A STUDY OF CERTAIN SYMBIOTIC NITROGEN-FIXING SYSTEMS

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"The relations of matter and life are infinitely too complex for the human mind to understand. Science brings us face to face with creative power in the beginning of life on this earth and its continuance".

LORD KELVIN, Professor of Natural Philosophy in the University of Glasgow, 1846-1899.

From 'The life of William Thomson, Baron Kelvin of Largs' by Silvanus P.Thompson. (London, 1910).

PREFACE

The work described in this Thesis was carried out in the Department of Botany of the University of Glasgow. To Professor John Walton of that Department the author wishes to express his thanks for providing him with every facility to carry out the programme of research.

A debt of gratitude is due to Dr. George Bond who supervised the investigations with untiring patience. His guidance was always at the disposal of the author and the thorough training he has provided in all aspects of research, theoretical and practical, has been greatly appreciated.

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C O N T E N T S

G E N E R A L
I N T R O D U C T I O N

Nitrogen is the cornerstone of the proteins, the compounds that characterise and distinguish the living cell. As the majority of plants and all animals require this in combined form, the fixation of free atmospheric nitrogen can be classed with respiration and photosynthesis as one of the basic processes in nature. Owing to the action of denitrifying organisms in the soil and in the oceans there is a continual loss of combined nitrogen, for which compensation is provided by the biological fixation of atmospheric nitrogen, and to a lesser extent by fixation arising from electric discharges in the atmosphere. A cycle of this general type appears to have been operative for a substantial part of the period during which plants have existed on earth, although initially combined nitrogen of non-biological origin is believed to have been present.

Thus the greater part of the combined nitrogen now in circulation in nature is due to biological

synthesis and the following are the organisms chiefly responsible:-

(a) Not involving higher plants:

Azotobacter and Clostridium - bacteria which are the most important of the free-living nitrogen fixing organisms.

Certain Photosynthetic bacteria.

Certain Blue-green algae.

Lichens - with blue-green algal constituents.

Certain Bryophytes - e.g. Blasia (again with blue-green algal constituent).

Certain soil yeasts.

(b) Involving higher plants:

Legumes with root nodules.

Non-legumes with root nodules.

Possibly a few plants (e.g. Psychotria) which have bacterial nodules on their leaves.

The work of the present author has been concerned entirely with the symbiotic nitrogen fixing systems (category (b) above) found in the relationship between certain higher plants and soil micro-organisms producing nodules on their roots.

The great majority of the nodulated plants belong to the family Leguminosae and many genera and species of this large group have nodules produced on their roots

by species of the bacterium Rhizobium. These nodules are normally active in the fixation of free nitrogen and consequently the growing of legumes is of very considerable importance in Agriculture, where owing to crop removal the likelihood of depletion of soil nitrogen is accentuated, and the value of legumes in the maintenance of soil fertility has been recognised since antiquity. The pre-eminent position assumed by the legumes in agricultural economy is due to their ability, unique in nature, of combining the power of soil enrichment with a contemporaneous supply of edible products.

Besides the legumes, there are eight other genera of Angiospermous plants which develop root nodules inhabited by a micro-organism. These plants are widely and somewhat haphazardly dispersed among the Angiosperms. The following list shows these genera together with their systematic position according to Engler and Diels (1936):-

Genus	Family	Order
Hippophaë Shepherdia Elaeagnus	Elaeagnaceae	Myrtiflorae
Alnus	Betulaceae	Fagales
Myrica	Myricaceae	Myricales
Coriaria	Coriariaceae	Sapindales
Casuarina	Casuarinaceae	Verticillatae
Ceanothus	Rhamnaceae	Rhamnales

Certain Gymnosperms also bear root nodules but these will not be further considered here.

Owing to the unsuitability of these non-legumes for utilisation in many systems of Agriculture the significance of their root nodules has until recently remained little investigated. However, since the late war a considerable amount of original work on non-legume nodulated plants has been undertaken in the Botany Department of the University of Glasgow. Satisfactory evidence, based on long-term growth experiments and on short-term isotopic tests, has been advanced to show that fixation of nitrogen occurs in the nodules of Myrica, Alnus and Hippophaë to an extent sufficient to sustain normal growth of the plant in a solution free of combined nitrogen (Bond, 1951b; Ferguson and Bond, 1953; Bond, Fletcher and Ferguson, 1954; Bond, MacConnell and McCallum, 1956). The literature relating to nodule-bearing non-legumes not native to Britain provides some evidence of fixation by their nodules and it is highly likely that nitrogen fixation is associated with all eight genera.

Some uncertainty remains as to the identity of the nodule organisms in the non-legumes, and in this connection the inability of any investigator to make a satisfactorily confirmed isolation of the organism has been a stumbling-block to progress. On the basis of the examination of sections of nodules most investigators have concluded

the organisms to be Actinomycetes, though some have taken a different view. A recent review of the literature was provided by Hawker and Fraymouth (1951).

These non-legume genera are nearly all well known plants which occur in large numbers in appropriate regions. Some of them, such as Hippophaë, Alnus and Coriaria, are particularly widespread in their occurrence over the surface of the Earth. It cannot be doubted that, taking the world as a whole, these non-legume genera make a substantial contribution to the general stock of combined nitrogen on which non-nodulated plants rely. Possibly means will be found in the future of putting to more immediate practical use the special properties of these plants.

Interesting evolutionary problems arise in connection with root nodule plants, as pointed out by Bond, MacConnell and McCallum (1956). The non-legume examples are without exception woody plants. The presence of nodules on herbaceous members of the Leguminosae may merely be because they are descended from woody legumes in which nodule formation was already established. It seems possible that at some stage in evolution, when the Angiosperms were represented by woody types, conditions favoured the initiation of symbiotic associations or were such as to make such associations particularly

valuable. The nodulated genera still existing today may represent remnants from that time.

Since the property of nitrogen fixation is somewhat unique, the further investigation of any organism or symbiotic system shown to possess that property is obviously desirable. Moreover the elucidation of various aspects of the legume symbiosis, including the mechanism of fixation, is proceeding very slowly, and there is always the possibility that other nitrogen fixing material, such as non-legumes with nodules, will present more favourable opportunities for the investigation of symbiotic fixation. The present Thesis is devoted in part to detailed studies of certain aspects of the development and functioning of non-legume nodules.

PART I.

The effectiveness in nitrogen fixation of isolations of the nodule organisms of *Ulex europaeus* L. and *Medicago lupulina* L..

PART I.

C O N T E N T S

INTRODUCTION

Within a comparatively short time of the realisation that the nodules on the root system of legumes were benefiting their hosts by supplying fixed atmospheric nitrogen, it was observed that the presence of such nodules was not always beneficial to the plant. Fred, Baldwin and McCoy (1932) have summarised and discussed the experimental work following on the early observations which led to the discovery, within a given species of the nodule organism, of 'strains' which varied in their nitrogen fixing powers in association with their host. Some strains, termed 'effective', supply ample fixed nitrogen to their host plants, while other strains, termed 'ineffective', supply little or none.

It was further noted that the different types of nodule organism strain produce a different and characteristic type of nodulation on their hosts. The effective strain usually, though not always, produces relatively few, large, red nodules situated on or near the main roots while the ineffective strain produces numerous, small, white nodules scattered over the entire root system. Such a differentiation between strains is however limited

to the association of a particular strain with its host, and effective and ineffective strains are not distinguishable by cultural or serological characteristics. For example in a recent investigation, Jordan (1952) reported that in Rhizobium cultures no consistent differences in biochemical requirements or growth responses occurred between effective or ineffective strains.

In more recent years it has come to be appreciated that host plant factors may be in part responsible for the production of ineffective nodules. The simple view that the occurrence of such nodules is due to an inherent impotency on the part of certain strains in fixation is no longer satisfactory in all cases. Thus Helz, Baldwin and Fred (1927) showed that strains which were ineffective with one genus of legumes were effective with a different genus within the same cross-inoculation group (that is whose nodules were caused by the same species of Rhizobium). A similar effect within one plant genus was reported by Strong (1937, 1940) who showed strains effective with red and white clover to be ineffective with subterranean clover and vice-versa. Jensen and Vincent (1941) postulated however, the existence of a strain effective with all the common clovers, maintaining that the effectiveness pattern found by Strong (loc.cit.) did not always hold. Bond

and McGonagle (1951) added support to the view of Jensen and Vincent (1941) by demonstrating consistent strain performance by the clover nodule organism on three clover species, red, white and alsike. However Vincent (1954) showed root nodule bacteria isolated from four species of clover to be generally effective in association with white and red clovers but almost always ineffective on subterranean and crimson clover. Effective and ineffective responses to the same isolation of the nodule organism within one species was reported by Boyes and Bond (1942) working with different varieties of Soya bean. Finally, difference in response to one strain of Rhizobium between plants of the one variety have been reported. Nutman (1946b) showed that in a set of clover plants of the one variety supplied with a generally effective strain, individual plants may show a completely ineffective association although bacteria reisolated from such plants have remained unaltered and produce normal effective nodules on other plants. Furthermore, with a strain of bacteria which normally gives a response intermediate in effectivity towards plant material of mixed genetical constitution, Nutman (1946a) reports that selection of the host plant to increase the effectivity response is possible, but so far selection for responsiveness to totally ineffective strains of bacteria have been unsuccessful.

Thus any statement as to the effectiveness or ineffectiveness of a strain may only apply when the strain is associated with a given species or variety of host plant, while within these limits any estimate of effectiveness may only be of the nature of an average value, covering a considerable variation in individual plant response.

Under natural conditions legumes have been shown to be usually infected by more than one strain of a Rhizobium species. Thus Hughes and Vincent (1942) showed that different nodules on the same plant are often inhabited by distinct serological types. Baird (1951) reported multiple infection on clover, the plants examined being associated with at least two strains of the nodule organism, usually an effective and an ineffective type. The latter finding offers an explanation of why ineffective strains are not normally to be detected in the field by reference to the condition of the host plant, though as pointed out by Allen and Baldwin (1954) the amount of soil nitrogen is usually sufficiently high to eliminate differences in strain efficiency. However, Vincent (1954) recorded that in one area examined by him, the contrast between effectively and ineffectively nodulated plants was most striking. The number, kind and distribution of nodules on the roots

conformed to the classical picture and the above-ground parts permitted ready diagnosis. That ineffective strains can be detrimental to field plant development was shown at an earlier date by Leonard (1930) who studying the failure of a pea crop in Louisiana U.S.A. concluded that this was due to ineffective strains of the nodule organism.

The distribution of effective and ineffective strains under natural conditions has received some attention and Wilson (1940) from a survey of work on the matter suggests that many of the strains found in the natural habitat, either in soil or in the nodules of wild legumes are of the poor type. In a survey of strains isolated from soya beans in the Wisconsin area, Umbreit (1944) found 25% of those examined were ineffective. Thornton (1946) examined 463 rhizobia strains from clover growing in Great Britain. Of 290 isolations from Scotland, Wales and North and West England, 28% were ineffective, 12% intermediate and 60% effective. Of 173 from South, Central and East England, 9.2% were ineffective, 4.6% intermediate and 86.2% effective. The ineffective strains came largely from hill pasture areas. Isolates from species of Trifolium growing in the New South Wales wheat belt were shown by Purchase and Vincent (1949) to exhibit considerable variation in characteristics based on

serological behaviour and effectiveness patterns and such characteristics were found to be localised within the area only to a slight extent. This work was extended by Purchase, Vincent and Ward (1951) to include species of Medicago (M.sativa, M.hispida and M.laciniata), and as with clover isolates, the strain types found, were largely distributed in a random fashion within the area investigated.

In contrast to the findings in Australia, Thornton (1950) reports on an investigation made by Dr. Read of his department involving 100 isolates of clover rhizobia from 17 localities, which showed that strains tend to be highly localised in their distribution, so that no rapid spread of a new strain whether of natural origin or introduced seems likely to occur under field conditions. This survey was however based on serological differentiation between strains.

In a study of the distribution and effectiveness of nodule bacteria of clover in the soils of the Latvian S.S.R., Kalnin'sh (1951) found effective, ineffective and slightly effective strains occurring. Slightly effective and ineffective strains were isolated primarily from nodules of plants growing in light sandy and loamy soils, expecially where the plants were wild species of clover.

In the following investigation an attempt has been made to assess the distribution of effective and ineffective strains harboured in the nodules of two 'wild' legumes from localities in the West of Scotland. The first of the species examined was Ulex europaeus L., Gorse, Furze or Whin. No previous work of this nature appears to have been done on this legume which is a native generally distributed throughout the British Isles. Today in some parts of Europe it is still used as fodder, a use to which it was formerly put in this country when to render it palatable to farm stock, its spines were crushed by heavy stone rollers, known in Scotland as 'whin mills'. When on occasion it was deliberately cultivated (Edlin, 1951) it was harvested in the second year after sowing, and thereafter annually as a rich source of winter keep. Strains more succulent and less spiny than the common wild forms were chosen for this purpose. The soft young shoots rising above the spines of wild Gorse are browsed by rabbits, sheep and ponies though a toxic alkaloid occurs in its seeds and it is under suspicion as a potentially poisonous plant (Whyte, Nilsson-Leissner and Trumble, 1953). Being highly inflammable, burning furiously even when green, Gorse may become a serious fire hazard and was one of the prime causes of the destruction of the town of

Brandon, Oregon, U.S.A., by a forest fire in 1936. At one time it was commonly gathered for kindling and for firing old-fashioned bread ovens and it was again so used in the Channel Islands during the German occupation from 1940 to 1945.

Today in this country Gorse is generally regarded as a pest and is frequently burned as such. The possible ecological value of the nitrogen fixing power associated with its nodules has been largely disregarded.

The second species to be examined was Medicago lupulina L., Black Medick or Yellow Trefoil, a native generally distributed throughout the country. According to Robinson (1947), Black Medick has been cultivated in this country since the middle of the fifteenth century and its use is widespread especially on dry, light land. It is not however a satisfactory hay plant and is never sown alone on account of its weak stems. It has been used in Norfolk in alternation with red clover for the Medick is more resistant to clover 'sickness'. Although the herbage is not very palatable, Black Medick is occasionally sown as an annual fodder crop for sheep on chalky soils (Edlin, 1951). Being of limited agricultural importance this plant has not received the attention directed to another species of Medicago, M.sativa, Lucerne, which although not a

native is of major agricultural importance in this country. It was found advantageous to inoculate Lucerne seed with suitable effective strains of the nodule organism and such strains have been made available to farmers (Thornton, 1931).

In the experiments to be described test plants were grown on agar slopes in test-tubes, a method which is suitable for testing many strains at once, which lessens the risk of contamination and which provides uniform conditions to all plants with the minimum of positional variation. Isolations of the nodule organism made from field plants were reinoculated into these plants growing under aseptic conditions on a nitrogen-free medium. An effectiveness value was calculated for each plant using a formula to be detailed later, based on plant development as measured by dry or fresh weight. Alternative methods of assessing effectiveness have been reported such as that based on nodule colour. As noted already, effective nodules are usually red in colour due to the presence of haemoglobin and are thus distinguishable from ineffective nodules which are found to be normally pale green or white. Nicol and Thornton (1941) distinguished between effective and ineffective strains inoculated into soya bean plants in sand culture by this means, effective nodules being red or olive green

in colour and soft in the centre while the ineffective nodules were pale green or white and hard in the centre. Jordan and Garrard (1951) also adopted this method and showed that the maximum haemoglobin content in nodules of field plants was attained just prior to blossoming and much sooner in greenhouse plants. However, as pointed out by Virtanen, Laine and Linkola (1945), when growth conditions were unfavourable nodules of even effective bacteria turn green at an early stage. It may also be noted that confusion could arise due to immature nodules not yet fully pigmented.

In the present investigations the term 'isolation' is preferred to that of 'strain' as eventual comparison is based entirely on host plant development and some of the isolations tested may have been identical.

It was at first supposed that a single species was responsible for nodule formation in all the legumes. Later however it was recognised that permanent and significant differences existed between the organism causing nodulation on certain groups of leguminous plants. The differentiation of species within the bacterial genus Rhizobium, the organism responsible for the nodules, was then based on what was termed the cross-inoculation groups of host legumes. Thus one species of Rhizobium is able to nodulate any one

of a certain group of legumes. Another species in a similar manner may nodulate another group of related legumes. Fred, Baldwin and McCoy (1932) have provided a detailed account of this classification and it has been recently summarised by Whyte, Nilsson-Leissner and Trumble (1953). This convenient form of classification is not however too sound and Wilson (1939a) has shown that the boundaries surrounding plants from each proposed group are irregular. No cross-inoculation tests have been made in the following investigation.

The precise position of Gorse in the above scheme is uncertain. Fred, et al.(loc.cit) placed it tentatively in the Cowpea group along with such genera as Arachis, Acacia, Lotus and Phaseolus. No specific name was attributed to the rhizobia concerned.

The nodule organism in the case of Black Medick is Rhizobium meliloti, the species which is responsible for the nodulation of the genera Medicago, Melilotus and Trigonella.

(1) Ulex europaeus L.. Gorse, Furze or Whin.

METHODS.

(a) Isolation of the nodule organism.

Isolations of the organism were made from Gorse nodules collected from various localities in the West of Scotland to be detailed later. In order to obtain as wide a range of types as possible from within each area each isolation was made from a different plant, a short length of nodulated root being cut from the chosen plant in the field and transported back to the laboratory for the isolation to be made from one nodule. In the case of the more mature and well established plants a considerable amount of root system had often to be unearthed before nodules were located. Nodules were found to persist on old roots but the numbers were much greater on young roots. Presumably over a period the majority of nodules decay as the roots become older and more woody. The mature Gorse nodule was found to be an elongated structure somewhat bulbous at the distal end. The writer has found them up to

two centimetres in length, though in such cases the junction to the root was often withered. It is debatable whether such nodules are still functional and they were avoided when making isolations.

