PHYSIOLOGY AND BIOLOGY OF NON-LEGUME ROOT-NODULE PLANTS

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Anne Hamilton Mackintosh, B.Sc.

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C O N T E N T S

A C K N O W L E D G E M E N T S

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GENERAL INTRODUCTION

For many years while the legume nodule symbiosis was receiving attention and investigation because of its agricultural importance, the root nodules present on a number of non-leguminous Angiosperms were largely ignored, despite the fact that evidence was obtained before the end of the last century that some of these nodules, e.g. those of alder, could fix atmospheric nitrogen. More recently, however, as the potential ecological importance of these plants has been realised, more and more time has been devoted to their study, and investigations have revealed that, apart from the identity of the endophyte, the nodule symbiosis of these non-legumes is basically similar to that of legumes.

The plants under discussion are those bearing what can conveniently be termed Alnus-type root nodules, by which is understood a frequently branching, tuberous, modified root whose cells contain a finely filamentous endophyte which fixes nitrogen. Rodriguez-Barrueco (1969) listed all the species that have been recorded up to the present to have the ability to form nodules

of this type, and his findings are included in the summary given on page 5. It will be noted that on present information there are thirteen genera of these plants. Of the 342 species classified as belonging to these genera, only 118 have been recorded as able to form nodules, the rest being apparently unexamined, often because they grow in very remote places. It is hoped that this knowledge will soon be extended since a survey of nodulation in non-leguminous Angiosperms has been promoted by Section PP (Production Processes) of the International Biological Programme in which botanists from many countries are participating.

As shown on page 5, three species of Dryas, namely D. drummondii, D. integrifolia and D. octopetala have been found to bear root nodules in North America. Hitherto the only particular feature of the roots of these species which had been reported was the presence of mycorrhiza. The paper of Lawrence, Schoenike, Quispel and Bond (1967) showed that these new-found structures, in D. drummondii at least, are definitely root nodules of the Alnus type, with the ability to fix nitrogen. It was originally intended that the present author should investigate the root nodules of D. octopetala, and as a first step plants of this species

Non-legume genera with *Alnus*-type nodules

Genus	Species complement	Distribution	Species recorded to bear nodules
Coriaria	15	Mediterranean, Central America, Japan, New Zealand	12
Alnus	35	Europe, Siberia, North America, Andes, Japan	27
*Myrica**	35	Tropical, sub-tropical and temperate regions	12
Casuarina	45	Australia, tropical Asia, Pacific Islands	15
Elaeagnus	45	Asia, Europe, North America	10
Hippophaë	3	Asia, Europe	1
Shepherdia	3	North America	2
Ceanothus	55	North America	31
Discaria	10	South America, New Zealand, Australia	1
Dryas	4	Arctic and north temperate zone	3
Purshia	2	North America	2
Cercocarpus	20	North America	1
Arctostaphylos	70	North-west America, Europe, Asia	1

* Including *Comptonia*

growing at various sites in Scotland and at the Burren, Eire, were very carefully examined for the presence of nodules. The only swollen root structures found were examined in section under the light microscope and these were seen to be similar to the ectotrophic mycorrhiza described by Hesselmann(1900). In an attempt to induce nodules on plants of D. octopetala, seedlings were grown in D. drummondii habitat soil from Alaska. These grew well at first but failed to survive their second winter and within this time did not nodulate. It is possible that the plants were not mature enough or that the conditions provided were not suitable, since Lawrence et al. (loc. cit.) noted that D. drummondii plants did not nodulate until they were in their second or third year, and that nodules did not form near the soil surface as in Alnus, but about 13 cm. down, pressed close against rock surfaces.

Another species which has only recently been reported to bear nodules is Arctostaphylos uva-ursi. Although this is nodulated in North America the author has been unable to find nodules on plants growing at several sites in this country. It was also intended to include in this thesis an investigation of nitrogen fixation in this species, and to this end the dormancy of

A. uva-ursi seed was successfully broken and seedlings grown in water culture. However, the expected arrival of habitat soil from Alaska was forestalled by the occurrence of some natural disasters in that region so that work could not be carried out.

As already mentioned the study of these non-legumes is now receiving much more attention than previously. There are many centres throughout the world apart from Britain where investigators are involved in studies in this field, e.g. in Finland, Japan, South Africa and the United States (especially at Oregon State University, Corvallis). As a further illustration of the increasing interest shown in these plants, a monograph on "Biology of Alder" was published in 1968 in Portland, Oregon by the Pacific Northwest Forest and Range Experiment Station. In this book are papers given at a symposium devoted entirely to Alnus which deal with different aspects of the genus, many of the papers being in some way connected with the ability of nodulated plants to fix nitrogen.

Another genus which has recently been under investigation by various workers is Ceanothus, and a Section of this thesis has been devoted to studying the effect of combined nitrogen on nodulation and fixation in

C. velutinus.

A puzzling feature and a handicap in the study of the non-legume symbiosis is the fact that so far no one has been successful in isolating the endophyte, although there is evidence that the organism can survive in the soil for a considerable number of years in the absence of the host plant (Rodriguez-Barrueco, 1968). The lack of pure cultures of the endophyte has meant that investigations into its nature must be confined to microscopic examinations of nodule material. So far it has been found that although there are differences in appearance between the endophytes of certain genera, all of them are finely filamentous - as established by the electron microscope studies of Becking et al. (1964), Gardner (1965) and others - and are probably Actinomycetes. Further evidence of similarities or differences between the endophytes of different genera and species can be gained by means of cross-inoculation studies, and a detailed investigation of this type in the genera Alnus and Myrica is reported in this thesis.

One of the most important discoveries of the past few years concerning nitrogen fixation has been that of Dilworth (1966) and Schöllhorn and Burris (1966), who found that the nitrogen-fixing system will readily

reduce acetylene to ethylene at a rate proportional to that of nitrogen fixation. This has been used as a means of measuring fixation by several workers and in this Department, Wheeler (1969) has used it to investigate diurnal variations in nitrogen fixation. His paper, which is described in more detail in a later Section, is part of the work at present being carried out in this Department on diurnal fluctuations in fixation. The latter part of this thesis is concerned with investigations made by the present author into this problem.

Other work recently undertaken in this Department is described in a paper by Wheeler and Bond (1969). The amino acids in the nodules of nine species of non-legumes were analysed. In three foreign species of Myrica, and also in species of Hippophaë, Elaeagnus, Ceanothus and Casuarina, asparagine was the predominant acid, as previously reported for Myrica gale (Leaf, Gardner and Bond, 1959), and also for legume nodules. In Alnus inokumai, citrulline was prominent, as previously shown for A. glutinosa (Leaf, Gardner and Bond, 1958). The amino acids indicated may be presumed to be those into which much of the products of fixation become incorporated in the nodules.

To recapitulate, the biological part of this thesis consists of a study of the possibility of cross-inoculation among species of Alnus and Myrica. The physiological part comprises an investigation into the effect of combined nitrogen on the nodule symbiosis of Ceanothus, and a study of diurnal variations in fixation in Casuarina.

SECTION I

Host plant-endophyte

adaptation in *Alnus* and *Myrica*

with Addendum on

Observations on the structure and cytology

of ineffective nodules of *Myrica cerifera* L.

Host plant-endophyte adaptation in *Alnus* and *Myrica*

I N T R O D U C T I O N

As already noted, there is satisfactory evidence that the endophytes of non-leguminous root nodules are finely filamentous in nature, with dimensions similar to those of the Actinomycetes, and it is believed by most workers that they in fact belong to that group (see review by Bond, 1967b). In this Section of the thesis the author is concerned with the question of how many forms or kinds of the endophyte occur in these nodules. Is the endophyte the same in them all, or is there one for each host genus, or one for each species, or is there some intermediate situation?

Until it becomes possible to culture the endophytes separately from the host plant, the number of criteria available for judging these issues is limited. The appearance of the endophytes in sections of nodules under the light or preferably the electron microscope can be studied, and from this it is clear that there

are differences in their structure. Thus, on the basis of rather few species studied, it appears that in the nodules of some host genera (Alnus, Hippophaë) relatively large, clearly-defined, roughly spherical structures - the vesicles - form terminally on certain hyphae, while these are absent in other genera (Myrica, Casuarina). Again, small structures - bacteroids - may be produced (Alnus, Myrica), or may be absent (Coriaria, Ceanothus). However, the appearance of the endophyte within the nodule cells might not be entirely a fixed character of the organism, since the host plant may exercise some control over the development and morphology of the endophyte. This was recently demonstrated for legumes by Kidby and Goodchild (1966) who showed that the appearance of a given strain of Rhizobium differed markedly in the cells of the nodules of two different genera of host plants. Both associations produced completely effective nodules, but their gross morphology differed, as well as the appearance of the endophyte in the cells. For example, the bacteria in the nodules of one (Ornithopus sativus) exhibited large electron-translucent regions and were enclosed in groups by a membrane, while the bacteria in the other host (Lupinus luteus) had no such electron-

translucent areas and were enclosed singly.

A second approach is to study the ability of endophytes of particular host species to infect and symbiose with "strange" host species of the same or different genera. In the legumes much use has been made of the results of attempted cross-inoculations in efforts to classify the Rhizobia. Although early investigators considered that one organism caused nodulation in legumes, this theory was soon destroyed with the arrival of more precise techniques, and it appeared that the infective ability of a particular organism was limited to a group of host plants. And so the theory of cross-inoculation groups was established, i.e. "groups of leguminous plants which shared mutual infective receptiveness to the same rhizobia" (Allen and Allen, 1958). The phenomenon of cross-inoculation was regarded as a prime character in differentiation of Rhizobium species and in 1932, Fred et al. listed 16 groups, the Rhizobia associated with six of these being given species status. However from this time the theory began to be discredited. Non-reciprocal cross-infection, indeterminacy and overlapping of group boundaries, and promiscuity between members of different groups caused Wilson (1939) and others to propose abandonment of the group concept.

Lange (1966) in his review of the current situation, states that the practice of delimiting rhizobial species according to cross-inoculation phenomena has reduced Rhizobium taxonomy to chaos, while Allen and Allen (1958) suggest that other outlets of experimentation should be employed in the search for differentiating characteristics, although "infective ability as the primary differentiating characteristic warrants confirmation or denial". It appears then, that caution must be applied in using cross-inoculation tests for classification purposes. Premature conclusions based on too limited tests should be avoided.

Some cross-inoculation studies with non-legumes, using crushed-nodule inocula, are to be found in the literature. Apart from the three genera of the family Elaeagnaceae, i.e. Elaeagnus, Shepherdia, and Hippophaë, between which there is complete compatibility in respect of their endophytes (Roberg 1934, Gardner and Bond 1957, and Moore 1964), there is no clear evidence of cross-inoculation between different genera of host plants (see review by Bond, 1963). Although Rodriguez-Barrueco (1967) reported that the alder organism is able to induce nodules in bog myrtle, confirmation of this is required. There has also been some study by

previous workers on the question of cross-inoculation between different host species of a given genus. Mowry (1933) found that nine species of Casuarina could be cross-inoculated. On the other hand, Bond (1962) has shown that an Asian species of Coriaria failed to nodulate in a soil containing the nodule endophyte of a European species of that genus. Also when American species of Myrica (Bond, 1967a) and Alnus (Rodriguez-Barrueco, 1966) were inoculated from nodules of European species of the corresponding genera, nodules formed in great numbers but remained small and fixed little nitrogen. However, satisfactory symbiosis of the Alnus glutinosa organism with other European alders has been found by Roberg (1938) and confirmed recently by Rodriguez-Barrueco and Bond (1968), although Becking (1966) has reported that the same endophyte performed indifferently on plants of the North American A. rubra.

Thus there is already evidence that the non-legume endophyte exists in a number of forms which differ in symbiotic properties, that is, in ability to induce nodule formation on a given host species and to carry on nitrogen fixation at a normal rate within the cells of the nodules.

In the work now to be described, the writer has

made an extended study of cross-inoculation within the genera Alnus and Myrica using a number of combinations not hitherto attempted.

MATERIALS AND METHODS

Host species and inocula employed

Table 1 lists the plant species used as hosts or as endophyte sources, and shows their geographical distribution. Out of the 27 Alnus species and 12 Myrica species recorded as bearing nodules (Rodriguez-Barrueco, 1969), 10 of the former genus have been employed in the present work, their distribution ranging over four continents. Of the 6 species of Myrica studied here, one - M. californica - has not previously been recorded as bearing nodules. Again these species are distributed over four continents.

The sources of seed for those species which the writer grew are shown in Table 2; seed collected by the author was taken from a number of different trees or bushes in the areas indicated, and it is fairly certain that the same was true of the seed of foreign species. It has been assumed that the foreign species had been correctly identified by the various botanists who supplied seed, to whom the writer is greatly indebted.

Table 1

Plant species employed, and their distribution

Species	Distribution
Alnus	
A. glutinosa (L.) Gaertn.	Europe, N. Asia, N. Africa
A. incana (L.) Moench	Europe and N. America
A. cordata (Lois.) Desf.	Europe
A. rugosa (Du Roi) Spreng.	N. America
A. rubra Bongard	West of N. America
A. inokumai Murai & Kusaka	Japan
A. multinervis Matsum.	Japan
A. sieboldiana Winkl.	Japan
A. firma Sieb. & Zucc.	Japan
A. jorullensis Kunth	Central and South America
Myrica	
M. gale L.	Europe and East of N. America
M. cerifera L.	N. America
M. rubra Sieb. & Zucc.	Japan
M. pilulifera Rendle	Tropical Africa
M. cordifolia L.	South Africa
M. californica Cham. & Schlecht.	West of N. America

Table 2

Sources of seed

Species	Source
Alnus	
A. glutinosa	Seed collected from several adjacent trees growing near Milngavie, Dunbartonshire.
A. incana	Vilmorin-Andrieux, Seed Merchants, Paris.
A. cordata	"
A. rugosa	Bergianska Trädgarden, Stockholm, via Mr. E. W. Curtis, The Curator, Botanic Gardens, Glasgow.
A. rubra	The Director, Pacific Northwest Forest and Range Experiment Station, U.S.D.A., Portland, Oregon.
A. inokumai	Dr. S. Uemura, Government Forest Experiment Station, Meguro, Tokyo.
A. multinervis	"
A. sieboldiana	"
A. firma	"
Myrica	
M. gale	Seed collected from plants growing on Stockiemuir, Stirlingshire.
M. cerifera	Prof. W. S. Silver, then of the University of Florida.
M. rubra	Dr. S. Uemura, as above.
M. cordifolia	The Director, National Botanic Gardens, C.P., South Africa.
M. californica	University of British Columbia, Vancouver, via Mr. E. W. Curtis.

In Table 3 are shown the species used as sources of the inocula employed for nodule induction. It will be seen that such inocula were prepared from two species of Alnus and four species of Myrica. In previous experiments with these genera, inocula of two types have been used, namely (a) those prepared by crushing nodules in water, and (b) water extracts of soil collected from around the nodulated roots of field plants. With some species (Alnus spp., Myrica gale) both methods are effective, but in others (Coriaria spp., Ceanothus spp.) crushed-nodule inocula are unreliable, and soil inocula are used. As indicated in Table 3, in the present work nodule inocula were used with six of the species, but soil inocula were used for Myrica cerifera (since nodule inocula had not proved fully dependable) and for M. cordifolia (since no nodules were available at the time).

Table 4 shows the combinations of host plant species and endophytes studied, totalling 33. In the genus Myrica a few combinations had to be omitted because of shortage of seed or poor germination.

Table 3

Sources of inocula

Species	Source
Alnus	
A. glutinosa	Nodules of stock plants grown in the greenhouse at the Botany Department, University of Glasgow, from seed collected at Milngavie, and originally inoculated from field nodules.
A. jorullensis	Nodules of stock plants grown in the greenhouse from seed and inoculated from nodules sent from Colombia by Mr. G. S. Smit (F.A.O.).
Myrica	
M. gale	Nodules of stock plants grown in the greenhouse from seed collected at Stockiemuir, and originally inoculated from field nodules.
M. cerifera	Habitat soil sent by Prof. W. S. Silver.
M. pilulifera	Nodules of stock plants originally sent by Mr. M. Dale, Ministry of Agriculture, Rhodesia, and grown on in the greenhouse.
M. cordifolia	Habitat soil sent by the Director, National Botanic Gardens, C.P., South Africa.

Table 4

Combinations studied

Host species	Inocula	
Alnus	A. glutinosa	A. jorullensis
A. glutinosa	+	+
A. incana	+	+
A. cordata	+	+
A. rugosa	+	+
A. rubra	+	+
A. inokumai	+	+
A. multinervis	+	+
A. sieboldiana	+	+
A. firma	+	+

Myrica	M. gale	M. cerifera	M. pilulifera	M. cordifolia
M. gale	+	+	+	+
M. cerifera	-	+	+	-
M. rubra	+	+	+	+
M. cordifolia	+	+	+	+
M. californica	+	-	-	-

Pre-treatment of seed. Raising the plants

Seed of A. glutinosa, M. gale, M. cerifera and M. californica was stratified by being placed in slightly moist sand in a refrigerator at about +2°C for two months prior to sowing, since this was known, or thought likely, to aid subsequent germination. Before sowing, the seed of all species except M. rubra (see below) was surface-sterilised by being shaken in a 2% solution of calcium hypochlorite for 45 minutes and then rinsed thoroughly in distilled water. This was done as a precaution against the possibility that the seed was contaminated with the normal endophyte, as for example by contact with the soil during collection. Seed of M. rubra had been kept in moist, probably sterilised soil by Dr. S. Uemura since its harvest, as he has found that this promotes subsequent germination. It was thus found on arrival to be in a germinating condition and therefore impossible to surface-sterilise. However 21 control plants of that species remained free of nodules throughout the experiment, confirming that the seed was uncontaminated.

After the above treatments, the seed was sown during the period March to May, 1967 - with the

exception of A. firma and M. cordifolia which were sown in the same period in 1968 - in trays of Peralite moistened with 1/8th strength Crone's solution* (N-free formula). The seed trays had previously been swabbed with methylated spirits and then thoroughly rinsed. In long experience Peralite, without sterilisation, has been found to be free of non-legume nodule endophytes.

After germination, when the seedlings were at the 2-3 leaf stage, they were transplanted into 2 or $2\frac{1}{2}$ litre sterilised water culture jars covered by teak tops previously dipped in boiling paraffin wax and with holes for 6 or 7 plants. Full precautions were taken to avoid contamination with nodule endophytes during this transfer. The culture solution for Alnus species was

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* Formula for full-strength Crone's solution (N-free)

KCl	3.75 g.
$CaSO_4.2H_2O$	2.50 g.
$MgSO_4.7H_2O$	2.50 g.
$Ca_3(PO_4)_2$	1.25 g.
$Fe_3(PO_4)_2.8H_2O$	1.25 g.
Distilled water	10 litres
Minor elements added	

½-strength Crone's (N-free) with 2 ml. of 2N H_2SO_4 added to 10 litres of solution, giving a pH of 5.3. For Myrica species the Crone's solution was used at ¼-strength (pH 6.3), except with M. gale, where acid was added to give a pH of 5.5. To prevent loss of seedlings through nitrogen starvation, a small amount of combined nitrogen was added at this stage, namely 2 mg. nitrogen per litre as ammonium sulphate to Alnus jars, and 3 mg. nitrogen per litre as ammonium nitrate to Myrica jars.

The use of water culture greatly facilitated the inspection of root systems for nodule formation. No forced aeration of the culture solution was provided, since previous experience is that these plants grow satisfactorily without it.

Preparation of inocula. Inoculation

As noted above, with most donor species crushed-nodule inocula were employed. Surface sterilisation of the nodules to be used for this purpose was not attempted, since there is no known effective method that can be routinely used. It is a common experience that after the application of various chemical sterilants to the surface of these nodules, a wide range of obvious

contaminants still develops when the nodules are plated out, while more drastic treatment results in the loss of infective power. However it is a reasonable assumption that nodules of say A. jorullensis received from Colombia will not arrive superficially contaminated with infective bodies of the A. glutinosa endophyte. Further, previous experience and also the results obtained in the present work with control plants (below) testify that transfer of endophytes from jar to jar does not occur in the greenhouse, with the precautions that are routinely taken. Thus, nodules on plants of A. jorullensis which have been in cultivation in the greenhouse for a period are unlikely to have become contaminated with other endophytes.

Crushed-nodule inocula were prepared by grinding nodules in a sterilised mortar with distilled water and a little sterile sand, in the proportions 5 g. nodules to 100 ml. water. This has been found in previous work to be of a suitable strength.

Habitat-soil inocula were prepared by shaking the soil with distilled water in the proportions of 1:1 by weight. Obviously there was nothing that could be done in the way of eliminating contaminating endophytes with

these inocula; the same argument as above indicates that the presence of such contamination was very unlikely.

Inoculation was effected by application of several drops of the appropriate inoculum to the root system of each plant, by means of sterilised, fine glass capillary tubes or a small sterilised brush. Inoculation was carried out within a month of the original transplanting. The application of different inocula to young plants of a given host species was usually carried out on the same day. A few exceptions to this will be pointed out later.

For each host species a number of plants were left uninoculated to serve as control plants against the possibility of cross infection between jars, or ingress from other sources. The jars containing these plants were placed on the greenhouse bench among the inoculated jars. It may be said now that of 160 such plants of various host species set up in the present work, only one plant developed nodules.

Standard plants

For the assessment of the success of the symbiosis between a given host species and a particular, unusual

endophyte, plants of the same host species associated with its normal endophyte and grown under the same conditions would ideally be available for purposes of comparison, and wherever possible this was arranged. For a number of host species, however, the normal endophyte was not available, and in these cases uninoculated plants supplied with ample combined nitrogen (as ammonium nitrate) were set up to provide alternative standard plants. The supply of nitrogen was commenced at the time when the corresponding plants were inoculated. These plants also served to test, in the case of species not previously grown in the greenhouse, whether under the conditions provided, and with nitrogen supplied, the particular species could grow well. No standard plants of either type could be set up for A. rugosa as insufficient plants were available on account of poor germination; in A. cordata, where the trial was done in two parts owing to poor germination at a first sowing, no standard plants were available for the first part.

Subsequent cultural treatment

As will have been realised already, the plants were grown in a greenhouse, which was lit by daylight.

The culture solutions were changed regularly - more frequently as the experiments proceeded - and the concentration raised as the plants increased in size. Nitrogen was supplied regularly to the combined nitrogen plants, which in most cases had to be thinned to prevent overcrowding of the jars, as was also the case with some nodulated plants.

Precautions were taken at all stages of the experiment to prevent contamination between jars.

At harvest, which was before leaf fall at the end of the first season of growth, all the plants of a given host species were dealt with at the same time. The dry weights of shoot and roots and of nodules were determined for each plant, and also the number of nodule clusters. The latter term is used because, in these non-legumes, the original simple nodule quickly develops into a many-branched structure or cluster. Kjeldahl analyses of total plant nitrogen were carried out for a few combinations, and for this purpose dry plant material from jars of the same treatment was bulked and milled.

Assessment of symbiotic performance

In an effort to secure an objective assessment of performance, a numerical basis was sought. Ideally data on the amount of nitrogen fixed would have been used in the calculations, but this would have entailed the making of several hundred Kjeldahl analyses. As an alternative basis, the dry weight attained by the plants has been used. This is justifiable since growth and the accumulation of dry matter by the plants was undoubtedly governed mainly by the rate of nitrogen fixation in the nodules. The following formula was employed to obtain what will be termed the percentage effectiveness of a particular symbiosis:-

$$\text{Percentage effectiveness} = \frac{DWx - DWc}{DWs - DWc} \times 100$$

where DWx = mean dry weight of the plants in the combination being studied;

DWc = mean dry weight of corresponding non-nodulated control plants growing in nitrogen-free culture;

DWs = mean dry weight of standard plants.

This was the formula employed by MacConnell and Bond (1957) to express the effectiveness of different

Rhizobium isolates on legume host plants. Obviously, the plant growth produced by the association of a given host species with an unusual endophyte is being compared with that produced by the same host species with its own endophyte, or with combined nitrogen supplied.

It will emerge later that in certain combinations some of the inoculated plants formed nodules, but others did not. This was interpreted as evidence of a degree of maladaptation between endophyte and host; in keeping with this attitude these non-nodulated plants were included in the calculation of mean plant dry weight, mean number of nodules per plant and mean nitrogen content per plant.

In those combinations for which analysis of nitrogen content was carried out, percentage effectiveness could be calculated additionally on a nitrogen content basis by substituting mean nitrogen content for mean dry weight in the above formula, the procedure followed by Bond and McGonagle (1951). The estimates yielded by both methods of calculating percentage effectiveness are assembled in Table 5, and show a satisfactory degree of agreement, so that estimates based solely on dry matter accumulation can be regarded with confidence.

Table 5

Comparison of percentage effectiveness values

Host species	Inoculum applied	Effectiveness of symbiosis, dry weight basis	Effectiveness of symbiosis, N content basis
A. incana	*A. glutinosa*	51	83
	A. jorullensis	9	14
A. rubra	*A. glutinosa*	26	36
	A. jorullensis	12	17
A. multinervis	*A. glutinosa*	15	20
	A. jorullensis	13	15
M. gale	*M. cerifera*	42	43
M. rubra	*M. gale*	0	0
	M. cerifera	2	4
	M. pilulifera	0	1
	M. cordifolia	4	6

As a measure of simplification, facilitating subsequent discussion, the figures for percentage effectiveness of the symbiosis have been graded as follows:-

Where comparison was against plants nodulated by the normal endophyte:

Percentage effectiveness	Grading
Over 50%	Satisfactory
25 - 50%	Fair
Less than 25%	Poor

Where comparison was against non-nodulated plants fed with combined nitrogen:

Percentage effectiveness	Grading
Over 25%	Satisfactory
10 - 25%	Fair
Less than 10%	Poor

The reason for having two sets of values for grading the success of symbiosis, depending on the type of standard plants used, is that experience has shown that under conditions similar to those of the present experiments, plants supplied with combined nitrogen

grow better than those dependent on nodule nitrogen. It is realised that the limiting values between the three grades were chosen rather arbitrarily, introducing an element of approximation into the gradings. This, however, was unavoidable.

D A T A O B T A I N E D

The results for the genus Alnus will be taken first, and it seemed better, before presenting the considerable array of numerical data, to refer first to the photographs of typical plants provided in Figs. 1-9, to be found at the end of this Section (prior to the Addendum). They were taken shortly before harvest and will allow the reader to gain a clear general picture of the over-all results.

The more important numerical findings for Alnus species are presented in Table 6. Firstly are shown the number of plants that were grown for each combination, and the proportion that formed nodules; it will be noted that in most combinations practically all the plants formed nodules, a notable exception being plants of A. rubra inoculated from A. jorullensis. Next is shown the mean number of nodule clusters present per plant at harvest, followed by the mean dry weight per nodule cluster; by a simple calculation from these two values the mean weight of nodules present per plant

Table 6

Summary of results for Alnus species

Host species	Inoculum applied	No. of plants grown[x]	Mean no. of nodule clusters	Mean dry wt. per nodule cluster mg.	Effectiveness of symbiosis	Grading
A. glut.	A. glut.	12 (12)	72	3.4	100*	(Standard plants)
	A. jor.	21 (20)	10	3.9	16*	Poor
A. incana	A. glut.	12 (12)	154	1.2	51**	Satisfactory
	A. jor.	10 (9)	21	1.7	9**	Poor
A. cordata	A. glut.	14 (14)	78	0.4	27**	Satisfactory
	A. jor.	12 (12)	28	3.6	X	Satisfactory by visual inspection
A. rugosa	A. glut.	6 (6)	53	1.3	X	Satisfactory by visual inspection
	A. jor.	5 (5)	9	2.8	X	Fair by visual inspection
A. rubra	A. glut.	19 (19)	169	0.8	26**	Satisfactory
	A. jor.	20 (13)	3	12.3	12**	Fair

(Table contd. over)

Table 6 (contd.)

Host species	Inoculum applied	No. of plants grown‡	Mean no. of nodule clusters	Mean dry wt. per nodule cluster mg.	Effectiveness of symbiosis	Grading
A. inok.	A. glut.	12 (12)	205	0.4	40**	Satisfactory
	A. jor.	10 (10)	20	2.9	29**	Satisfactory
A. mult.	A. glut.	12 (12)	132	0.2	15**	Fair
	A. jor.	10 (10)	17	0.7	13**	Fair
A. sieb.	A. glut.	11 (11)	79	0.3	21**	Fair
	A. jor.	11 (9)	13	0.6	0**	Poor
A. firma	A. glut.	12 (12)	148	0.2	46**	Satisfactory
	A. jor.	12 (12)	104	0.2	29**	Satisfactory

‡ Number of plants nodulated is shown in parentheses.

* Plants inoculated with the normal endophyte used as standard plants.

