by deducting the values recorded at the preliminary harvest of comparable plants at the commencement of gas-flow from those obtained at the final harvest. Mean preliminary harvest data (based on 10 plants) in this experiment were as follows:-

Shoot length 10.4 cm; nodule dry wt 0.017 g; whole plant dry wt 0.287 g; total N per plant 6.2 mg.

+Dry matter of all plants from each series bulked prior to Kjeldahl analysis.

of the flow of 2% CO_2 at the end of the week was followed in due course by the re-appearance of the previous symptoms, a state of affairs which continued until harvest. In view of the advanced season fluorescent tubes were fixed above these plants for the last three weeks, to supplement daylight, giving a 16 hr day.

Growth data are shown in Table 20, while a photograph is provided in Figure 56. Significant superiority on the part of the air plants in respect of height and dry weight is recorded. The substantial difference observed in respect of %N in the previous experiment is not repeated here, though there is a tendency in the same direction. But there is again a very substantial difference in N fixed. The same calculation as before yields 511 mg N fixed during the formation of 1 g nodule dry matter for the plus-CO_2 plants, and 668 for the air plants, the rather smaller difference this time being perhaps because of the week's interruption in the CO_2 treatment.

An experiment with bog myrtle was carried out with a gas-flow period 10 July to 8 August, 1973. Over the four weeks of gas-flow no visual differences between the two series appeared. All plants grew extremely satisfactorily. A photograph at harvest time is shown in Figure 57, and data are provided in Table 21. Both confirm the lack of any appreciable difference. This stands in marked contrast with experience in the last experiment with alder (above). The usual calculation yields 383 mg for the CO_2 plants, 474 mg for air plants, but there is some doubt as to whether the difference is significant.

Table 20. Mean growth and nitrogen data for alder plants grown in jar culture 13 August–8 October, 1973*.

Gas-flow	No. of plants	Shoot height cm	Dry wt per plant, g		%N in whole plant dry matter	Total N per plant mg
			Nodules	Whole plant		
Normal air	18	14.6	0.157	4.290	2.385	102.3
Air with 2% CO_2	18	6.4	0.130	2.886	2.238	64.6
Result of 't' test						
$P = 0.05$		Sig.	N.S.	Sig.	Not+ available	Not+ available
$P = 0.01$		Sig.		Sig.		

*Except in respect of %N, the values above indicate the increases in the various attributes shown during the period of gas-flow. They were obtained by deducting the values recorded at the preliminary harvest of comparable plants at the commencement of gas-flow from those obtained at the final harvest. Mean preliminary harvest data (based on 6 plants) in this experiment were as follows:-

Shoot height 12.8 cm; nodule dry wt 0.026 g; whole plant dry wt 0.545 g; total N per plant 10.4 mg.

+Dry matter of all plants from each series bulked prior to Kjeldahl analysis.

Fig. 56. Typical alder plants at end of experiment of 13 August-8 October, 1973 (x 1/4). Left; plants grown with air + 2% CO_2 bubbled through the culture solution. Right; plants with normal air bubbled through the culture solution.