In making the actual isolation the nodulated roots were first washed free of soil and other debris. Suitable nodules were then detached, placed in a Gooch crucible and rinsed in acidic mercuric chloride (1 gm. mercuric chloride, 2.5 ml. concentrated hydrochloric acid, 500 ml. tap water) for four minutes to remove any soil micro-organisms contaminating the nodule surface. Following sterilisation the nodules, still in the Gooch crucible, were rinsed in a chain of six beakers containing sterile water, the crucible being held throughout with sterile forceps. Finally individual nodules were transferred to a drop of water in a petri-dish and there crushed with the flattened end of a sterile glass rod. The latter was then streaked on to another petri-dish containing yeast-mannitol agar (detailed below). The above procedure was performed within a steam-sterilised cabinet. The cultures were incubated at 24°C. and replated as required, the isolate finally being transferred to test-tubes containing the same medium. Established cultures were stored at 2°C. and fresh slopes grown when required for actual experiments.

Bacterium radiobacter is by far the most frequent organism found as a contaminant in isolations made from legume nodules (Fred, Baldwin and McCoy, 1932) and it is difficult to distinguish from the Rhizobium due to similarities in cultural characteristics. Several differentiating tests are available and in the present case forty of the Gorse nodule isolations were tested for purity using Koser's uric acid medium as described by Hofer (1941). No contamination of the cultures with B. radiobacter was detected.

The yeast-mannitol agar used was prepared according to the formula originating in Wisconsin and quoted by Fred, Baldwin and McCoy (1932):-

Agar	15 gm..
Mannitol	10 gm..
Dipotassium phosphate . .	0.5 gm..
Magnesium sulphate . . .	0.2 gm..
Sodium chloride	0.1 gm..
Calcium carbonate . . .	3.0 gm..
Yeast water	100 ml..
Distilled water . . .	900 ml..

The yeast water was prepared by adding 10 gm. of fresh baker's yeast to 100 ml. of distilled water. This was shaken and allowed to stand at room temperature for two hours. The solution was autoclaved and then allowed to stand for several days. The clear liquid separating off was pipetted out for use in the medium.

(b) Seed source and sterilisation.

Gorse seed was collected locally in the autumn of 1953, but tests showed that germination was poor and moreover that the seed showed a heavy fungal contamination due it was assumed to a wet summer and autumn; surface sterilisation did not remove this contamination. Consequently it became necessary to use other seed obtained from Thompson and Morgan (Ipswich) Ltd.. It was confirmed that this seed was of British origin.

Comparative trials on the surface sterilisation and subsequent germination of Gorse seed using 0.1% mercuric chloride or alternatively concentrated sulphuric acid as sterilising agents resulted finally in the adoption of the latter. Following Nutman (1949) the seed were shaken in the acid for thirty minutes and thereafter given ten rinses in sterile distilled water over a period of forty-five minutes. In the course of the operation the seed arils almost invariably became detached but with no apparent effect on subsequent development. As noted by Nutman (loc.cit.) this method has the advantage of softening and sterilising the seed coat in one operation, so that the proportion of 'hard' seed is much reduced.

(c) Tube culture of Gorse plants.

Several experiments were carried out in the autumn

of 1953 to determine the most satisfactory method of cultivating Gorse plants in sterile tube culture and the technique described below was developed from these experiments.

The term 'seedling agar' used hereafter refers to the mineral agar medium used by Chen and Thornton (1940) for the culture of clover and is prepared as follows:-

Agar	15.0 gm..
Dipotassium phosphate . .	1.0 gm..
Calcium phosphate, di-acid .	0.5 gm..
Magnesium sulphate . .	0.2 gm..
Sodium chloride . . .	0.1 gm..
Ferric chloride . . .	0.01 gm..
Tap water	One litre.

Seed surface sterilised as described above was transferred aseptically to petri-dishes containing seedling agar, the petri-dishes being then placed under fluorescent lights for twelve hours daily. A full fortnight was required for substantial germination and in the meantime the culture tubes were prepared. Large "Samco" test-tubes 8" x 1¼" were used, their uniformly thin glass being considered suitable for plant culture, and 40 ml. of seedling agar was sloped in each. Thus prepared the tubes were stood in wooden racks (see Plates 1 and 2) which shielded the greater part of the agar slope from daylight.

Shortly after germination and before the plumule had emerged, seedlings were transferred, using a platinum needle and working in a steam sterilised box, from the petri-dishes to the tubes. One seedling only was set up in each tube as preliminary trials had shown that more than one seedling per tube resulted in unequal development. Root hairs were noted to develop very quickly after the transfer and after a few days most of the seedlings were fairly firmly established on the agar surface.

(d) Inoculation of seedlings and the use of control plants.

To inoculate the plants in the tubes, 10 ml. of sterile water was added to the appropriate isolation culture and a suspension of the organism thus obtained. Using sterile glass pipettes approximately 0.5 ml. of this suspension was added aseptically to each plant tube and allowed to drain down the agar slope over the root system of the enclosed seedling. Inoculation was normally carried out 3 - 4 days after the transfer of seedlings to tubes, five plants being inoculated with each available isolation.

Nodulation was somewhat slow, taking from three to five weeks to be completed. A close check showed this to be randomised throughout the experiments and not typical of any particular isolation.

Two types of control plants were grown in each experiment, (1), non-nodulated plants without a supply of combined nitrogen, to provide a basic comparison and (2), non-nodulated plants grown on seedling agar with ammonium sulphate added at a rate of 5 mg. of nitrogen per tube, to provide comparison as plants grown under favourable conditions with respect to nitrogen supply. Control tubes, ten of each kind in each experiment, were designated by the use of coloured wool plugs. In addition to these controls fifteen plants inoculated with a standard strain were set up in each experiment. This standard strain was chosen as being an isolation which in preliminary trials had produced plants comparable to those supplied with combined nitrogen. The use of these plants in subsequent calculations will be explained below.

(e) Subsequent management of plant cultures.

Following inoculation the tubes were randomised throughout the racks and the whole experiment placed in the greenhouse (Plate 1). The relative position of the racks was changed at weekly intervals and apart from checks on nodulation no further attention was required and at no time was there any indication of the agar medium drying out.

Little or no change was found to take place in

PLATE 1.

The 1955 Gorse experiment as set up in the greenhouse. Though the tubes were normally randomised amongst the racks this photograph was taken just before harvest when the tubes of each isolation were grouped together and it may be noted that the sixth rack from each end contains tubes with coloured wool plugs these being non-nodulated controls with and without nitrogen.

the initial pH of the medium (pH = 6.5) during the course of plant growth except in the case of those plants supplied with ammonium nitrogen where the pH fell to approximately 4.2. This pH level however, had no detrimental effect on the development of the plants.

While every effort was made to maintain aseptic conditions occasional contamination of the agar slopes in the tubes was experienced. Such contamination was greatest in the 1954 experiment when 19 out of 265 tubes showed some degree of contamination. Each case noted was watched for further development and the contaminant always died out before the close of the experiment due presumably to lack of suitable nutrients. In no case was there any obvious interference with the growth of the plant in a contaminated tube. The contamination, fungal in nature, was believed to have occurred when opening the rather large tubes at planting or inoculation.

During the course of these investigations approximately 950 Gorse plants have been cultured in tubes employing the techniques described above. Of these, 155 were set up as non-nodulated controls with and without combined nitrogen supplied and at no time did a plant develop nodules unless inoculated in the prescribed manner. Effective control of the nodule organism

was thus established and maintained.

Plants were harvested individually after some four months of growth and dry weights determined after drying plants overnight in an oven at 95°C.. Nitrogen content of a small proportion of the plants was determined using the standard Kjeldahl method.

(f) Assessment of effectiveness.

The Gorse isolations were tested over two experiments, one in 1954 and the other in 1955. In order that the experiments could be compared, a common factor was required and this was provided by the use of a standard strain against which the performance of other strains was judged on each testing occasion. The choice of such a strain has already been noted. Plants, non-nodulated and supplied with combined nitrogen might have been used for this purpose but factors operative only on symbiotic nitrogen fixation may arise so that the use of nodulated reference plants is preferable.

The mean dry weight of plants inoculated with the standard strain was used in the formula to calculate percentage effectiveness, the value used to compare isolations in the experiments to be described below. This formula was originally obtained from Dr.H.G.Thornton of Rothamsted Experimental Station by Dr.G.Bond, who

communicated it to the present author.

The calculation is as follows:-

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

where DWx = Dry weight of a plant inoculated with Isolation x.

DWc = Mean dry weight of non-nodulated control plants growing on nitrogen-free medium.

DWs = Mean dry weight of plants inoculated with the standard strain.

It will be seen that the standard strain is assigned a percentage effectiveness of 100. The value for an ineffective strain, conferring no benefit on the plant, will be zero. The percentage effectiveness was calculated separately for each plant. Thus, to give a typical example, in the 1954 experiment the dry weight of a plant inoculated with Isolation 23 was 87 mg., that of the non-nodulated control plants being 49 mg.(mean of 10 plants), and of the plants inoculated with the standard strain 85 mg.(mean of 15 plants). The calculation of the percentage effectiveness of Isolation 23 based on this plant is thus:-

$$\frac{87 - 49}{85 - 49} \times 100 = \underline{106\%}.$$

Since five plants were grown in association with each isolation, five separate estimates of percentage effectiveness were obtained for each isolation.

In each experiment an analysis of variance was carried out, and on the basis of this the strains, or isolations, have been grouped as follows:-

Effective isolations: Percentage effectiveness not significantly less than 100.

Ineffective isolations: Percentage effectiveness not significantly greater than zero.

Intermediate isolations: Percentage effectiveness falling in between the above limits.

EXPERIMENTAL RESULTS

Altogether 78 isolations were obtained and tested from Gorse nodules, each nodule being from a different plant. These isolations were made from nine different localities detailed in Table 1, the collections being made in the late summers of 1953 and 1954. Testing was carried out in two experiments which will be considered individually in the first instance.

In the summer of 1954 a total of 45 isolations, excluding the standard strain, were tested. Following the procedure already described, seed was sown on the 7th.April and seedlings transferred to tubes on the 23rd. April. Inoculation was performed three days later. This experiment was harvested in late August after approximately seventeen weeks growth in the tubes. By this time the shoot in many cases was pressing against the cotton-wool plug of the tube.

In the case of thirty isolations all five plants established themselves satisfactorily in the tubes and were included in the harvest. In the case of twelve isolations only four plants were available for harvest, and in the remaining four isolations only three plants

TABLE 1.

Details of localities from which isolations of the Gorse nodule organism were made.

	Locality.	Isolation numbers.	Total no. isolations.
(a)	Lossit, at foot of Kilsyth Hills. Steep bank running down to stream. Ground soft.	1-5	5
(b)	Roadside embankment on Campsie Muir north-east of Dungoil. Ground hard and exposed.	6,7	2
(c)	Portkil, near Kilcreggan. Roadside bank. Road cut within recent years and small Gorse growing in loose, dry, red sandy soil.	8-13	6
(d)	Road leading out of Glen Fruin to Helensburgh. Open moorland on lower slopes of Tom na h-Airidhe. Fairly firm, dry ground, very exposed.	15-28	14
(e)	Elmhurst, near Langbank, Renfrewshire. Ground possibly once cultivated and now grazed. Some difficulty here in finding plants with nodules.	29-39	11
(f)	Disused quarry near Hardgate in Dunbartonshire. Plants growing in rock clefts and stabilised dumpings.	40-43 45-48	8
(g)	Firm roadside bank, Auchincruive, Ayrshire.	50-52	3
(h)	Kirkfieldbank, by Lanark. Very steep hillside rising up from the river Clyde.	53-66	14
(i)	Coulport, Loch Long, Firth of Clyde. Plants growing among very large rocks on lochside. Apparently exposed to salt water splash at high tide (at least in bad weather).	67-81	15
			78

were available in each case. These plant losses were due mainly to failure of the seedlings to make satisfactory contact with the agar medium or in a few cases were the result of damage sustained during the initial transfer of the seedling to tube culture.

As was anticipated, the non-nodulated control plants not supplied with combined nitrogen made poor growth and developed signs of severe nitrogen deficiency. Most of the nodulated plants grew strongly and attained a size comparable with that shown by the non-nodulated plants supplied with ample combined nitrogen. Primary data, including nitrogen contents, for typical plants are presented in Table 2, and show that fixation of nitrogen in the nodulated plants in some cases approached 3 mg. per plant. On comparing nitrogen content of seeds and non-nodulated plants grown without added combined nitrogen it would appear that traces of nitrogen have been obtained from the medium by the latter though the quantity is insignificant.

Photographs of representative plants are provided in Plates 2, 3 and 4.

Considerable variation in the size and dry weight attained by the plants inoculated with a given isolation was found and to give some indication of the extent of this variation, as reflected in the separate estimates

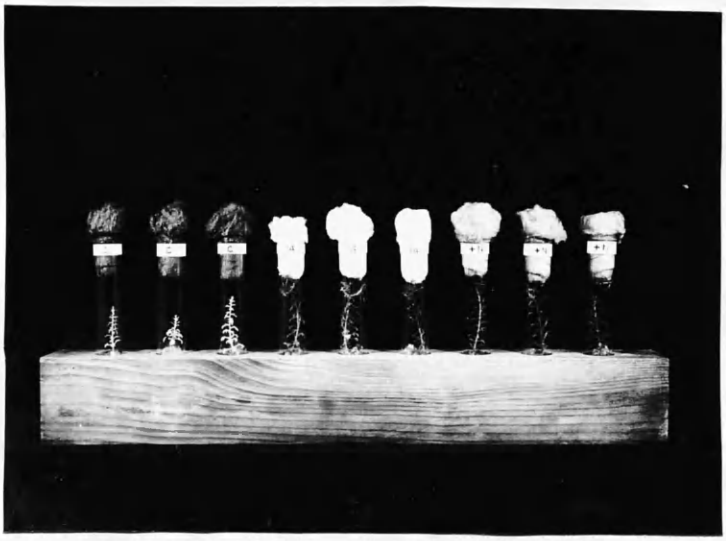
TABLE 2.

Examples of primary data obtained in the 1954 experiment.

The values for non-nodulated and standard strain plants are means. The remainder refer to individual plants.

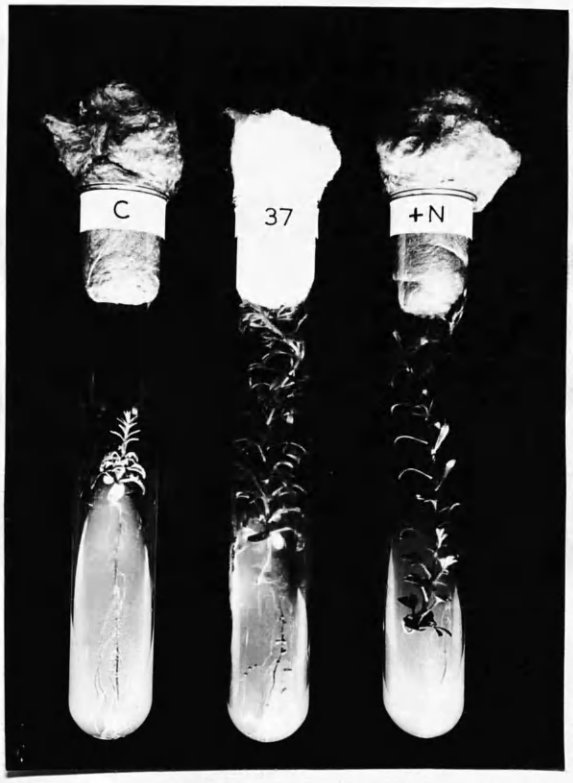
Type of plant	Number of plants	Dry weight mg..	Nitrogen content mg..	Percentage nitrogen content
Non-nodulated plus NH_4-N	10	88	3.27	3.71
Non-nodulated without N	10	49	0.52	1.06
Standard strain	15	85	2.55	3.00
Isolation 7	1	105	3.47	3.30
Isolation 10	1	99	2.91	2.94
Isolation 39	1	67	1.66	2.48
Isolation 45	1	106	3.47	3.27
Original seed	100	-	0.29 (per seed)	-

PLATE 2.



Rack of typical Gorse plants from the 1954 experiment. The three tubes on the left contain non-nodulated plants on nitrogen-free medium, the centre three are nodulated plants also on nitrogen-free medium and the three on the right are non-nodulated plants supplied with combined nitrogen.

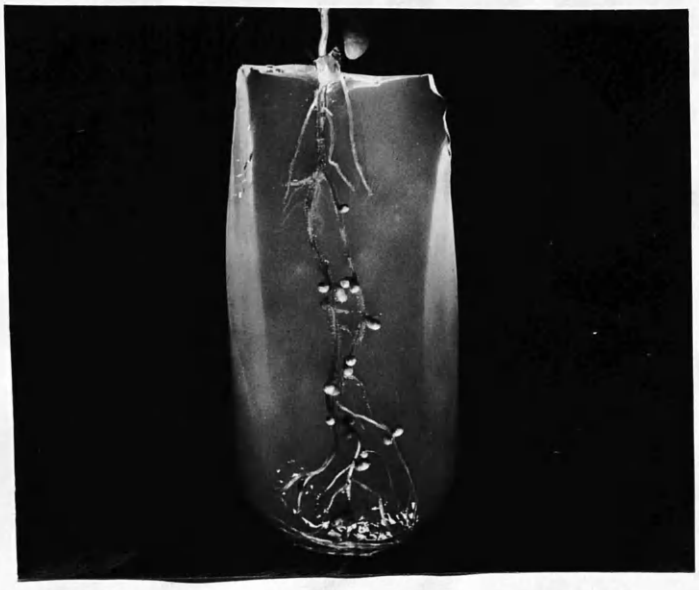
PLATE 3.



Typical Gorse plants at close of 1954 experiment. Left to right: non-nodulated plant supplied with nitrogen-free medium; plant inoculated with an effective isolation also on nitrogen-free medium; non-nodulated plant supplied with ammonium nitrogen.

X $\frac{3}{8}$.

PLATE 4.



Gorse 1954 experiment. Nodulated root system on an agar slope. These particular nodules proved to be fully effective in nitrogen fixation.

X 1.

of the percentage effectiveness values for a given isolation, the values for the first ten isolations tested in the 1954 experiment are presented in Table 3. The factors contributing to this variation include:-

(a) the differing degrees of success with which the individual plants established themselves in tube culture.

(b) differences in size of seed, with corresponding differences in seedling vigour, possibly persisting in later stages.