** Non-nodulated plants supplied with combined nitrogen used as standard plants.

X No standard plants available.

can be derived. It will be seen that with most host species the A. jorullensis inoculum produced relatively few nodule clusters, and though these tended to be relatively large - a notable example being the nodule clusters of A. rubra - it is obvious that in most cases the weight of nodules per plant was smaller than when the A. glutinosa inoculum was used. Also recorded, but not shown in the Table, was the interval for each combination between inoculation and the first appearance of macroscopic nodules. Under the conditions of the present experiments inoculation with the normal endophyte for a particular host species has usually been found to result in the appearance of nodules within three weeks. This was also true in these unusual combinations, with the exception of A. sieboldiana where nodules did not appear until five weeks from inoculation.

Next in Table 6 are the calculated effectiveness values for the symbioses, and the final grading. As will be indicated in more detail later, there was considerable plant-to-plant variation in growth in given combinations; analysis of variance shows that at $P = 0.05$ any two estimates of mean effectiveness (the values shown in the Table) must differ by at least 20

for significance. In the calculation of this significant difference it was assumed that there were 12 plants per treatment, a figure which is close to the average number for all the treatments. It is obvious that because of this statistical situation there is some unavoidable uncertainty in the grading of certain combinations into the categories set out on page 33. With these reservations in mind, the over-all position is that of the total of 17 unusual combinations tested, nine were graded "Satisfactory", five "Fair" and three "Poor". When the symbioses involving the A. glutinosa endophyte and the A. jorullensis endophyte are considered separately the position is as follows:-

Endophyte	Total no. of combinations	Symbiotic grading		
		Satisfactory	Fair	Poor
A. glutinosa	8	6	2	0
A. jorullensis	9	3	3	3

Obviously the A. jorullensis endophyte gave a relatively inferior performance, and one reason for this is undoubtedly the above-reported finding that the weight

of nodules formed per plant was usually smaller where this endophyte was involved. The question also arises as to whether the nodule tissues containing this endophyte were, weight for weight, as active in fixation as those containing the A. glutinosa organism, and to test this, the formation of plant dry matter associated with unit weight of nodule tissue formed was calculated as a measure of nodule efficiency. This again is based on the consideration that under the prevailing conditions the accumulation of dry matter by the plants is governed by the rate at which fixed nitrogen is provided by the nodules. The results are shown in Table 7, together with the gradings for symbiotic performance repeated from Table 6. Considerable variation in nodule efficiency between different combinations is indicated, but there is clearly no consistant relation between these values and the grading of the symbioses. The nodules induced by A. jorullensis inoculum on A. sieboldiana showed no ability to fix nitrogen, and this was obviously responsible for the "Poor" symbiotic performance of this combination, but apart from this there is no evidence that low nodule efficiency contributed to "Poor" or "Fair" gradings for the symbioses.

Table 7

Nodule efficiency data for Alnus*

Host species	Inoculum applied	Grading	G. plant dry matter formed per g. nodule dry matter**
A. glutinosa	A. glut.	(Standard plants)	21.8
	A. jor.	Poor	22.1
A. incana	A. glut.	Satisfactory	15.2
	A. jor.	Poor	14.3
A. cordata	A. glut.	Satisfactory	15.8
	A. jor.	Satisfactory	13.8
A. rugosa	A. glut.	Satisfactory	8.9
	A. jor.	Fair	12.7
A. rubra	A. glut.	Satisfactory	9.4
	A. jor.	Fair	15.1
A. inokumai	A. glut.	Satisfactory	15.3
	A. jor.	Satisfactory	17.6
A. multinervis	A. glut.	Fair	7.5
	A. jor.	Fair	14.7
A. sieboldiana	A. glut.	Fair	5.9
	A. jor.	Poor	0.0
A. firma	A. glut.	Satisfactory	6.8
	A. jor.	Satisfactory	5.4

* For nodulated plants only.

** Calculated from the formula:-

$$\frac{\text{Mean plant dry wt.} - \text{Mean dry wt. of non-nodulated control plants}}{\text{Mean dry wt. of nodules per plant}}$$

Thus in these Alnus trials the weight of nodules forming per plant was the factor mostly determining symbiotic performance.

A circumstance worthy of note is that the data in Tables 6 and 7 show that in Alnus rather numerous, small nodule clusters (e.g. on A. inokumai, A. firma, both inoculated from A. glutinosa) gave as satisfactory results as did fewer, larger clusters (e.g. A. glutinosa, A. rugosa again inoculated from A. glutinosa).

A noticeable feature which occurred in a few combinations in Alnus was the unusually large differences between the growth of individual plants. There is always quite considerable variation in the growth of nodulated plants raised from unselected seed and inoculated with the usual endophyte. This can be attributed to differences in seed size and genetic differences in the growth potential of the plants, these initial differences tending to be accentuated when the plants are grouped together in pots, by a shading effect of the stronger plants. The variation which was noticed in some unusual combinations, especially where the over-all grading was "Fair" or "Poor", was much greater, and can be observed in some

of the photographs. It is confirmed by the data showing the range in plant dry weights for some combinations in Table 8 and also by the standard deviations included in the same Table. It seems possible that the plants in these combinations differed for genetic reasons in their ability to symbiose with the endophytes concerned, as demonstrated by Nutman (1946) for clover. Variation of this kind in non-legumes has previously been reported by Rodriguez-Barrueco (1966) in plants of A. jorullensis inoculated from A. glutinosa, and also by Rodriguez-Barrueco and Bond (1968) in three European alders inoculated from A. rubra. Experiments using clonal host plant material would ensure populations with identical genotypes, although in the particular problem being studied in this investigation more representative results are probably to be found by use of plants raised from unselected seed.

An additional incidental observation was that in most of the Alnus cross-inoculations the nodules produced nodule roots - see Fig. 14. Earlier such nodule roots were believed to be confined to Casuarina and Myrica species - see Fig. 15 - but they have recently been described by Rodriguez-Barrueco for Alnus

Table 8

Dry weight and variation data for Alnus*

Host species	Inoculum applied	Grading	Mean whole plant dry wt., g.	Range, g.	Standard deviation** %
A. glut.	A. glut.	(Standard plants)	5.378	2.376-9.705	37
	A. jor.	Poor	0.966	0.124-3.455	83
A. incana	A. glut.	Satisfactory	3.039	0.931-5.703	59
	A. jor.	Poor	0.624	0.086-1.777	77
A. rubra	A. glut.	Satisfactory	1.296	0.420-2.125	38
	A. jor.	Fair	0.618	0.041-4.076	158

* All the plants of each treatment included, irrespective of whether nodulated.

** Expressed as a percentage of mean dry weight.

jorullensis (1966) and A. cordata (1967), and by Becking (1966) for A. rubra. The present author found that the nodule roots were mostly downward growing as did Rodriguez-Barrueco, and although Becking considered them to be typically upward growing his photographs scarcely confirm this.

The results of the Myrica trials will now be presented. Photographs of typical plants are provided in Figs. 10-13, to be found at the end of this Section, while the main data are presented in Table 9. Two inocula failed to produce any nodules on plants of M. gale, and the M. pilulifera inoculum caused nodulation in only half of the test plants of M. rubra. The combination of M. cerifera host plants inoculated from the M. gale endophyte was not attempted since Bond (1967a) had already carried out this cross (see Introduction). In the present trials a very similar result was obtained when the same inoculum caused the formation of extremely numerous, minute nodule clusters on M. rubra plants, the clusters being too tiny for their separation from the roots to be practicable. Fig. 15 (at the end of this Section) shows some of these clusters and also, for comparison, a cluster of more normal

Table 9

Summary of results for Myrica species

Host species	Inoculum applied	No. of plants grown‡	Mean no. of nodule clusters	Mean dry wt. per nodule cluster mg.	Effectiveness of symbiosis	Grading
M. gale	M. gale	16 (16)	12	12.8	100*	(Standard plants)
	M. cerif.	15 (14)	20	3.8	42*	Fair
	M. pilul.	16 (0)	0	0.0	-	Poor
	M. cord.	6 (0)	0	0.0	-	Poor
M. cerif.	M. cerif.	10 (10)	41	1.3	100*	(Standard plants)
	M. pilul.	10 (10)	4	18.3	125*	Satisfactory
M. rubra	M. gale	14 (14)	314	Not found, minute	0**	Poor
	M. cerif.	14 (14)	25	0.5	2**	Poor
	M. pilul.	14 (7)	1	0.7	0**	Poor
	M. cord.	14 (14)	25	1.3	4**	Poor

(Table contd. over)

Table 9 (contd.)

Host species	Inoculum applied	No. of plants grown‡	Mean no. of nodule clusters	Mean dry wt. per nodule cluster mg.	Effectiveness of symbiosis	Grading
M. cord.	*M. cord.*	12 (12)	13	0.7	100*	(Standard plants)
	M. gale	12 (12)	80	0.1	5*	Poor
	M. cerif.	7 (7)	43	0.1	0*	Poor
	M. pilul.	12 (10)	3	1.0	1*	Poor
M. calif.	*M. gale*	3 (3)	22	Not found, minute	X	Poor by visual inspection

‡ Number of plants nodulated is shown in parentheses.

* Plants inoculated with the normal endophyte used as standard plants.

** Non-nodulated plants supplied with combined nitrogen used as standard plants.

X No standard plants available.

appearance. The M. gale inoculum also induced minute nodule clusters on M. californica, and quite numerous, small nodule clusters on M. cordifolia. In contrast, a few extremely large nodule clusters were induced by the M. pilulifera endophyte on plants of M. cerifera.

Unlike the results for Alnus species, in Myrica ten of the twelve unusual combinations showed delayed nodulation, the two exceptions being M. gale plants inoculated from M. cerifera, and M. cordifolia plants inoculated from M. gale.

In respect of the effectiveness of the Myrica symbioses, analysis of variance indicates that at $P = 0.05$ the least significant difference is 40. Again it was assumed that there were twelve plants in each treatment as this was the average number for all the treatments. A picture very different from that for Alnus is shown, since of the 12 unusual combinations tested only one was assessed as "Satisfactory", only one as "Fair", while no less than ten were "Poor", including the two where no nodules at all were formed. It will be noted that the effectiveness of the combination of M. cerifera plants inoculated from M. pilulifera appears to have exceeded that of M. cerifera

with its normal endophyte; though this is not an impossible situation, the statistical analysis shows that the difference is not significant, and if it had been there would still have been a simple reason for it. This is that the M. cerifera crushed-nodule inoculum proved uninfective, and the plants had to be re-inoculated with soil inoculum, with the result that the post-nodulation time for growth was a few weeks shorter for these plants than for those nodulated from M. pilulifera. This delay in application of the infective M. cerifera inoculum occurred in all the species inoculated from this endophyte, and probably resulted in the symbiosis produced with plants of M. gale failing to be graded "Satisfactory".

The data in Table 9 suggest that in all the nodulating combinations graded "Poor" the nodule tissues were ineffective in fixation. This is confirmed by the nodule efficiency data, calculated as for Alnus, and presented in Table 10. This is clearly the main reason for the poor performance of these combinations. It will also be noted from Table 10 that plants of M. cerifera, inoculated from both M. cerifera and M. pilulifera endophytes, produced extremely

Table 10

Nodule efficiency data for Myrica*

Host species	Inoculum applied	Grading	G. plant dry matter formed per g. nodule dry matter**
M. gale	M. gale	(Standard plants)	19.8
	M. cerif.	Fair	17.5
M. cerifera	M. cerif.	(Standard plants)	56.5
	M. pilul.	Satisfactory	52.1
M. rubra	M. gale	Poor	0.0
	M. cerif.	Poor	7.3
	M. pilul.	Poor	7.5
	M. cord.	Poor	5.6
M. cordifolia	M. cord.	(Standard plants)	12.7
	M. gale	Poor	1.0
	M. cerif.	Poor	0.0
	M. pilul.	Poor	2.0
M. californica	M. gale	Poor	-

* For nodulated plants only.

** Calculated from the formula:-

$$\frac{\text{Mean plant dry wt.} - \text{Mean dry wt. of non-nodulated control plants}}{\text{Mean dry wt. of nodules per plant}}$$

efficient nodules. Nodules of this same species were found by Bond (1967a) to be more efficient in nitrogen fixation than any other species previously examined by him.

The large plant-to-plant variation shown by several of the Alnus combinations was not found in Myrica. In most cases the symbioses were very poor with ineffective nodules being formed. Little growth was made by the plants which therefore hardly varied in size. An example of this was M. cordifolia where the standard deviations of unusual combinations were lower than the value for normal nodulated plants:-

Host	Inoculum	Standard Deviation %
M. cordifolia	M. cordifolia	34
	M. gale	26
	M. cerifera	22
	M. pilulifera	20

D I S C U S S I O N

The results presented in the previous section suggest that host plant-endophyte adaptation has occurred in the genera Alnus and Myrica, especially in the latter since a satisfactory symbiosis often, though not always, failed to materialise when unusual combinations of host plant and endophyte were set up. Thus, as noted already, 8 out of 17 unusual combinations in Alnus were to a greater or lesser degree unsatisfactory, while in Myrica the proportion was 11 (or possibly 10 - see page 49) out of 12.

Incompatibility leading to unsatisfactory symbiosis showed itself, in the present studies, in the following ways:-

(1) The applied inoculum failed to induce any nodulation, at least not within the period of the experiment. This was the situation in plants of Myrica gale inoculated from M. cordifolia and M. pilulifera.

(2) The inoculum failed to cause nodulation in

all the test plants, resulting in the mean effectiveness - in the calculation of which all the plants were included - being low. Examples of this were plants of Alnus rubra inoculated from A. jorullensis, and Myrica rubra plants inoculated from M. pilulifera.

(3) Nodules were unusually slow to appear, so that even if they were efficient in fixation, they had less time, within the duration of the experiment, in which to promote the growth of the plants. This delay was noted in many of the Myrica combinations.

(4) Whether or not the above delay in the first development of nodules occurred, the mean weight of nodules formed per plant during the first season of growth was relatively low. This was true of several Alnus species inoculated from A. jorullensis.

(5) Nodules, although formed, were to a greater or lesser degree ineffective in fixation. This was especially true in several unusual combinations in Myrica, and was sometimes associated with the formation of numerous, very small nodule clusters.

At this point the possibility that errors of technique contributed to the results obtained will be considered. Thus, in those unusual combinations - nine in Alnus and one in Myrica - where a satisfactory symbiosis was obtained, it might be suggested that the normal endophyte had in some way gained access to the plants. The contamination would have to be on a substantial scale to account for the results. Reasons for believing that the inocula would not be contaminated with 'foreign' endophytes were given on page 26 and they become still stronger in view of some of the present results. Thus Alnus rugosa and the two Japanese species - A. inokumai and A. firma - had never been grown in the greenhouse before, and it is impossible to contemplate that the satisfactory symbioses set up after inoculation of these species with crushed-nodule inocula from A. glutinosa and A. jorullensis, were really due to the former species' own endophytes being present as contaminants in the inocula. The finding already reported that only one out of 160 uninoculated control plants developed nodules, points to the adequacy of seed sterilisation and the virtual absence of any contamination between jars during the experiment.

In the symbioses assessed as only "Fair" or "Poor" it might be suspected that the inoculum applied was for some local reason low in activity. Obviously this would be disproved if the same inoculum gave satisfactory results with some other species, and in fact inspection of the results already presented shows that every inoculum employed produced a satisfactory symbiosis with at least one species.

In view of the above considerations the findings reported can be viewed with considerable confidence. It is agreed that it would be much better if pure cultures of the various endophytes were available for use in cross-inoculation trials, but until that situation arises other methods must be used.

It should be noted that all the results are based on findings for the first year of growth, and that if the experiments had been continued for several years, differences between some of the treatments might have diminished.

Some of the present results confirm or are closely related to those of earlier authors. The finding that the Alnus glutinosa endophyte symbioses satisfactorily with other European alders (A. incana, A. cordata) confirms the earlier results of Roberg (1938) and

Rodriguez-Barrueco and Bond (1968), but the satisfactory symbiosis of the A. glutinosa endophyte with A. rubra plants differs from the results of Becking (1966) who found that only 28 per cent of the plants inoculated formed nodules. In 1966, Rodriguez-Barrueco found that A. jorullensis plants inoculated from A. glutinosa produced many small nodules which fixed little nitrogen. The reciprocal cross tried in this work produced nodules which were the same size and as efficient as normal A. glutinosa nodules, yet because there were so few, the symbiosis was graded as "Poor". Another reciprocal cross, of work previously carried out by Bond (1967a), was the inoculation of Myrica gale with the M. cerifera endophyte. The normal, effective nodules formed on M. gale contrasted with the numerous, small, ineffective nodules found by Bond on M. cerifera. It would appear that, as in the legumes, non-reciprocal cross-infection can occur between non-legume species. Bond (unpublished), has found that plants of M. cordifolia inoculated with M. gale produce small, ineffective nodules, results which are confirmed in this work. Thus plants of M. cerifera, M. cordifolia and M. rubra have been found to produce numerous, tiny, ineffective nodules on inoculation from M. gale. Plants of M. californica

inoculated with the same endophyte, produced similar nodules, although they were not numerous. It may be that there is a hormonal control of nodule numbers in non-legumes as was demonstrated by Nutman (1952) for legumes. However, as was pointed out earlier, in certain Alnus combinations the many small nodules which were produced proved to be effective in fixation.

In view of the lack of understanding of the processes of root infection, nodule development, and the initiation of successful symbiosis within the nodule, even in legumes, there would be little purpose in discussing the immediate reasons for the failure of many unusual combinations to establish a satisfactory symbiosis. Briefly, however, it may be surmised that features (1) and (2) on page 52 are due to inability of the endophyte to enter the root tissues of the unusual host species, or to do this very readily. Features (3) and (4) might also arise in part for the latter reason, but in addition there may be a failure of the endophyte to stimulate nodule growth in the strange host - a process which presumably has an auxin basis. Feature (5) may arise through metabolic incompatibility; alternatively, if fixation is the function of some morphologically distinct part of the endophyte, it is possible

that these nodules are inactive in fixation because endophyte development is incomplete. Becking (1966) has suggested that in Alnus glutinosa the vesicles might be responsible for the fixation of nitrogen. In the Addendum to this Section of the thesis, a light microscope investigation of the structure of effective and ineffective nodules of Myrica cerifera is reported.

It will, however, be profitable to discuss whether there is any rational basis for the finding that some unusual combinations give good results and others poor ones. A first possibility is that it is due to the geographical distribution of the species. The long association of one endophyte with a particular host species may have led, by processes of selection, to a loss of ability to infect and symbiose satisfactorily with a host species differing somewhat in its physiology from the usual host. Evidence of this geographical adaptation is mentioned in the review of previous work in the Introduction, and some of the findings in the present work support it also. Thus a degree of incompatibility was found between the endophyte of the South American species, Alnus jorullensis and two European, two North American and two Japanese alders. The A. glutinosa organism symbiosed satisfactorily with the

European alders, as would be expected, but not so well with two Japanese species. In the genus Myrica where host plant-endophyte adaptation was so marked, it appears that distribution of the species tends to be rather restricted. Chevalier (1900-1902), in the only authoritative monograph of the Myricaceae, stated that the family is now reduced to a small number of species spread all over the world, but for the most part each having only a small area of dispersion, Myrica gale covering an area as great as all the other Myricaceae together. He described how in the mio-pliocene the Myricaceae were abundant in Europe. However, when glaciation forced them towards the south into Africa, they became established round the Cape and on summits of the tropical zone of Africa, where they are found today, presenting a small number of very localised species. Results from the present experiments show that North American, African and European endophytes proved incompatible with the Japanese M. rubra, as did European, North American and tropical African endophytes with the South African M. cordifolia. Also, the two African endophytes failed to nodulate the European M. gale, while the organism of the latter gave poor results on M. californica.

However, some results do not support the geographical theory of adaptation. The endophyte of the South American Alnus jorullensis symbiosed well with the European A. cordata and the two Japanese species, A. inokumai and A. firma. Inocula from A. glutinosa proved compatible with two North American and two Japanese species. The African Myrica pilulifera organism symbiosed as well with plants of M. cerifera (North America) as did the normal endophyte, and finally, the endophyte of M. cerifera did well on plants of M. gale.

A second possible explanation is that the results have a taxonomic basis. Host species symbiosing satisfactorily with a given unusual endophyte might prove to be ones recognised by taxonomists as being relatively closely related to the normal host species of that endophyte. Although taxonomists depend mainly on morphological characters for classification it is likely that species morphologically similar will also have physiological similarities. In his classification of Alnus, Murai (1964) divided the genus into two subgenera - Alnaster and Gymnothyrsus - and these again into various sections. One of the sections of the subgenus Gymnothyrsus was Glutinosae, and in this he placed the following species:- A. inokumae (A. inokumai),

A. rugosa, A. rubra, A. glutinosa and A. incana, as well as six other species. The fact that A. glutinosa is closely related to the above species may explain why its nodule endophyte symbiosed satisfactorily with all of these. The two remaining species with which this endophyte symbiosed well were A. cordata, belonging to section Japonicae of the subgenus Gymnothyrsus, and A. firma belonging to section Bifurcatus of the subgenus Alnaster. The former can be explained on the basis of the geographical theory, the latter, however, cannot. The two species with which the A. glutinosa endophyte showed a degree of incompatibility were Japanese and not of the same section Glutinosae, i.e. A. sieboldiana, section Bifurcatus (Alnaster), and A. multinervis which is not mentioned by Murai. Alnus jorullensis is classified as a member of the section Japonicae (Gymnothyrsus). The three "Poor" and three "Fair" symbioses of this endophyte were all with host species from other sections. Of the three "Satisfactory" symbioses, one was with a species from the same section, i.e. A. cordata.

In respect of Myrica, experience in this department is that the seed form and foliar characters of Myrica species are more variable than in Alnus (see photographs). Early botanists placed M. gale into a separate genus and

called it Gale palustris (Lamk.), although present-day botanists, e.g. Engler (1964) generally refer to the species as M. gale. Chevalier (1900-1902) placed the species in the genus Gale because of various morphological and anatomical differences between the two genera, e.g. in Gale, the spikes which bear the male and female catkins die after the pollen or seed is shed, while in Myrica they do not. Chevalier's classification of the other Myrica species with which the present author is concerned was as follows: he divided the genus into three sections, to which the following species belong:-

Morella	Faya	Cerophora
		M. pilulifera
M. Nagi (M. rubra)	M. californica	M. cordifolia
		M. cerifera

Thus, of all the Myrica species used by the present author, only the three in the section Cerophora are considered to be closely related and these are geographically distinct, although the close relation of M. pilulifera and M. cerifera could explain why the endophyte of the former symbiosed so well with host plants of the latter.

Thus it would appear that it may be a combination of both geographical distribution, and whether or not species of a genus are closely related, which determines whether a satisfactory symbiosis will result from a particular cross-inoculation.

In the Introduction to this Section the question was posed as to whether there was a distinct endophyte for each host species or for each host genus, or whether there was some intermediate condition. From the results of the present experiments which dealt with only a proportion of the species in each genus, the only generalisation which can be made of both genera is that there is not one endophyte which will symbiose satisfactorily with all the species within each genus.

In order to be able to say that the endophytes of two species of a genus are probably identical in respect of cross-infection properties, reciprocal cross-inoculations must have proved satisfactory, since it is sometimes found that while the endophyte of one species symbioses well with another species, the endophyte of the latter does not do well on host plants of the former. On the other hand, to be able to say that the endophytes of two species are not identical in respect of cross-infection, if a poor symbiosis has occurred

from the infection of one host with the endophyte of the other, then the result of the reciprocal cross need not be known. Applying this to the findings for the Alnus species, apart from the European alders which are known to be cross-inoculable, further reciprocal crosses would have to be carried out to discover whether the A. glutinosa endophyte was identical to the endophytes of the species with which it symbiosed satisfactorily. The same can be said for the A. jorullensis endophyte, although it can be seen that it is not identical with those of A. glutinosa, A. incana and A. sieboldiana. More can be deduced about the endophytes of the Myrica species. All but two of the unusual combinations ~~had~~ showed poor symbioses. The reciprocal of one of these two, M. gale plants inoculated from M. cerifera, has been described earlier, and was found to be unsatisfactory (Bond, 1967a). The endophyte of M. pilulifera symbiosed satisfactorily with M. cerifera, and yet if the two endophytes were identical, then the inoculation of M. gale plants from both of these should have had similar results. This was not the case. It can therefore be said that the endophytes of M. gale, M. cerifera, M. pilulifera, M. cordifolia and M. rubra all appear to be different in respect of cross-infection, as are M. californica

and M. gale. Thus any proposal such as to call the Myrica endophyte "Frankia myricae", as was made informally by a speaker at the meeting of the Society for Experimental Biology in Glasgow, July 1968, would imply that there is an organism which will infect all species of Myrica, and no such organism has been found by the present author. On the contrary, it appears that the endophytes of all the species of Myrica which were studied must be different.

Since only a proportion of the species in each genus has so far been studied, it is therefore suggested that premature classification of the endophytes should be avoided, lest the chaos existing in the Rhizobium classification be repeated in the non-legumes.