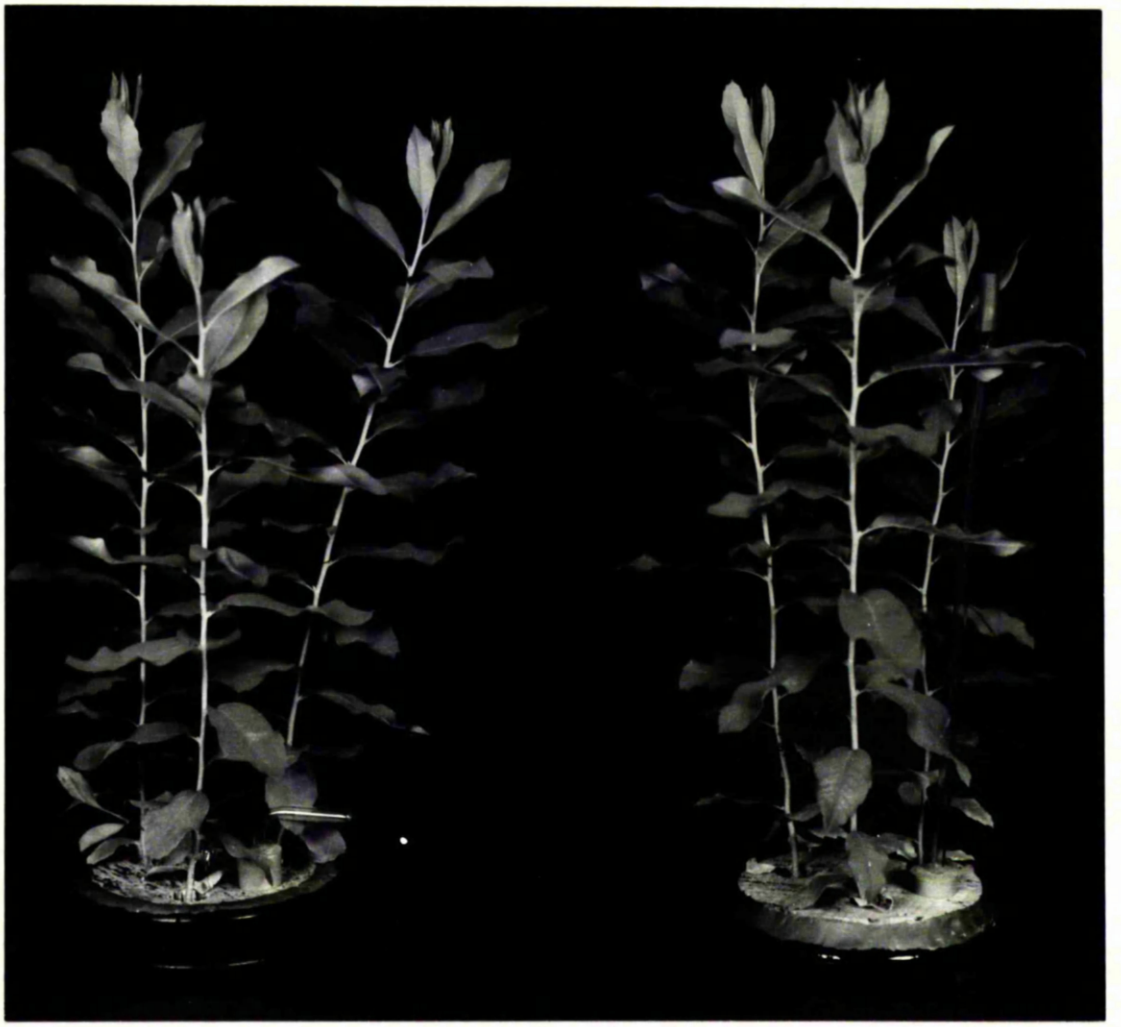


Fig. 57. Typical bog myrtle plants at end of experiment of 10 July-8 August, 1973 (x 1/3). Left; plants grown with normal air bubbled through the culture solution. Right; plants with air + 2% CO_2 bubbled through the culture solution.

Table 21. Mean growth and nitrogen data for bog myrtle plants grown in jar culture 10 July-8 August, 1973*.

Gas-flow	No. of plants	Shoot height cm	Dry wt per plant, g		%N in whole plant dry matter	Total N per plant mg
			Nodules	Whole plant		
Normal air	18	22.4	0.081	1.316	2.956	38.9
Air with 2% CO_2	18	22.2	0.087	1.182	2.860	33.8
Result of 't' test $P = 0.05$		N.S.	N.S.	N.S.	Not+ available	Not+ available

*Except in respect of %N, the values above indicate the increases in the various attributes shown during the period of gas-flow. They were calculated by deducting the values obtained at the preliminary harvest of comparable plants at the commencement of gas-flow from those obtained at the final harvest. Mean preliminary harvest data (based on 10 plants) in this experiment were as follows:-

Shoot height 8.5 cm; nodule dry wt 0.013 g; whole plant dry wt 0.140 g; total N per plant 4.5 mg.

+Dry matter from all plants from each series bulked prior to Kjeldahl analysis.

^{15}N Tests on Detached Alder Nodules

An experiment was carried out on 24 August, 1973, following the procedure described under Materials and Methods. The day was warm and sunny, which it was hoped would encourage rapid fixation in the nodules. The results are shown in Table 22. For three samples of alder nodules not exposed to excess ^{15}N, normal values of ^{15}N content were returned (0.366-0.368 atom %), indicating no enrichment and that the technique was generally satisfactory. The level of enrichment shown in the nodule which had been exposed to the special atmosphere was rather low - an enrichment of the order of 0.1 atom % had been hoped for, Mean enrichment in the nodules incubated without CO_2 is seen to be slightly greater than in the other series, but the difference is not significant by the 't' test, and is obviously due to one rather aberrant value.