(c) genetical differences between plants, leading to variations in rate of growth.

(d) possible differences, again of genetical origin, in the success with which different plants can symbiose with a given isolation of the nodule organism.

The analysis of variance carried out on the data of the 1954 experiment is summarised in Table 4a. On the basis of the calculated significant differences the grouping of the isolations is as follows:-

Effective isolations: Percentage effectiveness not less than 100 - 47 = 53.

Ineffective isolations: Percentage effectiveness not more than 0 + 50 = 50.

The above applies to isolations for which five estimates

TABLE 3.

Individual values of percentage effectiveness calculated for the first ten isolations tested in the 1954 experiment.

Isolation No..	% Effectiveness values obtained	Mean
1	158, 128, 92, 106.	121
2	189, 53, 58, 114.	104
3	147, 139, 114.	133
4	111, 36, 61, 64, 83.	71
5	25, 100, 103, 122, 139.	98
6	67, 108, 169, 147, 169.	132
7	106, 161, 92, 156, 144.	132
9	189, 81, 150, 125, 197.	148
10	50, 139, 133, 131, 186.	128
11	133, 158, 136, 53, 128.	122

TABLE 4a.

Summary of analysis of variance on the percentage effectiveness values obtained in the 1954 experiment.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	351844	46	7649
Within groups	386599	183	2113

Variance Ratio = 3.62
which is significant at P = 0.001

Minimum differences required for significance. P = 0.05.

In comparison of mean percentage effectiveness of a given isolation with the standard strain (5 and 15 separate estimates respectively):-

$$\text{Difference required} = \sqrt{2113} \times t_{183}0.05 \times \sqrt{1/5 + 1/15}$$
$$= 47$$

In comparison of mean percentage effectiveness of a given isolation with the theoretical ineffective strain, equated here with non-nodulated plants without supplied combined nitrogen (5 and 10 separate estimates respectively):-

$$\text{Difference required} = \sqrt{2113} \times t_{183}0.05 \times \sqrt{1/5 + 1/10}$$
$$= 50$$

TABLE 4b.

Summary of analysis of variance on the percentage effectiveness values obtained in the 1955 experiment.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	293825	39	7534
Within groups	96718	153	632

Variance Ratio = 11.92

which is significant at P = 0.001

Minimum differences required for significance. P = 0.05.

In comparison of mean pereentage effectiveness of a given isolation with the standard strain (5 and 15 separate estimates respectively):-

$$\text{Difference required} = \sqrt{632} \times t_{153}0.05 \times \sqrt{1/5 + 1/15}$$

$$= \underline{26.}$$

In comparison of mean percentage effectiveness of a given isolation with the theoretical ineffective strain, equated here with non-nodulated plants without supplied combined nitrogen (5 and 10 separate estimates respectively):-

$$\text{Difference required} = \sqrt{632} \times t_{153}0.05 \times \sqrt{1/5 + 1/10}$$

$$= \underline{27.}$$

of effectiveness are available. In cases where less than five are available the differences required for significance are slightly greater. Allowance for this is made in the subsequent consideration of results.

The distribution of the mean percentage effectiveness values for the isolations tested in 1954 is shown in Table 5, column (a). Of these isolations forty-four are not significantly inferior in performance to the standard strain, and may thus be classed as effective. Only in the case of one isolation (No.39) was the mean effectiveness value (32%) significantly inferior to the standard value, and not significantly different from zero. Unfortunately only three plants inoculated with this isolation survived (because of this the actual difference from the standard strain required for significance was 57) but the separate estimates of effectiveness were all low viz., 14, 31 and 50%. Thus from the 1954 experiment only one isolation of ineffective type emerged.

The 1955 experiment was conducted along identical lines, in so far as was possible, to that of the previous year. General procedure was precisely the same when seed was sown on the 25th. March and the seedlings transferred to tubes on the 8th. April. After inoculation three days later the tubes in their racks were transferred

TABLE 5.

Distribution of mean percentage effectiveness values.
(The 1955 totals include six isolations being retested).

Range of % Effectiveness	(a) 1954.	(b) 1955.
0-20	0	0
21-40	1	0
41-60	1	2
61-80	2	8
81-100	7	9
101-120	10	9
121-140	12	9
141-160	6	1
161-180	5	0
181-200	1	0

Classification.

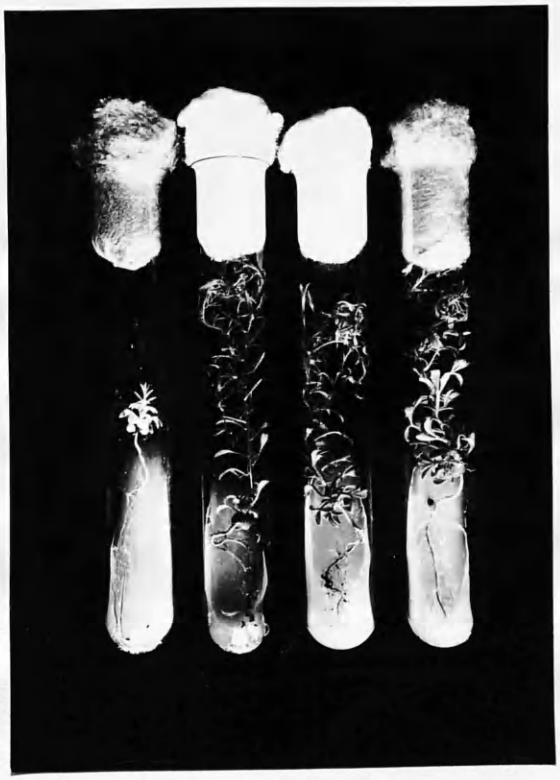
Experiment	Number of isolations:- Effective	Intermediate	Ineffective
(a) 1954	44	0	1
(b) 1955	33	5	0

to the greenhouse. Harvest took place in early August after approximately seventeen weeks of growth in tubes. On this occasion 38 isolations were tested and of these, six had already been tested in the 1954 experiment. In addition, fifteen plants inoculated with the standard strain were set up as in 1954. Typical plants of the 1955 experiment are shown in Plate 5.

From the analysis of variance on the effectiveness values in this experiment, summarised in Table 4b, it was found that the mean percentage effectiveness value for a given isolation must differ by 26 from that of the standard strain before significance was attained and by 27 from zero before significance was attained. These values, lower than in the 1954 experiment, indicate that the plants were more uniform than in the 1954 experiment. This may be due in part to the more favourable growing conditions during 1955 when the summer was unusually brilliant and warm. Also with the experience of the former large experiment it is likely that in 1955 the plants were more skilfully placed during the initial transfer to tube culture and were able to become established on the agar slope relatively quickly and more uniformly.

From the statistical data already given for the 1955 experiment the following grouping of the isolations

PLATE 5.



Gorse, 1955 experiment. From left to right: non-nodulated control without combined nitrogen supply; two plants of Isolation 8, the standard strain; a non-nodulated plant supplied with ammonium nitrogen.

X 1/3.

is possible.

Effective isolations: Percentage effectiveness not less than 100 - 26 = 74.

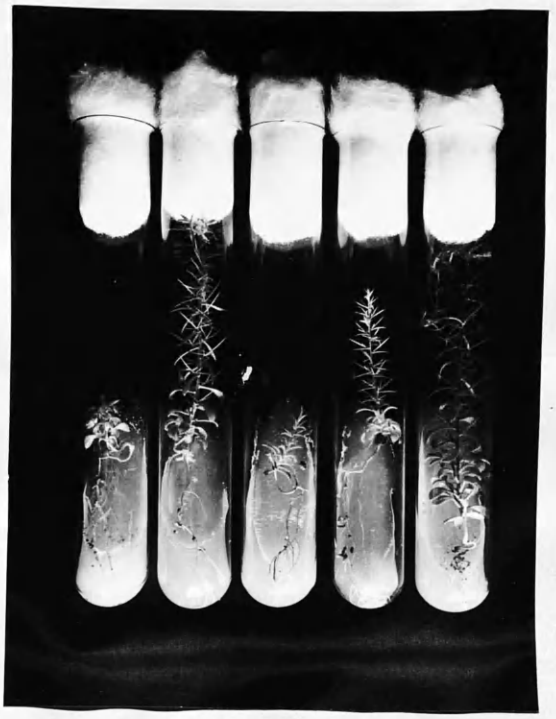
Ineffective isolations: Percentage effectiveness not more than 0 + 27 = 27.

Intermediate isolations: Percentage effectiveness in range 28 - 73.

The distribution of the mean percentage effectiveness values on this occasion is given in Table 5b. Among the isolations being tested for the first time twenty-eight were classed as effective and four as intermediate in effectiveness. The six isolations tested in 1954 and retested in this experiment gave five isolations in the effective class as on the first occasion. The remaining isolation retested was No.39 which gave an ineffective response in 1954. In 1955 this fell into the intermediate class (actual value was 57%). The five plants inoculated with this isolation (in 1955) are shown in Plate 6 and considerable plant to plant variation may be noted. In contrast a strongly uniform set of plants is shown in Plate 7, the isolation in this case being an effective one though such uniformity was not characteristic of effective isolations in general.

The overall result is that of the 78 Gorse isolations

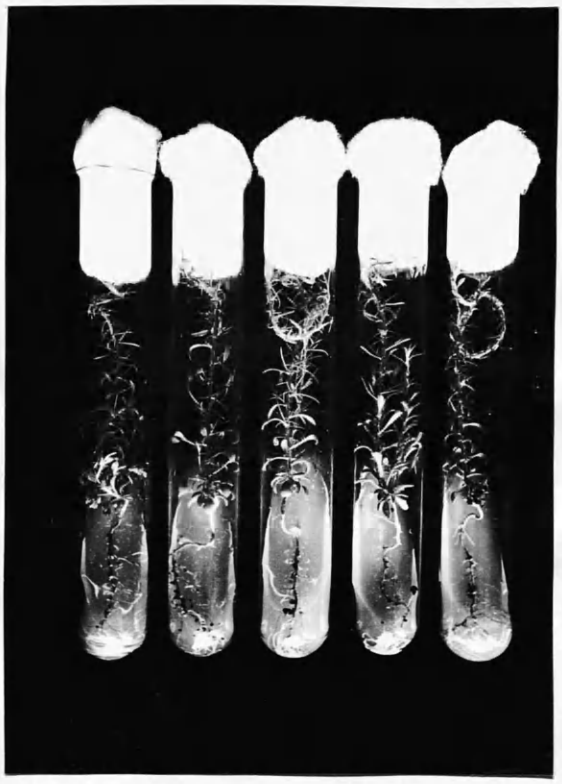
PLATE 6.



Gorse 1955 experiment. Five plants each inoculated with Isolation 39 and showing an extreme type of plant to plant variation.

X 1/3.

PLATE 7.



Gorse 1955 experiment. Plants inoculated with an effective isolation showing relatively uniform development. (Compare Plate 6).

X 1/3.

examined, 72 (plus the standard strain) may be classed as effective and four as intermediate. The position of the remaining one is somewhat doubtful as it has shown itself as ineffective on one occasion but capable of giving a better response (intermediate) on another. In the localities surveyed therefore it would appear that the Gorse nodule organism occurs almost invariably in an effective form.

Comparison of the growth of control and standard strain plants in the two years is of interest. Thus the non-nodulated plants supplied with combined nitrogen gave on the first test, a mean dry weight of 88 mg. and on the second, 141 mg.. Similar plants without combined nitrogen gave mean dry weights of 49 and 52 mg., and plants inoculated with the standard strain gave 85 and 110 mg. in the 1954 and 1955 tests respectively. The increases found in the second experiment are presumably due largely to the more favourable growing conditions in that year. This would apply especially to those non-nodulated plants supplied with combined nitrogen which with an immediately available nitrogen supply could take advantage of the good weather to develop strongly while the others awaited the formation of nodules.

Nodule size and number was very varied among the

isolations tested and the type developed gave no positive correlation with effectiveness. Nodules produced by the ineffective-intermediate isolation showed no marked difference in size or number from those produced by the other isolations. Nodule development was relatively uniform within an isolation and Plate 8 shows about as wide a range of nodule form as was found with any isolation. The nodules produced by Isolations 7 and 13 were directly opposite in type (Plate 9) yet both proved effective in fixatory powers. It may be noted however, that the many small nodules of Isolation 13 are intermingled with a few larger ones.

PLATE 8.

Gorse 1954 experiment. Three plants each inoculated with an isolation which gave an effective response and showing the range of nodule size.

X 4/5.

PLATE 9.

Gorse 1954 experiment. Comparison of type nodulation produced by different isolations. The two plants on the left were inoculated with Isolation 13 and bear many small nodules. The two on the right, inoculated with Isolation 7, bear a few large nodules. There was however no significant difference between the size of the plants developed in the two instances.

X 3/5.

(2) Medicago lupulina L.. Black Medick or Yellow Trefoil.

METHODS

Essentially the same procedure as previously described for Gorse was employed for Black Medick and only points of difference will be noted here.

(a) Isolation of the nodule organism.

Black Medick nodules were found in abundance on field plants and as for Gorse each isolation was made from a different plant. Isolation and culture was performed as before and it was noted that the organism in this case grew much faster in culture than did that of Gorse. Nodules were collected from two localities which will be detailed later.

(b) Seed source and sterilisation.

In the areas accessible to the author, Black Medick was not sufficiently abundant to permit of any considerable harvest of seed and consequently the seed used in this investigation was obtained from Edward Webb and Sons Ltd..

Not having the hard testa found on Gorse seed, surface sterilisation of Black Medick seed was carried

out as follows:- a three minute shaking of the seed in absolute alcohol was followed by a four minute shaking in 0.1% mercuric chloride and finally the seed were given twelve rinses in sterile water. Excellent germination was obtained with this seed sown on petri-dishes of seedling agar as previously described.

(c) Tube culture of Black Medick plants.

This was carried out precisely as for Gorse except that a smaller size of test-tube was used viz., 6" x $\frac{3}{4}$". Each contained 10 ml. of seedling agar and one plant.

(d) Inoculation of seedlings and use of control plants.

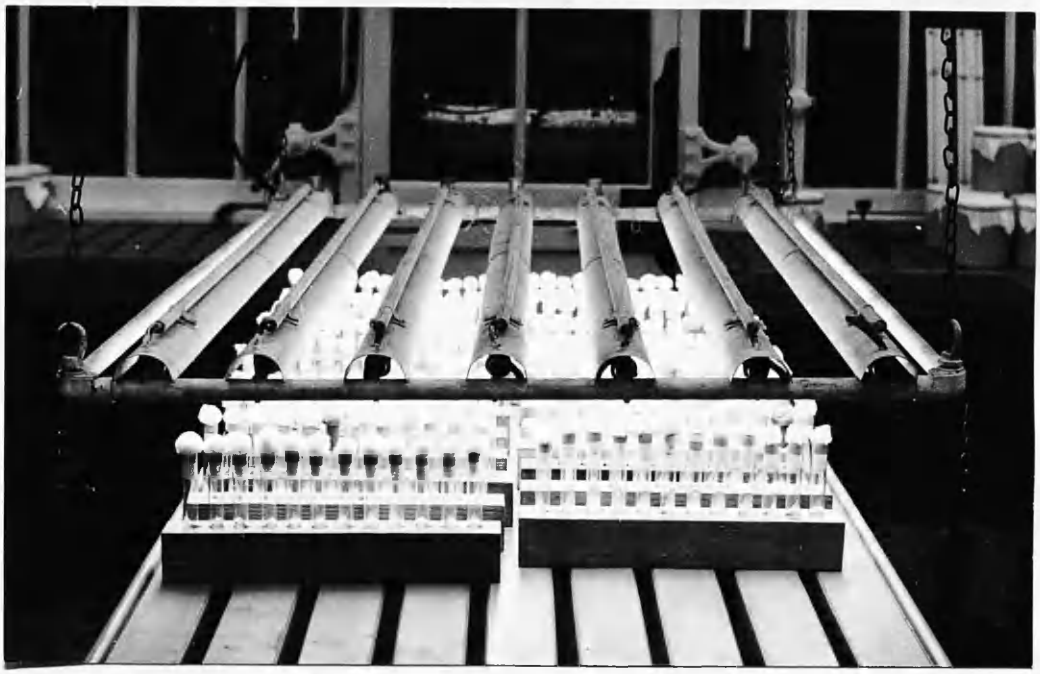
This was as for Gorse except that in the absence of preliminary trials no standard strain was selected prior to the experiment. Two types of control plant were again used, non-nodulated with and without a combined nitrogen supply.

(e) Subsequent management of plant cultures.

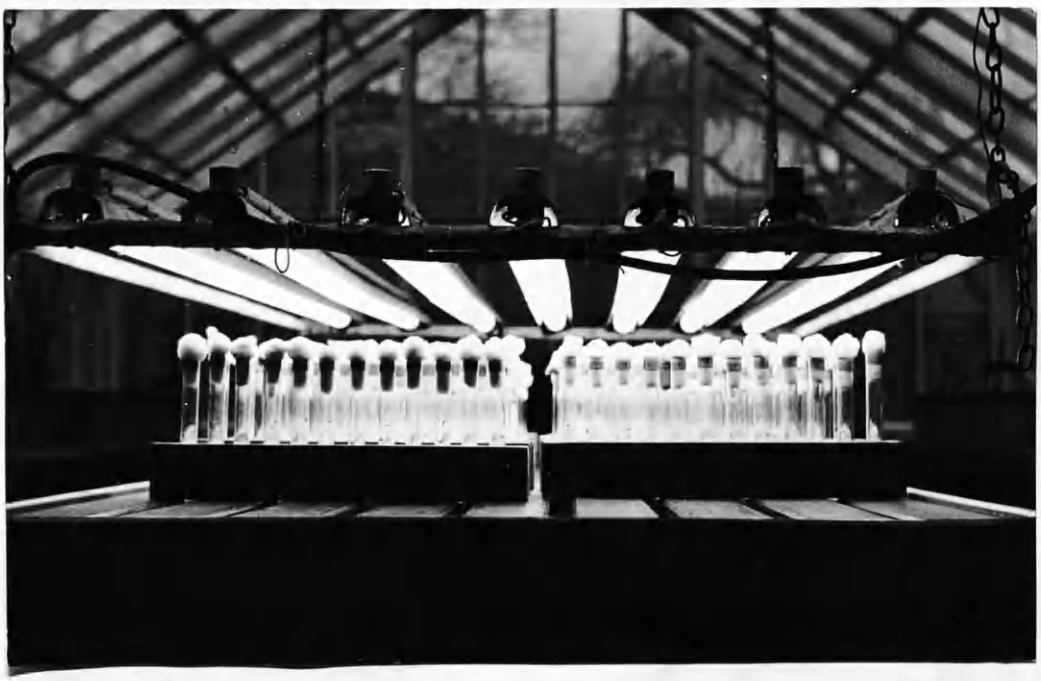
Only one experiment was performed and that in winter time. The racks of tubes were set up, in a heated greenhouse, under a bank of fluorescent tubes (Plate 10) which were lit daily from 06.00 to 22.00 hours. Growth was satisfactory under these lights. Their use was discontinued when the daylight became stronger and of longer duration in the early spring.