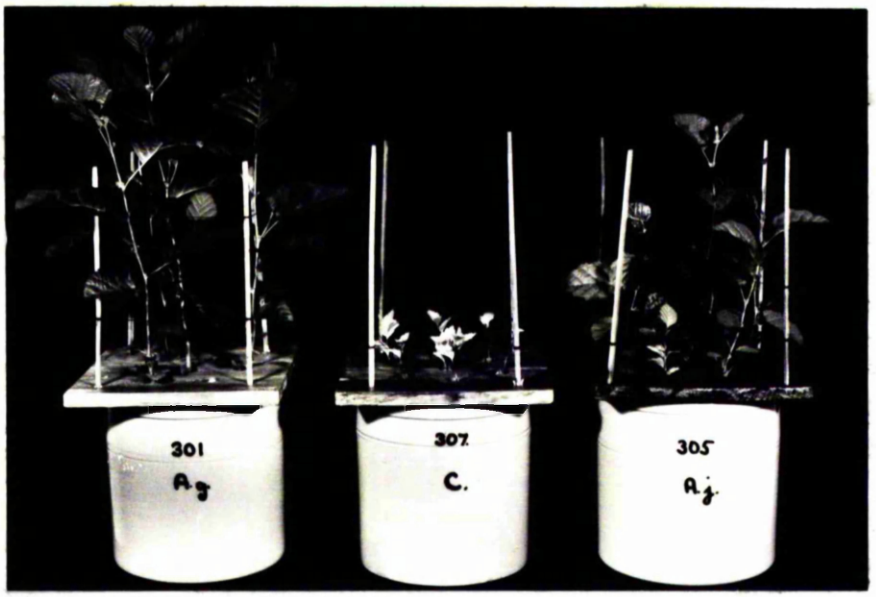


Fig. 1. Alnus glutinosa plants. Left to right: inoc. from A. glutinosa; control; inoc. from A. jorullensis. (x 1/7)

Fig. 2. Alnus incana plants. Left to right: inoc. from A. glutinosa; inoc. from A. jorullensis; control; non-nodulated plants supplied with combined nitrogen. (x 1/10)

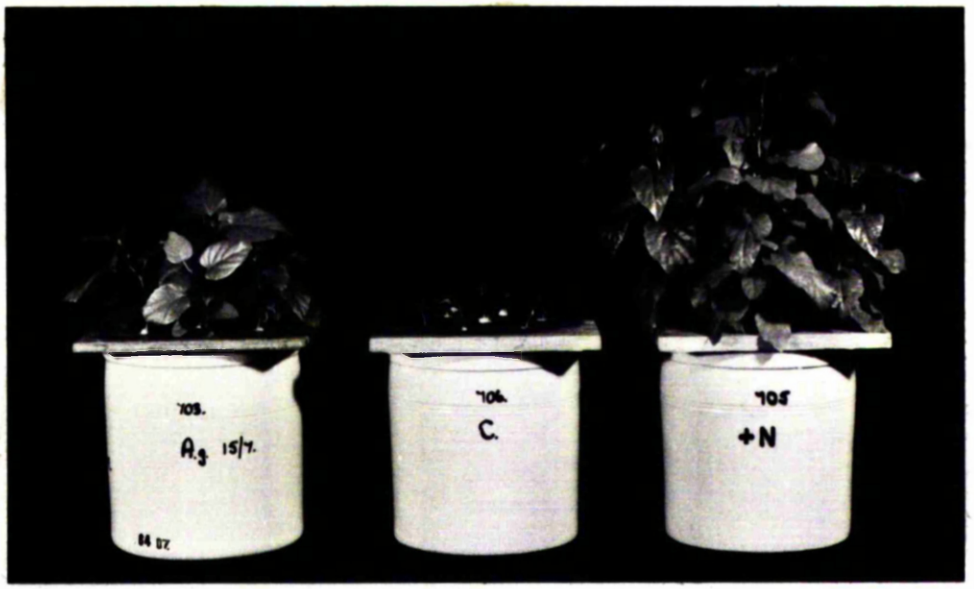


Fig. 3a. Alnus cordata plants. Left to right: inoc. from A. glutinosa; control; non-nodulated plants supplied with combined nitrogen. (x 1/7)

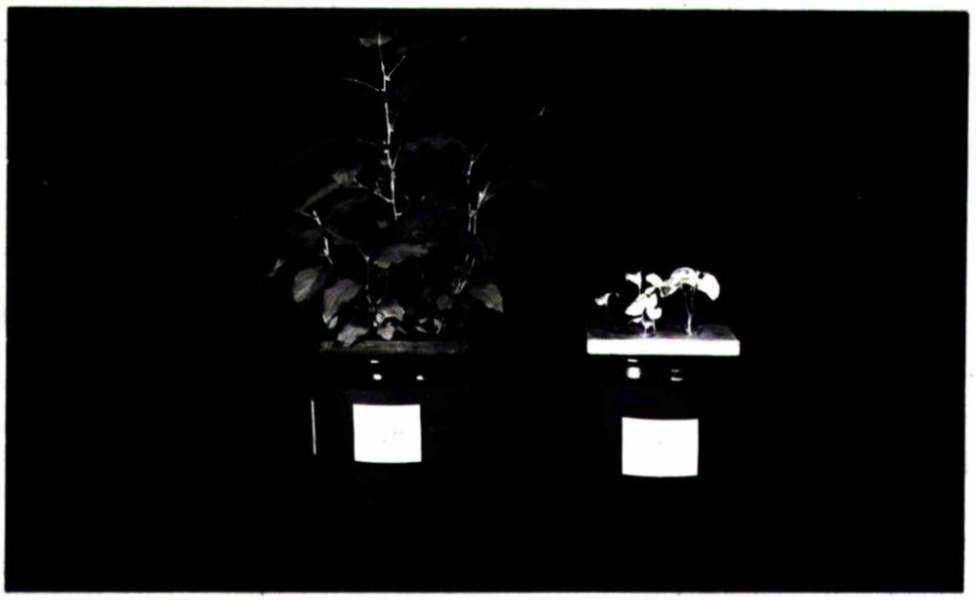


Fig. 3b. Alnus cordata plants. Left to right: inoc. from A. jorullensis; control. (x 1/7)

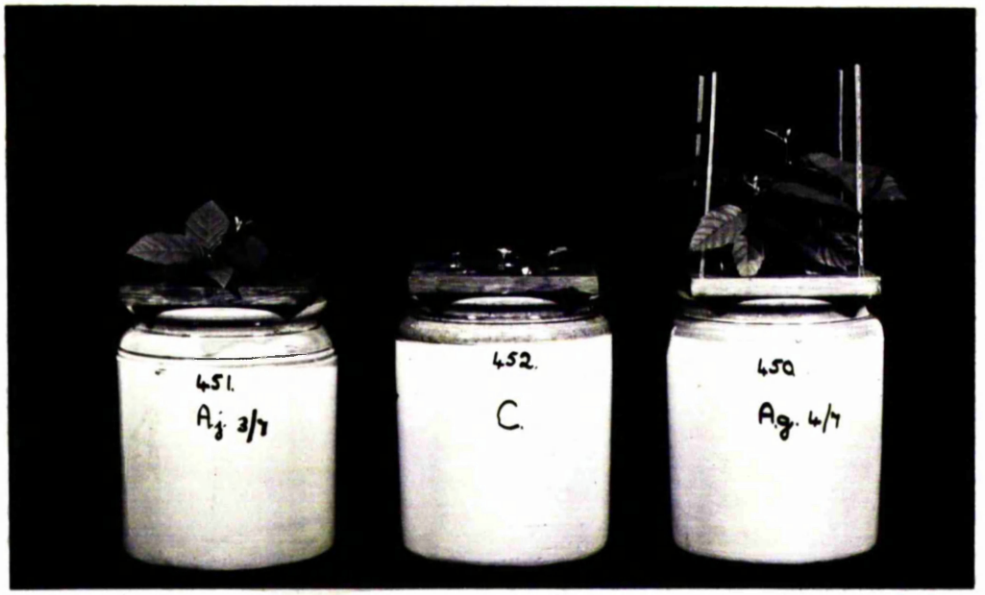


Fig. 4. Alnus rugosa plants. Left to right: inoc. from A. jorullensis; control; inoc. from A. glutinosa. (x 1/6)

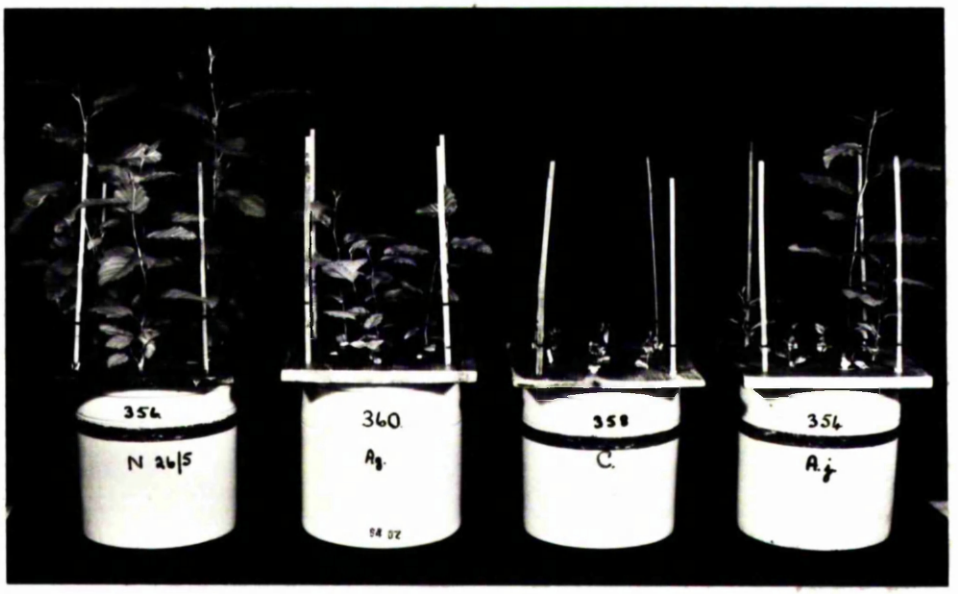


Fig. 5. Alnus rubra plants. Left to right: non-nodulated plants supplied with combined nitrogen; inoc. from A. glutinosa; control; inoc. from A. jorullensis. (x 1/8)

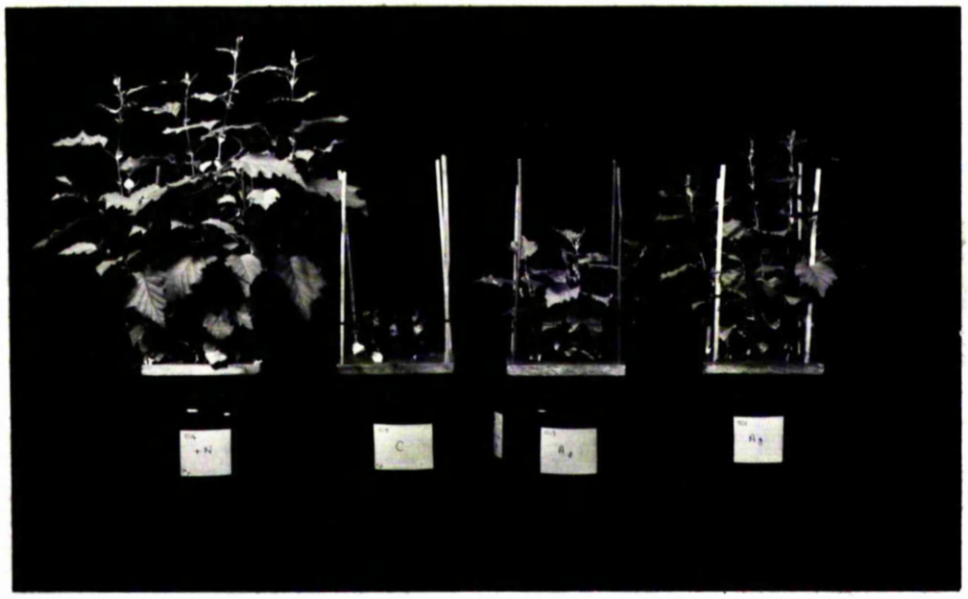


Fig. 6. Alnus inokumai plants. Left to right: non-nodulated plants supplied with combined nitrogen; control; inoc. from A. jorullensis; inoc. from A. glutinosa. (x 1/10)

Fig. 7. Alnus multinervis plants. Left to right: inoc. from A. glutinosa; inoc. from A. jorullensis; control; non-nodulated plants supplied with combined nitrogen. (x 1/7)

Fig. 8. Alnus sieboldiana plants. Left to right: non-nodulated plants supplied with combined nitrogen; inoc. from A. jorullensis; control; inoc. from A. glutinosa. (x 1/7)

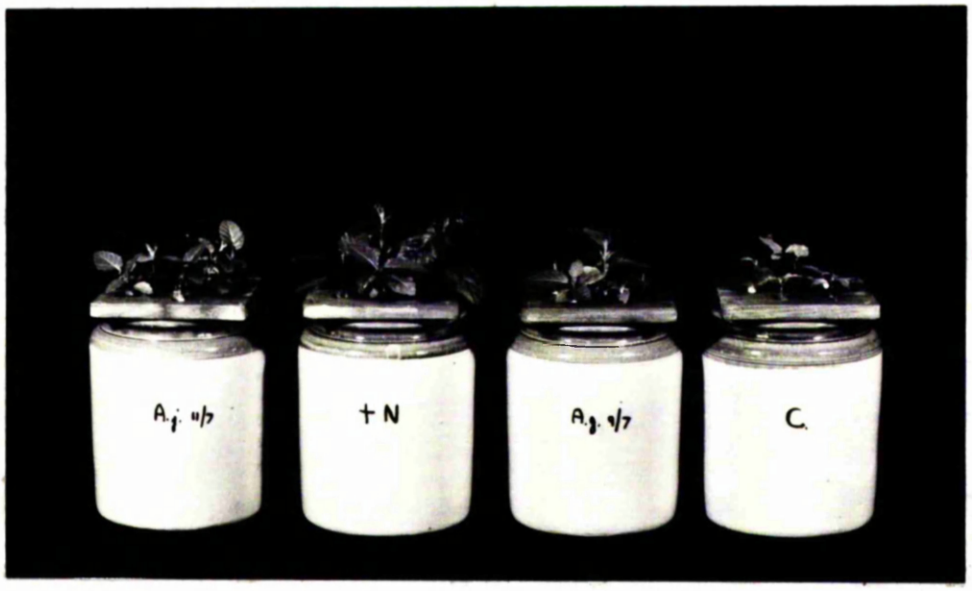


Fig. 9. Alnus firma plants. Left to right: inoc. from A. jorullensis; non-nodulated plants supplied with combined nitrogen; inoc. from A. glutinosa; control. (x 1/7)

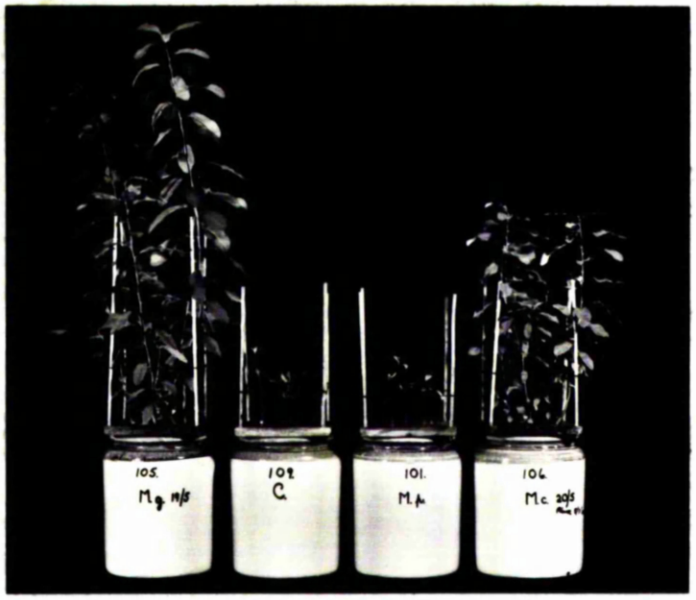


Fig. 10. Myrica gale plants. Left to right: inoc. from M. gale; control; inoc. from M. pilulifera; inoc. from M. cerifera. (x 1/12)

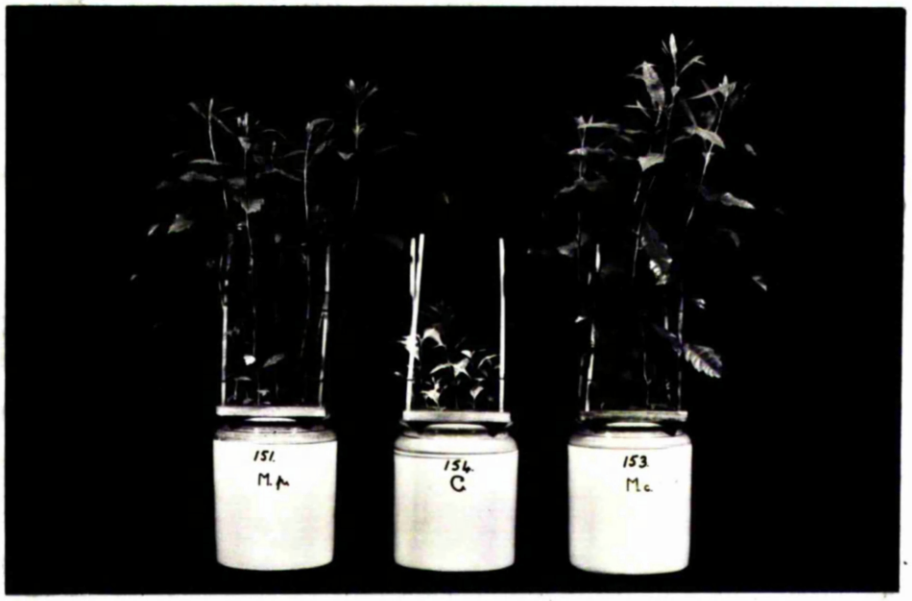


Fig. 11. Myrica cerifera plants. Left to right: inoc. from M. pilulifera; control; inoc. from M. cerifera. (x 1/10)

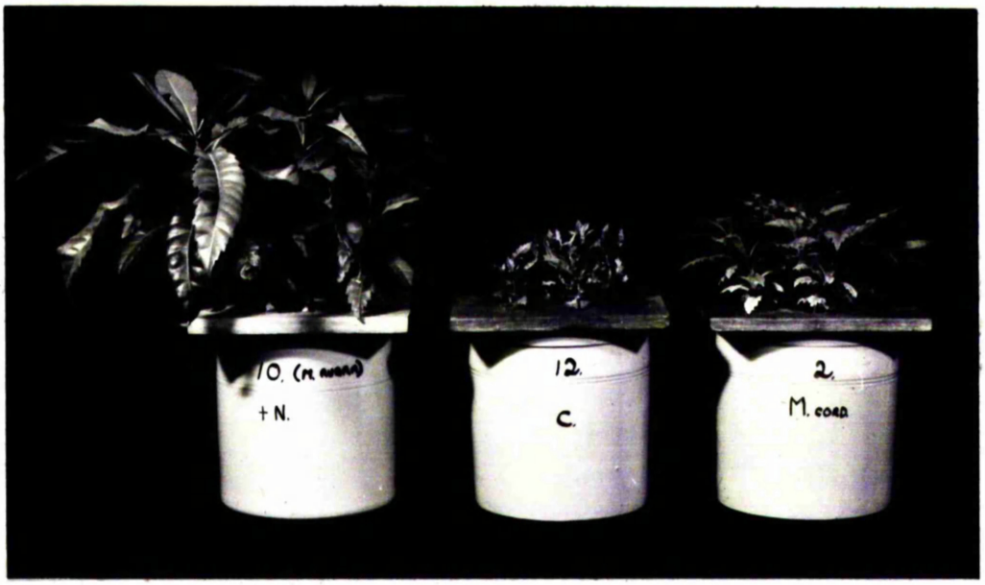


Fig. 12a. Myrica rubra plants. Left to right: non-nodulated plants supplied with combined nitrogen; control; inoc. from M. cordifolia. (x 1/7)

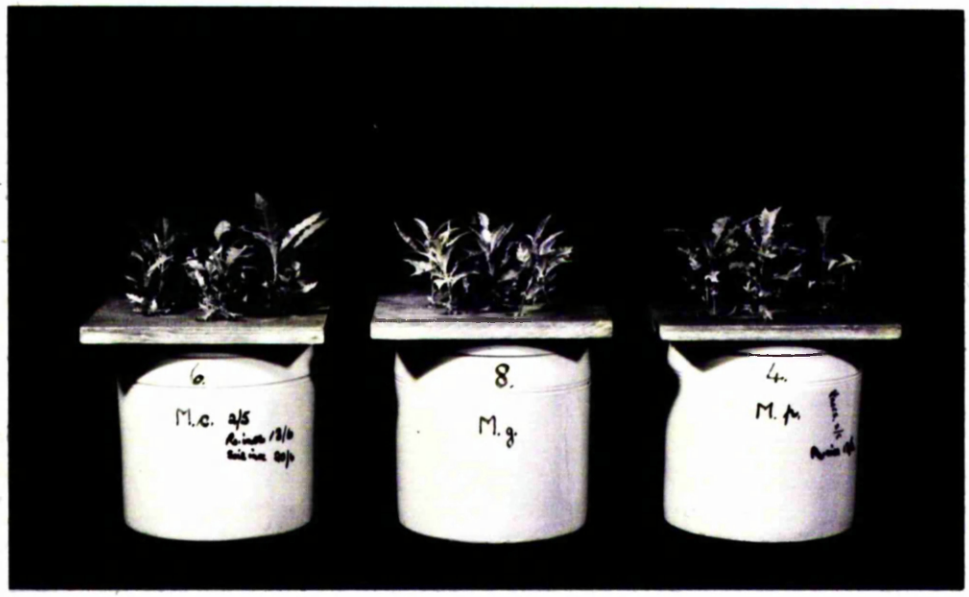


Fig. 12b. Myrica rubra plants. Left to right: inoc. from M. cerifera; inoc. from M. gale; inoc. from M. pilulifera. (x 1/6)

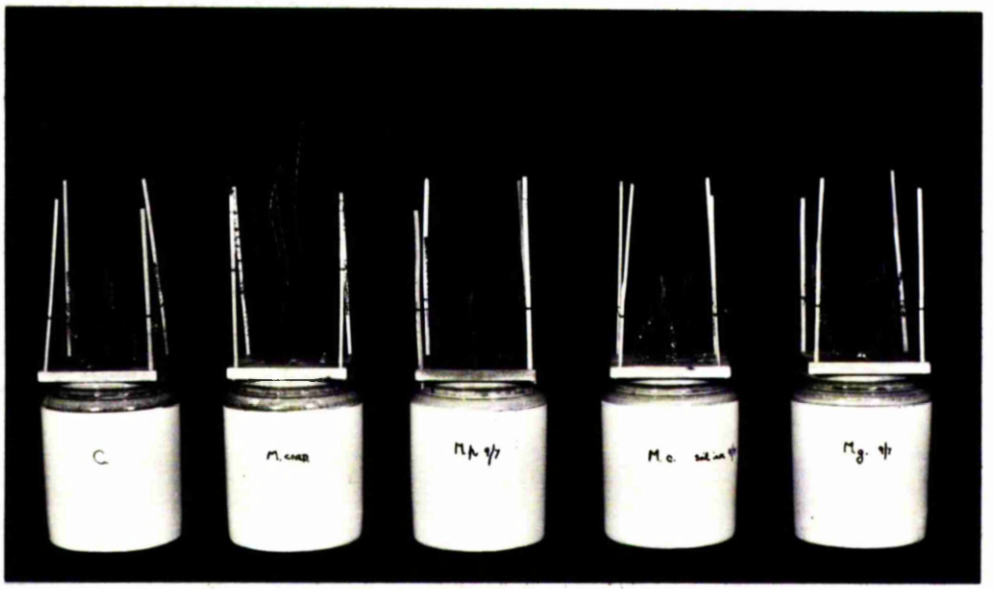
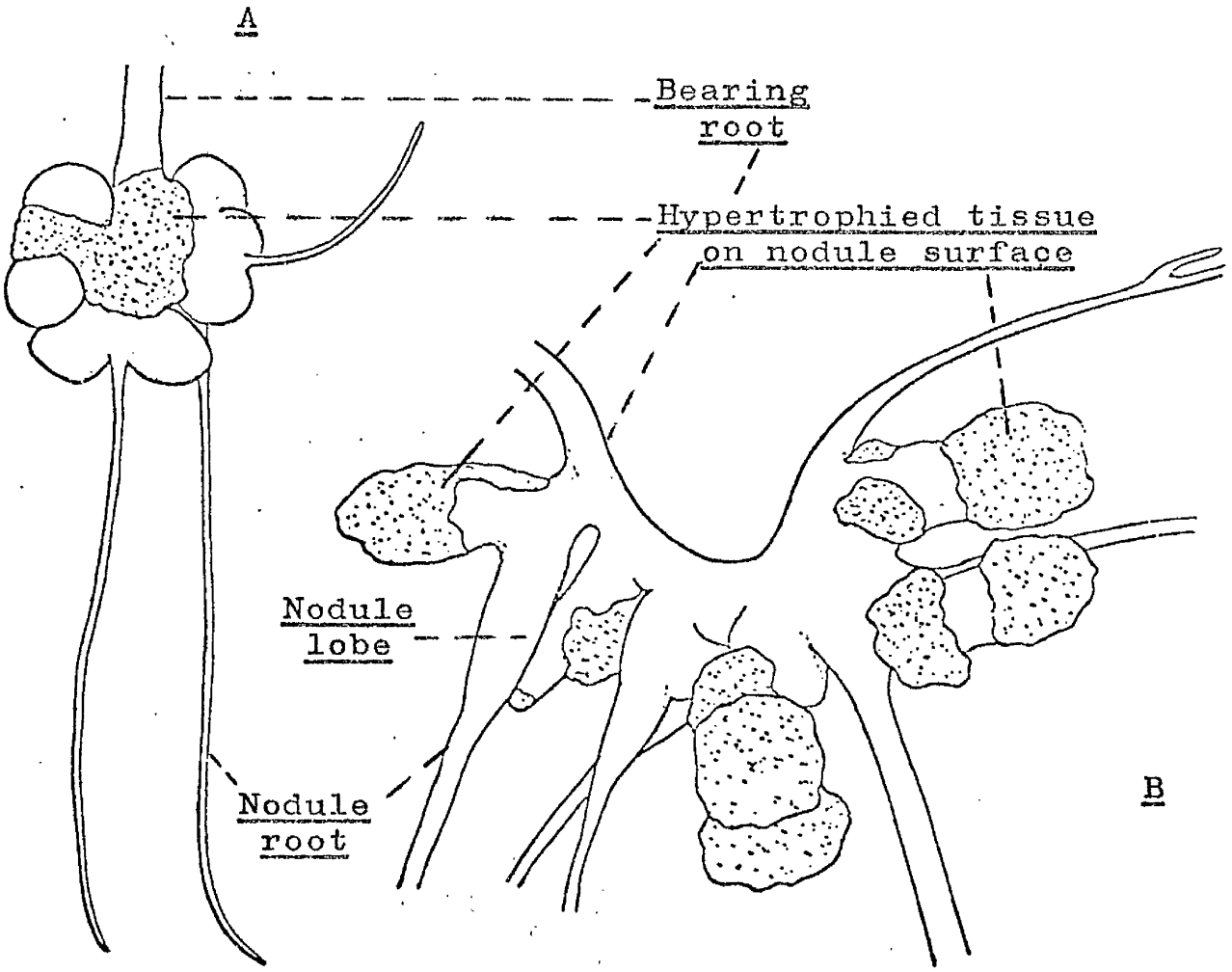


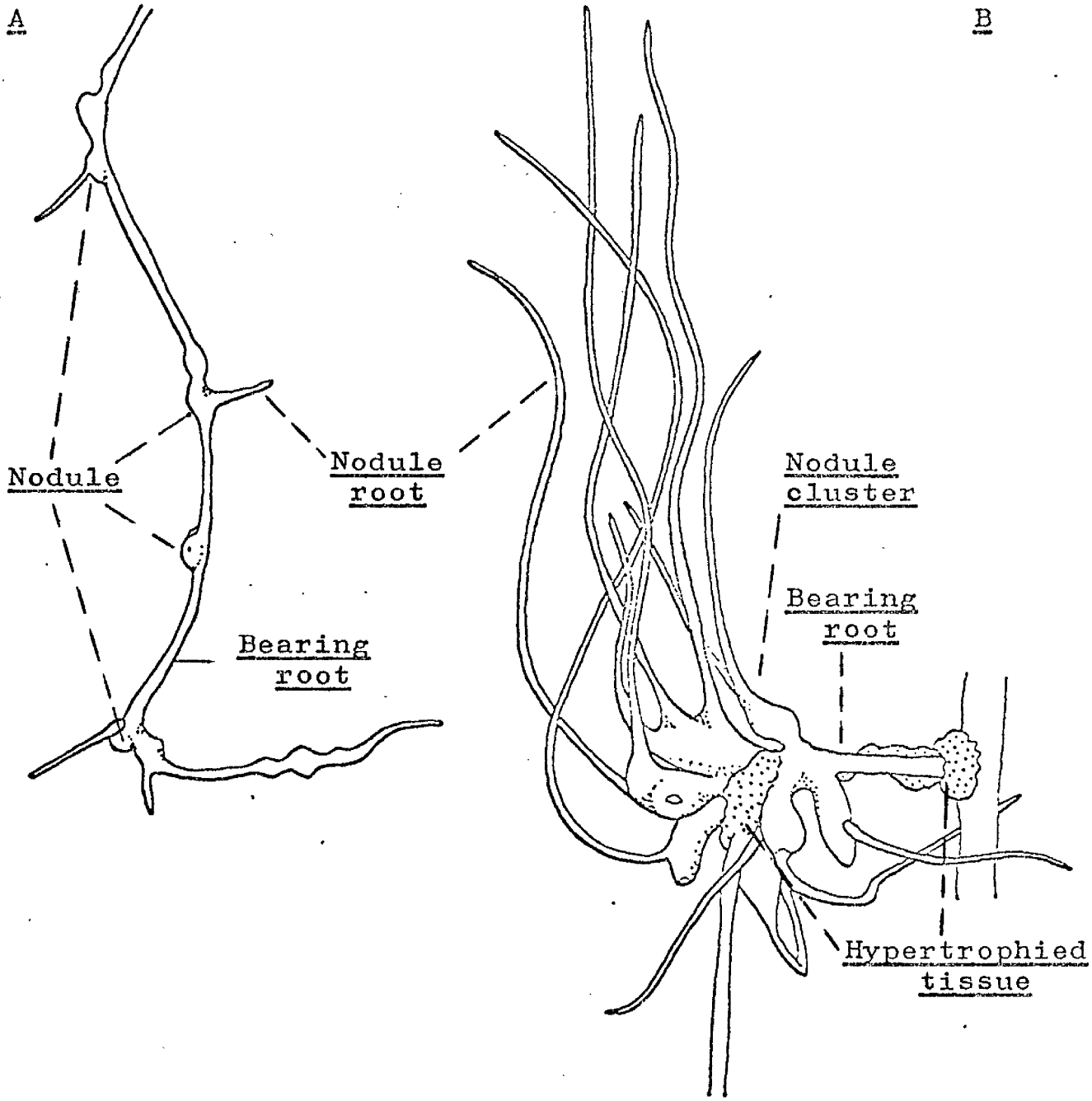
Fig. 13. Myrica cordifolia plants. Left to right: control; inoc. from M. cordifolia; inoc. from M. pilulifera; inoc. from M. cerifera; inoc. from M. gale. (x 1/10)

Figure 14



Nodule roots on A - Alnus rubra (x 6) and B - A. multinervis (x 10), both showing the original orientation.

Figure 15



A. Nodules induced by the Myrica gale endophyte on M. rubra (x 7)

B. Nodule cluster induced by the M. cordifolia endophyte on M. rubra (x 7)

Both figures show the original orientation of the roots and clusters.

S U M M A R Y

(1) Cross-inoculation studies were carried out in the genus Alnus between the endophytes of two different species and host plants of nine species, and in the genus Myrica between the endophytes of four different species and host plants of five species. For inoculation purposes crushed nodule and soil inocula were used.

(2) Several different types of response to inoculation were noticed, ranging from complete compatibility to complete absence of nodules. The endophyte of A. glutinosa was found to be more able to symbiose effectively with the "foreign" host species provided than was the endophyte of A. jorullensis which gave a relatively inferior performance. There was no evidence that in Alnus a low nodule efficiency contributed to low effectiveness of the symbioses. Thus the weight of nodules forming per plant was the factor mostly determining symbiotic performance. Most

of the unusual Myrica combinations were poor and this could be accounted for by the ineffectiveness of nodule tissues in the fixation of nitrogen.

(3) Many of the results can be explained on the basis of the geographical distribution of the two particular species involved, or on the basis of their taxonomic affinity. In both genera there is not one endophyte which will symbiose satisfactorily with all the species within each. On the basis of the combinations attempted, it can be said that incompatibility is more marked in Myrica than in Alnus.

SECTION I

(continued)

Addendum on

Observations on the structure and cytology of ineffective nodules of *Myrica cerifera* L.