Information on the levels of CO_2 which may be presumed to have been present at the end of the incubation in tubes of each type is given below.

Acetylene-Reduction Assays on Detached Alder and Bog Myrtle Nodules

Two experiments were carried out with alder nodules, with results as shown in Table 23. The general level of ethylene production was, as will be seen, generally much higher in the second experiment. This was probably because weather conditions on the two days prior to this experiment had been good, whereas this was not the case with the first experiment. In both experiments ethylene production was

Table 22. Effect of CO_2 on fixation in detached alder nodules, using ^{15}N technique.

CO_2 level	Sample number	N content of sample, mg	Atom % excess of ^{15}N in nodule N after exposure to labelled atmos.	
Absent or in low amount	1	6.6	0.046	Mean = 0.041
	2	6.1	0.044	
	3	6.4	0.029	
	4	6.8	0.046	
2% initially, rising	5	6.8	0.014	Mean = 0.035
	6	7.0	0.041	
	7	6.6	0.043	
	8	6.6	0.043	

Table 23. Effect of CO_2 on acetylene reduction in detached alder nodules.

Date of expt	CO_2 level	Sample number	Dry wt of nodule sample, mg	µmoles ethylene formed per hr per g nodule dry matter		Significance between means by 't' test
28 Aug. 1973	Absent or in low amount	1	175	3.01)		
		2	188	2.16)		
		3	182	1.41)	Mean = 2.28	
		4	173	3.83)		
		5	170	0.99)		Signif. at $P = 0.05$
	2% initially, rising	6	167	0.51)		
		7	170	0.28)		
		8	167	1.18)	Mean = 0.83	
		9	176	1.29)		
		10	166	0.91)		
31 Aug. 1973	Absent or in low amount	1	167	6.60)		
		2	162	4.84)		
		3	179	5.22)	Mean = 6.56	
		4	170	9.48)		
		5	175	6.67)		Signif. at $P = 0.01$
	2% initially, rising	6	170	0.88)		
		7	182	1.77)		
		8	173	1.67)	Mean = 1.62	
		9	168	2.93)		
		10	164	0.83)		

markedly reduced in the tubes where 2% CO_2 was provided initially and respiratory CO_2 was allowed to accumulate; in the first experiment the reduction was to less than a half of the ethylene production in tubes provided with soda-lime, and to a quarter in the second experiment.

In a similar experiment with bog myrtle nodules (Table 24) evidence of an inhibiting effect of CO_2 was again obtained. The effect was much smaller than with alder nodules, but was still significant. It will be noted that ethylene production was in general considerably higher than in the alder nodules of the preceding experiments. This tallies with previous comparisons of the activity of detached nodules of the two species in fixing nitrogen in ^{15}N tests.

Analyses of the CO_2 content of the gas within the tubes after the incubation period were made for the alder experiment of 31 August described above. For this purpose the residual gas was combined from all the five tubes in each treatment and analysed in an Orsat gas analyser. The analyses were made approximately 2 hr from the start of the incubation period. The gas from tubes containing soda-lime proved to have 0.8% of CO_2. That from tubes containing glass beads instead of soda-lime, and where 2% CO_2 was present initially, showed 6.0% CO_2. The latter agrees reasonably well with calculations based on the respiration rates given earlier. The presence of some CO_2 in tubes containing soda-lime shows that the latter reagent fails to keep the atmosphere entirely free of the gas, doubtless owing to the relative slowness of

Table 24. Effect of CO_2 on acetylene reduction in detached bog myrtle nodules.

Date of expt	CO_2 level	Sample number	Dry wt of nodule sample, mg	µmoles ethylene formed per hr per g nodule dry matter		Significance between means by 't' test
5 Sept. 1973	Absent or in low amount	1	166	15.21)		Signif. at $P = 0.05$
		2	170	12.21)		
		3	169	8.72)	Mean = 11.45	
		4	167	10.75)		
		5	175	10.36)		
	2% initially, rising	6	173	9.20)		
		7	178	8.39)		
		8	175	7.45)	Mean = 8.44	
		9	174	9.13)		
		10	184	8.04)		

gaseous diffusion.

Similar analyses of final CO_2 content were made in connection with the bog myrtle experiment. The results were almost identical with those for alder.