At harvest, after fifteen weeks of growth, the plants

PLATE 10.



Two views of the Black Medick experiment under a bank of seven fluorescent tubes. These photographs were taken on the 2nd.January 1955, two days after inoculation and setting up of the experiment in the greenhouse.



were comparatively small in stature and in view of this and to conserve time it was decided to obtain the fresh or green weight of individual plants rather than dry weight as measured in Gorse. Thus when a plant was removed from its tube the roots were gently but firmly dried with blotting paper and the plant immediately weighted.

No contamination of the agar in the tubes took place in this experiment. This was believed due in part to the smaller size of tube which was more readily handled in an aseptic manner.

In the whole investigation no plant developed nodules unless inoculated in the usual manner. Thus, as in Gorse, efficient control of the nodule organism was obtained.

(f) Assessment of effectiveness.

Again as in Gorse the percentage effectiveness was calculated for each plant, using fresh weight instead of dry weight data.

In the absence of preliminary trials, as already noted, no standard strain was selected prior to the experiment. An isolation was eventually chosen which gave five relatively uniform plants comparable in development to the non-nodulated plants supplied with combined nitrogen and which could therefore be described as an effective strain.

EXPERIMENTAL RESULTS

As already indicated all the isolations made from the nodules of Medicago lupulina were tested in one winter experiment. Seed was sown on petri-dishes of seedling agar on the 24th.December 1954 and rapid germination allowed transfer of seedlings to test-tubes four days later. Five tubes were set up with each isolation when inoculation was performed on the 31st. December. Twenty racks each containing twelve tubes were placed under fluorescent lights which burned continuously for sixteen hours daily from the 1st. January 1955 until the 18th.March, a period of eleven weeks, and from then until harvest on the 15th.April the plants were grown under natural lighting conditions. Plants were in tube culture for a total of fifteen weeks and typical examples at harvest are shown in Plate 11.

Of the 42 isolations (from two localities detailed in Table 6) tested, a total of nine failed entirely to produce nodules on the plants on to which they were inoculated in the usual manner. This is a similar finding to that reported by Purchase, Vincent and Ward

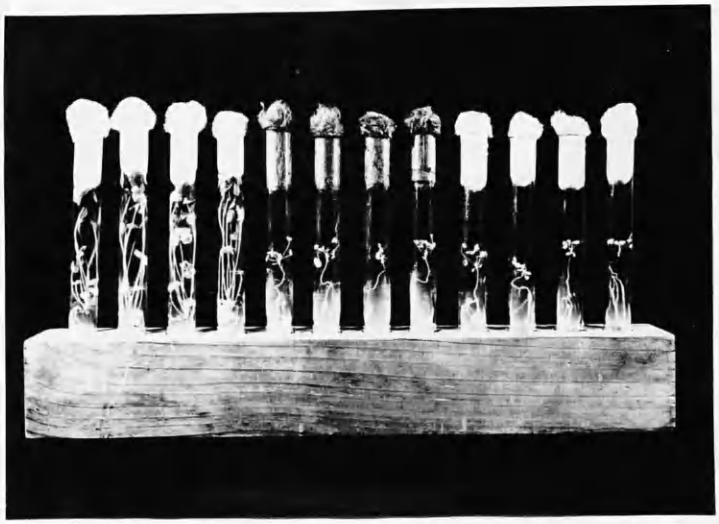
TABLE 6.

Details of localities from which isolations of the Black Medick nodule organism were obtained.

	Locality.	Isolation numbers.	Total no. isolations.
(a)	Troon, Ayrshire. Open ground bordering golf course. Soil very sandy and fairly coarse grained.	M1 - M14	14
(b)	Milngavie, Dunbartonshire. Verge at side of footpath, soil very coarse and stony.	M15 - M42	28
			42

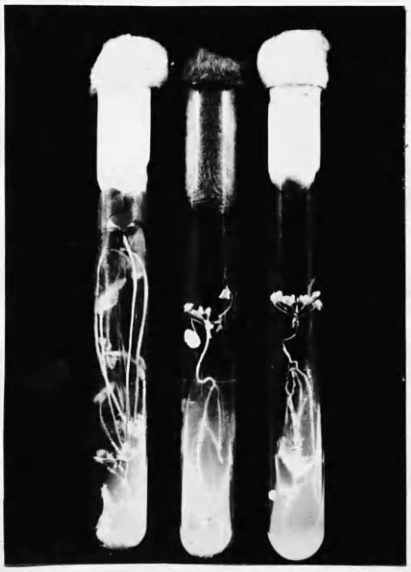
In both localities plants were found in fairly large colonies. Nodule collections were made from as many different colonies as possible within each area.

PLATE 11.



Above: A rack of typical Black Medick plants at the close of the experiment. The four plants on the left were inoculated with an effective isolation, the centre four are non-nodulated controls on nitrogen-free medium and the four on the right were inoculated with an ineffective isolation.

Below: One tube from each of the above groups.



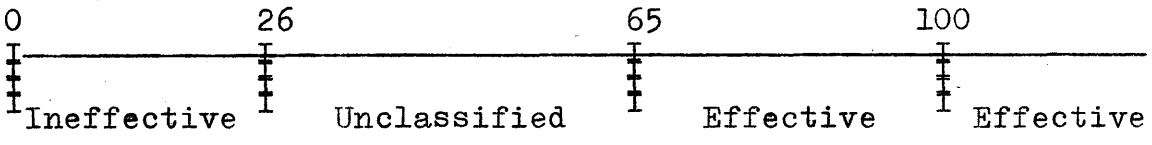
(1951), who working with species of Medicago, not including M.lupulina, encountered strains which failed in the laboratory to nodulate the host from which they were isolated. These authors gave their support to the possibility that when the isolation was made "the nodule chosen might have been a case of successful invasion in a relationship that is at best sporadic and perhaps partially susceptible to environmental influences". Thornton (1955) reporting on experiments relative to strain variation arising in Rhizobium cultures in liquid and agar media and in sterilised soil, noted two types of variant arising from effective strains, those producing ineffective nodules and those no longer producing nodules at all.

Of the remaining isolations, fifteen provided five plants at harvest, eight provided four plants, eight provided three plants and in the case of one isolation only two plants were harvested. These losses, 27 plants in all, were due in part to the causes already outlined for Gorse. In this case however, eleven of these plants were discarded solely because they had failed to form nodules, a feature not encountered in Gorse.

Individual percentage effectiveness values showed considerable variation as may be seen from the examples

given in Table 7. This is further reflected in the analysis of variance, summarised in Table 8, where very large differences were found to be required for significance. Thus a difference from the standard strain (100%) of 74, 79 and 86% in the case of five, four and three plants per isolation respectively, is required for significance. Similar values were calculated, viz. 65, 69 and 77% for a difference significant from the zero point.

No isolation could be classified as intermediate in this experiment due to the large differences required for significance causing an overlap and the classification is based on the following plan:-



These values apply to isolations with five plants only. As in the case of Gorse suitable adjustment was made for instances where fewer plants were available.

The distribution of the mean effectiveness values is detailed in Table 9 and is obviously very different from that for Gorse. Fourteen isolations were found

TABLE 7.

Individual values of percentage effectiveness calculated for the first ten isolations tested in the Black Medick experiment.

Isolation number.	% effectiveness values.	Mean.
M 2	306, 125, 239.	223
M 6	223, 96, 120, 135.	144
M 9	20, -4, 34, 16, 62.	26
M 10	152, 96, 144.	131
M 12	17, -11, 0, 5, -2.	2
M 14	4, -1, -3, -13, 13.	0
M 17	11, -8, 28, 19, 26.	15
M 18	8, -5, -22, -10, 3.	-5
M 20	61, 7, 3, 49.	30
M 24	98, 5, 139, 58, 165.	93

TABLE 8.

Black Medick experiment. Summary of analysis of variance on percentage effectiveness values.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	516268	33	15644
Within groups	399034	114	3500

Variance Ratio = 4.47
which is significant at P = 0.001

Minimum differences required for significance. P = 0.05.

In comparison of mean percentage effectiveness of a given isolation with the standard strain (5 and 5 separate estimates respectively):-

$$\text{Difference required} = \sqrt{3500} \times t_{114}0.05 \times \sqrt{1/5 + 1/5}$$
$$= 74.$$

In comparison of mean percentage effectiveness of a given isolation with the theoretical ineffective strain, equated here with non-nodulated plants without supplied combined nitrogen (5 and 10 separate estimates respectively):-

$$\text{Difference required} = \sqrt{3500} \times t_{114}0.05 \times \sqrt{1/5 + 1/10}$$
$$= 65.$$

TABLE 9.

Distribution of mean percentage effectiveness values.

Range of % Effectiveness	Number of isolations.
0 - 40	16 [x]
41 - 80	4
81 - 120	5
121 - 160	3
161 - 200	3
201 - 240	1

[x] This total includes two isolations giving negative mean values.

Classification.

Number of isolations:-

Effective.	Unclassified.	Ineffective.
14	7	11

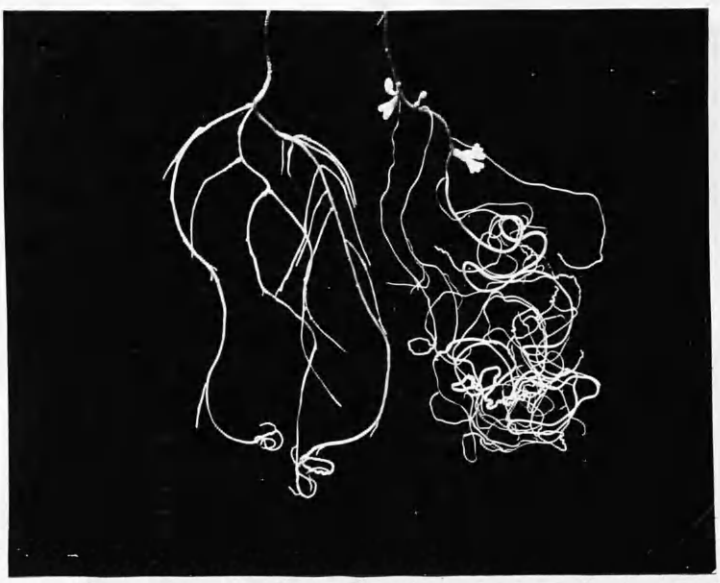
to give effective responses, eleven ineffective and seven were unclassified. The latter on retesting could theoretically fall into either of the two main classes. Of the ineffective isolations two gave negative mean percentage effectiveness values while among the others negative values for individual plants were commonly found. The proportion of effective isolations was similar in both localities from which isolations were made.

A 'beaded' type of nodulation was commonly noted with ineffective and unclassified isolations and an example is shown in Plate 12. Larger nodules were on occasion also found with such isolations as illustrated in Plate 13.

Right: Root system of a Black Gaddie plant
with an effective isolate. Note
while few in number are large and

The curling of the roots clearly seen here

PLATE 12.

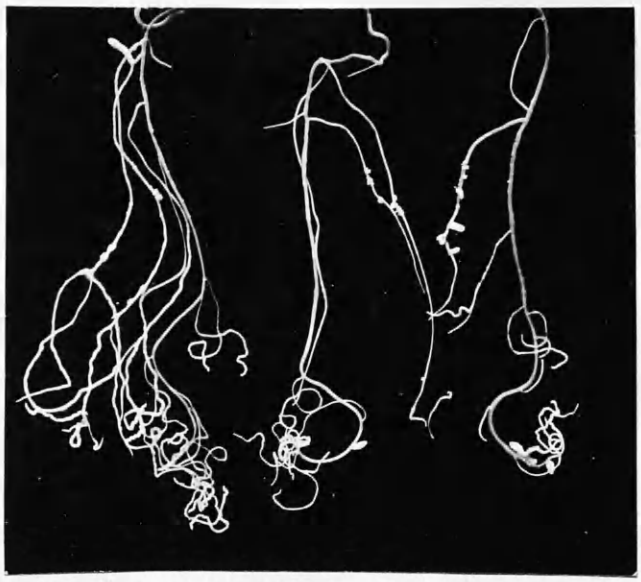


Left; Root system of a Black Medick plant inoculated with an ineffective isolation showing parts of the roots with beaded nodules.

Right; Root system of a Black Medick plant inoculated with an effective isolation. Note that nodules while few in number are large and branched.

The curling of the roots clearly seen here is characteristic of tube culture and is caused by the roots growing round the outer surface of the agar medium.

PLATE 13.



Black Medick experiment. Root systems of three plants inoculated with an ineffective isolation. In spite of its ineffectiveness this isolation produced several large nodules mixed with small ones more typical of such an isolation.

X 1.

D I S C U S S I O N

As previously noted, of the 78 Gorse isolations tested 73 proved effective, 4 intermediate and one was ineffective or intermediate. Thus within the limits of the investigation the organism associated with the nodules of wild Gorse plants is shown to be almost always fully effective in nitrogen fixation. While Gorse may be ranked as a troublesome weed on hill pastures it is no doubt able to contribute to some extent to the level of nitrogen in the soil.

A striking contrast to the Gorse result is seen with Black Medick. Considering only those isolations which produced nodules (33 out of a possible 42), 11 proved ineffective, 7 were unclassified and 15 were effective. There is little doubt that Black Medick in the wild state does harbour strains of poor nitrogen fixing ability in its nodules in a great many cases.

In both species, effective isolations produced large, pink nodules though in a few cases small nodules were intermingled with them. Ineffective isolations, in Black Medick at any rate, produced the classical type of small, white and often 'beaded' nodule formation. 'Beading' was not observed on Gorse.

The possible origin of ineffective strains such as were detected in Medicago lupulina has been considered by Nutman (1946b). According to him storage of an effective substrain in sterilised soil resulted in the production of a high percentage of ineffective types. Apparently in the soil environment a strong selection occurred in favour of the ineffective variant. A similar selection possibly occurs in soil under field conditions. Thornton (1955) reported that the development of ineffective variant forms of the clover Rhizobium when grown in pure culture in certain soils was apparently not related to soil acidity as had been suggested by preliminary trials. He indicated that it should be possible to select genetically stable strains of Rhizobium unlikely to produce ineffective variants in the soil. It may be the case that the Rhizobium of Gorse tends to be more genetically stable in soil than that of Black Medick and does not readily give rise to ineffective variants.

In studies concerned essentially with the ability to produce nodules, Wilson (1939b) postulated a relationship between the pollinating character of a leguminous plant and the number of rhizobial strains with which it could symbiose. It is conceivable that this relationship could be extended to account

for the presence of more variation in the nodule organism of one species than in that of another. In the scheme of Wilson, two principles were involved; (a) self pollinating legumes tend to comprise pure lines in which the inherent character permitting symbiosis is absent or carried as a recessive, and (b) cross pollinating species have either maintained or developed in a dominant state those characters which make possible promiscuity with diverse rhizobia. In a similar manner it is suggested that the plant character allowing successful symbiosis with one particular species of rhizobia is lost or retained. Thus the self pollinating species being more stable in this character are more likely to show response to slight variation in the nature of the invading organism and in time are liable to increase such variation in the organism itself. The cross pollinating species on the other hand are more adaptable to changes in the invading organism and by making compensation for such change tend to restrict it from becoming greater. This is in keeping with the present observations in that Gorse, with a great majority of effective isolations, is insect pollinated and Black Medick, with isolations

of mixed effectiveness, is mainly self pollinated (Clapham, Tutin and Warburg, 1952). The greater instability of the Black Medick nodule organism in the soil, tentatively suggested in the previous paragraph, would further increase the tendency for ineffective strains to develop.

Mention must also be made of the rhizosphere factor which is of an abnormal nature in sterile tube culture. Kalnin'sh (1951) on inoculating plants simultaneously with two different strains concluded that successful invasion was made by the strain with the greater degree of adaptation to the rhizosphere. Bacteria capable of stimulating the growth of rhizobia in the soil have been described and termed 'activators' by Krasilnikov and Korenyako (1944). Harris (1953) extended this work and showed increased virulence in rhizobia strains in the presence of various fungi and bacteria. Thus one strain examined by him gave an ineffective reaction in sterile soil due to scanty nodulation but in the presence of other micro-organisms was increased sufficiently in virulence to give an effective response. This has led him to consider that many of the reports of large numbers of ineffective strains of rhizobia found in soils under natural conditions may in fact have been made on the

basis of poorly virulent strains giving apparently ineffective plant reactions when tested under sterile conditions. Such lack of virulence is possibly the explanation of the number of isolations which failed entirely to produce nodules on Black Medick plants. However at no time did ineffectiveness appear due to scanty nodulation and in fact three nodules on one plant was sufficient in one case to give an effective response.

Both species investigated were grown from seed originating in this country though not in the particular locality from which the Rhizobium isolations were made. There is always the possibility therefore that the genetic constitution of the plants was favourable or unfavourable to the various isolations to a somewhat different degree from what might have been found with seed from the same localities as the isolations. There is however, no reason to suppose that the performance of the isolations would have been substantially different with seed of other origin.

The abundance of ineffective strains of rhizobia in wild legumes is confirmed in Black Medick and is a further indication of the need of seed inoculation for Lucerne already noted and indeed for Black Medick

itself when used for agricultural purposes. Gorse on the other hand presents an entirely different picture and indicates that under natural conditions some wild legumes at least play a substantial part in the nitrogen cycle, as in the case of agricultural legumes.