Observations on the structure and cytology of ineffective nodules of *Myrica cerifera* L.

As noted in the previous part of this Section, the nodules which form in response to certain cross-inoculations among non-legumes, especially in the genus *Myrica*, are minute, numerous and completely ineffective in the fixation of nitrogen. Nodules of this type were obtained by Bond (1967a) when young plants of *Myrica cerifera* were inoculated from *M. gale*, and by the present author when the same endophyte was applied to the roots of *M. rubra* plants (see Fig. 15). It seemed to be of interest to undertake a preliminary light microscope study of the structure and cytology of such nodules, and the results of this will now be described.

Nodules of the above type from *M. cerifera* plants inoculated as described by Bond (*loc. cit.*), were removed from first year plants in September, and fixed in Craf III solution (Sass, 1958, p. 18). They were then dehydrated in "dioxan" (diethylene dioxide) and embedded in paraffin wax. Sections of the nodules,

4-8μ thick, were cut with a microtome, and then stained with tannic acid, iron alum, safranin and orange G in the manner described by Sharman (1943). Bright field light microscopy was used for the investigation, and photomicrographs were taken with a Zeiss photomicroscope. The author wishes to thank Dr. B. G. Bowes and Mr. T. N. Tait for assistance with the photomicroscopy.

For comparative purposes, normal, effective nodules from first year plants of M. cerifera and M. gale inoculated with their own respective endophytes were similarly fixed, also in September, sectioned and stained. All the nodules were taken from plants growing in water culture in the greenhouse.

Since the structure and cytology of the ineffective nodules is to be compared with that of normal nodules it will be better to describe the latter first. It was thought that normal nodules of both M. cerifera and M. gale should be described, for the reason that there is one rather notable difference between the nodules of these two species (see below), and it cannot be assumed that this difference is wholly due to the influence of the host plant. The structure and cytology of these normal nodules has been described by a number of previous authors, among them Harshberger

(1903), Arzberger (1910), Shibata and Tahara (1917) and Silver (1964) in the case of M. cerifera, and Fletcher (1955) for M. gale. In the present work only brief descriptions are provided, attention being largely limited to those features of special interest in relation to the ineffective nodules.

A longitudinal (but not fully median) section traversing three lobes of a normal nodule cluster of M. cerifera is shown at low magnification in Fig. 16. It will be noted that the infected cells are confined to two or three rows in the outer cortex, the inner cortex being uninfected. This feature was observed by Arzberger (1910) and by Shibata and Tahara (1917). Fig. 17 shows the infected cells at higher magnification; it will be seen that the hyphae of the endophyte, together with host cell cytoplasm, completely fill the cell. The swollen, club-shaped hyphal tips extending to the periphery of the cell are visible, as described by previous authors.

Fig. 18 shows a longitudinal section traversing two lobes of a normal nodule cluster of M. gale, median in respect of one lobe and median also to the basal, original lobe. The infected cells are seen to be

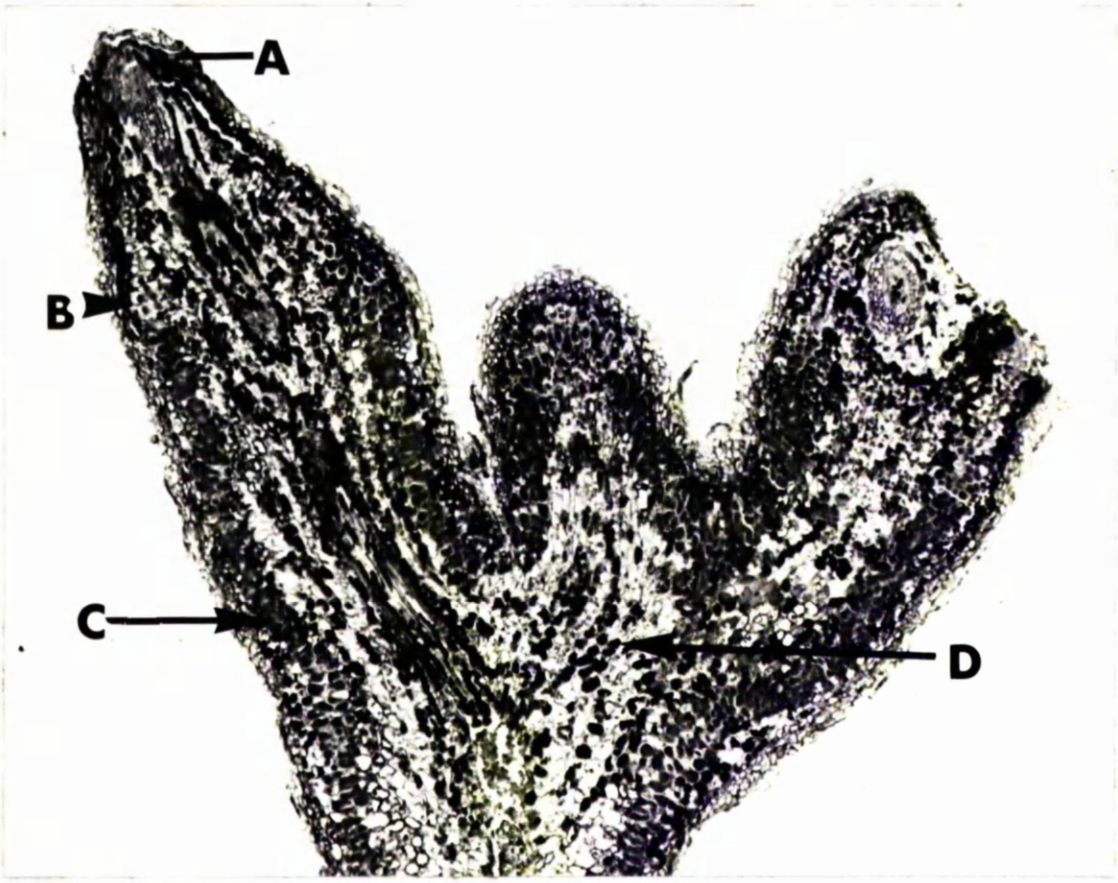


Fig. 16. L.S. of M. cerifera normal nodule (x 45). A - base of nodule root. B - newly infected cells. C - infected cells confined to outer cortex. D - tannin.

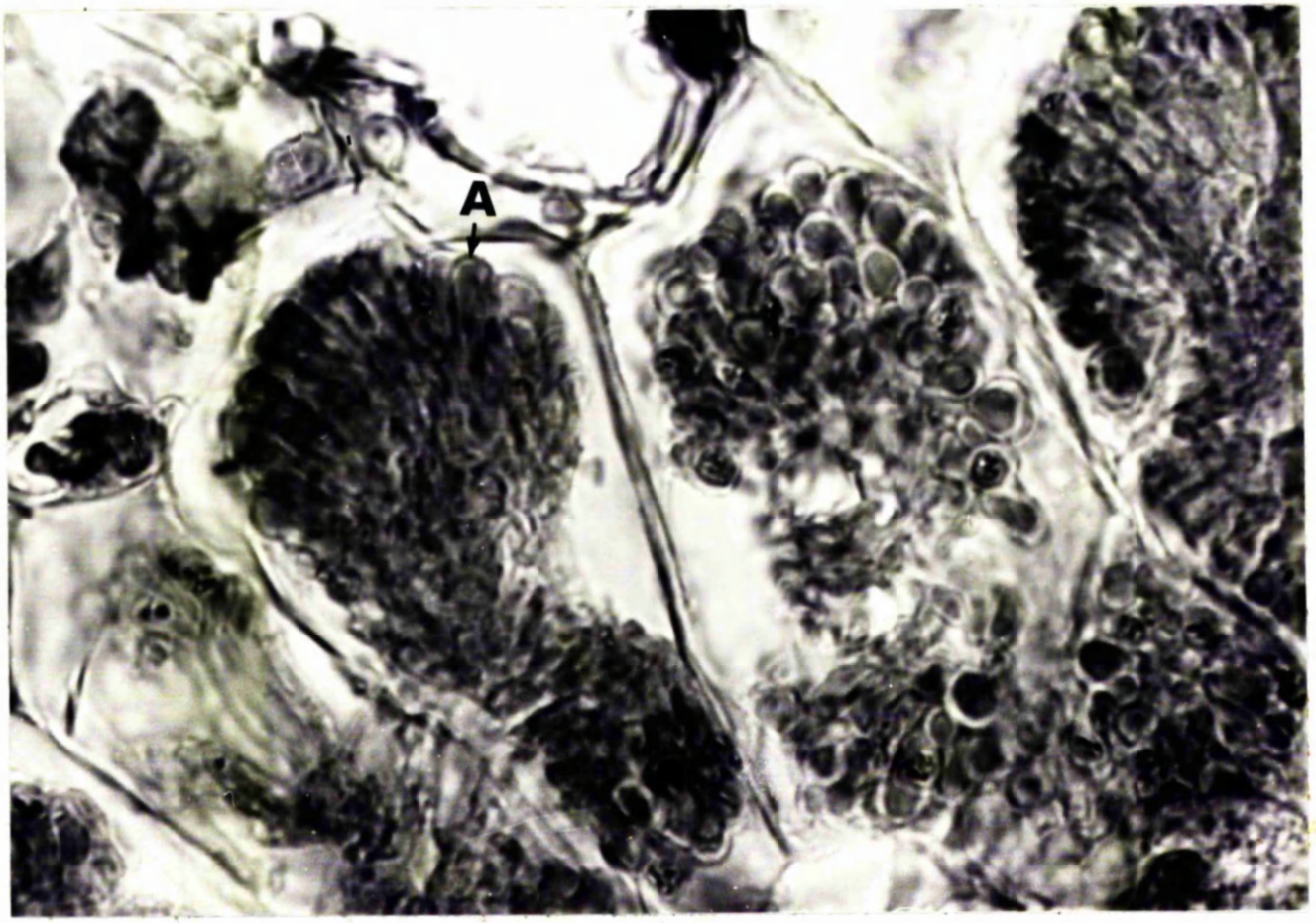


Fig. 17. Infected cells of M. cerifera normal nodule (x 1800). A - swollen hyphal tips.

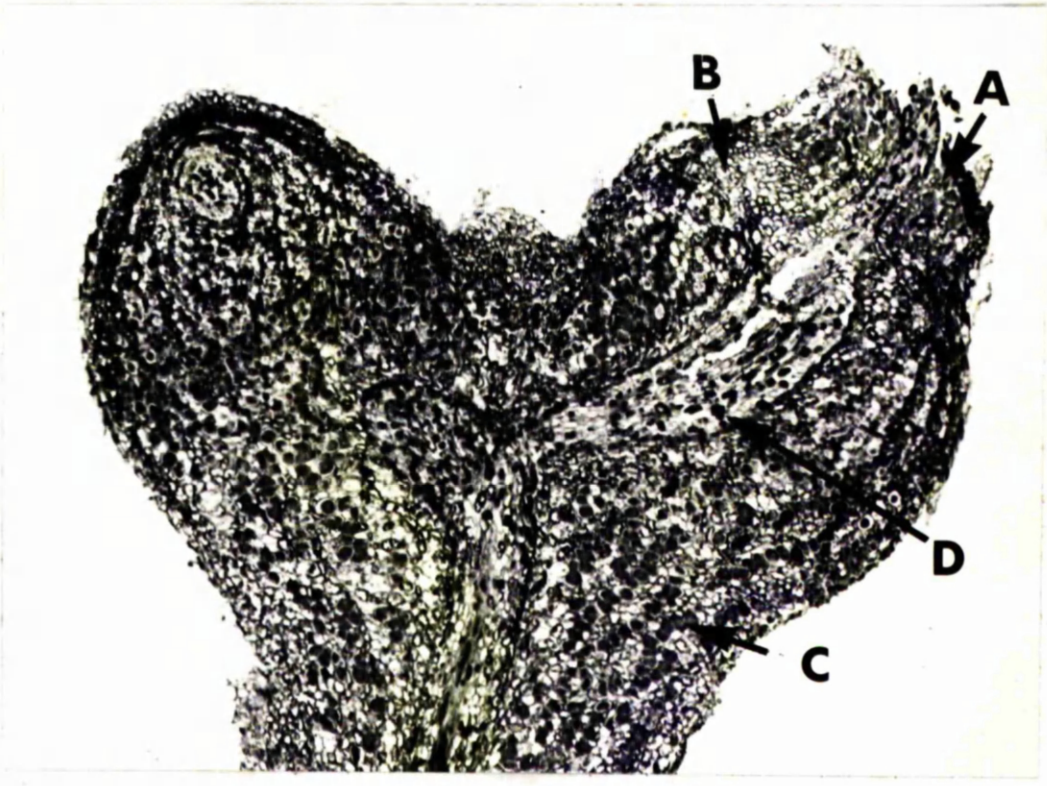


Fig. 18. L.S. of M. gale normal nodule (x 45). A - nodule root. B - region where new nodule lobe will emerge. C - infected cells distributed throughout cortex. D - tannin.

dispersed throughout the cortex, and thus differ sharply in this respect from M. cerifera. Shibata and Tahara (1917) noted that M. rubra and M. adenophora showed a distribution of the infected cells similar to M. cerifera, and the present author has found this to be true also of M. pilulifera and M. cordifolia. Thus on the present evidence, M. gale differs from other Myrica species in this respect. It has already been noted in this Section that earlier botanists placed M. gale in a separate genus, Gale. Although they did not use this present feature as a reason for this, it is perhaps additional evidence in support of their claim. Fig. 19 shows typical infected cells; in these also can be seen hyphae with swollen ends, although they are less obvious than in M. cerifera.

The ineffective nodules produced on M. cerifera plants by the M. gale endophyte will now be considered. The two most obvious differences between these and effective nodules are nodule number and nodule size. In respect of the former, several hundred ineffective nodules may appear on each plant, a number many times greater than that of effective nodules. On the other hand ineffective nodules are many times smaller than effective ones, and do not branch to form lobed clusters.

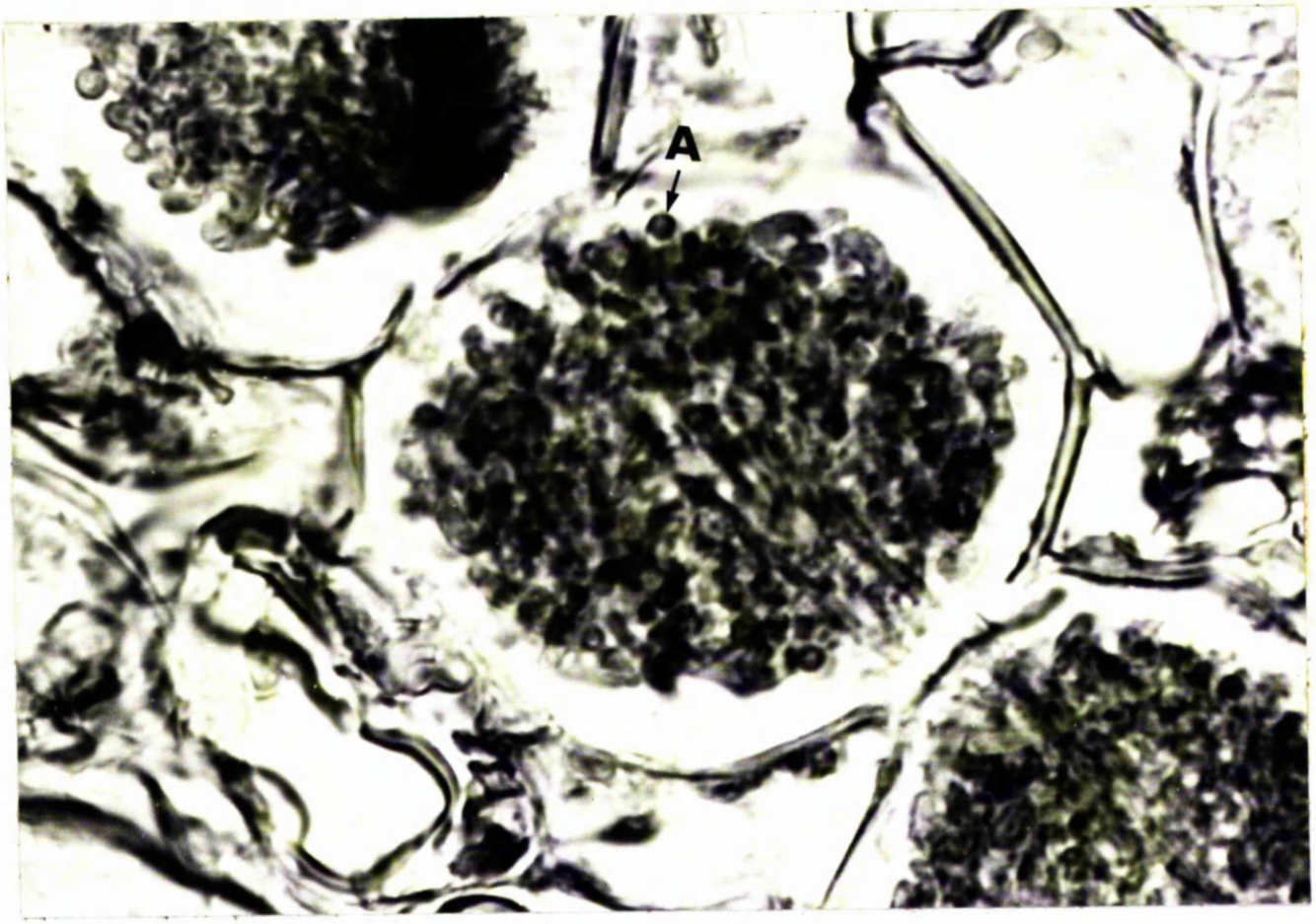


Fig. 19. Infected cells of M. gale normal nodule (x 1800). A - swollen hyphal tips.

This can be seen from Fig. 20, a low power view of an off-median section of such a nodule. The infected cells are seen to be restricted to the outer cortex as in normal M. cerifera nodules and hence it appears that this is a feature of the nodules that is determined by the host and not the endophyte. Fig. 21 shows an infected cell under high power. The endophyte forms a tangled knot of hyphae restricted to the central part of the host cell and occupying only about one third of the cell area shown. Hyphae can be seen extending to the host cell walls, but no obvious swollen hyphal tips are to be seen. These cells thus have a similar appearance to the newly infected cells found near the apex of effective nodules (Fig. 16).

The nodules just described resemble the ineffective nodules long known in legumes in being unusually numerous and of minute size. Chen and Thornton (1940), in their examination of ineffective legume nodules, attributed the small size of these to the fact that the nodule meristem ceases to function only a short time after nodule formation, while in effective nodules meristematic activity continues. They considered that this was due to lack of stimulation from the endophyte, which in turn was due to an unfavourable environment

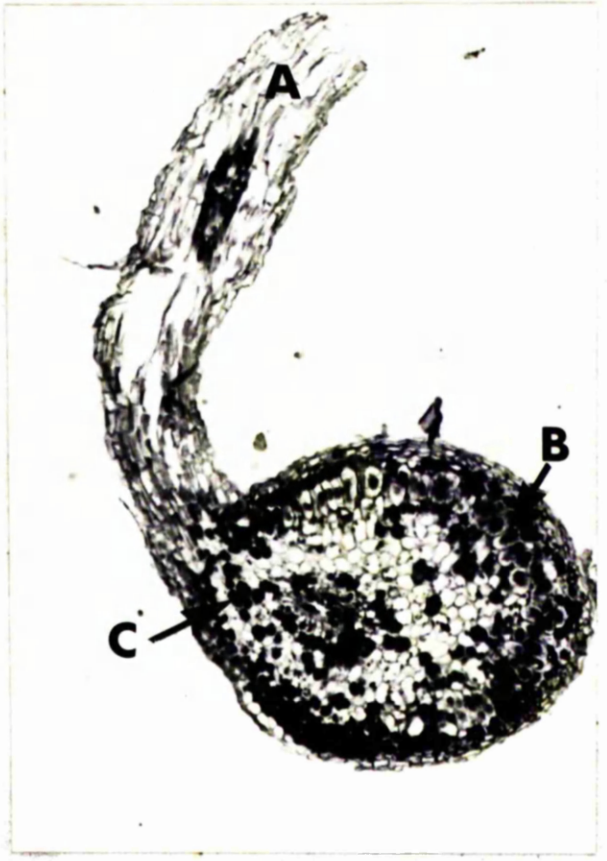


Fig. 20. L.S. of M. cerifera ineffective nodule (x 60). A - nodule root. B - infected cells confined to outer cortex. C - tannin.

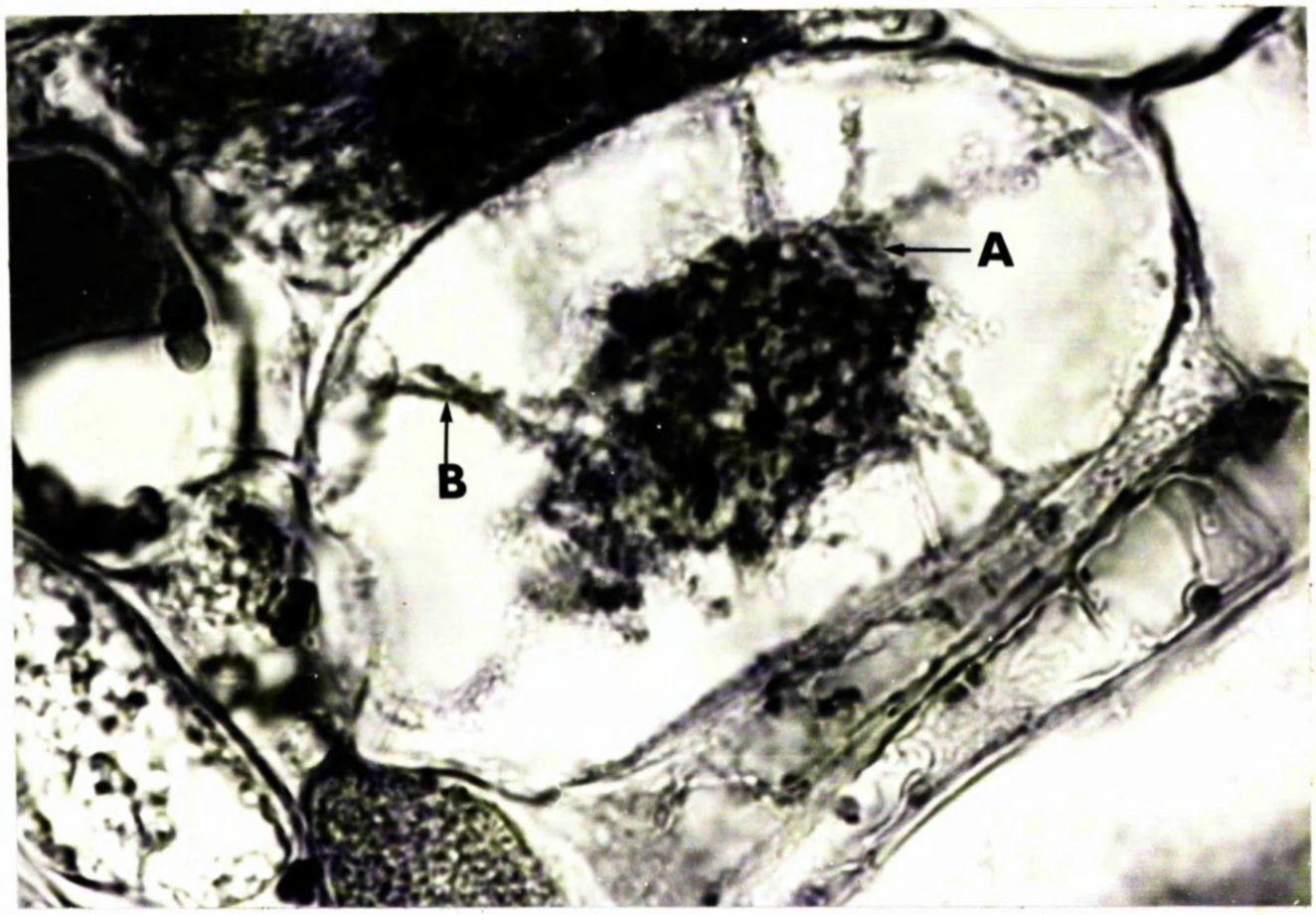


Fig. 21. Infected cell of an ineffective nodule (x 1800). A - endophyte. B - hyphae.

preventing normal bacterial growth.

The large number of ineffective nodules produced per plant in the legumes can be explained by the findings of Nutman (1952) who showed that nodule meristems produce a substance which inhibits further nodule production, and since the meristems of ineffective nodules are short-lived, there is thus no such inhibition. This effect may well apply to non-legumes also.

The most obvious difference between the infected cells of effective and ineffective nodules is the restricted proliferation of the endophyte found in those of the latter. Chen and Thornton (1940) found that in the particular legumes studied - which bore only partly ineffective nodules - the small volume of endophyte and its relatively short life could explain the small amount of nitrogen fixed per plant. They found no evidence that the ineffective strains were really less efficient in fixing nitrogen per unit of bacterial mass in unit time than the effective strains. In the ineffective nodules examined by the present author this is obviously not the case, since the complete absence of fixation indicates that the endophyte is unable to fix atmospheric nitrogen.

SUMMARY

(1) A light-microscope study has been made of the structure and cytology of ineffective nodules induced on *Myrica cerifera* by the *M. gale* endophyte, and these have been compared with normal nodules of *M. cerifera* and *M. gale*.

(2) The distribution of the infected cells in the ineffective nodules is similar to that in normal nodules of the same host species. The endophyte in the cells of ineffective nodules is not so well developed as in the cells of effective nodules.

(3) The reason for the absence of fixation in these nodules cannot be entirely explained by the lack of endophyte development.

SECTION II

The effect of combined nitrogen

on the nodule symbiosis of *Ceanothus velutinus*

The effect of combined nitrogen on the nodule symbiosis of *Ceanothus velutinus*

INTRODUCTION

Ceanothus is a large genus with 55 species, some of which are very prominent in natural associations in the United States, e.g. the so-called chaparral association, but although the occurrence of root nodules in the genus has been known for almost eighty years it is only recently that they have attracted much attention. Several workers have shown that during its growth *Ceanothus* exerts a soil-improving effect. Quick (1944) found that *C. cordulatus* had a favourable influence on the growth of Sierra gooseberry (*Ribes roezli*) when the two species were grown together, while Hellmers and Kelleher (1959) showed that when *C. leucodermis* was grown in three rather infertile Californian soils and the roots left in the soil, the growth of a succeeding tomato crop was greatly improved. Wollum and Youngberg (1964) showed that pine seedlings grown in low nitrogen soils benefited from the previous growth of *C. velutinus*, and also that the application of litter from this species

had a beneficial effect on the pines. However, although Zavitkovski and Newton (1968a) found that C. velutinus may be beneficial to infertile soils, they concluded that this was probably not the case in sites of moderate productivity.

While the above results are consistent with the occurrence of fixation in the nodules, it was left to Delwiche et al. (1965) to show this more directly by means of ^{15}N tests on nodules collected in the field from 12 species of Ceanothus. In a growth experiment Russell and Evans (1966) found that the mean nitrogen content of nodulated plants of C. velutinus brought in from the field and set up in nitrogen-free culture increased from an initial 45 mg. to 331 mg. per plant during 18 weeks. With the same species, Bond (1967a) found that plants raised from seed grew well and accumulated substantial amounts of nitrogen, provided they bore nodules, and by means of ^{15}N confirmed that the nodules were the site of the fixation. Webster, Youngberg and Wollum (1967) also demonstrated fixation in excised nodules by use of ^{15}N.

It is of interest to know what happens to the Ceanothus symbiosis when, through the action of the nodules, the nitrogen content of the soil is increasing

in the manner demonstrated in the first paragraph above. Is the continued growth of the nodules affected, and do they maintain their effectiveness in fixation? In previous work at Glasgow by Stewart and Bond (1961), Stewart (1963) and Rodriguez-Barrueco (1967), this question has been investigated with Alnus, Myrica and Casuarina by supplying young, already nodulated plants with different amounts of ammonium-nitrogen, and observing the effect on the further growth of the nodules and the plant, and also on the fixation of nitrogen. In all cases there has been a tendency for the weight of nodules per plant to increase at low levels of combined nitrogen, but to decrease at higher levels. Fixation of nitrogen per plant - the measurement of this being made possible by the use of ammonium labelled with ^{15}N - was sometimes increased at a low level of combined nitrogen, but in all cases fell at higher levels, and the plant utilised the supplied combined nitrogen to an increasing extent. Plant growth was usually enhanced in the presence of combined nitrogen. Somewhat similar responses were found by Zavitkovski and Newton (1968b) for Alnus rubra and by Daly (1966) for Alnus rugosa.

Three publications on the above aspects in Ceanothus

have appeared during the course of the present author's experiments. Wollum (1967) in a brief summary of a read paper reported that in Ceanothus velutinus the addition of ammonium to the rooting medium, even in low concentration, reduced the number and weight of nodules per plant. In a similarly brief abstract Webster, Youngberg and Wollum (1967) reported that nodules from plants grown with added ammonium-nitrogen "showed no difference in fixation" from those from plants in nitrogen-free medium when both types were excised and exposed to gaseous nitrogen labelled with ^{15}N. Zavitkovski and Newton (1968a) on the basis of greenhouse pot cultures concluded that the nitrogen fixing capacity of C. velutinus decreased, owing to reduced nodulation, when the fertility of the soil used was raised by addition of nitrogen in the form of pulverised broom (Cytisus) litter.