In reviewing the results of these acetylene assays, attention was given to the possibility that soda-lime might have a hygroscopic property which would lead to a drying-out of nodule samples enclosed over the substance. There is evidence that the precise state of wetness of the surface of legume nodules at least can markedly affect the rate of reduction of acetylene, and in particular that in the presence of a film of water on the nodules the rate of acetylene reduction is depressed. In a first test of the possible possession of hygroscopic properties, weighed samples (in the region of 14 g) of soda-lime were exposed to laboratory air for 2½ hr. The three samples all showed losses of 0.03%. Allowing for the fact that the samples would be tending to increase their weight through the uptake of CO_2, it was concluded that they were losing weight at a slightly greater rate through loss of moisture by evaporation. Confirmation of a lack of hygroscopicity was sought by placing weighed samples (1 g) of alder nodules (initially blotted as described in Materials and Methods) in the tubes used for the ^{15}N and acetylene assays, again with some over soda-lime and others over glass beads. After enclosure for 1 hr, re-weighing the nodules showed that the two samples over soda-lime had lost 1.8% and 2.0% in weight respectively, while those over beads showed very similar decreases of 1.7% and 1.8%. It was concluded that

in all tubes the nodules had equilibrated with the initially rather dry air in the tubes, and that the soda-lime had exerted no desiccating effect.

Finally, it is of interest to attempt to compare the estimates of nitrogenase activity in detached alder nodules obtained by these acetylene assays with those indicated in the already-described ^{15}N tests. From the equations

$$C_2H_2 + H_2 = C_2H_4 \quad \text{and}$$

$$N_2 + 3H_2 = 2NH_3$$

it follows that if the supply of hydrogen is limiting nitrogenase activity, then simultaneous measurements on given material by the two techniques should indicate a reduction of three molecules of acetylene for each molecule of nitrogen reduced. In such comparisons made by previous workers on various nitrogenase-containing samples, ratios of approximately 3:1 have often been obtained, but in some instances much higher ones have been reported, suggesting that reducing power is not always the limiting factor. No reliable ratio has been reported for alder nodules; from data provided by Akkermans (11) ratios varying from 1.3:1 to 3.3:1 may be calculated, but the author himself did not consider the data to be very dependable. The present author's data cannot be used to calculate such a ratio, since the tests were done on different days, and it is known that nitrogenase activity in nodules from greenhouse alder plants varies from day to day depending on the weather conditions (Wheeler, 63). Suitable calculations

from the data presented in Table 22 and other data provided in the 6th Section of Materials and Methods, shows that in the minus CO_2 tubes fixation amounted to 0.5 μmole N_2 per g nodule dry matter per hr, corresponding to a production of 1.5 μmole C_2H_4 if the theoretical ratio of 3:1 is assumed to apply to alder nodules. This figure is fairly close to the ethylene production actually observed in the minus CO_2 tubes in the first of the acetylene experiments shown in Table 23, which was carried out four days after the ^{15}N tests, but is considerably lower than that observed in the second experiment, performed seven days after the ^{15}N tests.

DISCUSSION

Since culture in jars gave overall better growth of alder than in tubes, discussion of growth experiment results in that species will be confined to those obtained in jar culture. The 1972 experiment with alder indicated a favourable effect of 2% CO_2. The benefit appeared to be due to a more rapid fixation of nitrogen in the nodules. As noted already, however, the two experiments with alder in 1973, which it was hoped would confirm that result, in fact, indicated a harmful effect of the CO_2 treatment. Now, it seemed, the nodules failed to supply fixed nitrogen in the amounts required for proper plant growth. It is believed that the 1973 result was the correct one, partly because the writer was able to give them constant personal attention. It is possible that in 1972, when gas supply was rather limited, the rate of gas-flow through the jars was too slow to bring the solution in the CO_2-air jars into equilibrium with the gas. This pre-supposes that at some CO_2 level below 2% fixation and growth in alder are better than in air, a supposition for which there is at present no evidence.

In the one growth experiment with bog myrtle, extending over four weeks of gas-flow, no significant difference was found between the two treatments, though the data suggested that with a longer growth period a harmful effect of 2% CO_2 might here also have been shown.

Thus the results of growth experiments with non-legumes reported here are very different from those found by

Mulder & van Veen with various legumes (see Introduction to this Chapter).

The results of the ^{15}N tests were disappointing. It has already been pointed out that alder nodules do not retain their nitrogen-fixing capacity very strongly after detachment, presumably due to a developing shortage of photosynthates, and of the derived ATP and reducing power. Under these circumstances it is conceivable that any promoting effect of CO_2 might tend to have on fixation would not, in fact, be realised, but there is no obvious reason why an inhibiting effect could not be revealed.