S U M M A R Y

The suggestion that strains of the legume nodule organism ineffective in nitrogen fixation are harboured in the nodules of wild legumes has been investigated.

Isolations made from the nodules of two wild legumes were tested for their effectiveness in nitrogen fixation. Such tests were carried out using a sterile tube culture technique. Percentage effectiveness values were obtained for each isolation, these values being based on the comparison of isolations with the performance of a selected isolation which produced plants comparable to those non-nodulated plants supplied with combined nitrogen.

Eventual classification of isolations was based on statistical analysis of the data.

Of the 78 Gorse nodule isolations, 73 were classed as effective, 4 as intermediate and 1 (whose performance varied in different tests between ineffective and intermediate) as ineffective-intermediate.

Of 42 Black Medick isolations, 15 were classed as effective, 11 as ineffective and 7 were unclassified. The remaining nine isolations failed to produce nodules.

It is concluded that within the limits of the investigation, wild Gorse does not generally harbour ineffective strains in its nodules but that with respect to Black Medick this is very often the case.

P A R T II.

The effect of combined nitrogen on nodulation in *Myrica gale* L., *Alnus glutinosa* (L.) Gaertn., and *Ulex europaeus* L..

PART II.

C O N T E N T S

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

It is well known that in the case of the free-living nitrogen-fixing organisms such as Azotobacter and the blue-green algae the fixation of elemental nitrogen is suppressed in the presence of adequate combined nitrogen, that is, combined nitrogen is assimilated preferentially over elemental nitrogen. In such instances there is of course no visible morphological change in the organism, though there may be alterations in the enzyme components and thus in protoplasmic structure.

A similar preference for combined nitrogen exists in the case of the dual organism presented by the root nodule symbiosis. Moreover it has long been known that in the legumes the extent of the formation of the particular parts that are responsible for fixation - the nodules - is reduced when combined nitrogen is present in the rooting medium. Thus here the substitution of the assimilation of combined nitrogen for that of elemental nitrogen is accompanied by morphological changes. Large amounts of combined nitrogen, greatly in excess of those likely to be present naturally in soils, are required to produce complete suppression of nodules: thus it is a matter of common observation that plants such as broad beans and peas show very abundant nodulation in the garden, even although the soil may have been well

manured and dressed with fertiliser. The explanation of this reduced nodule formation is imperfectly known and has been the subject of much confused thinking. This aspect will be taken up again in the Discussion.

In view of the previous neglect of the non-legume root nodule plants, it is not surprising that until quite recently there was no information on the extent to which these plants run parallel to the legumes in their responses to combined nitrogen. The first observations, those of Bjorkman (1942), were incidental to an investigation concerned mainly with factors controlling the formation of mycorrhiza in Pine and Spruce. Inoculated Alder plants were grown in a humus-sand mixture to which, in certain cases, ammonium nitrate equivalent to 100, 200 or 400 mg. nitrogen per litre of added culture solution was supplied. The assessment of the effects of this combined nitrogen is complicated by accompanying variations in phosphate supply, but on comparing instances where the phosphate level was similar it appears that the addition of 100 mg. combined nitrogen reduced the volume of nodule tissue from 33 cu. mm. (the value for plants with no added nitrogen) to 15 cu. mm. per plant. The higher levels of nitrogen suppressed nodulation almost completely. It should

be noted that the nitrogen levels employed were greatly in excess of plant requirements or of those likely to occur in nature.

Bond, Fletcher and Ferguson (1954) reported on the effect of ammonium nitrogen on nodulation in Alnus, Myrica and Hippophaë growing in water culture. The results of the Alnus experiment (in which substantial assistance was given by the present author) are reproduced in Table 10. Various levels of combined nitrogen were provided at the time of inoculation with the nodule organism. Since the formation of nodules and the initiation of substantial fixation takes about a month, it is obviously to be expected that the plants supplied with combined nitrogen will in their growth get well ahead of those in nitrogen-free solution. Thus, as shown in Table 10, the dry weight of plants supplied with 10 mg.nitrogen per litre was, after 12 weeks of growth, ten times greater than that of plants in zero nitrogen. The absolute weight of nodules formed per plant was greater at all nitrogen levels than with zero nitrogen, but evidently had not kept pace with the greater development of the plant as a whole, as is indicated by the value of the nodule weight expressed as a percentage of the weight of the whole plant (right hand column). The root systems of typical plants from this experiment, grown at 0 and 10 mg. nitrogen levels, are shown in Plate 14.

TABLE 10.

Effect of added combined nitrogen on nodulation and growth of Alnus plants grown for twelve weeks in water culture at pH 6.3.

(Data of Bond, Fletcher and Ferguson, 1954).

Mg. NH_4-N added per litre of culture soln..	Mean dry weight per plant in gm.. Nodules	Whole plant	Nodule weight as % of whole plant weight.
0	0.008	0.131	6.1
10	0.042	1.322	3.2
50	0.021	1.734	1.2
100	0.016	1.581	1.0

At the 0, 10, 50 and 100 mg. nitrogen levels the number of plants harvested was 19, 19, 18 and 14 respectively.

PLATE 14.

Nodulated roots of Alder grown for 12 weeks in nitrogen free solution.

X 8/7.

Nodulated roots of Alder grown for 12 weeks in culture solution containing 10 mg.of ammonium nitrogen per litre.

X 8/7.

Photographs from Bond, Fletcher and Ferguson (1954)

Bond et al. found that Hippophaë was much more sensitive to combined nitrogen than Alnus, since the greater growth of plants supplied with combined nitrogen was not accompanied by increased absolute nodule dry weight. The latter fell sharply and was completely suppressed by 50 mg. nitrogen per litre. The data presented for Myrica were scantier, but permitted a tentative conclusion that this genus resembled Alnus in its response to combined nitrogen.

Quispel (1954) reported on the effect of two levels of ammonium and nitrate nitrogen nn nodulation in Alder growing in water culture, an extract of his data being provided in Table 11. Unfortunately the only type of datum common to this work and to that of Bond et al. (loc. cit.) is weight of nodules per plant. However it is quite clear that Quispel's findings differ sharply from the Glasgow findings in that, in the presence of ammonium nitrogen the absolute weight of nodules per plant is markedly reduced. On the other hand, a small amount of nitrate nitrogen produced the opposite effect, attributed by Quispel to unspecified secondary effects operating in this case. It is important to note that there was a significant difference between the procedure followed by Quispel and that of the Glasgow experiment. Quispel grew

TABLE 11.

The influence of combined nitrogen on nodule formation in Alder. Data extracted from those obtained by Quispel (1954). Phosphate concentration was also studied by Quispel but the values quoted here were all obtained at the same phosphate level.

Nitrogen type.	Mg.N per litre of culture sol..	Number of nodules.	Weight of nodules Per plant.	Per nodule
-	0.0	106.5	56.2	0.53
NH_4	5.25	9.1	29.2	3.21
NH_4	52.50	0.3	0.3	1.00
NO_3	5.25	176.8	84.0	0.49
NO_3	52.50	0.4	0.7	1.60

12 plants were grown at each nitrogen level for a period of seven weeks following inoculation.

Units of weight are not stated but from comparison with the present authors experiments they are most likely mg. dry weight.

his plants to a height of 8 cm. on a liberal nitrogen diet before inoculating and commencing the experiment proper; Bond et al. started their experiment with much smaller plants which had grown essentially on their seed nitrogen, and thus were very low in that element. The fact that Quispel grew his plants for only seven as compared with twelve weeks after inoculation is probably of smaller importance.

Quispel claimed that his findings were in agreement with those of Bjorkman (1942), but actually the inhibiting effect of ammonium nitrogen shown in the experiment of Quispel is much greater than that detected by Bjorkman.

Quispel also provides data for numbers of nodules, though he explains in the text that the data refer to the total number of nodule branches or lobes present. A count of the number of nodule clusters would have been more informative, since each cluster rather than each lobe represents an original point of entry of the endophyte into the root. It is clear from the data given, however, that in the presence of ammonium nitrogen the number of infection points must have been greatly reduced. Further comments on Quispel's findings will be offered in the Discussion relative to the Alder experiments.

Three lines of investigation have been followed in the experiments to be described below.

(1) The effect of combined nitrogen on nodulation in Myrica gale has been studied to supplement the preliminary data provided by Bond, Fletcher and Ferguson (1954).

(2) An attempt has been made to explain the discrepancies between the results of Bond et. al. (1954) and those of Quispel (1954) with respect of Alnus glutinosa.

(3) In order to facilitate the comparison of the effect of combined nitrogen on nodulation in non-legumes and legumes, data have been secured for Ulex europaeus using precisely the same technique with this legume as used in the experiments with non-legumes.

GENERAL EXPERIMENTAL PROCEDURE.

The same general pattern of treatment and culture was followed with all three species investigated. Seedlings after being raised to a suitable stage of development in peat, sand or a simple agar medium (a detailed description of the treatment of each species will be given later) were transferred into water culture. A nitrogen-free form of Crone's solution prepared as follows was used:-

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

A minor element concentrate based on Hoagland's A-Z solution with molybdenum added was supplied to the above at 1 ml.per litre. This basic culture solution differs from the full Crone's formula only in the replacement of KNO_3 by KCl.

This solution was placed in glazed earthenware jars of two-litre capacity topped with waxed teak squares each square having seven holes, each of which in turn had an inset of thick walled rubber tubing.

The seedlings were fitted into these insets and supported by small rubber wedges. When combined nitrogen was to be supplied it was added in the form of ammonium nitrogen, the source of this being a solution of ammonium sulphate containing 20 mg. nitrogen per ml.. Appropriate amounts of this solution were added to the two-litre jars to give the required nitrogen levels.

To inoculate the non-legume plants the appropriate nodules were ground up with water and a suspension of the nodule organism thus obtained. In the case of the legume a similar suspension was prepared from pure cultures of the nodule organism. In all cases the suspension was brushed on to the roots of individual plants and in addition a further amount of the inoculum was added to the culture jars.

In the case of the non-legumes, experience has shown that the nodule organism is not carried on the seed, and that the organism does not appear to be introduced into the greenhouse and into culture vessels on wind-blown dust, since without deliberate inoculation cultured plants do not develop nodules. Hence seed sterilisation is not necessary in order to gain full control over nodulation. Jars and teak tops were sterilised before use in a new experiment. The cultures were of a non-aseptic type.

The experiments were conducted in a cool greenhouse and the relative position of the jars in any one experiment was changed at weekly intervals during the course of the experiment. Due to the frequent attention required and the necessity of avoiding confusion especially when nitrogen was being added to the cultures, complete randomisation was not attempted and the group of jars containing a particular nitrogen level was always kept as one unit when positional changes were made.

Active growth in the presence of ammonium nitrogen results in rapid falls in the pH of the culture solution. To maintain the pH level suitable to the particular species it was therefore necessary to check and if required, to adjust, this value every two days. Such an adjustment was made by the addition of sodium hydroxide, the critical amount being determined by the titration of a small sample of the solution from each jar. Maintenance of nitrogen levels will be detailed later.

At harvest in all experiments plants were treated individually. Nodules were removed for dry weight determinations separate from those of root plus shoot. Dry weights were obtained by heating the material overnight in an oven at 95°C..

In the following pages a period of growth in the presence of combined nitrogen prior to inoculation with the nodule organism will be referred to as 'pre-treatment'.

I. EXPERIMENT WITH MYRICA GALE L.. BOG MYRTLE, SWEET GALE.

METHODS

Seed collected from Stockiemuir, Dunbartonshire, in the autumn of 1953 was stored at 2°C. for twelve weeks prior to sowing in trays of horticultural peat in March 1954. Ten weeks after sowing, seedlings of approximately 1.5 cm. in height and bearing 3-4 true leaves were selected from the trays and transferred to water culture. The Crone's solution was adjusted to, and maintained at, a pH of 4,5.

The same day as the transplanting, ammonium-nitrogen was added to the culture solution giving five jars at each of four levels, viz., 0, 10, 50 and 100 mg. nitrogen per litre.

Nodules were collected in the field two days after transplanting and inoculation was carried out that same day. The inoculum was prepared by grinding 2 gm. nodules in 40 ml. distilled water with the addition of a little sand and the brown suspension thus obtained was brushed on to the root system of individual plants. One ml. of

the inoculum was also stirred into each jar. A second inoculation was carried out five days later using the same strength of inoculum and the same procedure as before.

At intervals small additions of nitrogen were made to the solution containing initially 10 mg. per litre as active plant growth would rapidly reduce this level. Accurate estimations of nitrogen levels in later experiments indicate that the system adopted in this case kept the nitrogen level reasonably close to that required. Culture solution was renewed completely twice during the experiment.

EXPERIMENTAL RESULTS

At the conclusion of the experiment the plants had been in water culture for a period of fourteen weeks, from the 22nd, May until the 28th. August 1954. During this time considerable dying off of the plants had occurred. Miss I.C. Gardner in unpublished work, has since found that these losses of young Myrica gale plants can be avoided by using diluted Crone's culture solution. In the present case the reduced numbers did not confuse the result.

The first nodules were observed two weeks after inoculation. Subsequently nodule development was rapid and the upwardly growing nodule-roots made their appearance.

The initial advantage conferred on those plants supplied with combined nitrogen became clear early in the experiment when they made markedly better growth than those awaiting the formation of nodules as their sole nitrogen source. This waiting period would appear to be at least two to three weeks from inoculation.

Results obtained at harvest are presented in Table 12. Plant development has been greatly enhanced in the presence of nitrogen as dry weights indicate.

TABLE 12.

Data (means per plant) obtained at harvest of *Myrica gale* plants supplied during the period of 14 weeks of growth with different amounts of ammonium nitrogen.

Mg. NH_4-N per litre culture soln..	Height of shoot cm..	Mg. dry weight		Nodule wt. as % of plant wt..
		Nodules.	Whole plant.	
0	3	4	33	12.2
10	8	17	304	5.4
50	12	24	561	4.2
100	10	19	488	3.6

At the 0, 10, 50, and 100 mg. nitrogen levels the number of plants harvested was 9, 12, 6 and 5 respectively.

An analysis of variance was carried out on the value 'Nodule weight as % of plant weight' and is summarised in Table 12a.

TABLE 12a.

Summary of analysis of variance on nodule weight as a percentage of whole plant weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	37395	3	12465
Within groups	8789	28	314

Variance Ratio = 39.7
which is significant at P = 0.001

Differences between means required for significance:

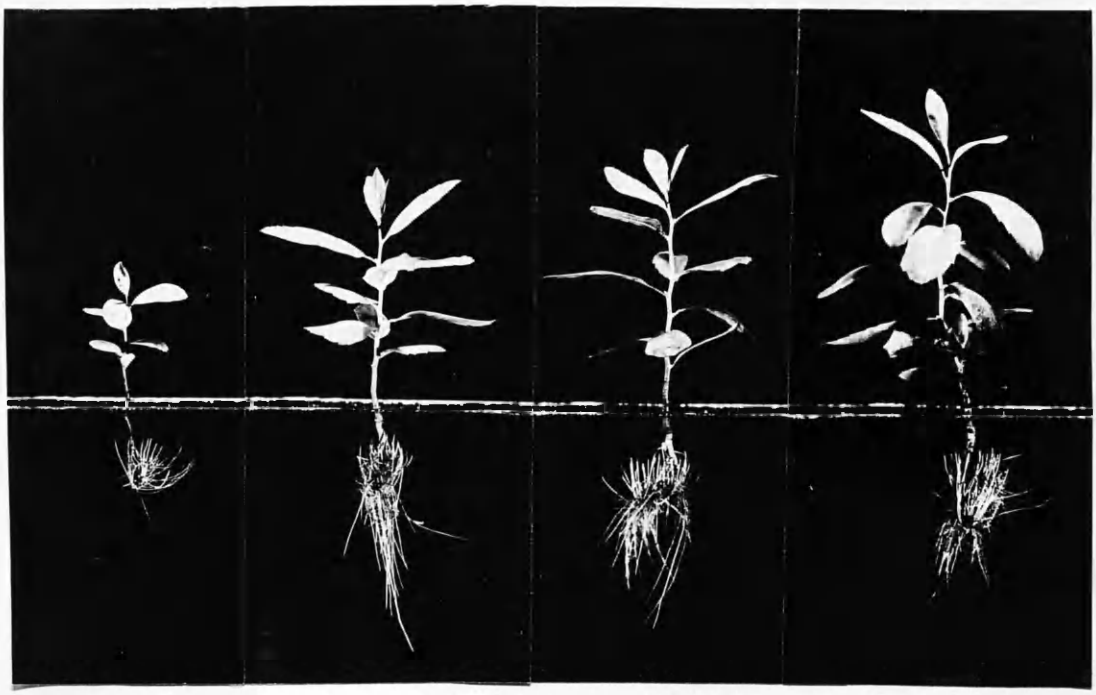
P = 0.05

Comparison	Required difference	Observed difference
0 and 10 mg.N	1.6%	6.8%
10 and 50 mg.N	1.8%	1.2%
50 and 100 mg.N	2.2%	0.6%

The primary interest in the results lies in the values obtained when the nodule dry weight is expressed as a percentage of the total dry weight. At 0 mg. of nitrogen this value is more than twice as great as at the 10 mg. level and more than three times that at the 100 mg. level. However an analysis of variance (Table 12a) shows that although the value at 0 mg. nitrogen is significantly greater than at other nitrogen levels, significance is not attained between the plants supplied with 10, 50 and 100 mg. of nitrogen per litre of culture solution.

Typical plants at harvest are shown in Plate 15.

PLATE 15.



Typical Bog Myrtle plants after 14 weeks of growth in water culture. From left to right they received 0, 10, 50 and 100 mg. of ammonium-nitrogen per litre of culture solution. Although the 100 mg. plant shown on the extreme right is the largest in the line, dry weight mean values indicate that the plants at 50 mg. nitrogen made somewhat better growth. Note the clearly displayed negatively-geotropic nodule roots, the density of which is an indication of the degree of nodulation. The nodules themselves are largely hidden.

X 1/3.