In the experiments now to be described the effect of added ammonium or nitrate-nitrogen to the rooting medium on the further growth of nodules present at the commencement of the treatment has been studied with Ceanothus velutinus. The ammonium-nitrogen was labelled with ^{15}N, so that in this case the effect of the combined nitrogen on fixation could be measured. In the

nitrate experiment more limited information on the fixatory activity of the nodules was obtained by the acetylene reduction method.

METHODS

Source of plants

The author took over young nodulated plants of *Ceanothus velutinus* Dougl. var. *laevigatus* Torr. & Gray (varnish-leaf ceanothus) which had been raised by Professor G. Bond by sowing heat-treated seed in *Ceanothus* habitat soil. Details have been published (Bond, 1967a). Sowing in habitat soil had been adopted since the application of crushed-nodule inoculum to the root system had proved an unreliable method of securing nodulation in this genus. The seed had been sown in April, and in August of the same year, when most of the plants had nodulated, they had been moved into Peralite watered with Crone's solution (nitrogen-free formula). The plants for use in the ammonium experiment were taken over by the present author in April 1967, and those for the nitrate experiment in April 1968. The subsequent treatment of the plants was as follows.

Setting up the plants

In outline the procedure was to set up the plants

in individual plastic pots containing Peralite moistened with initially nitrogen-free culture solution to which, in some cases, had been added a certain level of ammonium sulphate or sodium nitrate. The pots were flushed with fresh solution at frequent intervals as a means of correcting the pH of the rooting medium and of replenishing the level of combined nitrogen in the rooting medium. Peralite culture was used in preference to water culture since Bond (1967a) found that nodules of Ceanothus were very prone to decay in water culture, apparently as a result of fungal attack, but that this happened less frequently in Peralite.

The plants available for the ammonium experiment, commenced in April 1967, were judged to be too variable in size to be used without some preliminary segregation, and they were in fact divided into two series, A and B, the former comprising the larger plants. The manner of use of these two series is indicated below. Representative plants were harvested (Table 11), and the remaining plants, 20 in each series, set up in plastic pots, 13 cm. in diameter for series A and 10 cm. for B. These contained Peralite moistened with a nitrogen-free form of the culture solution specified by Hewitt (1966, Table 40), in preference to Crone's since, in view of

the flushing technique to be employed, a culture solution free of undissolved salts was necessary. The solution was used at half the strength indicated by Hewitt, the actual composition being as follows:-

Composition of culture solution

K_2SO_4	20 ml.	8.7 per cent stock solution*
$CaSO_4.2H_2O$	10 ml.	34.4 " " " "
$NaH_2PO_4.2H_2O$	5 ml.	20.8 " " " "
$MgSO_4.7H_2O$	10 ml.	18.4 " " " "
$FeC_6H_5O_7.5H_2O$	0.125 g.	
Distilled water	10 litres.	

* i.e. 8.7 g. K_2SO_4 made up to 100 ml. of solution with distilled water.

10 ml. of a minor element mixture, based on Hoagland's A-Z solution (Templeman, 1941) with molybdenum and cobalt added, was supplied.

Ferric citrate was prepared by heating the appropriate amount of flakes in a little of the distilled water until the flakes dissolved, and then adding this to the rest of the water. The pH of the culture solution was 4.7, while the pH of Peralite + solution allowed to stand overnight was 6.4. This difference in pH is due to the alkaline effect of the Peralite.

A weight of culture solution equal to three times the weight of the Peralite was added to each pot, equivalent to 360 ml. for the larger pots and 150 ml. for the smaller. Several layers of fine nylon mesh were placed at the base of each pot at planting in order to prevent any egress of Peralite during the flushing operation mentioned above.

The 20 plants for the nitrate experiment, commenced in April 1968, were set up as above, except that they were more uniform in size, needed no subdivision, and were all put in 13 cm. pots. Representative plants were again harvested (Table 11).

Table 11

Mean data per plant obtained at initial harvest

Type	No. of plants	Height of shoot cm.	No. of nodule clusters	Dry wt. of nodule clusters g.	Dry wt. of whole plant g.	N content of whole plant mg.
Ammonium-nitrogen experiment						
Series A	3	6.9	4.3	0.025	0.507	8.4
Series B	3	6.4	3.3	0.009	0.246	4.0
Nitrate-nitrogen experiment						
	3	5.1	6.0	0.016	0.359	-

Addition of combined nitrogen

Nitrogen in the form of ammonium sulphate or sodium nitrate was added to the culture solution supplied to a proportion of the plants. In each case two levels were employed, namely 10 and 50 mg. nitrogen per litre of culture solution. The remaining plants were supplied with nitrogen-free solution. In the ammonium experiment the series A plants (see above) were used to study the effect of the lower level of nitrogen, half (i.e. ten) of the plants being supplied with nitrogen-free solution, while series B plants were used in a corresponding fashion to study the effect of the higher level of nitrogen. In the nitrate experiment eight plants were grown in nitrogen-free culture and six plants were treated at each level of combined nitrogen. The combined nitrogen was first supplied to the plants a few days after they had been set up in individual pots.

As noted earlier, the nitrogen of the ammonium sulphate was labelled with ^{15}N, and a quantity of the salt sufficient for the whole experiment was prepared at the commencement by "diluting" a stock (purchased from the Office National Industriel de l'Azote, France) containing 4.78 atom per cent excess ^{15}N with ordinary ammonium sulphate to reduce the label to approximately

2 atom per cent. Actual analysis showed the label to be 2.021 atom per cent excess. For convenience in use the prepared salt was brought into the form of a stock solution containing 20 mg. nitrogen per ml.

Subsequent cultural treatment of the plants

The flushing treatment was at first applied twice-weekly, increased to thrice-weekly after approximately the first month of the experiments. Since the high moisture-holding capacity of the Peralite had not, by design, been fully exercised at the time of setting up the plants, a greater quantity of culture solution than was originally added had to be supplied at flushing in order to secure a free flow of displaced solution. The actual volumes used were 450 ml. per larger pot and 200 ml. per smaller pot. The result was that on average the Peralite was wetter than might have been thought desirable, but the vigorous growth of most of the plants suggested that they liked a wet medium. The displaced solution was allowed to drain into catchpots in the form of beakers in which the pots were stood permanently, the beakers being wrapped in black paper in order to prevent algal growth.

Regular measurements of pH were made on the displaced

solution. In the ammonium experiment the pH at the first flushing (when the combined nitrogen was first added) was 6.6, and in the zero nitrogen pots the value fell only slowly, remaining above pH 5.0 until very late in the experiment. As expected, the pH fell more rapidly in the pots supplied with ammonium, especially at the 50 mg. per litre level, and despite the frequency of flushing the pH of the solution displaced from these pots had fallen to 4.0 after three weeks from the start of the experiment, but thereafter fell to about 3.3 only. No detrimental effects on the plants were detectable.

In the nitrate experiment the tendency, as expected, was for the pH of the rooting medium to rise as the plants absorbed the nitrate ion. Thus after seven weeks the pH of the draining solution from the 50 mg. per litre pots had risen from the initial value of 6.4 to 7.2, and since it was feared that iron chlorosis might develop in the plants the precaution was taken of lowering the pH of the culture solution supplied at flushing from its normal value of 4.7 to 3.9 by suitable addition of sulphuric acid. This step was taken for all three treatments, but subsequently the use of acidified solution was stopped for the zero nitrogen

treatment since the rooting medium was becoming too acid. From the eleventh week onwards solution adjusted to pH 3.5 was supplied to the 50 mg. per litre plants. By these procedures the pH in the two nitrate treatments was maintained in the range 5.5 to 6.5 during most of the growth period, and that in the nitrogen-free pots somewhat lower.

In both years some algal growth occurred on the surface of the Peralite, especially in pots receiving combined nitrogen. In an effort to combat this, circles of black paper were at one stage placed around the stem bases of the plants so as to cover the Peralite, but these were discarded in view of the possibility that the ventilation of the rooting medium might be impaired. Several examinations of the algal growth showed that it consisted mostly of unicellular green algae of the Euglena type; no heterocystous blue-green algae were ever observed.

Plant harvest and analysis

The plants were harvested after the following periods of growth:

Ammonium experiment, series A - 12 weeks

" " " B - 9 weeks

Nitrate experiment - 23 weeks

Details were taken of the height of the shoot, number of nodule clusters, and dry weights of shoot, roots and nodule clusters. Dry weights were found by drying the plant material to constant weight in an oven at 95°C.

In the ammonium experiment the plant dry matter was subjected to Kjeldahl analysis to learn the total nitrogen content and also to prepare for the mass spectrometric assay of the ^{15}N content of the plant nitrogen in the various treatments. The Ranker modification of the Kjeldahl process (Ranker, 1925; Official Methods of Analysis of A.O.A.C., 1955) was employed. This method, unlike the standard Kjeldahl, is stated to include any nitrate-nitrogen in the material, and this was checked by carrying out the process on weighed amounts of sodium nitrate, recoveries of 95 and 93 per cent of the added nitrate being achieved in the two tests. This was considered satisfactory. It was thought advisable that the analyses of plant nitrogen should include nitrate since it was just possible that some nitrification of the supplied ammonium-nitrogen might have occurred in

the Peralite rooting medium, followed by uptake of the resulting nitrate by the plants. With plants of relatively small size (total dry matter not exceeding about two grams) the whole of the dry matter was Kjeldahled, being divided between two 800 ml. Kjeldahl flasks where necessary. The dry matter from larger plants was ground in a Christy & Norris mill and duplicate samples of approximately 0.45 g. taken for analysis; no single value differed by more than 0.04 per cent from the mean percentage nitrogen for the two analyses. In such cases, whole plant nitrogen was calculated from the mean percentage nitrogen of the duplicates and the total dry weight of the plant.

The distillates from the Kjeldahl process were re-acidified and evaporated down to a volume of a few ml., those from duplicate samples being combined after evaporation. Portions of these concentrates were sent to Dr. C. W. Crane, Queen Elizabeth Hospital, Birmingham, for ^{15}N analysis in the mass spectrometer.

Strict precautions were taken in the Kjeldahl process and the ensuing evaporation to prevent contamination between samples. The flasks used in both the combustion and distillation stages of the Kjeldahl process were cleaned in a mixture of chromic acid and

concentrated sulphuric acid before each occasion of use, as were also the evaporating basins used in the concentration of the distillates.

Acetylene reduction assay

Evidence that, weight for weight, nodule tissues in Alnus and Myrica are less active in fixation when combined nitrogen is available in the rooting medium was mentioned in the Introduction to this Section. To find whether this was true in Ceanothus also, it was decided to measure fixation in detached nodules over a short period, using material from the nitrate experiment. By this stage in the author's work, Dr. C. T. Wheeler was operating the acetylene-reduction assay for fixation, and offered to assist in the use of it for these Ceanothus tests. The method, used in extensive studies in legumes by Hardy et al. (1968), depends on the finding of Dilworth (1966) and Schöllhorn and Burris (1966) that the nitrogen-reducing system responsible for fixation will, if supplied with acetylene, readily reduce it to ethylene at a rate proportional to that at which nitrogen would normally be fixed by the same material. The method is less arduous and more sensitive than the ^{15}N method used elsewhere in the thesis, though less definitive.

The experiments were carried out on 1st and 2nd October 1968, and on each day a comparison was made between the acetylene-reducing activity of the nodules from two plants which had been supplied with 10 mg. nitrate-nitrogen per litre and that of nodules from two zero nitrogen plants. On the day before the plants were used, artificial light was employed to extend the rather short natural day and also to supplement the natural light through the day, as necessary.

For the assay, the nodule clusters were removed from the plant, but with short lengths of root still attached to them, and placed in an 85 ml. tube with a side arm. The tubes also contained 50 glass beads for a reason indicated below, and were sealed with vaccine stoppers. A control tube with beads only was included. The tubes were evacuated and the following gases admitted in turn to give the proportions indicated:-

Acetylene	20%
Oxygen	20%
Argon	60%

The tubes were inverted several times to mix the gases - the glass beads assisting in this - and then placed in an incubator at 23°C. Every 15 minutes they were shaken

to prevent accumulation of ethylene round the nodules, while after two hours gas samples were withdrawn by means of a syringe and analysed on an Aerograph 200 gas chromatogram. This was equipped with a 6' x 1/4" stainless steel column filled with Porapak R (120-150 mesh), and operating at room temperature. Helium was used as the carrier gas at a flow rate of 33 ml. per minute and 40 pounds per square inch. A flame ionisation detector at 150°C was used. Gas samples, 3 ml. in volume, were introduced into the chromatogram for analysis. The ethylene and acetylene peaks from the column were identified by comparison of retention times with those of standard samples of gas. Ethylene used for the preparation of standard curves was obtained from Hilger I.R.D. Ltd. By analysis of the gas in the control tube, a correction was made for the presence of any ethylene in the initial gas sample.

Finally the dry weights of the nodule samples were ascertained.

R E S U L T S

1. The Ammonium-nitrogen Experiment

Most of the plants grew well, better growth being obtained than by Bond (1967a) probably as a result of the flushing procedure. The plants mostly had dark green foliage with a good varnish and white healthy nodules. Fig. 22 shows the nodule clusters from a plant raised in nitrogen-free solution. However, several plants in nitrogen-free culture fell behind and showed symptoms of nitrogen deficiency, and when these were harvested they were found to have suffered nodule decay. They were therefore excluded from the results. A smaller number of plants grown in the presence of combined nitrogen were also excluded on account of their growth being greatly inferior to the remaining plants. Towards the end of the experiment, a few of the plants supplied with ammonium at the higher level developed foliar abnormalities, namely browning round the edges of the leaves. The most probable explanation was an excessive accumulation of ammonium-nitrogen in the tissues, and to prevent any further damage to the plants

Fig. 22. Nodule clusters of a zero nitrogen plant. (x 1)

Fig. 23. Ammonium experiment - series A. Left to right: two zero nitrogen plants; two plus nitrogen plants. (Diameter of pots = 13 cm.)

it was decided to commence the harvest of these after nine weeks of treatment. It will emerge later that the percentage nitrogen in these plants was relatively very high.

Growth and dry weight data for series A plants are provided in Table 12, the data being shown, with the exception of number of nodule clusters, as increases over the corresponding data at the initial harvest (Table 11). It will be seen that the provision of 10 mg. of ammonium-nitrogen per litre resulted in a reduction in the mean number of nodule clusters, an enhanced increase in nodule dry weight during the growth period, and also in the height and dry weight of the plants, all these differences being significant or very nearly so. The greater average size of the plus ammonium plants, indicated by the greater dry weight, is confirmed by Fig. 23. From the first two findings above it follows that the nodule clusters were larger in the presence of ammonium, and calculation shows that the mean weight per nodule cluster was 146 mg. compared with 40 mg. in the zero nitrogen plants. A further point is that the enhancement of nodule growth kept pace with that of plant growth, since when the means in column (3) are expressed as a percentage of those in

Table 12

Effect of 10 mg. per litre of ammonium-nitrogen on nodule and plant growth

Treatment	(1)* Increase in height of shoot cm.	(2)* No. of nodule clusters	(3)* Increase in dry wt. of nodule clusters g.	(4)* Increase in dry wt. of whole plant g.
Zero nitrogen	40.3	5	0.285	6.267
	13.0	6	0.098	2.442
	32.4	6	0.243	5.099
	6.5	2	0.102	1.743
	4.3	4	0.062	1.220
Mean per plant	19.3	4.6	0.158	3.354
10 mg. ammonium-nitrogen per litre	47.1	4	0.446	7.434
	42.1	1	0.275	6.346
	27.6	3	0.214	4.277
	51.4	2	0.437	8.247
	33.6	2	0.318	6.334
	21.1	3	0.344	4.644
Mean per plant	37.2	2.5	0.339	6.214

* The following indicates the Least Significant Differences between means (P=0.05) together with the observed differences:

Column	L.S.D.	Observed difference
1	19.0	17.9
2	1.9	2.1
3	0.130 g.	0.181 g.
4	2.557 g.	2.860 g.

column (4), values of 4.7 and 5.5 are obtained for the zero nitrogen and the 10 mg. nitrogen per litre treatments respectively. It will be noticed that the mean number of nodule clusters present on plants receiving ammonium-nitrogen was apparently lower than the initial value (Table 11). It is considered that this is due to the small number of plants included in the initial harvest.

The corresponding results for series B plants are presented in Table 13. In the presence of the higher level of ammonium-nitrogen the number of nodule clusters per plant was again decreased, but here the increase in the dry weight of the nodules was markedly depressed. Whole plant dry weight was again enhanced, though to a smaller extent than in series A. Mean shoot height was unaffected. Typical plants are shown in Fig. 24. There was now no increase in individual nodule cluster size in the presence of nitrogen, but rather the contrary, since the clusters had a mean dry weight of 9 mg. compared with 16 mg. for those of plants grown in zero nitrogen. When increase in mean nodule dry weight is expressed as a percentage of increase in whole plant dry weight, a value of 4.5 is obtained for the zero nitrogen plants, while with added nitrogen it falls to 0.6.

Table 13

Effect of 50 mg. per litre of ammonium-nitrogen on nodule and plant growth

Treatment	(1)* Increase in height of shoot cm.	(2)* No. of nodule clusters	(3)* Increase in dry wt. of nodule clusters g.	(4)* Increase in dry wt. of whole plant g.
Zero nitrogen	4.1	6	0.065	1.616
	2.1	6	0.091	1.934
	11.9	3	0.079	1.807
	10.1	4	0.097	1.642
	10.6	7	0.048	1.369
Mean per plant	7.8	5.2	0.076	1.674
50 mg. ammonium-nitrogen per litre	9.9	1	0.011	2.097
	6.6	3	0.018	2.470
	7.1	2	0.009	2.216
	8.6	3	0.005	2.530
	16.1	4	0.061	3.326
	2.1	2	0.000	2.074
	12.9	4	0.004	2.111
Mean per plant	9.0	2.7	0.015	2.403

* The following indicates the Least Significant Differences between means (P=0.05) together with the observed differences:

Column	L.S.D.	Observed difference
1	5.8	1.2
2	1.8	2.5
3	0.027 g.	0.061 g.
4	0.485 g.	0.729 g.

Fig. 24. Ammonium experiment - series B. Left to right: two zero nitrogen plants; two plus nitrogen plants. (Diameter of pots - 10 cm.)

Thus nodule growth failed by a long way to increase in proportion to that of the plant.

The findings concerning the effect of ammonium-nitrogen on the fixation of atmospheric nitrogen will now be considered. It was possible to calculate directly the amount of ammonium-nitrogen which had been absorbed by the roots of each plant during the experiment (X mg.), knowing the proportion of ^{15}N in the supplied ammonium salt (2.021 atom per cent excess), the nitrogen content of the plant at harvest (Y mg.) and the ^{15}N content of the plant nitrogen (Z atom per cent excess), as follows:

$$X \times 2.021 = Y \times Z$$

$$\therefore X = \frac{Y \times Z}{2.021} \text{ mg.}$$

By subtraction of this amount from the total increase in plant nitrogen content, the amount of atmospheric nitrogen fixed by the nodules during the experiment was found.

The results of these calculations are presented in Table 14 for series A plants. It will be noted that the increase in the mean nitrogen content per plant (column 1) was more than doubled in the presence of

Table 14

Effect of 10 mg. per litre of ammonium-nitrogen on fixation of atmospheric nitrogen

Treatment	(1)* Increase in N content of whole plant mg.	(2) Atom per cent excess ^{15}N	(3) NH_4-N absorbed per plant mg.	(4)* Atmospheric N fixed during expt. mg.	(5)* N fixed as per cent of total uptake of N
Zero nitrogen	82.6	0.000	0	82.6	100
	29.7	0.000	0	29.7	100
	65.9	0.000	0	65.9	100
	12.1	0.000	0	12.1	100
	11.8	0.000	0	11.8	100
Mean per plant	40.4	0.000	0	40.4	100
10 mg.** ammonium-nitrogen per litre	116.2	0.922	53.0	63.2	54
	95.5	1.111	52.5	43.0	45
	67.0	1.239	41.1	25.9	39
	129.8	0.900	57.8	72.0	55
	96.6	0.999	47.8	48.8	51
	76.1	1.093	41.2	35.0	46
Mean per plant	96.9	1.044	48.9	48.0	48

* The following indicates the Least Significant Differences between means (P=0.05) together with the observed differences:

Column	L.S.D.	Observed difference
1	38.2 mg.	56.5 mg.
4	34.3 mg.	7.6 mg.
5	6%	52%

** Nitrogen supplied to each plant = 99.5 mg.

Nitrogen uptake as a percentage of nitrogen supplied = 49

10 mg. per litre of ammonium-nitrogen. Percentage total nitrogen (not shown in the Table) attained a mean of 1.57 compared with 1.21 in the zero nitrogen plants. Atom per cent enrichments shown by the plant nitrogen are recorded in column (2). Three only of the zero nitrogen plants were analysed and proved to contain the normal level of ^{15}N, confirming that the precautions taken to prevent contamination had been successful. It was assumed that the other two plants would have shown the same result if analysed. The data in column (3) calculated as above, show that on average each plant provided with nitrogen absorbed about half of the total ammonium-nitrogen supplied to it during the experiment (see footnote to Table). Column (4), together with the statistical treatment appended to the Table, shows that the fixation of atmospheric nitrogen per plant was unaffected by the presence of ammonium-nitrogen, but on average now accounted for only 48 per cent of the total gain of nitrogen by the plant.

Table 15 presents the nitrogen data for series B. Again the increase in mean nitrogen content per plant doubled in the presence of nitrogen, while the percentage total nitrogen in the tissues rose from 1.63 in the zero nitrogen plants to the very high level of 2.31 in

Table 15

Effect of 50 mg. per litre of ammonium-nitrogen on fixation of atmospheric nitrogen

Treatment	(1)* Increase in N content of whole plant mg.	(2) Atom per cent excess ^{15}N	(3) NH_4-N absorbed per plant mg.	(4)* Atmospheric N fixed during expt. mg.	(5)* N fixed as per cent of total uptake of N
Zero nitrogen	26.9	-	0	26.9	100
	33.2	0.000	0	33.2	100
	32.2	0.000	0	32.2	100
	23.6	-	0	23.6	100
	21.2	-	0	21.2	100
Mean per plant	27.4	0.000	0	27.4	100
50 mg.** ammonium-nitrogen per litre	44.3	- ‡	-	-	-
	63.1	1.825	56.9	6.2	10
	55.7	1.887	52.0	3.7	7
	64.8	1.897	60.8	4.0	6
	63.6	1.762	55.4	8.2	13
	49.8	1.873	49.1	3.7	7
	55.4	1.967	53.9	1.5	3
Mean per plant	56.7	1.869	54.2	4.5	8

* The following indicates the Least Significant Differences between means (P=0.05) together with the observed differences:

Column	L.S.D.	Observed difference
1	8.9 mg.	29.3 mg.
4	5.3 mg.	22.9 mg.
5	4%	92%

** Nitrogen supplied to each plant = 220 mg.

Nitrogen uptake as a percentage of nitrogen supplied = 25

‡ No ^{15}N analysis carried out on this plant

the plants supplied with 50 mg. nitrogen per litre. As before the few zero nitrogen plants which were analysed for ^{15}N proved to contain no excess of this above the normal level. The plants grown in the presence of nitrogen absorbed on average about one quarter of the total nitrogen supplied (see footnote to the Table), and fixed very little atmospheric nitrogen, the amount fixed representing only 7.6 per cent of the total plant uptake of nitrogen.

2. The Nitrate-nitrogen Experiment

During the course of the experiment two of the plants in the zero nitrogen treatment were discarded as they were not growing well. They showed signs of nitrogen deficiency, and on examination their nodules were found to be decayed. All the other plants however were considered suitable for harvest.

Table 16 shows the results obtained, again presented as increases over the corresponding data of the initial harvest (Table 11). The increase in nodule dry weight (column 2) was unaffected by the presence of 10 mg. nitrate-nitrogen per litre, but was severely depressed at the higher level. Eye-judgement during the progress of the experiment suggested that plant growth was definitely promoted by the 10 mg. per litre level of nitrate-nitrogen, but not by the higher level, and this impression is also conveyed by the photograph of typical plants (Fig. 25). The mean height data (column 1) are in agreement with the above, but owing to variation within treatments (particularly in the plants supplied with 50 mg. nitrogen per litre) the quite large differences in dry weight increases fall just short of significance. It appears that some growth retarding

Table 16

Effect of nitrate-nitrogen on nodule and plant growth

Treatment	(1)* Increase in height of shoot cm.	(2)* Increase in dry wt. of nodules g.	(3)* Increase in dry wt. of whole plant g.
Zero nitrogen	7.9	0.188	4.190
	24.9	0.220	5.863
	14.9	0.249	4.676
	16.4	0.294	5.913
	17.4	0.524	9.104
	7.5, 5.4	0.258	5.153
Mean per plant	15.9	0.289	5.817
10 mg. nitrate-nitrogen per litre	32, 29.9	0.292	8.153
	41.4	0.260	7.174
	58.9	0.367	16.677
	25.6, 26.9	0.460	11.283
	23.9	-0.008	5.880
	12.4, 10.5	0.035	5.126
Mean per plant	43.9	0.235	9.049
50 mg. nitrate-nitrogen per litre	2.9	0.007	1.316
	28.5, 41.9	0.177	10.200
	6.9	-0.013	2.633
	8, 11, 11, 13.9	0.218	8.015
	11.2	0.063	4.925
	9, 14.9	0.003	5.276
Mean per plant	26.9	0.075	5.394

* Statistical treatment of the data indicates that the differences between means necessary for significance at P=0.05 are as follows:

Column (1)	22.5 cm.
Column (2)	0.172 g.
Column (3)	4.046 g.

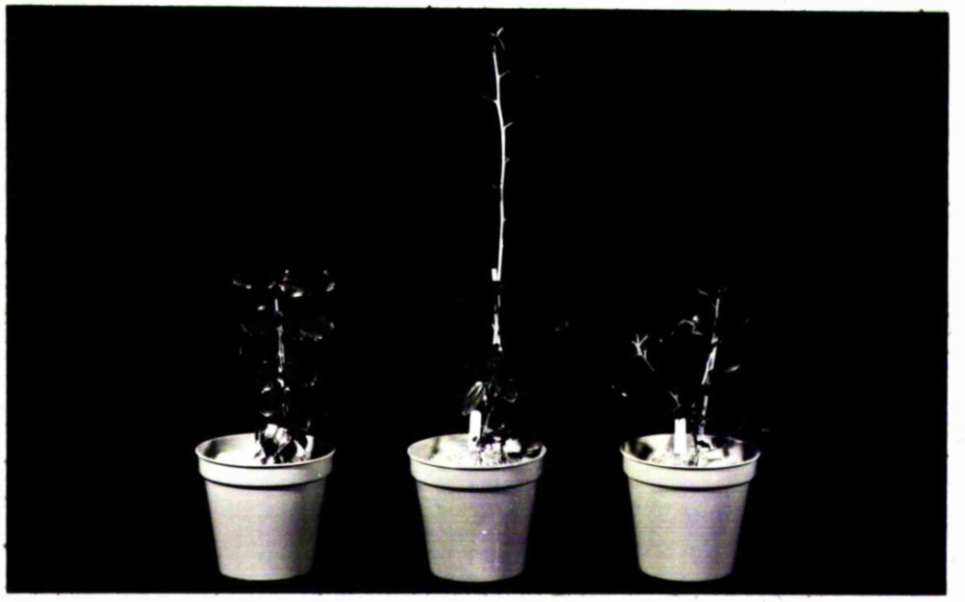


Fig. 25. Nitrate experiment. Left to right: plants supplied with 0 mg., 10 mg. and 50 mg. nitrogen per litre. (Diameter of pots - 13 cm.)

effect was exerted by nitrate at the higher level which cancelled out the benefit that might have been expected. When increase in nodule dry weight is expressed as a percentage of increase in whole plant dry weight, the following values are obtained: 5.0, 2.6 and 1.4 for the 0, 10 and 50 mg. nitrogen per litre plants respectively. Thus as nitrogen was added, the nodule clusters increasingly failed to keep pace with plant growth.

Since the nitrate supplied to plants in this experiment was not labelled with ^{15}N no information is provided on the effect of the nitrate on the fixation of atmospheric nitrogen over the experimental period as a whole. However, as noted earlier, the activity in nitrogen fixation of the nodule clusters from plants of the zero nitrogen and the 10 mg. nitrate-nitrogen per litre treatments of this experiment was compared by the acetylene-reduction assay. The measurements were made over two days, nodule material from two plants of each type being compared on each day.