As noted, the acetylene assays gave clear-cut results. In the two experiments with alder nodules (Table 23) ethylene production was 64% and 75% inhibited respectively in the presence of 2-5% of CO_2, as compared with that in nodule samples exposed to a maximum of about 0.5%. In bog myrtle (Table 24) the inhibition was 26%. The only previous observation on the effect of CO_2 on acetylene reduction by nodule nitrogenase seems to be that of Sprent (64) on soya bean nodules, arising from her attempt to explain the gradual fall in activity of nodules confined in a relatively small container. Oxygen depletion was shown not to be responsible, nor did the removal of the accumulated respiratory CO_2 restore activity. She concluded that hydrogen produced by the nodules was possibly the inhibiting factor. In these circumstances it seems to the present writer impossible to exclude that the CO_2 also could have been inhibitory in Sprent's experiments.

Until the situation has received further study the explanation of the results can only be hazarded. It is possible that the presence of CO_2 at the levels here involved retards respiration in the nodules. The effect of CO_2 on respiration has been little studied, but Kidd (65) showed that in white mustard seedlings respiration (measured by oxygen uptake) was 20% inhibited in the presence of an initial level of 10% CO_2 rising to 15% by the end of the 14 hr period. A depressing effect of CO_2 on fruit respiration was a reason for the incorporation of CO_2 in the atmosphere of apple gas stores; 5% of CO_2 retarded the respiration by 45% in apples (Thomas et al., 66). Carbon dioxide has been shown to inhibit the action of succinic dehydrogenase, an enzyme of Kreb's cycle; in the presence of 10% CO_2 the enzyme was 50% inhibited in Ricinus mitochondria, while in pea mitochondria the inhibition was 50% when only 5% of CO_2 was present (Thomas et al., loc. sit.). Lundegårdh (67) states that in the presence of unusual levels of CO_2 the oxidation of cytochrome b is inhibited. If, in fact, CO_2 does interfere with respiration in these nodules (although according to the results of Mulder & van Veen reviewed in the Introduction it does not do so in legume nodules), then a slowing up of acetylene reduction by the nitrogenase might be a secondary effect, since there is an ATP requirement for that process.

The question now arises of how far the results of the acetylene assays can be used to explain the findings of the writer's growth experiments, namely that the presence in the

air-stream of 2% CO_2 had a harmful effect on the growth of alders, probably through an interference with nitrogen fixation, while with bog myrtle it seems likely that the same effect would develop given a longer growth period. It must be recalled that the level of CO_2 in the acetylene assays eventually rose to about 5%, but it does not seem unreasonable to conclude provisionally that the growth results and the acetylene assay results have a common explanation, namely that relatively high levels of CO_2 inhibit nitrogenase activity, possibly indirectly following a retardation in respiration. If, as suggested by evidence reviewed in the Introduction, increased CO_2 supply promotes 'dark' fixation in these nodules, it seems at the particular levels of CO_2 used here other, now deleterious effects - possibly on respiration - prevent any benefit to nitrogen fixation which the 'dark' fixation of CO_2 might otherwise convey. It is possible that at some lower level of CO_2, such as 0.5%, nitrogen fixation and growth would be benefitted, but it remains for further work to test this. Further work should also be directed to testing whether respiration is, in fact, depressed by increased CO_2 levels in these nodules.

The results here reported indicate that the incorporation of some device for the removal of respiratory CO_2 might be advantageous in routine acetylene assays with non-legume nodules.

SUMMARY

1. In growth experiments extending over several weeks, a comparison was made between the growth of nodulated plants of alder and bog myrtle rooted in culture solution through which ordinary air or in other cases air enriched with 2% CO_2 was constantly bubbled. Results were somewhat variable, but in most cases a harmful effect of the extra CO_2 on plant growth and nitrogen fixation was observed in alder, while the data suggested that the same might have been true eventually for bog myrtle.

2. No effect of CO_2 on fixation in detached alder nodules could be detected in ^{15}N tests, but in the more sensitive acetylene assay the presence of 2% rising to 5% CO_2 resulted in a marked reduction in the activity of nitrogenase in detached nodules of alder, with a somewhat smaller reduction in those of bog myrtle.

3. It is concluded that although in the presence of an enhanced level of CO_2 an increased rate of 'dark' fixation of CO_2 by carboxylase enzymes in the nodules might tend to promote nitrogen fixation, some other deleterious effect of the CO_2 prevents this happening. Evidence in the literature suggests that the harmful effect may have been exerted on some part of the respiratory process.

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