D I S C U S S I O N

The results reported in the previous section show that the addition of various levels of ammonium-nitrogen two days prior to inoculation resulted in much more rapid growth of the Bog Myrtle plants and in much greater absolute development of nodules. But nodule weight did not increase proportionately with the weight of the whole plant. Thus when a nitrogen supply of only 10 mg. per litre of culture solution is provided the weight of nodules as percentage of whole plant weight is reduced from 12.2 (the value in zero nitrogen) to 5.4. Increase in the level of nitrogen probably tends to depress further the relative weight of nodules, though the reductions recorded did not attain statistical significance.

It might be argued that the depression in relative nodule weight in the presence of combined nitrogen is due entirely to the more advanced development of the plants resulting from the continuous access to combined nitrogen, and that given time all nodule percentage weights would fall. However Bond (1951b) gives the nodule dry weight as 7.4 per cent. of the whole plant dry weight in six months' old Bog Myrtle plants grown

in nitrogen-free culture solution. This certainly represents a fall from the value of 12.2 per cent. recorded in the present experiment after just three months of growth in zero nitrogen, but it is clear that the values for plants receiving combined nitrogen are already well below Bond's figure. Thus it may be concluded that the low relative nodule weights result from a direct effect of combined nitrogen on nodule development.

These results show a close resemblance to those previously reported for Alder by Bond, Fletcher and Ferguson (1954), and it is clear that Bog Myrtle and Alder respond similarly to combined nitrogen.

II. EXPERIMENTS WITH ALNUS GLUTINOSA (L.) GAERTN.. ALDER.

METHODS

Two types of experiment have been carried out with this species. In the first type the procedure was as with Myrica gale, the different levels of combined nitrogen being established and inoculation effected soon after the young plants had been transplanted from the seed tray into water culture, at a stage when only 1-2 leaves had been formed. This, as noted already, was also the procedure adopted by Bond, Fletcher and Ferguson (1954) in their Alder experiments. In the second type of experiment, immediately after transplanting all the plants were provided with an amount of combined nitrogen adequate for growth, and were allowed to grow on for approximately five weeks. Only then was the experiment proper commenced, that is the different levels of combined nitrogen were established and inoculation effected. The introduction of this period of pre-treatment was made in order to reproduce the procedure of Quispel (1954).

The seed used in these experiments was collected from Milngavie, Dunbartonshire, in November 1953 and stored at room temperature until required. Sowings were made in trays of sand watered with dilute nitrogen-free Crone's solution, and after 3-5 weeks seedlings were transferred from these trays into water culture in Crone's solution at pH 6.3. At this stage the young plants had an over-all height of 3-4 cm. and bore, as noted above, 1-2 leaves.

A small scale preliminary experiment was set up on the 7th.July 1955, using plants from a sowing made on the 2nd.June. Only four jars were included, two being with nitrogen-free solution and the other two supplied with 10 mg. ammonium nitrogen per litre of solution. In this experiment there was no period of pre-treatment, and the reason for setting it up was that, due to other commitments, the main experiment (detailed below) could not be set up till later in the season, and it was desired to confirm the main findings of Bond, Fletcher and Ferguson (1954) in an experiment carried out at the same time of year as in their case. The plants were inoculated on the 8th.July using an inoculum prepared by grinding 20 gm. of nodules from other greenhouse plants in 100 ml. of water. This inoculum was brushed on to each root system and 1 ml. was added to the solution

in each jar.

The main experiment comprised two parts. With one series of plants the experiment proper was commenced directly after transplanting into water culture, as in the preliminary experiment. With the other series a period of pre-treatment with combined nitrogen was given. Further details are as follows:-

No pre-treatment. Fifteen jars of young plants, five plants per jar, from a sowing of the 29th.June, 1955, were set up in water culture (Crone's solution at pH 6.3) on the 29th.July, the plants being at a stage of development already indicated. On the same date appropriate additions of ammonium sulphate were made so that in different jars levels of combined nitrogen of 0, 10, 50 and 100 mg. per litre of solution were established. On the following day (30th.July) these plants were inoculated, the inoculum being prepared and applied as in the preliminary experiment.

With pre-treatment. Twelve jars of young plants, five plants per jar, from a sowing of the 2nd.June were set up in water culture on the 27th.June and 50 mg. ammonium-nitrogen per litre of culture solution then added to all jars. On the 30th.July the solution in the jars was replaced by fresh Crone's solution, and a differential addition of ammonium-nitrogen was now made to the jars so that the four levels already specified for the first

part of the experiment were established. At this stage the plants had an over-all height of 7-9 cm. and bore 4-5 true leaves. On the same date the plants were inoculated, the inoculum being identical with that used for plants of the first part of the experiment.

Samples of plants at the stage of development at which inoculation was carried out were taken for both parts of the experiment for dry weight and total nitrogen determination.

As noted previously, the pH of the culture solution was tested and if necessary adjusted (to pH 6.3) every two days. Steps were also taken to maintain the desired levels of combined nitrogen in the culture solution. Samples of the latter were made alkaline and the ammonia distilled over into standard acid, the amount of ammonia being then ascertained by titration. These tests were made at approximately weekly intervals. When necessary appropriate additions of further ammonium-nitrogen to the culture jars were made. On the basis of these estimations it can be stated with confidence that during the period of the experiment the contents of combined nitrogen did not deviate from the range 10-6, 50-47, 100-95 mg. nitrogen per litre of culture solution.

The culture solution was entirely renewed at intervals of four weeks.

EXPERIMENTAL RESULTS

In the preliminary experiment the first nodules were detected two weeks after inoculation. The plants were harvested some ten weeks after the date of inoculation, the data obtained being displayed in Table 13. It will be observed that the presence of 10 mg. per litre of ammonium-nitrogen benefited the growth of the plants very considerably, as indicated by comparison of the data for height of shoot and dry weight of whole plant. The dry weight of nodules per plant in the presence of combined nitrogen was over four times as great as in its absence, but relative to the dry weight of the plant as a whole that of the nodules was much reduced (right hand column).

Nodulation was very rapid in the main experiment, the first nodules being detected only ten days after inoculation. Both parts of this experiment were harvested between the 4th. and 9th. October 1955, again approximately ten weeks from inoculation. The data for the plants which did not receive pre-treatment with combined nitrogen are provided in Table 14, while typical plants are shown in Plate 16. The data indicate that growth of the plants was considerably reduced as

TABLE 13.

Preliminary experiment with Alder. Mean data (per plant) obtained at harvest.

12 plants were harvested at each nitrogen level.

Mg.NH_4-N supplied	Height of shoot cm..	Mg. dry weight. Nodules	Mg. dry weight. Whole plant	Nodule wt.as % plant wt..
0	5	8	127	7.2
10	19	36	1264	2.9

Nitrogen level is expressed as mg. N per litre of culture solution.

The minimum difference between the means of 'Nodule weight as % of plant weight' required for significance at P = 0.05 is 1.8%. From the above table the actual difference is seen to be 4.3%.

TABLE 14.

Main experiment with Alder, first part (plants not given pre-treatment with combined nitrogen). Mean data (per plant) obtained at harvest.

Mg.N per litre of solution	Number of plants	Shoot height cm..	Dry weight in mg..		Nodule wt. as % of plant wt..
			Nodules	Whole plant	
0	30	3	3.3	65	5.3
10	15	9	5.5	394	1.7
50	15	9	3.6	436	1.0
100	15	9	2.6	403	0.7
—	—	—	—	—	—

Statistical treatment of these data is summarised in Table 14a.

TABLE 14a.

Summary of analyses of variance on data given in Table 14.

(a) Nodule dry weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	69	3	23
Within groups	104	71	1.5

Variance Ratio = 15.3

which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (N levels)	Difference (in mg.) Required	Observed
0 and 10	0.8	2.2
10 and 50	0.9	1.9
50 and 100	0.9	1.0

(b) Nodule weight as percentage of plant weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	31895	3	10632
Within groups	9178	71	129

Variance Ratio = 82.4

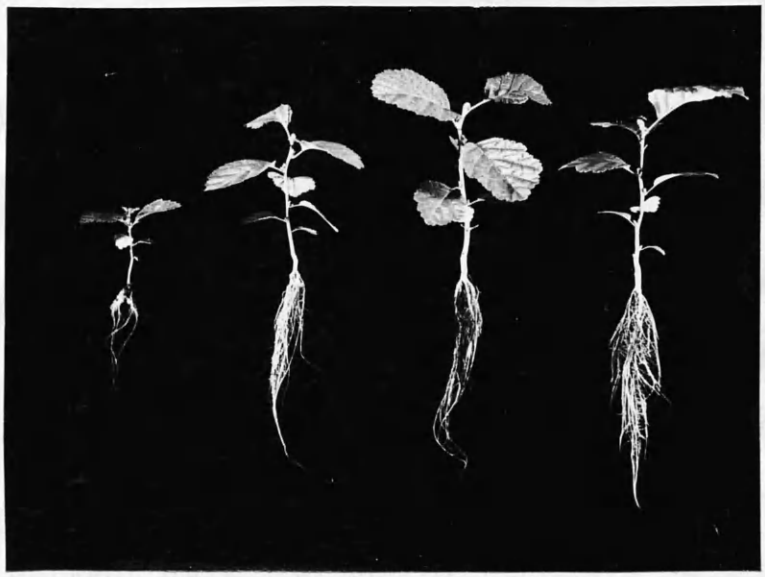
which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (N levels)	Difference (in %) Required	Observed
0 and 10	0.7	3.6
10 and 50	0.8	0.7
50 and 100	0.8	0.3

PLATE 16.



Alder experiment, first part. Plants given no pre-treatment. Above are shown typical plants after ten weeks of growth in water culture at different nitrogen levels. From left to right they were supplied with 0, 10, 50 and 100 mg. ammonium-nitrogen per litre of culture solution respectively.

X 1/5.

compared with the corresponding plants of the preliminary experiment, due to the later stage in the season at which the main experiment was started. The effect of combined nitrogen on nodule development is however, similar to that shown in the preliminary experiment in that in the presence of 10 mg. combined nitrogen the absolute weight of nodules per plant is increased significantly, while the relative development (right hand column) is decreased significantly. In the presence of larger amounts of combined nitrogen absolute dry weight of nodules is reduced to a level comparable with that in nitrogen-free solution. Relative development tends to be further depressed, though full statistical significance is not attained.

The harvest data for the second group of plants which received pre-treatment with combined nitrogen prior to inoculation, are shown in Table 15, and typical plants are illustrated in Plate 17. As was to be expected these plants were much larger than the corresponding plants of the first group since they were set up in water culture five weeks ahead of the first group, and during the period of pre-treatment made rapid growth at a very favourable time of year. Turning to consider the effect of the differential treatment with combined nitrogen from the time of inoculation it will be seen that the dry weight of nodules per plant is approximately doubled

TABLE 15.

Main experiment with Alder, second part (plants given pre-treatment with combined nitrogen prior to inoculation). Mean data (per plant) obtained at harvest.

12 plants were harvested at each nitrogen level.

Mg.N per litre of solution	Shoot height cm..	Dry weight in mg..		Nodule wt. as % of plant wt..
		Nodules	Whole plant	
0	13	28	878	3.4
10	23	54	2275	2.4
50	23	20	2363	0.8
100	20	7	2116	0.4

Statistical treatment of these data is summarised in Table 15a.

TABLE 15a.

Summary of analyses of variance on data given in Table 15.

(a) Nodule dry weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	14439	3	4813
Within groups	6272	44	143

Variance Ratio = 33.7

which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (N levels)	Difference (in mg.) Required	Observed
0 and 10	10	26
10 and 50	10	34
50 and 100	10	13

(b) Nodule weight as percentage of plant weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	5813	3	1938
Within groups	744	44	17

Variance Ratio = 114

which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (N levels)	Difference (in %) Required	Observed
0 and 10	0.3	1.0
10 and 50	0.3	1.6
50 and 100	0.3	0.4

Plate 17.

Alder experiment, second part. Plants given pre-treatment. After three weeks growth in culture solution containing 50 mg. ammonium nitrogen per litre these plants were grown for a further ten weeks in culture solution at different nitrogen levels. Above, from left to right, are typical plants which were supplied with 0, 10, 50 and 100 mg. ammonium nitrogen per litre of solution respectively.

x $\frac{1}{8}$.

by the presence of 10 mg. nitrogen per litre, but at higher nitrogen levels is much reduced so that in the presence of 100 mg. the dry weight is only one quarter of the corresponding value in nitrogen-free solution. Thus in this respect the effect of combined nitrogen is considerably more marked than in the first group of plants (Table 14). In the right hand column of Table 15 it is noticeable that in these pre-treated plants the relative development of nodules on plants in nitrogen-free solution is considerably smaller than in the corresponding plants of the first group (3.4% against 5.3%). The introduction of combined nitrogen depresses this relative value, all the successive reductions being significant, though the initial depression resulting from the presence of 10 mg. nitrogen is much less severe than in the first part of the experiment (Table 14).

As noted already, representative plants at the stage when inoculation was carried out were reserved for dry weight and nitrogen determination. In view of the presence of appreciable amounts of very firmly-fixed sand particles on the root systems it was decided to use only the shoots of these plants. The percentage nitrogen content proved to be 1.3 for the plants which received no pre-treatment and 3.4 for the pre-treated set.

DISCUSSION

It has been shown in the preliminary experiment and in the first part of the main experiment that in the presence of 10 mg.of ammonium nitrogen per litre of culture solution the absolute dry weight of nodules per plant is markedly increased as compared with the corresponding value for plants grown in solution devoid of combined nitrogen. This is in agreement with the previous finding of Bond, Fletcher and Ferguson (1954) with Alder. The fact that in the first part of the main experiment (Table 14) the dry weight of nodules per plant in the presence of 50 or 100 mg. combined nitrogen per litre was no greater than in the absence of combined nitrogen, whereas Bond et al. found that it was still considerably greater, may be attributed to the somewhat later stage in the year at which the present author's experiment was carried out, with consequently a poorer general nodule development. As in the experiment of Bond et al., there is a continuous depression in the nodule dry weight expressed as a percentage of the whole plant dry weight as the level of combined nitrogen is increased: in other words the

enhanced growth of the plants shown in these short term experiments as a result of the provision of combined nitrogen is not attended by a proportionate increase in nodule growth, so that in a relative sense nodule development is depressed.

Turning now to consider the second part of the main experiment (Table 15) in which the plants were grown for a period in the presence of ample combined nitrogen prior to inoculation, certain differences in the effect of combined nitrogen on nodulation are shown as compared with those in the experiments already reviewed. Attention was drawn to the salient differences in the previous section. The absolute weight of nodules per plant is again increased when 10 mg. is supplied, but at 50 mg. and still more at 100 mg. nitrogen the weight is below that of the zero nitrogen plants. This is reflected in a steeper fall in the percentage value. It is also important that the percentage weight of nodules on pre-treated plants grown in nitrogen-free solution from the time of inoculation is markedly less than on plants inoculated immediately, without pre-treatment. Obviously there is a continuing effect of the combined nitrogen supplied to the time of inoculation and then withdrawn. It has been noted that the percentage nitrogen content of these plants at the time of inoculation was

3.4 against 1.3 in the plants without pre-treatment. The higher nitrogen balance in the pre-treated plants reacts unfavourably on the subsequent development of nodules. The more severe depression of nodule development resulting from the presence of combined nitrogen after inoculation shown in this experiment can be explained in the same way - in addition to the effect of the nitrogen currently supplied there is also a persisting effect due to the period of pre-treatment with combined nitrogen.

It will be recalled that the main object in performing the experiment in which the plants were pre-treated with nitrogen prior to inoculation was to reproduce the procedure of Quispel (see Introduction) and to attempt to elucidate the differences in results between his experiment and that previously carried out in Glasgow. The procedure for pre-treatment adopted by the present author resembled that of Quispel closely. A level of approximately 50 mg. combined nitrogen per litre was supplied to the plants in both instances, and the pre-treatment was continued until the plants had attained an over-all height of 8 cm.. The only material difference was that while Quispel supplied nitrate-nitrogen the present author supplied ammonium-nitrogen, but either form is readily accepted by the Alder plant. Despite

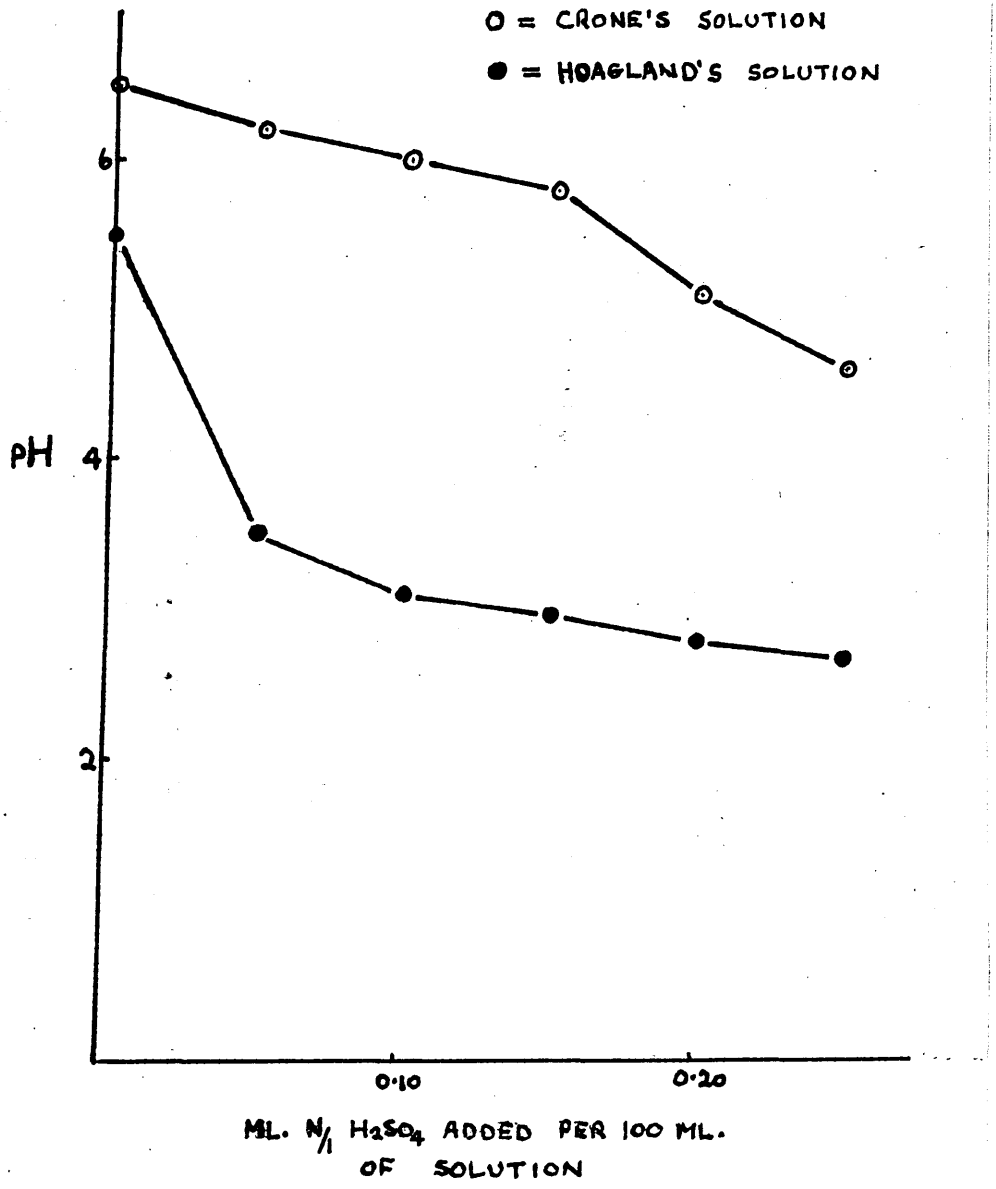
the similarity in procedure there are notable differences in results obtained, as will be seen by comparing Tables 11 and 15. Thus in the presence of only 5 mg.(approx.) of ammonium-nitrogen Quispel found that the weight of nodules per plant fell to about half that present on plants in solution with zero combined nitrogen, while with about 50 mg. of nitrogen nodulation was virtually completely suppressed. This latter finding is in sharp contrast to the present author's finding that in the presence of 50 mg. nitrogen the weight of nodules per plant was not significantly less than in zero nitrogen plants, while as regards the effect of small amounts of nitrogen, although the present author did not include a 5 mg. level, in his experience 10 mg. doubled the weight of nodules and it cannot be contemplated that 5 mg. would have failed to increase nodule weight.