The results are provided in Table 17, and columns (3) and (4) show that on average the acetylene-reducing capacity of nodules from plants grown in the presence of nitrate-nitrogen was only half that of those from zero nitrogen plants. The difference in means, however,

Table 17

Results of acetylene-ethylene tests

Treatment of plants	(1) Date of expt.	(2) Sample number	(3) µmole C_2H_4 per g. dry nods./2 hrs.	(4) Means	(5)* Relative value	(6)** Mean relative value
Zero nitrogen	1.10.68	1	15.4	11.8	131	100
	"	2	8.2		69	
	2.10.68	3	4.2	6.3	67	
	"	4	8.3		133	
10 mg. nitrate-nitrogen per litre	1.10.68	1	6.7	6.5	57	49.3
	"	2	6.3		53	
	2.10.68	3	3.7	2.8	59	
	"	4	1.8		28	

* In calculating relative values for each day, the mean result of the zero nitrogen samples for each particular day was taken as 100.

** Least Significant Difference between means (P=0.05) is 48.5.

falls slightly short of significance. Comparison of the results obtained on the two days shows that the activity of the nodules from both types of plant was consistently lower on the second day and this is almost certainly due to the inferior conditions experienced by these particular plants in the greenhouse on the previous day which was very dull. The fixatory activity of nodules determined at 11.00 hours (see Methods) will be affected by the amount of photosynthesis achieved by the plants during the previous light period. On these grounds the data have been transferred to a relative basis by assigning a value of 100 to the mean value for the zero nitrogen nodules on each day (column 4), and recalculating the individual results for these and also the plus-nitrate samples accordingly. These relative values are provided in column (5), and the difference between the mean relative values (column 6) is significant. Thus the acetylene-reducing activity of the plus-nitrate nodules, and by inference their nitrogen fixing activity, was only about half that of the zero nitrogen nodules.

The explanation of this finding will be considered in the Discussion. Here it will only be noted that the nodules of the plants supplied with 10 mg. nitrate-

nitrogen per litre were definitely fixing nitrogen during the growth period, though with decreased efficiency.

D I S C U S S I O N

In experiments of the type being considered here the control of two factors of the environment is difficult. The first is the maintenance of the desired level of combined nitrogen in the rooting medium. A constant level could only be obtained by some type of drip culture, which would be costly when labelled nitrogen was being used. All that can be claimed in the present experiments is that the combined nitrogen was restored to the desired level at frequent intervals. Obviously the level would fall between these occasions, especially at the 10 mg. per litre level, but even there the plants absorbed only half of the total supplied in the ammonium experiment (Table 14). The second factor is the pH of the rooting medium which would ideally be kept constant at some value known from previous work to be favourable both to nodule growth and activity, and to the uptake of combined nitrogen by the plant. Control of pH is more difficult when solid rooting media such as Peralite are used than with water culture, and as noted earlier substantial drifts in pH occurred during these experiments. However Ceanothus velutinus

is obviously a species of wide tolerance to pH and there is no reason to suspect that pH was a factor contributing appreciably to any of the effects attributed to the combined nitrogen.

In the Introduction to this Section the question was asked how continued nodule development would be affected by a rising level of combined nitrogen in the soil. It has been shown that under the conditions of the present experiments a low level of ammonium-nitrogen promoted the weight of individual nodule clusters as well as the total weight per plant, while the same level of nitrate-nitrogen, although it did not have this promoting effect, did not deter nodule growth. At the higher level both forms of nitrogen reduced nodule growth severely. Both levels of ammonium reduced the number of nodule clusters per plant. Wollum (1967) also found that individual nodule size was greatest at low levels of ammonium-nitrogen, and that all levels of ammonium lowered cluster numbers, but he reported that ammonium and nitrate-nitrogen reduced nodule weight per plant in proportion to the level of added nitrogen. This presumably means that there was reduction at all levels, in which case his result differs from that of the present author. However lack of information

concerning conditions of growth in Wollum's experiments, e.g. the type of medium in which the plants were cultured, makes further comment difficult. On the whole the findings of the present author for Ceanothus agree with the previous work on other genera mentioned in the Introduction.

Further discussion of the above results will be limited to changes in nodule weight per plant. The promotion of the weight of nodules per plant (ammonium) or the lack of its depression (nitrate) at low levels of supplied nitrogen, and its severe depression at a high level, may indicate that two effects operate. The presence of external combined nitrogen will almost certainly make more nitrogen available for nodule growth, either because less fixed nitrogen is removed from the nodules or because the supplied nitrogen is used. On the other hand the more rapid growth of the plant when external nitrogen is available is very likely to reduce the amount of photosynthates entering the nodules. In fact Small and Leonard (1969) have shown by use of $^{14}CO_2$ that this was the case in two legumes, and concluded that the more active growth of the roots when provided with nitrate increased their "sink" value relative to the nodules. The first of the above effects predominates, it is suggested, at a low level of combined

nitrogen, especially in the ammonium form. The second is thought to predominate at higher levels of nitrogen where plant growth is expected to be still stronger, although in the present experiments this tendency was not maintained owing to presumed toxic effects of the combined nitrogen at the 50 mg. per litre level.

The second question asked in the Introduction was whether the efficiency of nodule tissues in fixation was affected by the presence of combined nitrogen in the rooting medium. If it remained unaffected then obviously fixation per plant in the presence of different levels of combined nitrogen would run parallel to the reported changes in nodule weight. If efficiency were to fall, fixation would be less than proportional to nodule changes. Inspection of the results of the ammonium experiments (Tables 12, 13, 14, and 15) suggests that the latter was the case. To express this more precisely, the mg. nitrogen fixed per gram nodule dry matter during the period of the experiment can be calculated, the results being shown in Table 18 along with comparable data for Alnus reproduced from Stewart and Bond (1961).

Table 18

Mg. nitrogen fixed per gram of nodule dry matter present

Genus	NH_4-N per litre of culture solution, mg. 0	10	50	L.S.D. (P = 0.05)
Alnus	438	410	315	101 mg.
Ceanothus - series A	197	130	-	73 mg.
Ceanothus - series B	331	-	227	126 mg.

It will be seen that in both series of Ceanothus the efficiency of nodules which had grown in the presence of combined nitrogen was lower than that of the corresponding nodules of zero nitrogen plants, though the differences do not quite attain significance. The higher over-all level of values for series B plants cannot be explained.

Further evidence of reduced nodule efficiency in the presence of nitrogen is provided by the acetylene reduction data for detached nodules from the nitrate experiment. Here the efficiency was calculated in a different manner, the amount of ethylene formed per unit weight of nodules being measured over a much shorter time - i.e. two hours at the end of the experimental

growth period - as opposed to the whole experimental period, and the formation of ethylene by the nodules of plants grown in the presence of 10 mg. nitrogen per litre was found to be only about half of that by nodules from zero nitrogen plants. The finding of Webster et al. (1967) that "nodules from plants grown with addition of up to 50 ppm ammonium-nitrogen showed no difference in fixation" is difficult to accept in the light of results of previous workers and also those of the present experiments, especially since the investigation was only briefly reported with little detail being given.

Possible explanations of the observed effect of combined nitrogen on nodule efficiency are as follows:-

(1) In the presence in the nodule cells of ammonia absorbed from the rooting medium or produced within the cell by the reduction of supplied nitrate, the endophyte uses this in its growth in preference to fixing nitrogen and the fixation process tends to be suppressed, possibly through a lack of ATP and reducing power which has been used up in the assimilation of the supplied nitrogen.

(2) As noted, Small and Leonard (1969) suggested

that in the presence of nitrogen the increased growth of the roots acted as a sink for photosynthates and the nodules were deprived of them. There might thus be an additional lack of reducing power and ATP due to lack of photosynthates.

(3) A microscopic examination of the nodules might show that the amount of endophyte was reduced in the presence of combined nitrogen.

The data on ethylene production (Table 17) can be converted into terms of nitrogen fixing activity from the theoretical expectation that three moles of ethylene correspond to one of nitrogen. When this calculation is made for the nodules from the zero nitrogen plants a mean fixation of 0.04 mg. nitrogen per gram of nodule dry matter per hour is indicated, which is reasonably consistent with a value of 0.02 mg. found by Bond (1967a) also for detached nodules, bearing in mind small differences in procedure. Webster et al. (1967) reported a much higher figure of 0.9 mg. for detached nodules of C. velutinus, "based on accumulation of excess ^{15}N in a short period after excision". It is presumed that they prepared a time-course curve for fixation in detached nodules and based their calculation on an inter-

polated value of ^{15}N accumulation, perhaps during the first 15 minutes after the commencement of exposure to labelled nitrogen, when fixation will be maximal. Russell and Evans (1966) calculated values ranging from 0.1-1.0 mg. nitrogen per gram of nodule dry matter per hour for C. velutinus nodules still attached to the plant, indicating that the figure of Webster et al. is definitely exceptional.

A further feature of interest in the results of these experiments is that the addition of combined nitrogen usually led to much stronger growth of the plants and also to higher percentage nitrogen in the tissues compared with those entirely dependent on nodule nitrogen. This same feature has been observed by investigators at Glasgow in several different species grown under similar conditions to the present experiment (Stewart and Bond, 1961; Rodriguez-Barrueco, 1967), though the only previous investigators to report this aspect in Ceanothus were Zavitkovski and Newton (1968a), who found much stronger growth in the presence of added organic nitrogen. The possible explanation of this feature will now be considered.

A first possibility is that under the conditions of the experiments the nodules of the zero nitrogen

plants failed to provide sufficient nitrogen. This might have been because the plants developed an insufficient nodule mass. In this respect the three sets of plants grown with zero nitrogen showed values of 4.7, 4.5 and 5.0 for nodule dry weight at harvest expressed as a percentage of whole plant dry weight. A corresponding value of 5 per cent, or possibly less, is indicated by the data of Russell and Evans (1966) who worked with C. velutinus plants grown in a greenhouse at Corvallis, Oregon (latitude 45°), presumably in an environment similar to optimum field conditions, and almost certainly more favourable to the plants than those provided in Glasgow (latitude 56°). Thus it can be assumed that the plants in the present experiments were normally supplied with nodules. Alternatively, conditions might not have been optimal for nodule activity. The frequently-wet rooting medium might have been detrimental to growth, and yet by comparison with the results of Bond (1967a) who did not use this technique, it appears that the flushing procedure must have been beneficial over all. From the results of Russell and Evans (loc. cit.) it can be calculated that 155 mg. nitrogen was fixed per gram of nodule dry matter, a value lower than those of the present author for zero nitrogen plants

(Table 18), so that it would appear that her conditions were such as to promote satisfactory nodule activity. Thus to explain the results along the lines just discussed it would seem necessary to assume that in a nodulated plant the supply of nitrogen from the nodules falls short of the full requirement for some inherent reason at least under greenhouse conditions.

There is a second and very different possible explanation. The inferior growth of the zero nitrogen plants might have been due to a shortage of photosynthates rather than of nitrogen. Although they showed a lower percentage nitrogen content that the plants supplied with combined nitrogen, they were very healthy in appearance and devoid of any visible symptoms of nitrogen deficiency, while the higher percentage nitrogen in the combined nitrogen series could merely have been due to an accumulation of unassimilated nitrogen in the tissues. The fixation of nitrogen is undoubtedly attended by a considerable consumption of photosynthates, but whether or not the requirements for nodule development, maintenance and nitrogen fixation is greater than for the formation and maintenance of roots and the uptake of nitrogen in the combined form is open to question. Gibson (1966) attempted to find the carbohydrate require-

ments for symbiotic nitrogen fixation in a legume by comparing the relative growth rates of clover plants, some nodulated in nitrogen-free medium, others non-nodulated and fed with ammonium nitrate in rationed amounts to give the same rate of nitrogen accretion in both series. He concluded that the carbohydrate requirements of fixation itself were similar to or only slightly greater than those for the uptake and assimilation of the combined nitrogen, but that a further quantity was required at times of active nodule growth. However Gibson's conclusions depend on the assumption that the photosynthetic rate of nodulated and non-nodulated plants are similar, and this was not verified. It has been found (Bond and MacConnell, 1955) that in alder at least the respiration of the nodules is two to three times greater than that of the roots.

If this second explanation, that the zero nitrogen plants are short of photosynthates, is correct, then factors affecting photosynthesis (e.g. light intensity, CO_2 concentration) might be of more importance to these plants which may require to photosynthesise faster than the plants supplied with combined nitrogen.

In conclusion it is suggested that under field conditions, as the level of soil nitrogen rises,

Ceanothus plants may respond in the following ways. Plant growth may be benefited by the presence of nitrogen in the soil. Initially, at low concentrations of combined nitrogen nodule growth may either be promoted (as in the presence of ammonium) or unaffected (in the presence of nitrate), but as the level of nitrogen increases, nodule growth will be retarded. The effectiveness in fixation per unit weight of nodules will be depressed, but at first the amount of nitrogen fixed per plant may be unaffected. This however will fall sharply as the concentration of soil nitrogen increases.

S U M M A R Y

(1) Nodulated plants of Ceanothus velutinus var. laevigatus, in their second year of growth, were grown in Peralite culture, a proportion of them being supplied with combined nitrogen as ammonium or nitrate at the levels of 10 mg. and 50 mg. nitrogen per litre of culture solution, the remaining plants being given nitrogen-free solution. The ammonium sulphate supplied was labelled with ^{15}N, so that after the experiment was completed the amount of combined nitrogen absorbed by the roots and the amount of atmospheric nitrogen fixed by the nodules could be calculated. At the time of harvest the fixation rates of nodules detached from zero nitrogen plants and plants supplied with the lower level of nitrate-nitrogen were compared by use of the acetylene-ethylene method.

(2) Plant growth was improved by the presence of 10 mg. nitrogen per litre, but at the higher level there was evidence of toxicity. At the lower level of

nitrogen the weight of nodules per plant was increased in the presence of ammonium and unchanged in the presence of nitrate, but at higher concentrations of both types of nitrogen this was retarded. Ammonium-nitrogen reduced the number of nodule clusters formed per plant.

(3) The efficiency of the nodules in fixation was depressed in the presence of nitrogen, although the amount of nitrogen fixed per plant was unaffected at the lower level. At the higher level, however, this dropped drastically, and the plants grew chiefly at the expense of the supplied combined nitrogen.

(4) The possible explanation of these effects is discussed.

SECTION III

Diurnal variations in nitrogen fixation in non-legumes with special reference to *Casuarina*

Diurnal variations in nitrogen fixation in non-legumes with special reference to *Casuarina*

I N T R O D U C T I O N

It is clearly possible that under natural conditions of growth nitrogen fixation in a nodulated plant will show diurnal changes in rate, on account of (a) variation in temperature, which is likely to affect the rate of fixation itself, and influence also the rates of photosynthesis and translocation, and (b) light intensity, acting through photosynthesis, because of the need for ATP, reducing power and carbon skeletons for fixation; the immediacy of any effect of light will depend on the distance between the photosynthetic organs and the nodules, and the speed of translocation.

It is only very recently that direct evidence of the existence of diurnal variations in fixation has been sought (see later), but the experiments of some earlier investigators have a bearing on the matter.

Thus Lindstrom *et al.* (1952), using a ^{15}N method, examined the effect of darkening on fixation in intact red clover plants growing in large test tubes. Plants

darkened at the time of exposure to ^{15}N and incubated for twelve hours, fixed nitrogen at about 75 per cent of the rate of plants which were kept in the light. The effect of different periods of predarkening on fixation rates found subsequently was also studied, and it was found that as this period was increased, so the rate of fixation decreased. The results of this paper would seem to indicate that in red clover the hours of natural darkness experienced by the plants at night might result in a certain reduction in fixation on that account.

Also working on the effect of darkening on nitrogen fixation, Virtanen et al. (1955) used excised nodules from pea plants and exposed them for two hours to an atmosphere labelled with 60 atom per cent excess ^{15}N, after which the acid soluble portion of the nodules was analysed. It was found that fixation in nodules removed at 12.00 hours was about three times greater than at 08.00 hours on the following day, but by midday had risen substantially. Plants which had been predarkened for 24 hours fixed nitrogen at a rate only five per cent of that of nodules from illuminated control plants.

In investigations by Bach et al. (1958), $^{14}CO_2$ was supplied to soybean plants and the incidence of

^{14}C-labelling in the nodules studied. It was found that, of the total ^{14}C in the nodules, the proportion in amino acids fell during the night, pointing, the authors suggested, to a reduction in fixation.

Pate (1962) analysed the bleeding sap from decapitated plants of field pea growing in nitrogen-deficient medium. Sap was collected for one hour from plants decapitated at two hour intervals and it was found that under natural conditions the level of organic nitrogen exuded per plant (attributed to nodule action) showed a well marked rhythm, rising to a maximum near noon. An additional observation was that favourable weather conditions promoted higher output of exudate and organic nitrogen.

Working at Glasgow, Rodriguez-Barrueco (1967) detached nodules from plants of Casuarina torulosa at different times of day and by means of ^{15}N measured their fixation over a period of $4\frac{1}{2}$ hours at a temperature of $24^{\circ}C$. The plants, which were growing in the greenhouse, were in their second year and stood about one metre in height. It should be carefully noted that Rodriguez-Barrueco did not attempt to measure the actual rates of fixation at different times of day, but rather what will, in this thesis, be termed the potential

for fixation, i.e. that shown when the nodules are incubated at a fixed, favourable temperature. It was found that for these particular plants the potential for fixation was greater in the evening than around midday. However, since the results only just reached significance, Bond (personal communication) repeated the experiment using second year C. cunninghamiana plants, with similar, but more significant results. The fixation potential at 11.00 hours was found to be less than half that at 07.00 and 23.00 hours. It was suggested by Rodriguez-Barrueco that his results could be explained by the probability that in such large plants as his, the evening was the time when the arrival of photosynthates in the roots would reach its peak; nodules detached at this time would then be better supplied with such substances. Obviously it does not follow that the actual fixation achieved in nodules in the greenhouse shows this pattern since temperature fluctuations will exert some control.

Recently a study of diurnal variations in actual fixation was made by Hardy et al. (1968), using the acetylene method. Excised, nodulated roots of soybeans held under field conditions were shown to exhibit maximum fixation rates between 12.00 and 20.00 hours,

and a minimum rate between 24.00 and 08.00 hours. The authors considered that this suggested a close relationship between light and nitrogen fixation, and dismissed temperature as relatively unimportant. The effect of darkening on fixation was also investigated by these workers, and it was found that after 17 hours the nitrogen fixing activity of plants grown in a growth cabinet had fallen to 30 per cent that of the controls, but after 64 hours 15 per cent of the control activity still remained. It was suggested that the rapid decline in the first period of darkness might reflect the depletion of photosynthate, while the residual activity might be due to the utilisation of storage products.

The most recent investigation of diurnal rhythms in nitrogen fixation has been carried out in Glasgow by Wheeler (1969). He studied variations in the rate of nitrogen fixation (mainly by means of the acetylene assay) in first year plants of Alnus glutinosa and Myrica gale, the height of the plants being in the order of 20 cm. To simplify the issue, on the day of the experiments the effect of temperature was eliminated by keeping this approximately constant at 21°C in the greenhouse in which the plants were growing. At intervals through the day, trimmed, excised root systems,

bearing most of the nodules, were taken and assayed for acetylene-reducing power, again at 21°C. This proved to rise sharply in the morning, and by midday was 2-3 times as fast as soon after sunrise, while it fell in the mid-afternoon. Supporting evidence of a midday peak in nitrogen fixation was obtained from exudation experiments with Alnus, in which the total nitrogen content of the exudate per plant was found to be highest in those decapitated at 13.00 hours, falling to only 30 per cent of this value by 22.00 hours, and also from studies of the ethanol-soluble nitrogen content of nodules of Alnus and Myrica which revealed that in both cases this was maximal at midday. These findings which, it should be noted, again involved the determination of potential rather than the fixation actually shown under normal greenhouse conditions, are in sharp contrast with those of Rodriguez-Barrueco and Bond for Casuarina reviewed above; the reasons for this will be considered in the Discussion. Wheeler pointed out that light intensity was the factor possibly responsible for the midday maximum in fixation, but the results of determinations of the gross carbohydrate content of the nodules at different times of day showed no correlation with the variations in fixation.

The work now to be described resembles in part that of Rodriguez-Barrueco (1967), in that second year plants of Casuarina were again used, and nitrogen fixation in nodules detached at different times of day was measured by ^{15}N assay. The original reason for the selection of Casuarina for this type of work was that its nodules, after detachment, continue to fix nitrogen at a steady, readily-measured rate (Bond, 1957). An important difference from the Rodriguez-Barrueco procedure is that the detached nodules were held in the greenhouse during exposure to ^{15}N, i.e. the fixation was measured at the temperature prevailing in the greenhouse at that time, rather than at a constant, favourable temperature. Thus the aim was to assess the actual rate of fixation rather than the potential for fixation. In addition the gross carbohydrate content of nodules at different times of day has been measured, while in other experiments, the effect of darkening and that of temperature on fixation in Casuarina and other genera have been studied.

MATERIALS AND METHODS

Plant culture

Plants of Casuarina cunninghamiana Miq., Hippophaë rhamnoides L. and Myrica cerifera L. used in the following experiments were from stocks available in the greenhouse. Seedlings had been transplanted in April or May into water culture with nitrogen-free solution, and in the first two species, had been inoculated with the appropriate nodule endophyte by application to the roots of an aqueous suspension of crushed nodules. In the case of the Myrica plants, a suspension of habitat soil was applied for inoculation purposes. The greenhouse is lit by daylight and has a good exposure. With Casuarina, the experiments were carried out in August of the second year of plant growth, when these had reached an average height of some 90 cm. Hippophaë and Myrica were used in August or September of their first year of growth, shoot height being then about 25 and 30 cm. respectively.

It was thought best that days when weather conditions were good should be selected for the actual

experiments. Since most experiments were commenced in the early morning, dependence had to be placed on the weather forecast.

Measurement of fixation

Where there was any lack of uniformity in the growth of plants available for a particular experiment, the plants were graded for size, and a similar combination of sizes was used at each time of sampling.

On each occasion when fixation was to be measured, a proportion of the nodules present was taken from each of several plants and combined to give a bulk sample of 4.5 grams fresh weight in the case of Casuarina. These plants with their remaining nodules were then discarded, or the nodules collected for use in carbohydrate estimations (see below). After mixing the bulk sample as thoroughly as possible, it was equally divided between three 28 ml. glass specimen tubes fitted with stopcocks and containing 1 ml. of culture solution. The tubes were then attached to a manifold, fully evacuated and re-filled to one atmosphere with a gas mixture containing 10 per cent nitrogen, 20 per cent oxygen and 70 per cent argon. The nitrogen was enriched with ^{15}N to an extent which will be indicated

later for each experiment, and had been prepared by the action of hypobromite on a suitable stock of ammonium nitrate. The interval between the commencement of nodule detachment and gassing was about 15 minutes.

With Hippophaë and Myrica nodule yields were smaller, and rather smaller samples were used for measurement of fixation.

The conditions under which the gassed samples were held, and the duration of the exposure to the gas, will be specified later. At the end of the exposure period the nodules were quickly transferred to Kjeldahl flasks and covered with an appropriate amount of concentrated sulphuric acid, preparatory to the use of the Kjeldahl process. After titration the distillates were re-acidified, evaporated down to a small volume, and then analysed for ^{15}N content by Dr. C. W. Crane. In certain experiments the distillates derived from the replicate nodule samples exposed to ^{15}N were combined prior to ^{15}N assay.

The provision of only 10 per cent of nitrogen in the gas mixtures was adopted as a measure of economy. Judging by alder (Bond, 1959) it may have been somewhat below the optimal level for fixation, so that some slight limitation of fixation may have resulted. As

regards the oxygen level, previous work (Bond, 1961) has shown that a level close to 20 per cent is optimal for fixation in detached Casuarina nodules.

Estimation of carbohydrate content

Nodules whose carbohydrate content was to be found were quick-frozen after collection in tubes immersed in an ethanol-dry ice mixture and stored at -20°C until required. The nodules were then freeze dried and triplicate sub-samples (0.1-0.5 grams, depending on the quantity available) of the dry matter from each original sample were weighed out and separately analysed for carbohydrates by the method of McCready et al. (1950) with some modifications suggested by Yemm and Willis (1954). This involves the successive extraction of the material with hot 80 per cent ethanol for the removal of sugars and glycosidic carbohydrates, and with dilute perchloric acid at room temperature for the removal of reserve polysaccharides, followed by colorimetric analyses of the extracts by the anthrone method. The two fractions will be referred to as "soluble" and "reserve" carbohydrates respectively, and both are expressed in terms of glucose, the colorimetric readings having been compared with a standard curve for glucose.

When the above procedure was followed with Myrica nodules, a lot of interference was found in the ethanol fraction when this was treated with anthrone. This was presumed to be due to phenolic compounds and these were removed by firstly taking portions of the ethanol extract to dryness on a rotary evaporator, the effect of concentrating the solution causing some of the phenols to precipitate out, and these failed to redissolve when the residue was made up to known volume with distilled water. This was shaken with powdered animal charcoal and then centrifuged, the supernatant being analysed by the anthrone method. Removal of interfering substances by adsorption on to charcoal was recommended by Ebell (1969). The present author found that a glucose solution treated with charcoal gave the same readings as an untreated sample, while Dr. C. T. Wheeler (personal communication) also found this to be the case with sucrose and fructose, so that it can be assumed that charcoal will remove very little if any of the sugars from the ethanol extracts.

Exudation studies

To measure diurnal fluctuations in weight and total nitrogen of the bleeding sap of Myrica cerifera

plants, exudates were collected in the following way. The plants which were growing in Peralite in plastic "transpots" were placed in trays of distilled water on the afternoon prior to the day of the experiment, to ensure saturation of the Peralite, and any leaves or side shoots at the base of the stem were removed. On each sampling occasion seven plants with an average height of 30 cm. were decapitated 1-2 cm. above the Peralite and the cut surface washed with distilled water. By means of latex rubber tubing a length of glass capillary tube shaped like an inverted "u" was attached to each stump, the rubber tubing being additionally secured to the stump and the glass tube with thread. For collection of the exudate a weighed 5 ml. bottle was placed on the Peralite with the free end of the tube projecting into it. The "transpots" were then placed in an incubator at 23°C and left for three hours. At the end of this period, the exudates remaining in the capillary tubes were transferred to the bottles by cutting the stem below the rubber tube, removing the small part of the stem and blowing the sap into the bottle. The bottles were re-weighed and the amount of exudate thus found. The exudates from each occasion were then combined and stored at -20°C

until required.

For estimation of total nitrogen, the exudates were transferred to 30 ml. semi-micro Kjeldahl flasks, a drop of concentrated sulphuric acid added in case there was any free ammonia in the sample, and then evaporated to dryness on a rotary evaporator. Total nitrogen was then found by the semi-micro Kjeldahl method described by Purvis et al. (1966), except that mercuric oxide replaced selenium dioxide in the catalyst.

Measurement of light intensity

Light intensity was measured with an "EEL" light meter which records wavelengths in the range 250-730 mμ. The photocell was fixed in the same horizontal position beside the test plants throughout the day. In retrospect, in experiments with Casuarina, the photocell should have been turned towards the maximum light source at each reading since the cylindrical photosynthetic organs of this genus are probably able to use oblique light rays quite efficiently.

All the times given are in British Standard Time (i.e. Greenwich Mean Time plus one hour).

D A T A O B T A I N E D

In connection with experiments involving the measurement of fixation by the ^{15}N method, since the label of the nitrogen to which the nodule samples were exposed varied in different experiments, to facilitate comparison of data the total fixation (^{15}N and ^{14}N) was calculated for each sample, using the enrichment shown by the sample on analysis, the enrichment of the supplied nitrogen, and the total nitrogen content of the sample.

It is difficult to decide which is the best basis on which to express the fixation. Several methods have been used by other workers, for example, on nodule nitrogen, on fresh weight or on dry weight of nodules, but it will be realised that diurnal changes might well occur in any one of these. In the present experiments it was decided to express fixation as nitrogen fixed per mg. of nodule nitrogen, since the latter was accurately measured, while it was also found that the total nitrogen content of each of the bulk samples of 4.5 grams fresh nodules of Casuarina did not vary throughout

the day. Additionally, it may be noted that the nodules of first year alder plants which had been harvested on four occasions during the day were found by the author to be unchanged in total nitrogen content.

The minimum enrichment shown by any sample, with the exception of the "darkening" experiment where the intention was to take fixation to extinction, was 0.018 atom per cent excess ^{15}N, which is well above the lower limit of significance for mass spectrometric readings.

A sample of Casuarina nodules unexposed to excess ^{15}N, collected on 28th August 1967, showed normal content of ^{15}N on analysis. Previous control samples of Casuarina nodules have also shown normal content (Bond, personal communication).

For statistical treatment of the data from fixation studies, in two of the Casuarina experiments the three samples of nodules exposed on each occasion of fixation measurement were separately analysed for ^{15}N content. The three results thus yielded were used in the statistical treatment, and the Least Significant Difference found. Appreciable variation was present between the replicates and this is believed to go back to variability in fixatory activity among nodule clusters

from one and from different plants, which the mixing of the bulk sample cannot wholly overcome. In the three remaining Casuarina experiments, however, the triplicate samples were pooled prior to analysis, since the analyses are done by contract, so that statistical analyses could not be carried out. Since the nodules used in all the Casuarina experiments were drawn from comparable plants, approximate inferences of significance are justifiable in the latter three experiments on the basis of the two Least Significant Differences actually calculated. In the Hippophaë experiments, the Least Significant Difference could be calculated, but in the Myrica ^{15}N experiment, the duplicate samples had to be combined for ^{15}N analysis as there the absolute nitrogen content was rather low.

Statistical treatment of the carbohydrate data was based on the three samples of nodules separately analysed for each occasion. As will be seen later, agreement here was considerably closer than in the fixation measurements. In all cases the Least Significant Difference values given are for $P = 0.05$.

Diurnal variations in fixation in Casuarina

In these experiments the tubes containing the

gassed nodule samples were placed for the requisite time (two hours) in a ventilated box on the greenhouse bench beside the remaining plants. The temperature within the box did not deviate by more than $1^{o}C$ from that in the greenhouse as a whole. It is assumed that the rate of fixation in these detached nodules would be proportional to that in attached nodules over the same two hours, except that any effect of changes in light intensity during the two hours could not be shown in the detached nodules.

The results of the first experiment are shown in Fig. 26, together with light intensity and temperature records. As noted, in planning these experiments considerable reliance had to be placed on weather forecasts, and the weather on the day of this first experiment was frequently dull, so that light intensity attained a maximum of 920 foot candles only and that for a brief spell. Nonetheless a clear variation in fixation is obvious, a doubling of the rate being shown during the morning, repeated in reverse in the evening.

A second experiment (Fig. 27) was spread over two days. The weather on the first day was again much inferior to that forecast, and for this reason the frequency of sampling was reduced and the experiment

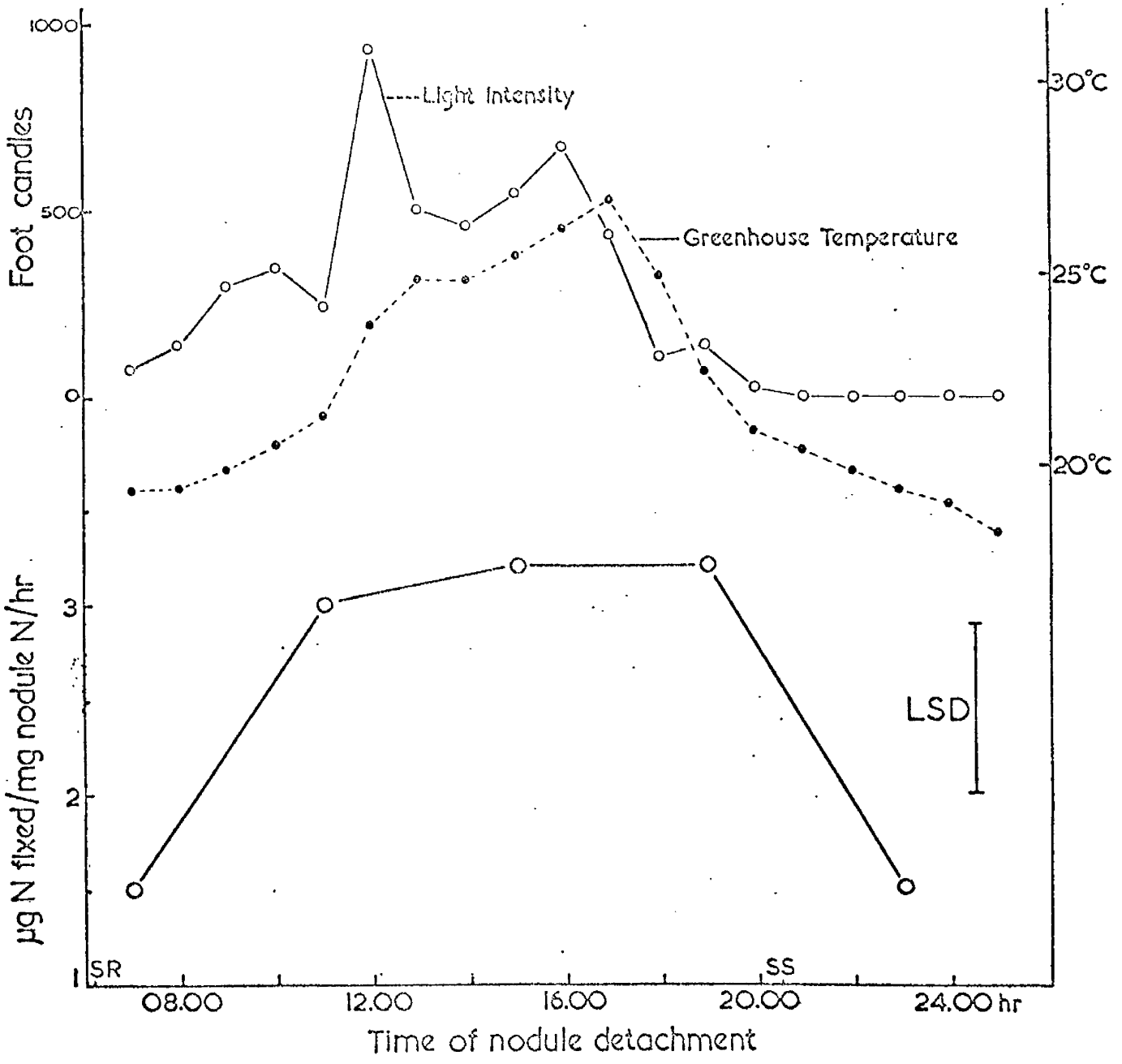


Fig. 26. Diurnal variations in fixation in Casuarina. Experiment carried out on 25th August, 1967. Samples of nodules, from three plants on each occasion, were taken every four hours from 07.00 hours until 23.00 hours. These were exposed to an atmosphere containing nitrogen labelled with 60 atom per cent excess ^{15}N, and left for two hours at greenhouse temperature.

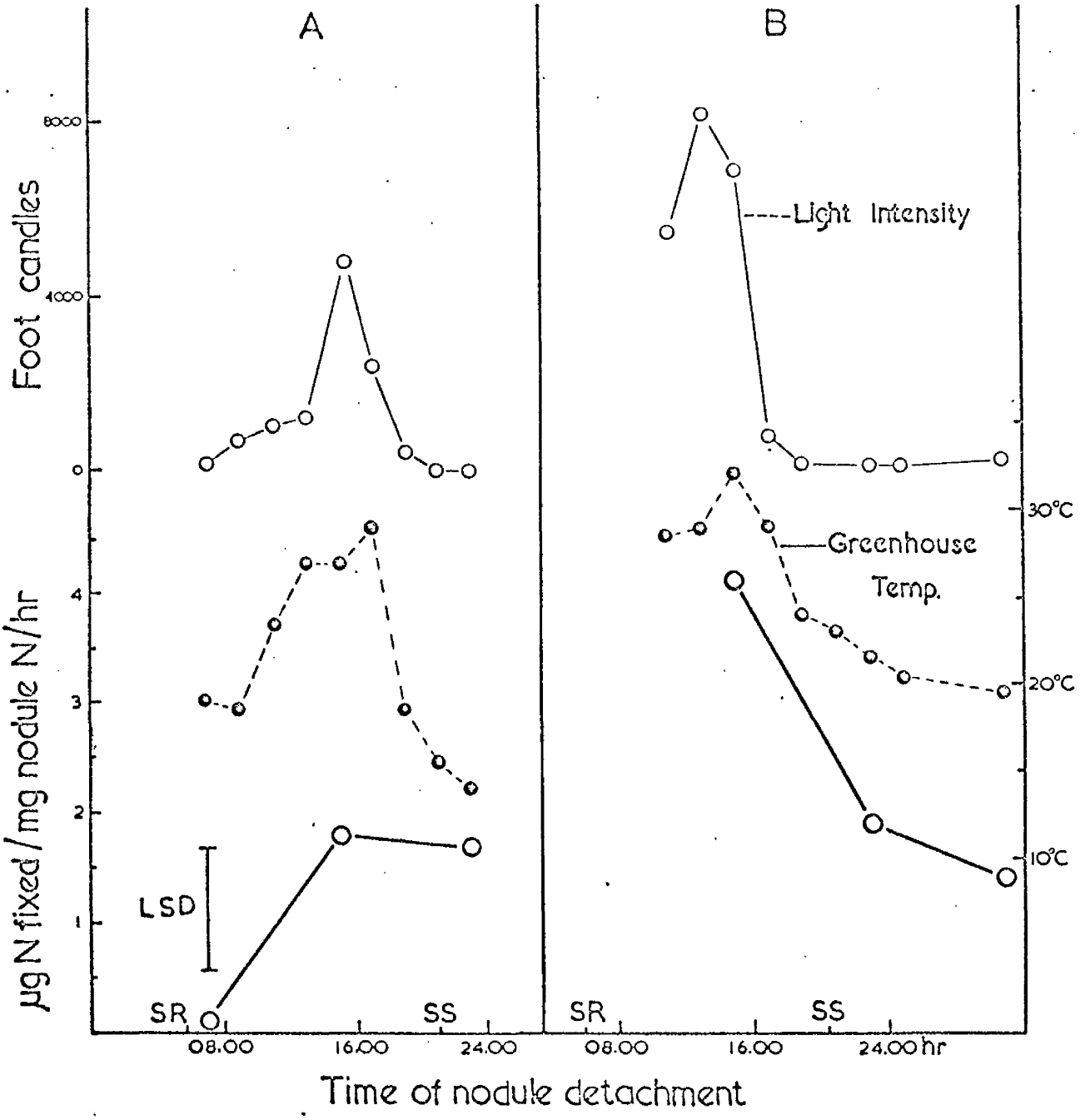


Fig. 27. Diurnal variations in fixation in Casuarina.

A. Experiment carried out on 14th August, 1968. Samples of nodules, from four plants on each occasion, were taken at eight hour intervals from 07.00 hours until 23.00 hours. Exposure as in Fig. 26.

B. Experiment carried out on 22nd August, 1968. Samples of nodules, from three plants on each occasion, were taken at eight hour intervals from 15.00 hours until 07.00 hours on the following day. Exposure as in Fig. 26.

continued on a later day when sampling was not commenced until the day had proved to be favourable. Fixation on the first day again showed a marked rise between 07.00 and 15.00 hours, but on this occasion did not fall in the evening. On the second day, when light intensity and temperature were higher, fixation in the sample taken at 15.00 hours considerably exceeded that shown on the previous day and in the first experiment (Fig. 26), but had fallen substantially by 23.00 hours. The nodules taken at 07.00 hours on the following morning still showed a low value.

Nodular carbohydrates

The carbohydrate data corresponding to Fig. 26 are presented in Fig. 28. The level of "soluble" carbohydrates showed no significant change over the main part of the day, but had fallen significantly by 19.00 hours, followed by a marked increase by 23.00 hours. The "reserve" carbohydrates showed a significant rise between 07.00 and 15.00 hours and a further rise by 23.00 hours. The "reserve" fraction is presumably the starch which is seen to be present in sections of Casuarina nodules.

The carbohydrate data corresponding to Fig. 27

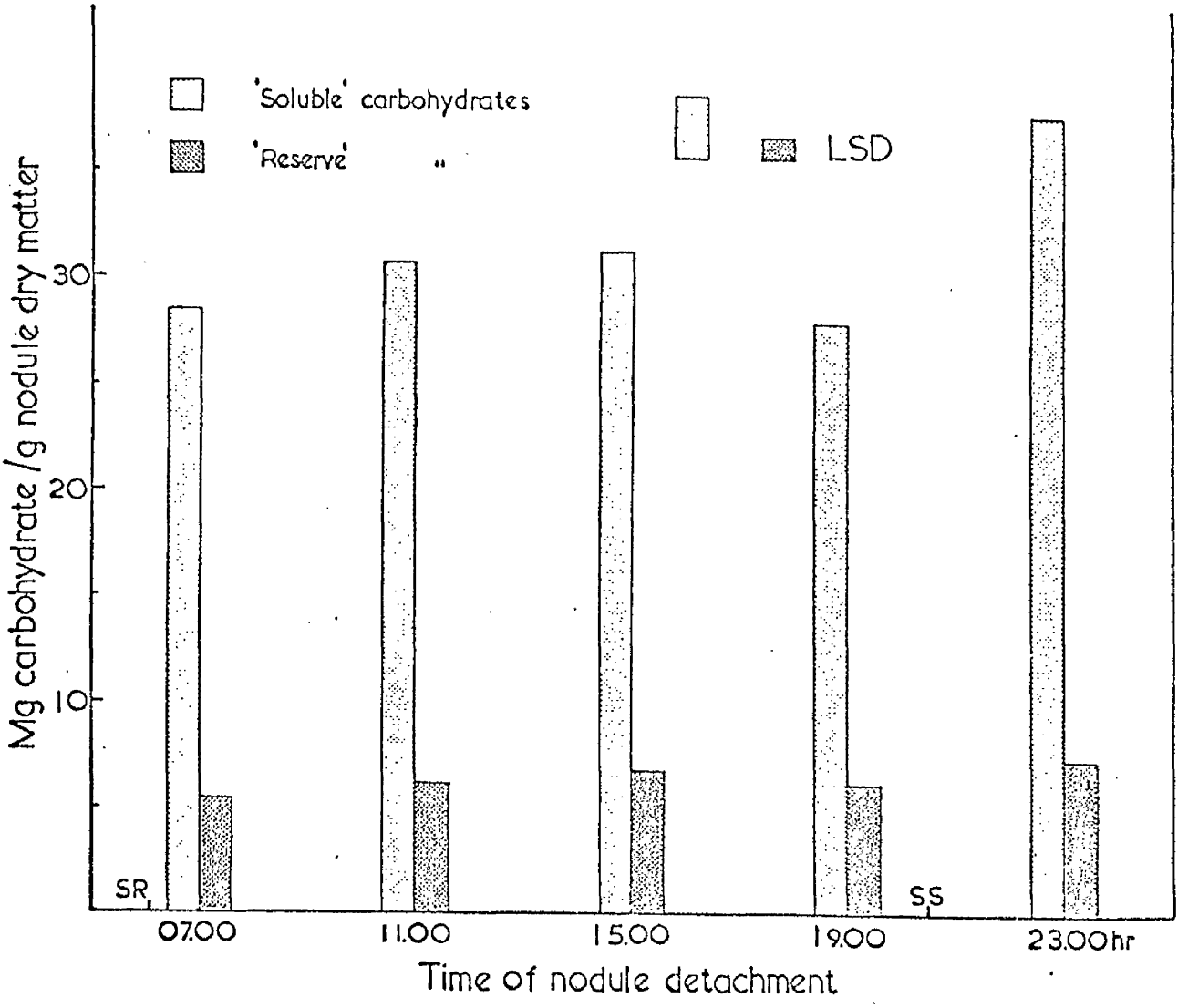


Fig. 28. Diurnal fluctuations in "soluble" and "reserve" carbohydrates in nodules of Casuarina. Nodules removed from same plants and at same times as in Fig. 26.

are shown in Fig. 29. In Part A the "soluble" carbohydrates again show a significant downward trend towards evening, but on this day the accumulation at 23.00 hours shown in Fig. 28 was not reproduced, although it was in respect of "reserve" carbohydrates. In Part B the "soluble" fraction was greatly increased by 23.00 hours as compared with 15.00 hours. The "reserve" fraction also rose at this time. It is of interest that the carbohydrate levels were still high early next day.

In order to summarise the findings for the three days, the values for each day were converted to a relative basis and then averaged. Relative values for the "soluble" fraction were calculated by calling the result at 23.00 hours on each day, 100, and determining the other values accordingly, while those for the "reserve" fraction were calculated as a percentage of the original result of the 23.00 hour "soluble" fraction for each day. Results were compared with the 23.00 hour samples since an analysis of the nodules at this time had been made on each of the three days. Fig. 30, a histogram of the relative values, shows that on average both the "soluble" and "reserve" carbohydrate content of the nodules was higher in the early

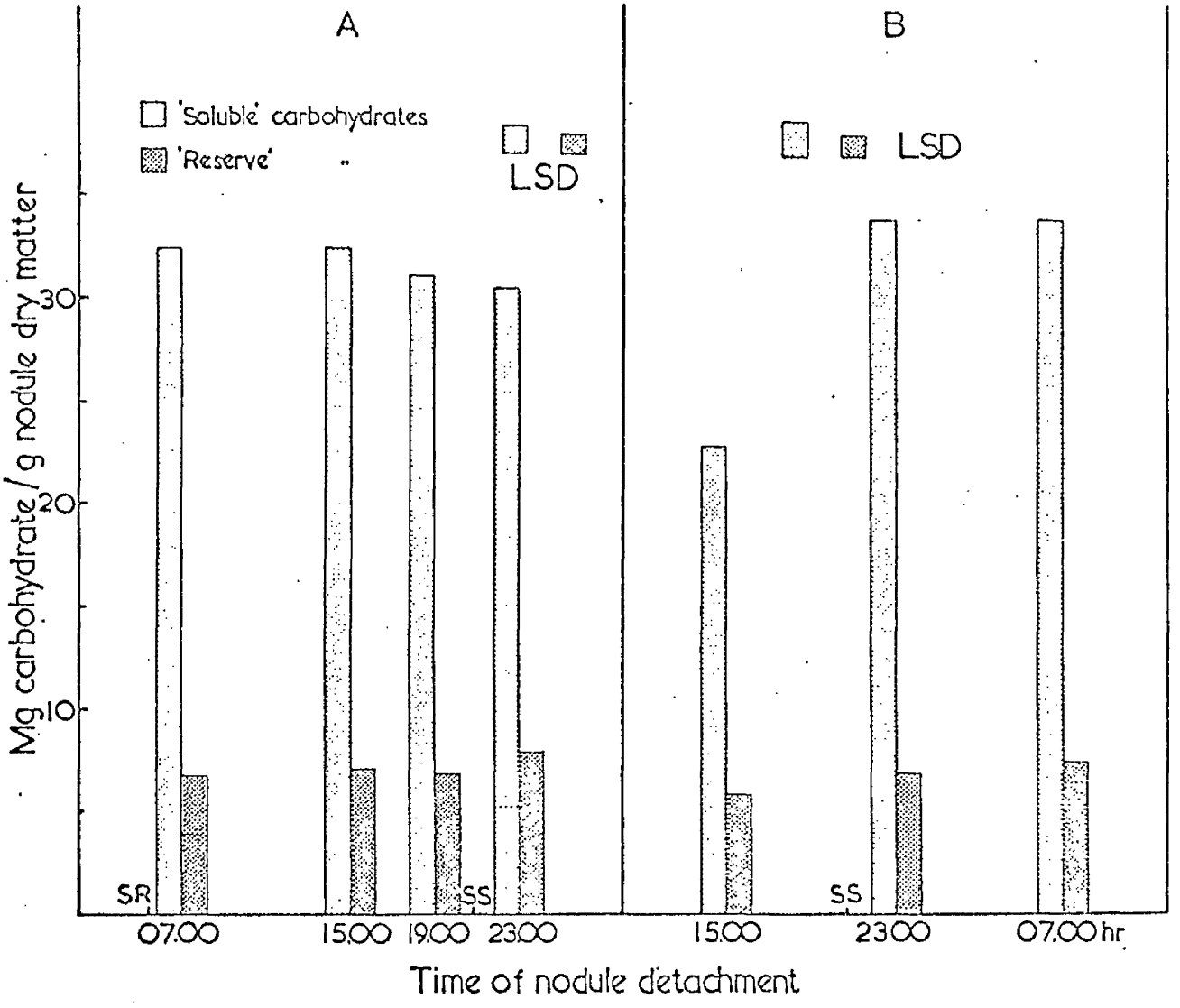


Fig. 29. Diurnal fluctuations in "soluble" and "reserve" carbohydrates in nodules of Casuarina.

A. Nodules removed from same plants and at same times as in Fig. 27A, with an additional sample taken at 19.00 hours.

B. Nodules removed from same plants and at same times as in Fig. 27B.

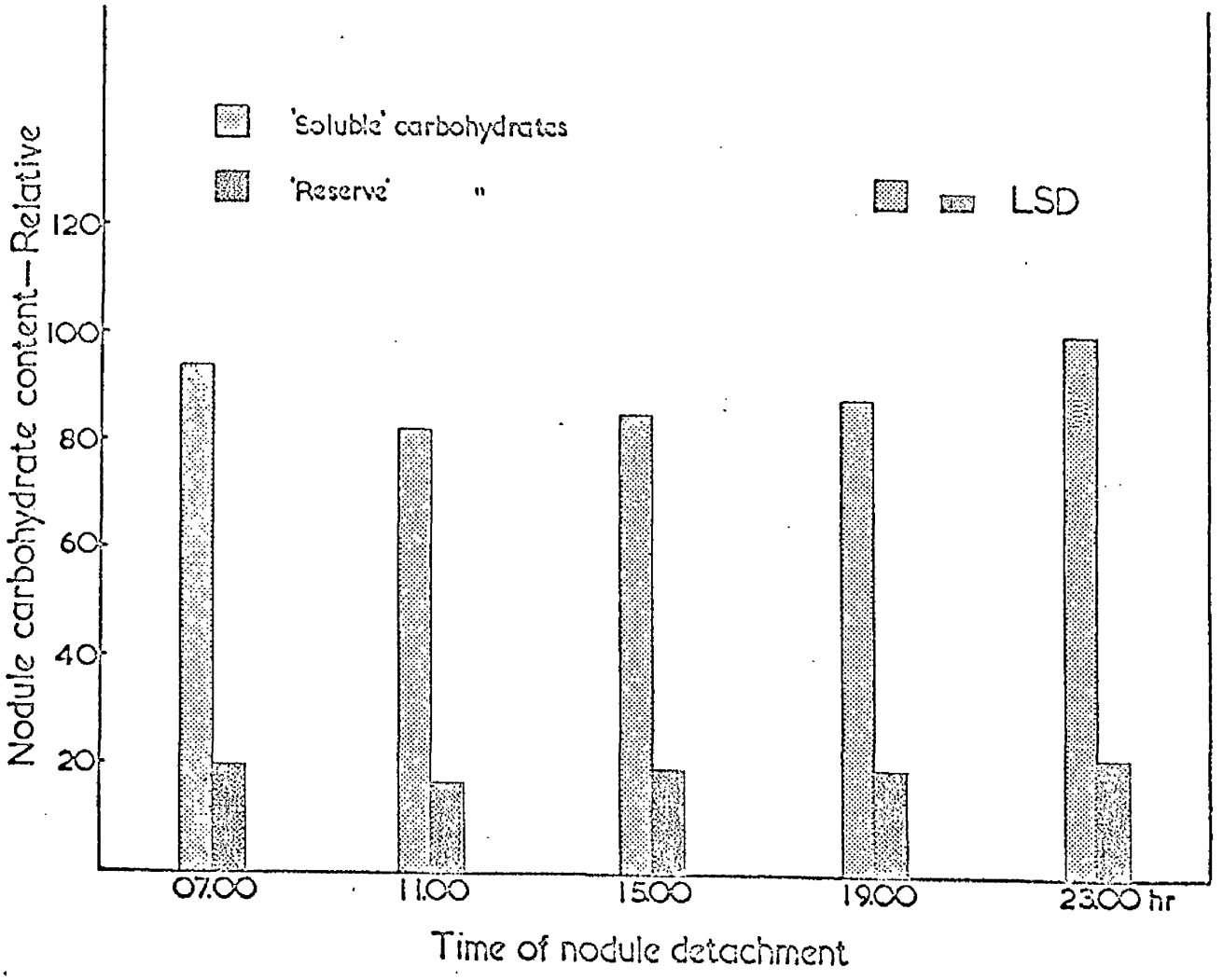


Fig. 30. Summary histogram of carbohydrate results from Figs. 28 and 29, expressed in relative terms.

morning and late evening than during the middle of the day.

It is clear that there is little relation between the diurnal changes in fixation recorded in Figs. 26 and 27 and the carbohydrate variations shown in Figs. 28, 29 or 30.

Effect of darkening plants on fixation potential and carbohydrate content of nodules

These experiments were carried out to try to secure further information on the extent to which light intensity could be responsible for the diurnal variations in fixation shown in Figs. 26 and 27, and for this purpose the effect of darkening the plants on the fixation potential and carbohydrate content of the nodules was studied.

On the day of commencement of the experiment (Day 0), the fixation potential of nodules detached at 12.00 hours from plants which had been under normal conditions was determined by measuring the fixation of nitrogen with ^{15}N during $4\frac{1}{2}$ hours incubation at a constant, favourable temperature ($24^{\circ}C$). In Casuarina the nodules were taken from second year plants as usual, but it was thought to be of interest to investigate first year plants also, and since in August

such plants of Casuarina are too small for use in experiments, first year plants of Hippophaë rhamnoides and Myrica cerifera were used instead.

Also at noon on Day 0 other similar plants were placed in large light-proof cartons in the greenhouse. In Casuarina, on each of the next three days and again on the fifth day, nodule samples were taken from two of the darkened plants at 12.00 hours, and the fixation potential determined. On the fourth day samples from further "light control" plants were assayed. The procedure was similar for the other two species, but continued for a shorter time. Temperature readings confirmed that the temperature inside the cartons and in the greenhouse were in close agreement, i.e. there was no over-heating. All the plants were of perfectly normal appearance when taken out of the dark.

With the exception of Hippophaë, nodule samples were also collected for carbohydrate analyses.

The fixation and carbohydrate data for Casuarina are presented in Fig. 31. Taking fixation potential first, some irregularity is shown in the results for Days 0, 1 and 2. This is thought to have arisen because the nodule samples were taken from two plants only on each occasion, this being in turn due to the

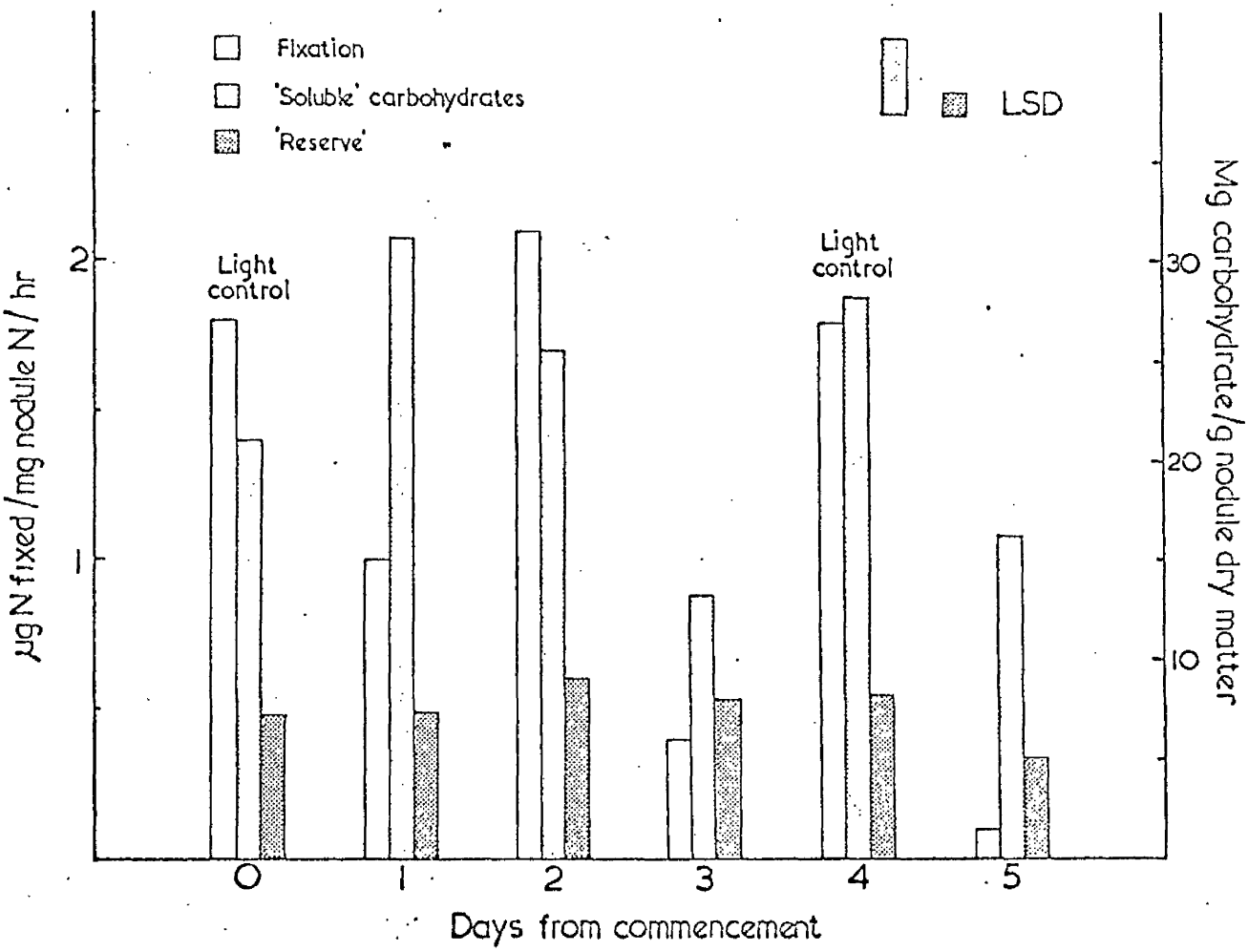


Fig. 31. Effect of darkening on fixation potential and carbohydrate content in nodules of Casuarina. Experiment commenced on 31st August, 1967 (Day 0). At 12.00 hours, on each day, nodules were removed from two plants, and samples for fixation measurements were exposed to an atmosphere containing nitrogen labelled with 31 atom per cent excess ^{15}N, incubated for $4\frac{1}{2}$ hours at 24°C. The measurements on Day 0 and Day 4 refer to nodules from plants kept under normal greenhouse conditions.

difficulty of finding dark, warm, storage space for still more of these large plants. This limitation to two plants would increase the sampling error. It can, however, be stated with considerable confidence that there is no evidence of a reduction in fixation potential until the third day in darkness, when potential was less than one quarter of the original value. By the fifth day fixation potential was almost extinguished. The result for the "light control" plants on Day 4 shows that fixation potential was not falling away for seasonal or other reasons.