Thus the position is that although the introduction of the pre-treatment procedure resulted in the effects of combined nitrogen being less unlike those of Quispel than were those obtained by Bond et al. in their earlier experiment, marked differences still exist which must have their origin in variations in procedure after the stage of inoculation.

Variations which may be of significance exist in respect of the particular culture solution employed,

in the ratio of number of plants to volume of solution, and in the arrangements for pH control. Quispel used a diluted form of Hoagland's solution, and in this diluted form this solution is markedly inferior to Crone's solution in its buffering power on the acid side of neutrality, as is shown by the data expressed graphically in Figure 1. In the present author's main experiment (pre-treated part) there were four or five plants per two-litre jar, i.e., a minimum of 400 ml. of solution per plant, whereas Quispel, with three plants per 300 ml. jar, had only 100 ml. of solution per plant. The use of such a high ratio of plants to solution is detrimental in various ways but particularly in connection with the control of pH. The prevention of falls in pH to levels likely to interfere with nodulation or with the general growth of the plants is a serious problem in experiments involving plants growing rapidly on ammonium-nitrogen. As stated already, the present author's procedure was to restore the pH in each jar to the original value (6.3) every second day, but even in the space of only two days the pH had frequently fallen to 4.5. Quispel relied on a weekly replacement of the culture solution for the correction of pH, but in view of the poorer buffering properties of his solution and of his higher ratio of plants to volume of solution, it is an obvious possibility

FIGURE 1.



A comparison of the buffering power, on the acid side of neutrality, of Crone's and Hoagland's culture solution.

that in his jars containing ammonium-nitrogen the pH fell to levels likely to interfere with nodulation. Information on the effect of pH on nodulation is provided by Bond, Fletcher and Ferguson (1954). In their experience only 45 per cent. of inoculated Alder plants actually formed nodules when the pH of the culture solution was maintained at 4.2, while at pH 3.3 nodulation failed entirely.

There is thus a real possibility that the poor nodulation shown by Quispel's plants supplied with ammonium-nitrogen, and attributed by him to a direct effect of the combined nitrogen, was in fact mainly a pH effect. This interpretation is supported by Quispel's finding that in the presence of a small amount of nitrate-nitrogen nodulation was benefited. With this source of nitrogen the pH would tend to rise, but would not attain levels inhibitory to nodulation so rapidly as in the case of falling pH.

The conclusion is then that this particular experiment of Quispel's is of doubtful significance, and that the results must be treated with reserve.

So far consideration has been mainly of the weight of nodules on plants exposed to various nitrogen treatments. Interest also attaches to the numbers of nodules formed,

since such data would permit conclusions as to the effect of the various nitrogen treatments on the extent of infection of the root system by the nodule organism, and also on the mean size of the nodules. As mentioned already, in the non-legumes (and in a few legumes) the originally simple nodule forming at each point of infection soon develops into a branched, clustered structure (see Figure 3 in Part IV of this Thesis). In the present connection it is the total number of such clusters that is of interest, but except in quite young plants this number is obtained only with considerable difficulty since the clusters are often packed tightly together. In the present author's personal experiments with Alder this count was not attempted, but in the experiment reported by Bond, Fletcher and Ferguson (1954 - see Table 10 of the present Thesis), a count was made, with the present author's assistance, of infection points seven weeks after inoculation. The results were as follows:-

Mg.NH_4-N present per litre of culture soln..	Number of plants examined.	Mean number of infection points per plant.
0	20	17
10	12	34
50	12	33
100	12	28

Obviously the number of infection points is greater in the presence of combined nitrogen, which differs sharply from Quispel's finding (see Table 11 of this Thesis). It should be noted however, that the increase is only two-fold, whereas the dry weight of nodules showed a five-fold increase at the 10 mg. nitrogen level (see Table 10). Thus at this nitrogen level the nodule clusters must have been much larger in mean size than in the zero nitrogen plants, as is in fact clearly shown in Plate 14. The beneficial effect of combined nitrogen on absolute weight of nodules is therefore due more to increased size of nodules than to increased number of nodule clusters.

III. EXPERIMENTS WITH ULEX EUROPAEUS L.. GORSE, FURZE, WHIN.

METHODS

The general plan of the Gorse experiment was similar to that of the main Alder experiment. With one group of plants different levels of combined nitrogen were established and inoculation effected soon after the young plants had been set up in water culture, whereas with a second group a period of growth in the presence of a uniform amount of combined nitrogen was allowed prior to the commencement of the experiment proper.

As a matter of convenience the Gorse seeds were germinated under aseptic conditions as described in Part I of this Thesis. Seed (obtained from Thompson and Morgan (Ipswich) Ltd.) was surface-sterilised by the use of concentrated sulphuric acid and thereafter sown on petri-dishes containing nutrient agar. Seed sterilisation, though found from experience to be unnecessary with non-legumes, is advisable with legumes

since the nodule organism is frequently testa-borne. In addition, as pointed out in Part I of this Thesis, the acid treatment helps to reduce the proportion of hard seeds in Gorse.

The petri-dishes were placed under fluorescent lights for two weeks, after which the seedlings were transferred to water culture in Crone's solution (pH 6.3). At this stage the seedlings had well developed cotyledons and tap root, with the plumule still in the bud form. In the case of the no pre-treatment part of the experiment ammonium nitrogen was added two days after transplanting, three jars being established at each of the levels 0, 10, 50, 100 and 150 mg. of nitrogen per litre of culture solution. Inoculation was carried out the following day using fresh cultures of an effective strain (No.37) of the Gorse nodule organism. The growth on each slope was taken up in 10 ml. distilled water and the suspension so obtained applied to each root system and a further 1 ml. pipetted into each jar.

In the case of the pre-treated part of the experiment, after the plants had been set up in water culture they were all supplied with ammonium nitrogen at a level of 50 mg. per litre, and left to grow under these conditions for a period of three weeks. The solution in the jars was then replaced by fresh solution, and the differential

additions of ammonium-nitrogen now made to give three jars at each of the levels 0, 10,50 and 100 mg. nitrogen per litre. On the same day the plants were inoculated as above. At this stage these plants showed an over-all height of 10-12 cm., with 3-4 trifoliate leaves present. Plate 18 allows of a comparison of the stage of development of the plants at the time of inoculation in both parts of the experiment.

As with the Alder, the timing of the two parts of the experiment was arranged so that the plants of both parts were all inoculated on the same date (23rd.May, 1955) with the same inoculum.

The testing and where necessary the adjustment of pH of the culture solution was again carried out at two-day intervals. The nitrogen-content of the culture solution was also examined regularly, and on the basis of the data so obtained it can be stated that the variations in nitrogen content during the period of the experiment did not exceed 10-5, 50-40, 100-87, 150-140 mg. per litre respectively in the different series of cultures receiving combined nitrogen.

Plate 18.

Gorse seedlings at time of inoculation. The plant on the left has received pre-treatment with 50 mg.ammonium nitrogen per litre of culture solution for a period of three weeks.

x 1.

EXPERIMENTAL RESULTS

Nodules began to appear on the Gorse plants within a week of inoculation, and all plants were nodulated within a fortnight. The superiority in size of the pre-treated plants at the time of inoculation persisted through the experiment, and is illustrated in Plate 19. Harvest of both parts of the experiment commenced on the 26th.July, that is, some nine weeks after inoculation, and was completed within a few days.

The data obtained for the first part of the experiment, in which the plants received no pre-treatment with combined nitrogen, are summarised in Table 16 and typical plants are illustrated in Plate 20. Firstly it may be noted that the superiority in size of the plants supplied with combined nitrogen over those entirely dependent on nodule nitrogen was much less marked here than in the case of Myrica and Alnus, doubtless because in the legume the nodules develop more rapidly, and become able to meet the full requirements of the plant for nitrogen at an earlier date. Next it should be noted that there is a significant increase in nodule number with the move from 0 mg. to 10 mg. nitrogen and

Plate 19.

General views of the Gorse experiment in progress. The twelve jars to the right contain the group of plants which have received pre-treatment and these are noticeably larger.

TABLE 16.

Gorse experiment, first part (plants not given pre-treatment with combined nitrogen).
Mean data (per plant) obtained at harvest.

Mg.N per litre of solution	Shoot height cm..	Number of nodules	Mg.dry weight		Nodule wt. as % of plant wt..
			Nodules	Plant	
0	15	38	11	166	6.6
10	20	82	10	304	3.4
50	21	69	9	342	2.8
100	21	95	8	409	2.1
150	21	79	8	355	2.3

At the 0, 10, 50, 100 and 150 mg. nitrogen levels, 17,18,21,18 and 18 plants were harvested respectively.

Statistical treatment of these data is summarised in Table 16a.

TABLE 16a.

Summary of analyses of variance on data given in Table 16.

(a) Nodule weight as percentage of plant weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	23506	4	5877
Within groups	10443	88	119

Variance Ratio = 49

which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (N levels)	Difference (in %). Required	Observed
0 and 10	0.7	3.2
10 and 50	0.7	0.6
50 and 100	0.7	0.7
50 and 150	0.7	0.5

From similar analyses the following minimum differences required between means for significance were calculated:-

(b) Nodule dry weight. P= 0.05

Comparison (N levels)	Difference (in mg..) Required	Observed
0 and 10	2	1
10 and 50	2	1
50 and 100	2	1
50 and 150	2	1
0 and 50	2	2

(c) Nodule number. P= 0.05

Comparison (N levels)	Difference (in no..) Required	Observed
0 and 10	36	44
10 and 50	34	13
50 and 100	34	26
50 and 150	34	10

PLATE 20.

Gorse, no pre-treatment group. Typical plants after ten weeks of growth in water culture. From left to right they received 150, 100, 50, 10 and 0 mg. nitrogen per litre of culture solution respectively.

X 1/7.

this remains high with further increase in nitrogen level. The dry weight of nodules per plant shows a significant fall at the higher nitrogen levels (see Table 16a for significant differences), but at lower levels remains unaffected. Nodule dry weight when expressed as a percentage of the whole plant dry weight (Table 16, right hand column) shows a marked fall in the presence of 10 mg. nitrogen and thereafter tends to continue to fall, not all the differences being significant (see Table 16a).

The data for the second part of the experiment (pre-treated plants) are presented in Table 17 and typical plants are shown in Plate 21. The number of nodules shows a significant rise in the presence of 10 mg. nitrogen and thereafter drops markedly away. The same applies to the dry weight of the nodules per plant, and in these respects the data differ from those for the first part of the experiment. The nodule weight as percentage of whole plant weight attains a value, in the absence of combined nitrogen, approaching that shown in the first part of the experiment, and falls by the same amount in the presence of 10 mg. nitrogen but thereafter falls to lower levels than were shown in the first part.

In order to show more clearly the variation in mean

TABLE 17.

Gorse experiment, second part (plants given pre-treatment with combined nitrogen prior to inoculation).
Mean data (per plant) obtained at harvest.

Mg.N per litre of solution	Shoot height cm..	Number of nodules	Mg.dry weight Nodules	Mg.dry weight Plant	Nodule wt. as % of plant wt..
0	14	97	12	200	6.1
10	22	148	16	468	3.6
50	24	76	8	600	1.3
100	21	60	6	544	1.1

At the 0, 10, 50 and 100 mg. nitrogen levels, 21, 20, 21 and 19 plants were harvested respectively.

Statistical treatment of these data is summarised in Table 17a.

TABLE 17a.

Summary of analyses of variance on data given in Table 17.

(a) Nodule weight as percentage of plant weight.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	33000	3	11000
Within groups	6042	77	78

Variance Ratio = 141

which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (N levels)	Difference (in %) Required	Observed
0 and 10	0.6	2.5
10 and 50	0.6	2.3
50 and 100	0.6	0.2

From similar analyses the following minimum differences required between means for significance were calculated:-

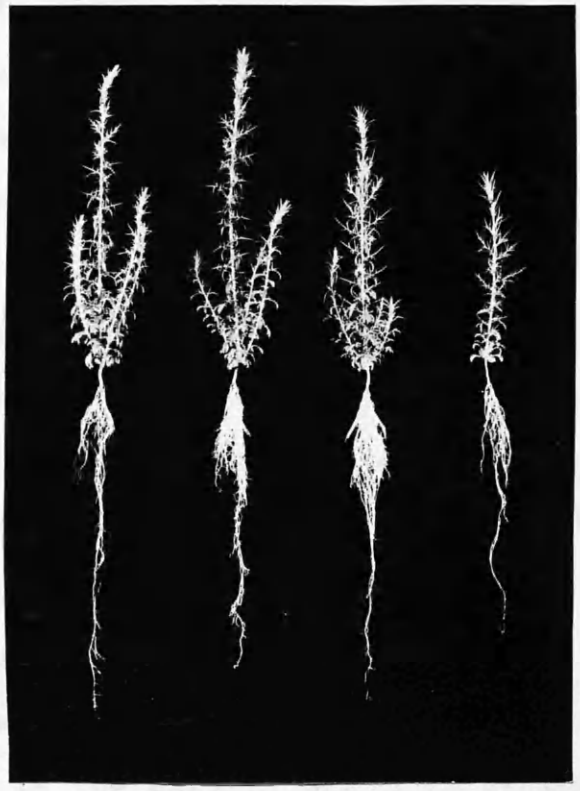
(b) Nodule dry weight. P = 0.05

Comparison (N levels)	Difference (in mg..) Required	Observed
0 and 10	2	4
10 and 50	2	8
50 and 100	2	2

(c) Nodules number. P = 0.05

Comparison (N levels)	Difference (in no..) Required	Observed
0 and 10	22	51
10 and 50	22	72
50 and 100	21	16

PLATE 21.



Gorse, pre-treated group. After three weeks of growth in culture solution containing 50 mg. ammonium-nitrogen per litre, these plants were inoculated and thereafter grown in varying amounts of ammonium-nitrogen for a period of nine weeks. From left to right are typical plants which have been grown with 100, 50, 10 and 0 mg. nitrogen per litre of culture solution respectively following inoculation.

X 1/6.

dry weight (and by implication in mean size) of individual nodules in the different treatments, the data in Table 18 (columns 2 and 3) have been calculated. It is seen that in both parts of the experiment the nodules developing in the absence of combined nitrogen are largest and heaviest; also it is seen that the effect of pre-treatment with combined nitrogen prior to inoculation is to more than halve the mean size of the nodules. Also in this Table nodule numbers are expressed per unit dry weight of plant as suggested by Wilson and Wagner (1935) to compensate for differences in plant size. In the case of the plants inoculated without pre-treatment the value obtained does not fall immediately in the presence of nitrogen as reported by Wilson and Wagner with clover, but rises with 10 mg. of nitrogen. A constant fall does occur in the case of the pre-treated plants, and here also the initial numbers are considerably larger.

TABLE 18.

A comparison of certain data from the two parts of the Gorse experiment.

Mg.N per litre of solution	Mg. dry weight per nodule		Nodule number per gram dry wt. of plamt.	
	A	B	A	B
0	.289	.124	229	485
10	.122	.108	270	316
50	.130	.105	202	127
100	.084	.100	232	103

A = No pre-treatment group.

B = Pre-treatment group.

D I S C U S S I O N

As noted already, the Gorse experiment described above was designed to provide a comparison with the non-legume experiments under similar cultural conditions, while at the same time using a legume previously uninvestigated with respect to the effect of combined nitrogen on nodulation.

As an example of previous authors' findings with other legumes the following extract from the results obtained by Thornton and Nicol (1936) is provided:-

Effect of nitrate-nitrogen on nodulation in Lucerne.

Mg.nitrate nitrogen supplied *	Whole plant dry weight gm..	Mean number of nodules per plant.	Mean length of nodules mm..
0	0.77	50	2.2
163	0.86	51	1.4
326	0.73	33	1.0
651	0.77	20	0.7
977	0.62	7	0.6

*Per pot of sand, each holding about 2 litres of culture solution.