In respect of the carbohydrate data there are again some irregularities over the first two days, but there is no clear evidence of a fall in "soluble" carbohydrates until Day 3, and in the "reserve" fraction until Day 5.

The results with the first year plants of the two other species - Fig. 32 - are in marked contrast with the above, especially in Hippophaë where fixation potential was at a low ebb after only 24 hours of darkness, and was almost nil after a further similar period. In Myrica the effect of darkness was less drastic, but potential was low after two days of darkness and was almost non-existent after three days. The relatively

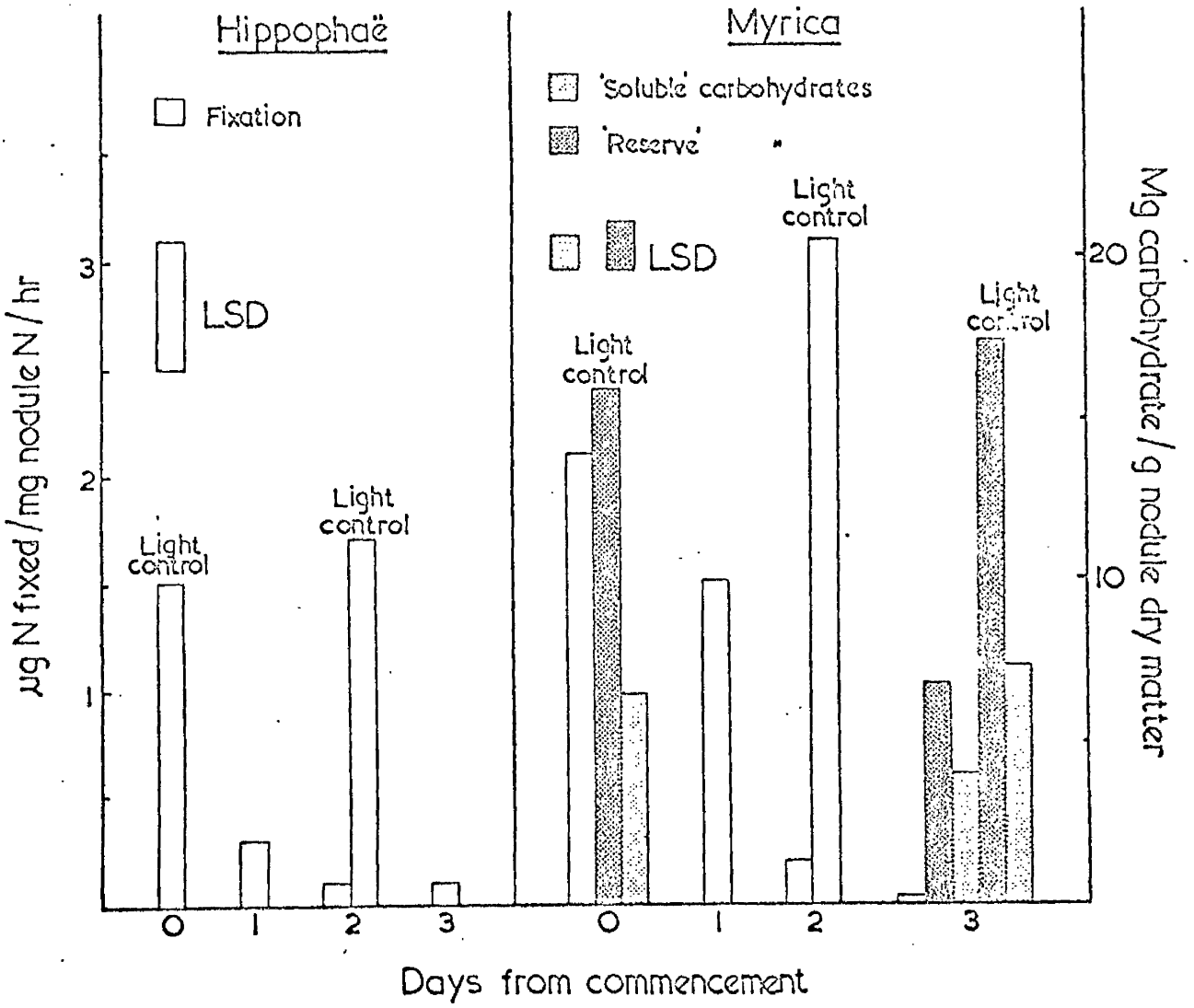


Fig. 32. Effect of darkening on fixation potential and carbohydrate content in nodules of Hippophaë and Myrica. Experiment commenced on 31st August, 1967 (Day 0) at 12.00 hours. On each day eight plants of Hippophaë yielded 1.5 g. nodules which was divided into two samples, while in Myrica, two samples were obtained from 1.5-3 g. nodules yielded from four plants. Exposure as in Fig. 31. Those columns labelled "light control" refer to nodules from plants kept under normal greenhouse conditions.

high fixation rates shown by nodules from "normal" plants of this species is in keeping with earlier results of Bond (1967a) and was also found in Section I.

Carbohydrate analyses are only available for Myrica plants and were made on two occasions, namely Day 0 and Day 3, "light" and "dark" plants being sampled on the latter day. It will be seen that in this species, in contrast with second year Casuarina plants, the "reserve" carbohydrates are more abundant than "soluble" ones, as was found by Wheeler (1969) in Myrica gale. In this respect it can be said that microscopic examination of sections of Myrica cerifera nodules shows the presence of abundant starch grains throughout the cortex, while in Casuarina, much less is obvious, being seen only in the outer cortex. After three days in the dark both fractions were significantly reduced, the "soluble" carbohydrates being less than two-thirds and the "reserve" being less than half of that of plants kept in the light.

Effect of temperature on fixation in Casuarina and Hippophaë

In order to be in a position to assess more clearly the importance of temperature in the daily variations in fixation rates (Figs. 26 and 27), an experiment was

carried out in which Casuarina nodules were held at different temperatures over the range 10-36°C during exposure to ^{15}N. The tubes for the samples were placed in the appropriate incubators one hour prior to the start, to allow them to come to the particular temperatures. The loading of the tubes was done with maximum speed, nodules being taken from four plants for each temperature.

The results of the experiment are shown in Fig. 33. The value for 24°C is aberrant, the reason being unknown, but there is nonetheless clear evidence that temperature has a marked effect on fixation, the rate at 30°C being some fourteen times greater than at 10°C.

For comparative purposes a similar experiment was carried out with Hippophaë rhamnoides, a species of temperate climates. The results are given in Fig. 34, and show a significant increase in fixation between successive temperatures from 10°C to 24°C, while values for the two higher temperatures are significantly lower than at 24°C. The results suggest that the optimum temperature for this species is several degrees lower than in Casuarina.

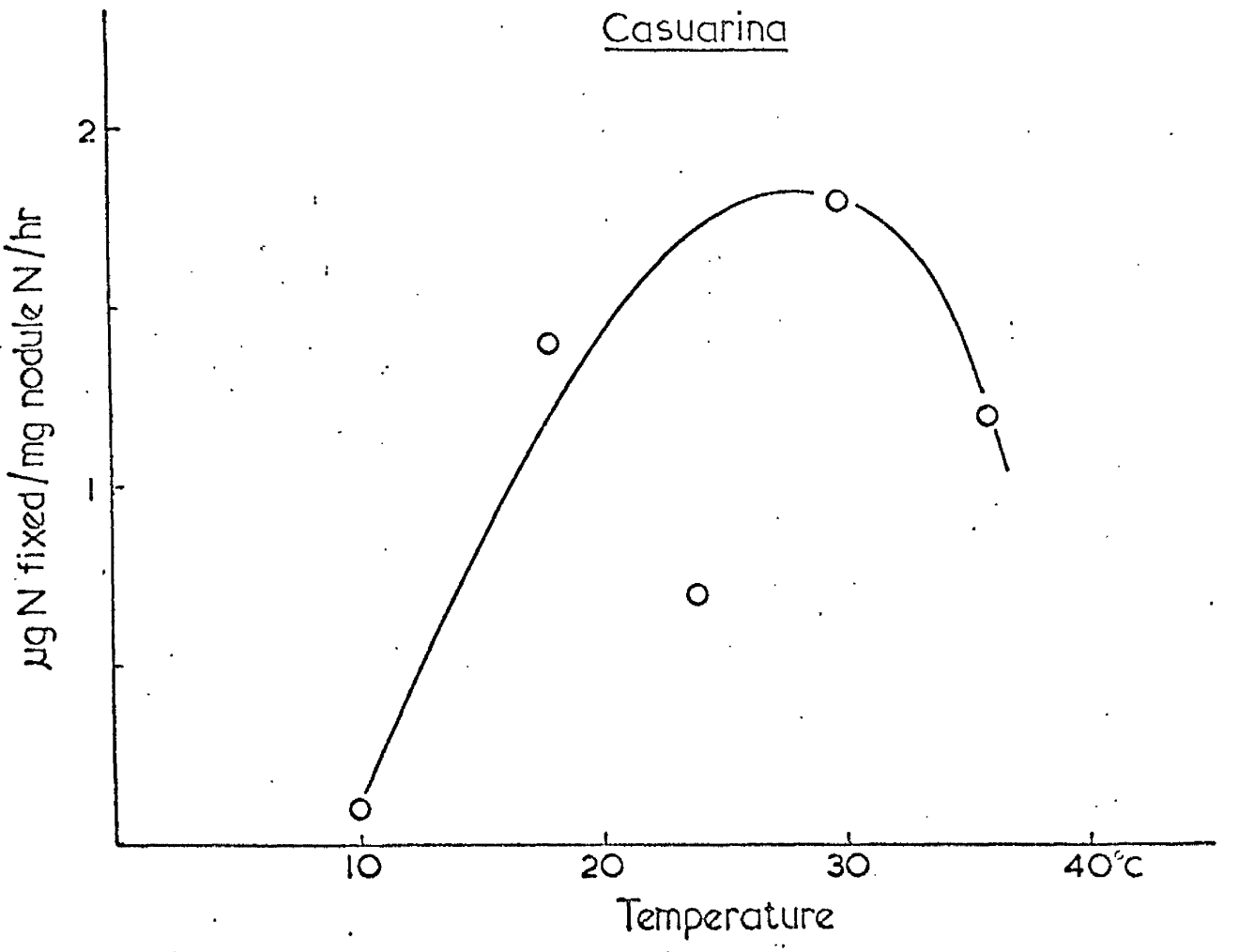


Fig. 33. Effect of temperature on fixation in Casuarina. Experiment carried out on 15th August, 1968 at 14.30 hours. Samples of nodules were taken from four plants for each temperature, and exposed to an atmosphere containing nitrogen labelled with 33 atom per cent excess ^{15}N for $3\frac{1}{2}$ hours.

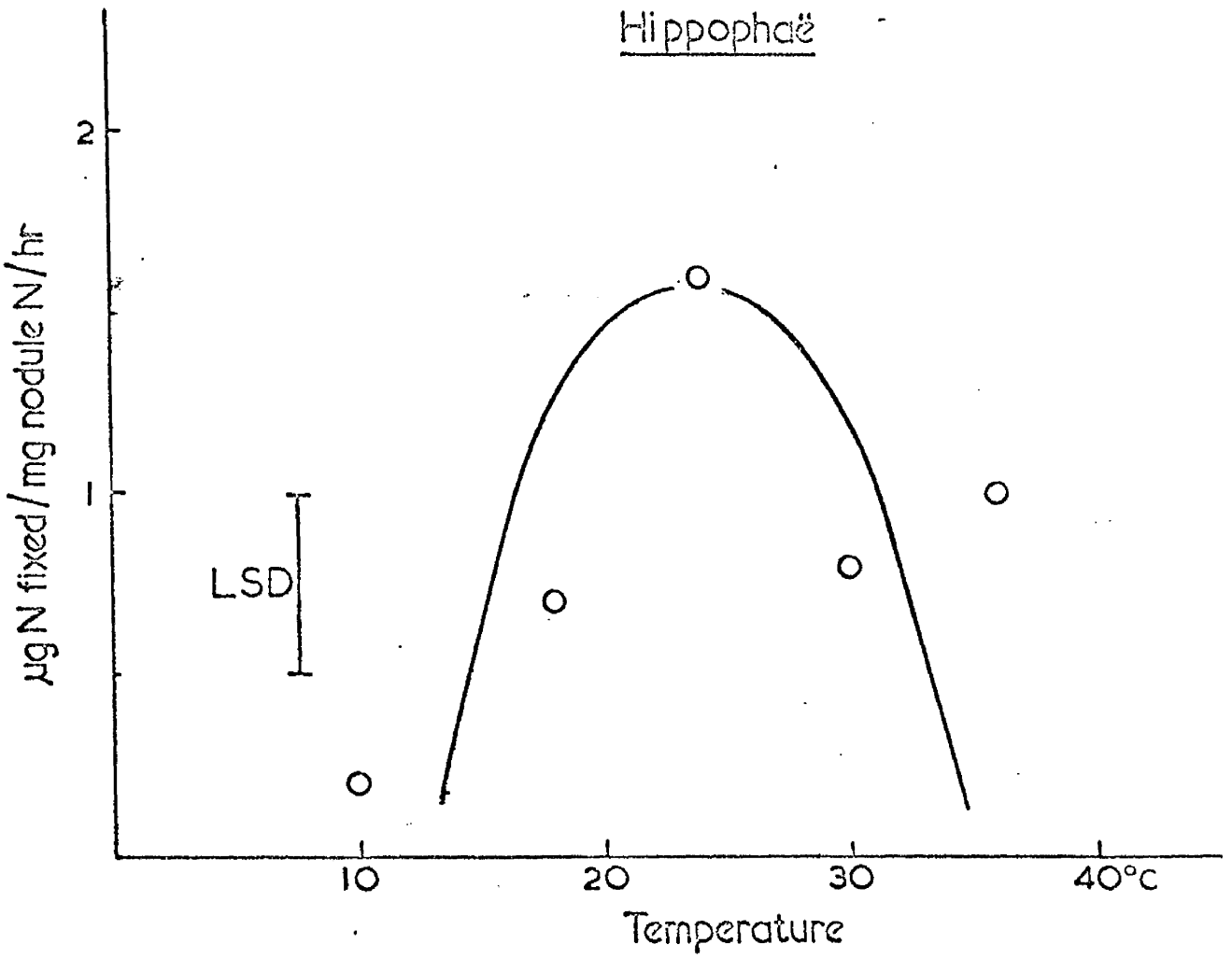


Fig. 34. Effect of temperature on fixation in Hippophaë. Experiment carried out on 19th August, 1968 at 14.30 hours. Three 1 g. samples of nodules were taken from about twelve plants for each temperature, and exposed to an atmosphere containing nitrogen labelled with 32 atom per cent excess ^{15}N for $3\frac{1}{2}$ hours.

Diurnal fluctuations in the bleeding sap of Myrica

Although the main concern in this work was with Casuarina, since first year plants of Myrica cerifera became available it was thought that a short study of diurnal fluctuations in them might be of use for comparative purposes. The method ~~used~~ was that used by Pate (1962) for legumes, and by Wheeler (1969) for alder ~~and bog myrtle~~, the argument being that since the translocation of fixed nitrogen from the nodules occurs without delay, the amount of nitrogen in the exudate per plant will be a measure of the current rate of fixation.

The results are shown in Fig. 35. Four of the 21 plants used in the experiment did not bleed and these were omitted from the number of plants per treatment in calculating the results. The weight of exudate per plant was high in plants decapitated at midday, fell to less than half during the afternoon, but recovered by morning; these changes are doubtless related to changes in the transpiration rate and the water content of the plants. Of more immediate interest is that the nitrogen content of the exudate per plant was high at midday, but fell during the afternoon and continued to fall through the night. This

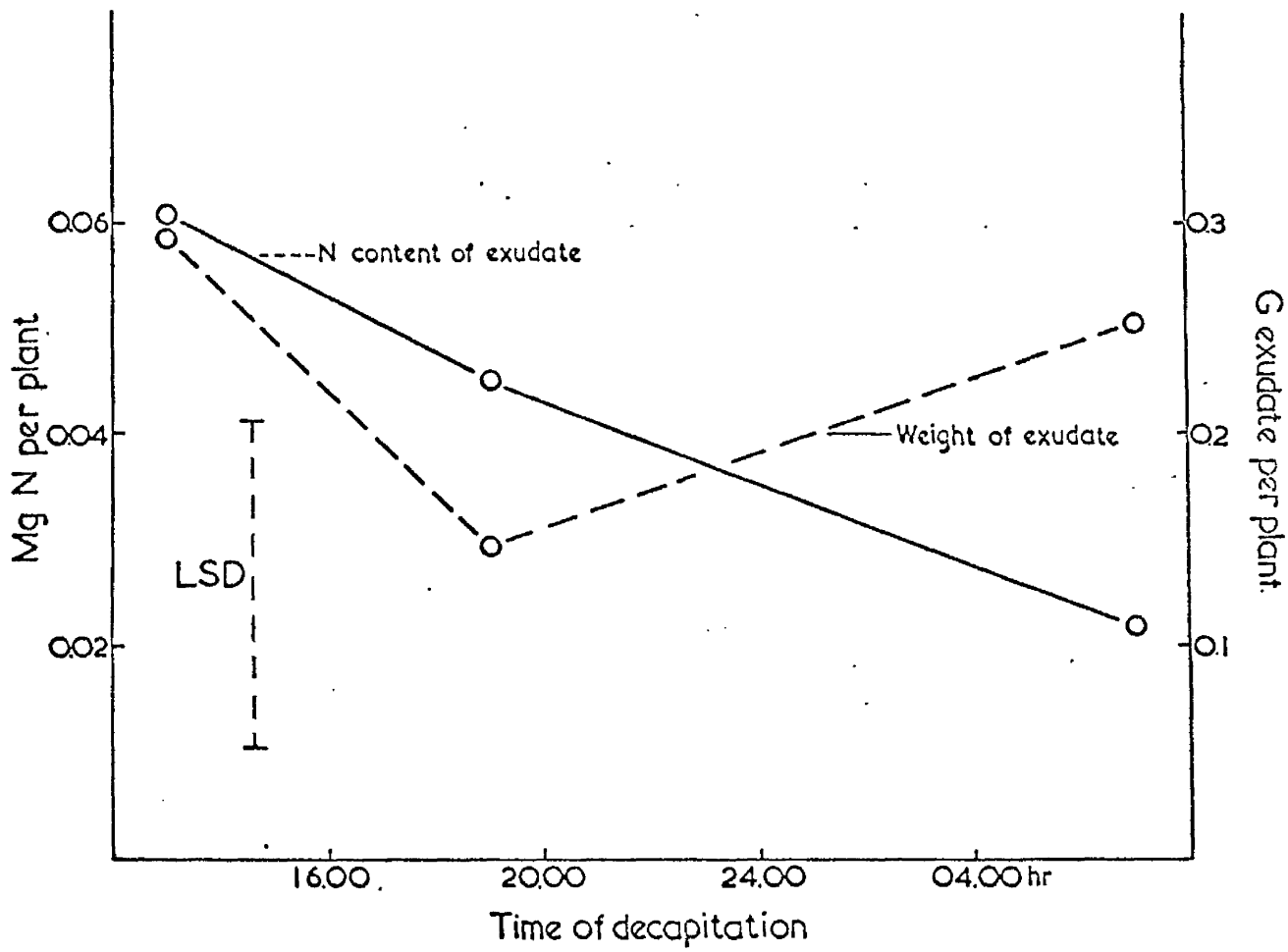


Fig. 35. Diurnal fluctuations in the amount and nitrogen content of bleeding sap from Myrica. Experiment carried out on 5th and 6th September, 1968, seven plants being decapitated on each occasion.

suggests that in these first year plants of Myrica cerifera fixation potential changes diurnally in a manner quite different from that in Casuarina (see Introduction), but similar to that found by Wheeler in Myrica gale and Alnus glutinosa (see Introduction).

D I S C U S S I O N

In this investigation it is assumed that the fixatory activity of detached nodules, tested over a period of usually two hours commencing almost immediately after detachment, will provide a reliable indication of the rate of fixation just prior to detachment. Hardy et al. (1968) and Wheeler (1969), who used trimmed excised nodulated systems in combination with the acetylene assay, were able to represent, after calculations involving various approximations, that rates of fixation shown in their material were of the same order as those reported by previous workers for corresponding intact plants, i.e. that over a period of one hour following excision and separation from the photosynthetic organs fixation was not much reduced. At present, data for fixation rates in intact plants of Casuarina, against which to compare those in detached nodules do not exist, and it can only be argued that it is obviously likely that for a short time after detachment the fixation rate in nodules will reflect, though perhaps not fully equal, that immediately prior

to detachment, and that the general consistency of the results reported bear out that belief.

The results presented indicate that in second year Casuarina plants exposed to normal greenhouse conditions there is a well-marked diurnal fluctuation in fixation, maximal rates being shown during the early afternoon, and relatively low ones in the early morning and evening and, presumably, through the night.

The question then arises as to which factor in the changing environment is responsible for these diurnal variations. As pointed out in the Introduction to this Section, the most likely contenders are temperature and light intensity, both of course controlled by solar radiation.

The separate study of the effect of temperature on fixation showed the process to be markedly temperature-sensitive, and when Q_{10} values are calculated from Fig. 33 for the ranges 10-20°, 15-25°, 20-30° and 25-35°, the values obtained are 13, 2.2, 1.4 and 0.7 respectively. The only other experiment of this type known to the writer is one carried out by Hardy et al. (1968) on the effect of temperature on acetylene reduction in nodulated root systems of soybean. Here also, temperature was found to have a marked effect,

Q_{10} values of 7.4 and 1.2 being obtained for rates of ethylene formation between the temperatures 10-20° and 20-30° respectively.

When suitable calculations are made on the data presented in Figs. 26 and 27, utilising the above values for the Q_{10} ratios for Casuarina, it emerges that the observed changes in the fixation rates can be very largely explained by the fluctuations in greenhouse temperature.

Though it is thus scarcely necessary to implicate light intensity in the explanation of the observed results, some discussion of its possible importance is desirable. In the long run light of appropriate intensity is obviously a necessary condition for the continuance of nodule activities, but there is little evidence that under the prevailing conditions it exercised much control. The complete absence of any parallel between the diurnal changes in fixation (Figs. 26 and 27) and those in the total carbohydrate content of the nodules (Fig. 30) militates against light intensity being much concerned with the former. This belief is reinforced by the results of the darkening experiment with Casuarina, where fixation appeared to be unaffected by the retention of the plant in darkness

for 48 hours. This indicates that changes in light intensity during a given day can have little effect on the rate of fixation on that day, under the conditions of the experiment.

Although the diurnal changes observed in the gross carbohydrate content of the nodules of Casuarina do not, as already concluded, appear to affect the rate of fixation, the reason for them is a matter of interest. As summarised in Fig. 30, the carbohydrate level tended to be high in the morning and evening, and presumably through the night, but fell by about 20 per cent during daytime. It is obvious that the level of carbohydrates at any time will depend on the balance existing between the rate of consumption in the nodules - primarily perhaps in fixation - and the rate of their ingress in translocation. The fall in nodule carbohydrates during the morning indicates that utilisation in fixation - the latter stimulated by rising temperature - is outstripping ingress (if any), while it must be presumed that later in the day the falling fixation and the continuance of ingress of carbohydrates result in increasing carbohydrate level.

In the latter connection it should be noted that at present there is no knowledge of the characteristics

of translocation in Casuarina. It is not known whether there are diurnal fluctuations in the amounts of carbohydrates reaching the root system and the nodules, or the time taken on the journey. The carbohydrate data in the darkening experiment with Casuarina (Fig. 31) could be interpreted to mean that there is a very considerable lag, of the order of 48-72 hours in translocation, since it was only after that duration of darkening that carbohydrate levels in the nodules removed at the same time each day began to fall. Until that stage, carbohydrates already "in the pipeline" may have maintained normal levels in the nodules. Alternatively it is possible that in these rather large plants there are substantial carbohydrate reserves, and that as soon as levels tend to fall, through the cessation of photosynthesis, the reserves are mobilised and for a time suffice to maintain levels in the nodules close to their normal values. If such a mechanism is operative then translocation can be envisaged as a normally rapid process. However, it has to be presumed that a slight fall in carbohydrate level - too small to be revealed in analyses - or some other factor as yet undetermined causes mobilisation of reserves.

The diurnal variations in nodular carbohydrates

observed in the present investigations (Fig. 30) are also of interest in relation to the experiments of Rodriguez-Barrueco and Bond (see Introduction to this Section) on diurnal changes in the potential of the nodules for fixation, i.e. the fixation shown by nodules detached at different times of day and held at a near-optimal temperature (24°C) during the fixation measurement. This potential, it may be recalled, fell during the morning and rose later in the day, and thus showed a distinct parallel to the changes in nodular carbohydrates observed by the present author. A quantitative comparison shows that by late morning potential had fallen by some 50 per cent and carbohydrates by just less than half as much. It is possible that there is a causal relation here, in that the carbohydrate content of the nodule determines its fixatory potential. This would mean that under conditions of consistently favourable temperatures, fluctuations in light intensity might be important in the control of diurnal changes in fixation, though the extent of the lag period between the two cannot at present be estimated even for these second year plants and still less in older plants.

In summary, it is concluded that under the con-

ditions prevailing the diurnal variations demonstrated in the fixation of nitrogen by Casuarina nodules are due mainly to the diurnal progress of temperature in the greenhouse. Fixation is severely restricted by this factor in the early morning and evening, and doubtless overnight also, but is stimulated by the higher temperatures prevailing over the middle part of the day. Although there is evidence that nitrogen fixation is basically controlled by the carbohydrate content of the nodules and thus, after a time lag of uncertain length, by light intensity, it is concluded that under the prevailing conditions such effects were masked by those of temperature, which was thus the limiting factor. Such a conclusion is not surprising in respect of a sub-tropical species growing, in the present investigation, at temperatures below normal for it.

The discussion has dealt so far with second year plants of Casuarina. The results obtained with first year plants of Hippophaë rhamnoides and Myrica cerifera, though brief, stand in considerable contrast with those for Casuarina. The effect of artificial darkening of these species on fixation is more immediate, possibly because in these smaller plants translocation from the

photosynthetic organs to the nodules is more rapid, so that the cessation of photosynthesis affects nodular activities more quickly, or because these younger plants have less carbohydrate reserve to fall back on, or both. In either case it can be envisaged that in these species light intensity changes during a day could affect fixation on the same day. This is borne out by the implication of the bleeding sap experiment with Myrica cerifera that in this species fixation potential is greater at midday than in the evening or early morning, as found by Wheeler (1969) in first year alder and bog myrtle, and in contrast to the situation in Casuarina (see Introduction). The lower temperature requirements shown for fixation in Hippophaë at least will tend to enhance the effect of light intensity.

SUMMARY

(1) Investigations were carried out into several aspects of diurnal variation in nitrogen fixation, the ^{15}N method being mainly employed. Most of the experiments involved second year plants of Casuarina cunninghamiana, but in several cases first year plants of Hippophaë rhamnoides and Myrica cerifera were used as well. Fluctuations in nitrogen fixation and the carbohydrate content of the nodules during the day were studied under greenhouse conditions. The effects of temperature and plant darkening on the fixation process were also investigated.

(2) A well marked diurnal rhythm in fixation, with maximal rates during the early afternoon and minimal rates in the early morning and late evening, was found in second year Casuarina plants held under greenhouse conditions. Temperature was found to have a marked effect on the fixation rate, and it was concluded that this was mainly

responsible for the fluctuations in fixation during the day. The effect of light intensity was less immediate, since darkening of the plants had no effect on fixation until after three days. It was concluded that changes in light intensity during a particular day would have little effect on the rate of fixation on that day.

(3) Fluctuations in the level of nodular carbohydrates did not parallel fluctuations in the nitrogen fixation rate during the day.

(4) The effect of darkening on the fixation of first year plants was more immediate and it was considered that in these light intensity on a particular day might well have some control over fixation on that day.

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B I B L I O G R A P H Y

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