It should be noted that Thornton and Nicol's levels of combined nitrogen should be reduced by half for

comparison with the present author's levels. In respect of number of nodules Lucerne appears to be more susceptible than Gorse to combined nitrogen. Thus with 326 mg. nitrogen supplied per pot in Lucerne the number of nodules is below that for plants with no combined nitrogen supplied, whereas with Gorse (Table 16) at the corresponding nitrogen level (150 mg.) the number is significantly above that for the zero nitrogen plants. Both experiments agree in showing a reduction in mean size of the nodules in the presence of combined nitrogen (see Table 18 for Gorse data). The Lucerne plants however, do not show the marked benefit, as measured by whole plant dry weight, of growth in the presence of combined nitrogen as is seen in the case of Gorse. Closer comparison of the experiments is prevented by differences in method and type of data obtained. In general it may be concluded that a fairly typical reaction for legumes has been shown by Gorse in this experiment.

The effect of pre-treatment with combined nitrogen prior to inoculation on the responses shown to a subsequent differential treatment with combined nitrogen have been noted in the previous section. It was shown that at the higher levels of combined

nitrogen (50 and 100 mg.) inhibition of nodulation was now greater. Also the mean individual weight of the nodules developing in nitrogen-free solution is now only half of that in the corresponding plants not pre-treated. Thus as with Alder it is again clear that the effect of combined nitrogen supplied prior to inoculation is similar to that of nitrogen currently supplied, and it may be noted how similar the pre-treated plants, grown after inoculation in 0 mg. nitrogen, are to those grown without pre-treatment in the presence of 10 mg. nitrogen, in respect of nodule number and size.

GENERAL DISCUSSION AND CONCLUSIONS

A comparison can now be made between the effect of combined nitrogen on nodule formation in the three species examined.

In the first instance the data for plants grown without pre-treatment with combined nitrogen prior to inoculation will be considered, the relevant figures being brought together in Table 19.

In section (a) of this Table it is seen that while in the legume the total weight of nodules per plant only changes significantly by dropping at the higher nitrogen levels, in the non-legumes the weight at first shows significant increase, reaching a maximum value at the 10 mg. level in Alder and at the 50 mg. level in Bog Myrtle. The fall which sets in subsequently would no doubt have continued had still higher levels of nitrogen been employed. It is shown in the case of Alder that the initial beneficial effect of combined nitrogen on nodule development is due more to increase in size of the nodule clusters than to increase in their number. The legume showed an increased number of nodules per plant in the presence of combined nitrogen,

TABLE 19.

Comparison of data obtained from experiments on different plant species receiving no pre-treatment with combined nitrogen prior to inoculation with their respective nodule organisms.

(a) A comparison of mean dry weight of nodules per plant.

Mg.N per litre of solution	Mg. dry weight of nodules per plant.		
	Bog Myrtle	Alder	Gorse
0	4	3	11
10	17	6	10
50	24	4	9
100	19	3	8

(b) A comparison of mean percentage weight of nodules per plant.

Mg.N per litre of solution	% weight of nodules.		
	Bog Myrtle	Alder	Gorse
0	12.2	5.3	6.6
10	5.4	1.7	3.4
50	4.2	1.0	2.8
100	3.6	0.7	2.3

Significant differences between means are listed in Tables 12a, 14a and 16a for Bog Myrtle, Alder and Gorse respectively.

but here the size of the nodules was much reduced so that there was no increase in weight of nodules.

This difference between legumes and non-legumes in respect of nodulation is doubtless bound up with the the further difference in respect of the effect of combined nitrogen on the growth of the plant as a whole. In Gorse the dry weight of plants supplied with 10 mg. combined nitrogen per litre was only about twice that of plants dependent on nodule nitrogen alone, whereas in Alder there was a six-fold and in Bog Myrtle a ten-fold difference. This can be attributed to the tardier completion of nodulation and attainment of full activity in the fixation of nitrogen in the non-legumes, the effects of this being particularly marked in relatively short-term experiments such as those under discussion. Obviously unless combined nitrogen exerts a very strong inhibitory effect on nodulation it is to be expected that this great increase in plant size will be attended by a higher total weight of nodules.

In Table 19b the data are assembled for the weight of nodules expressed as a percentage of total plant weight. As indicated earlier in the relevant tables, not all the successive reductions in the percentage value which accompany increases in the level of

combined nitrogen are statistically significant, but it is obvious that the general effect is much the same in all three species. In all cases combined nitrogen has relatively an inhibitory effect on nodule development, since the latter fails to keep pace with the enhanced development of the plant as a whole.

In passing it may be noted how much higher the percentage weight of nodules is for Bog Myrtle plants growing in a solution free of combined nitrogen than it is for corresponding plants of the two other species. This is not to be regarded as advantageous to the plants, since Ferguson and Bond (1953) showed that Bog Myrtle nodules, weight for weight, are less efficient in fixation than those of Alder and of Legumes.

In Table 20 the data for pre-treated plants, relatively high in nitrogen content at the time of inoculation, are gathered together for Alder and Gorse. In respect of Alder the effect of pre-treatment is that the higher levels of combined nitrogen (50 and 100 mg.) now have a more drastic depressing influence on the total weight of nodules formed. The same is true of Gorse, but there is here the new and rather puzzling feature that with 10 mg. nitrogen supplied the total dry weight of nodules is significantly increased, which was not the case without pre-treatment. The increase is due to a

TABLE 20.

Comparison of data obtained from experiments where plants of Alder and Gorse received pre-treatment with combined nitrogen prior to inoculation.

(a) A comparison of mean dry weight of nodules per plant.

Mg.N per litre of solution	Mg. dry weight of nodules per plant.	
	Alder	Gorse
0	28	12
10	54	16
50	20	8
100	7	6

(b) A comparison of mean percentage weight of nodules per plant.

Mg.N per litre of solution	% weight of nodules	
	Alder	Gorse
0	3.4	6.1
10	2.4	3.6
50	0.8	1.3
100	0.4	1.1

Significant differences between means are listed in Tables 15a and 17a for Alder and Gorse respectively.

greater number of nodules combined with only a slight reduction in the weight of individual nodules.

The data for percentage nodule weight indicate the enhanced inhibition of nodules by all levels of combined nitrogen following the pre-treatment in Alder, the effect being the same in Gorse so far as the higher nitrogen levels are concerned.

At the commencement of this section of the Thesis a comparison was attempted between the effects of combined nitrogen on the free-living nitrogen-fixing organisms and on root nodule plants. It was pointed out that in the former the effect is a suppression of fixation of elemental nitrogen. Presumably this is because the more abundant supplied ammonia dominates the catalytic machinery in the cell which was previously concerned with fixation. A similar consequence can be invisaged as occurring in root nodules, though Fogg (1955) has pointed out that here the combined nitrogen supplied in the rooting medium will have greater difficulty in reaching the nodule cells. Bond (1955) has shown by isotopic techinque that in fact nodules of Alder and Bog Myrtle continue to fix substantial amounts of elemental nitrogen despite the presence of combined nitrogen in the rooting medium. Though direct data for legumes are scanty

agricultural experience leaves little doubt that the position is similar here.

But even if there were a complete suppression of fixation, this could not of itself explain a reduction in nodule development, since it is not clear why this could not continue at the expense of supplied combined nitrogen. Much consideration has been devoted by previous authors to this problem in connection with the legumes. The literature was reviewed by Wilson (1940). The evidence indicates that the reduction in nodule development is due to internal changes in the correlative balance between the different parts of the plant, and that these are closely connected with the carbohydrate-nitrogen balance within the plant. Thus a number of authors have suggested that when combined nitrogen is available to a plant a rapid synthesis of protein takes place in the plant, lowering the level of carbohydrate in the tissues, which in turn is thought to restrict the development of nodules. Wilson (loc.cit.) himself prefers to suppose that the carbohydrate-nitrogen balance has an intrinsic effect on plant development, comparable to that exerted by a hormone.

Since there is ample evidence that in all essential respects, except for the identity of the

organism, the non-legume root nodule plants show a close similarity to the legumes, it is highly probable that the generally inhibiting effect of combined nitrogen on the relative development of nodules now demonstrated for the former plants originates as in legumes. The present experiments provide evidence that in the non-legumes the internal nitrogen balance of the plants is again of vital importance. Thus there are clear instances where nitrogen previously supplied affected the subsequent response of the plant to inoculation. To cite only one example, it is shown that in Alder whereas nodules develop to the extent of 5.3% of total plant weight when no combined nitrogen has been previously supplied, this figure falls to 3.4% when combined nitrogen is supplied to the plant for a period prior to inoculation. Both figures refer to plants supplied with nitrogen-free solution from the time of inoculation onwards.

Finally the relevance of the present experiments to plants growing under field conditions will be noted. Alder trees are commonly found growing in mixed woodland, and the ability of the other tree species to flourish indicates that plentiful combined nitrogen is available in the soil. Bog Myrtle has a greater tendency to occur in fairly pure stands,

but quite frequently these are interspersed by clumps of Willows. Despite the presence of combined nitrogen in the soil, indicated by the successful growth of the accompanying species, both Alder and Bog Myrtle appear to be well nodulated under field conditions, although data based on the examination of complete plants are not available and would be very difficult to obtain. This good nodulation is understandable in view of the present experiments, which show that moderate amounts of combined nitrogen lead to enhanced absolute development of nodules, although in a relative sense the nodulation is depressed.

SUMMARY

(1) The effect of combined nitrogen on the nodulation of Bog Myrtle, Alder and Gorse has been investigated by growing the plants in water culture to which varying amounts of ammonium nitrogen were added.

(2) In Bog Myrtle and Alder, in experiments in which ammonium-nitrogen was first supplied in differential amounts at the time of inoculation, the dry weight of nodules formed per plant was markedly increased in the presence of combined nitrogen. This increase was due more to greater nodule size than to an increased number of infection points. The plants themselves showed greatly increased growth in the presence of combined nitrogen and relative to the dry weight of the plants the proportion of nodules was increasingly depressed.

(3) In a corresponding experiment with Gorse the number of nodules per plant was increased in the presence of combined nitrogen but the dry weight of nodules per plant was reduced. This difference in behaviour between the non-legumes and legumes is considered to be bound up with the much greater increase in the size of the plant as a whole in the case of the non-legumes in the presence of combined nitrogen.

(4) In another experiment Alder plants were grown for

a 'pre-treatment' period in the presence of combined nitrogen prior to inoculation and the establishment of differential nitrogen levels. The higher levels of nitrogen had now a more drastic depressing influence on the total weight of nodules formed. The effect of pre-treatment was thus essentially similar to that of nitrogen currently supplied, the higher nitrogen balance in the pre-treated plants reacting unfavourably on the subsequent development of nodules. Apart from an unexplained initial increase in nodule weight the effect of such pre-treatment on Gorse was similar to that on Alder.

(5) The present experiments provide evidence that, as shown by previous investigators in legumes, in the non-legumes also the internal nitrogen balance of the plants is of vital importance with respect to nodulation.

(6) The results with the non-legumes are consistent with those of previous experiments in Glasgow and differ from those obtained by another investigator in which a much greater depression of nodulation by combined nitrogen was reported. A possible explanation of this latter result is given.

(7) Field observations are shown to be understandable in view of the present results.

PART III.

The importance of the oxygen factor in the initiation, growth and function of the nodules on the roots of Alder plants.

PART III.

C O N T E N T S

INTRODUCTION

Both in legumes and non-legumes the development and functioning of the root nodules, and thus of plants dependent on nodule nitrogen, is subject to control by a number of external factors, including the pH of the rooting medium, the relative abundance of combined nitrogen and the availability of mineral nutrients. The factor to be considered here is that of oxygen supply. There were three reasons for the decision to investigate the effect of this factor on nodular activity and plant growth in Alder. Firstly that the results might be of ecological interest by explaining field observations, secondly that light might be thrown on the infection and on the fixation processes, and thirdly that information on the effect of oxygen supply was needed in connection with the culture of Alder plants.

There have been several investigations on the effects of growing nodulated leguminous plants at different levels of oxygen supply. Sometimes the whole plant has been exposed to adjusted oxygen tensions and in other cases the root system only. Effects specific to nodulated plants can be identified by comparison with

parallel experiments with non-nodulated plants supplied with ample combined nitrogen. It is a reasonable deduction that such effects are due primarily to responses of the nodules to varying oxygen supply. In such long term experiments the oxygen effect is liable to represent an integration of several separate effects, including that on the infection and nodule initiation stage, on the subsequent growth of the nodules and on the efficiency in nitrogen fixation of the mature nodules.

Wilson and Fred (1937) grew clover wholly enclosed in large aspirator bottles, the atmospheres within which were adjusted to contain different levels of oxygen. Growth of nodulated and combined nitrogen plants was reported to be essentially unaffected by reduction of oxygen level until a partial pressure of 0.05 atmospheres of oxygen was attained. Below this value growth decreased similarly for both types of plant, and it was concluded that nodulated plants and the nodules themselves have no special requirement for oxygen. These findings were widely accepted and led to the view that the oxygen factor was relatively unimportant in nitrogen fixation.

The matter was further investigated by Bond

(1951a) and Ferguson and Bond (1954), an abstract of their results for Soya Bean and clover being provided in Table 21. A root enclosure-culture technique was found to be preferable to whole plant enclosure as previously used by Wilson and Fred (1937), since superior conditions for shoot growth are provided. The results show that nodulated plants are markedly more sensitive to oxygen reduction than plants supplied with combined nitrogen, leading to the conclusion that the effective functioning of nodules requires a higher external level of oxygen than does that of roots. In Soya bean the number of nodules per plant was unaffected by the lowering of the oxygen level to 5% while in Clover the number was greater at 5% than in air, suggesting that the early stages of nodulation are relatively insensitive to or actually favoured by reduced oxygen supply in these particular legumes.

Thus by providing improved growth conditions, responses to variation in oxygen supply were detected in the Glasgow experiments which had not shown themselves in the experiments of Wilson and Fred. The Madison workers have recently modified their original conclusions in the light of experiments using isotopic nitrogen with detached Soya bean nodules,

TABLE 21.

Effect of oxygen supply on the relative dry weight attained by clover and soya bean plants.

% oxygen supplied	Clover		Soya bean	
	Nodulated	Non-nodulated	Nodulated	Non-nodulated
21	100	100	100	100
12	74	100	84	93
5	50	71	64	94
1	9	21	-	-

Results from Ferguson and Bond (1954).

bringing them into line with those obtained in Glasgow. Thus Burris, Magee and Bach (1955) showed that fixation by these nodules fell markedly as the pO_2 was decreased below 0.20 atmospheres while above the latter value, fixation increased up to 0.50 atmospheres and was then reduced.

The oxygen relation of the nodulated non-legumes has received little previous attention. Observations on Alder trees growing under natural conditions have shown that nodulation of the root system is often so abundant in the upper layers of the soil as to lead to the conclusion that at deeper levels nodules must be much sparser. The question then arises as to whether such a condition is due to nodule development being highly aerobic or to the distribution of the nodule organism in the soil. A practical instance is given by McVean (1953), in a paper concerned chiefly with the distribution of Alder in the British Isles, who reports the case of an Alder tree in a habitat where the water table was above the surface, between Molinia tussocks, throughout the year. On this tree, nodules were found to be present only on the parts above the level of the water table.

Virtanen and Saastamoinen (1936) noted that Alder nodules in non-aerated water culture tended to develop

near to the surface of the solution, a fact which they interpreted as indicating a relatively high oxygen requirement on the part of the nodules. However it is within the present author's experience that on reinoculating an Alder plant in its second year of water culture, new nodules may be formed fairly low down in the culture solution though these never attain the size of the nodules near to the surface of the solution. With another nodulated non-legume, Myrica gale, Bond (1952) found, using a root enclosure technique, that nitrogen fixation was curtailed by a reduced external oxygen supply to nodulated roots and concluded that the fixation here was a distinctly aerobic process. Ferguson and Bond (1953) showed that forced aeration is specifically beneficial to the development of nodulated Alder plants. They concluded, in view of the fact that after eight weeks of growth the oxygen level in the non-aerated jars had fallen only slightly below that in aerated jars, that the initial effect of the air current was to disperse the organism over the newly formed parts of the root system and thus increase the number of nodules. A subsequent effect was probably that the additional oxygen favoured the activity of the submerged nodules. This experiment forms a background

to the work now to be described.

As noted already information on the effect of oxygen supply on the functioning of already-formed nodules is derivable from experiments on detached nodules. Such information may be of assistance in explaining the results of long-term growth experiments. A considerable advance in this connection was made in the recent demonstration by the Madison group of workers that by the use of an isotopic nitrogen technique a limited persistence of fixation after detachment of legume nodules could be detected and measured. At Glasgow this work has been extended to include non-legume nodules. These observations on detached nodules will be considered in Part IV of this thesis.

In the work now to be described information on the effect of varying oxygen supply on the initiation, development and functioning of Alder nodules has been sought by means of experiments similar to those of Ferguson and Bond (1954) on clover. The root-enclosure technique has been employed. Observations were made both on nodulated plants in nitrogen free solution and on non-nodulated plants supplied with ample combined nitrogen, to permit the detection of oxygen effects specific to the nodules, as already explained.

Principally for reasons connected with the supply of gas mixtures the investigation of nodulated plants was carried out in two stages. A first experiment related to the effect of oxygen supply on the initiation of nodules, while in a second experiment the effect of oxygen supply on the further growth of already nodulated plants was studied.

METHODS

The principle of the method employed in the following experiments is that the plants are grown in culture solution in such a way that their root systems are to a large extent sealed off from the natural atmosphere and exposed to gas mixtures of varying composition. Thus while the shoots grow in normal greenhouse conditions the roots are in culture solution through which is passing a continuous stream of an oxygen-nitrogen gas mixture of known proportions.

Raising of plants.

Alders for use in these experiments were raised following the same general procedure as previously described in Part II of this Thesis. Seed, collected from Milngavie in November 1953, was sown in sterilised sand and transferred to water culture in Crone's nitrogen free culture solution (previously detailed) when the seedlings reached the 1-2 leaf stage.

In preparation for the experiment on the further growth of already-nodulated plants, the plants were grown to a shoot height of approximately two centimetres in ordinary water culture jars. Inoculation, except

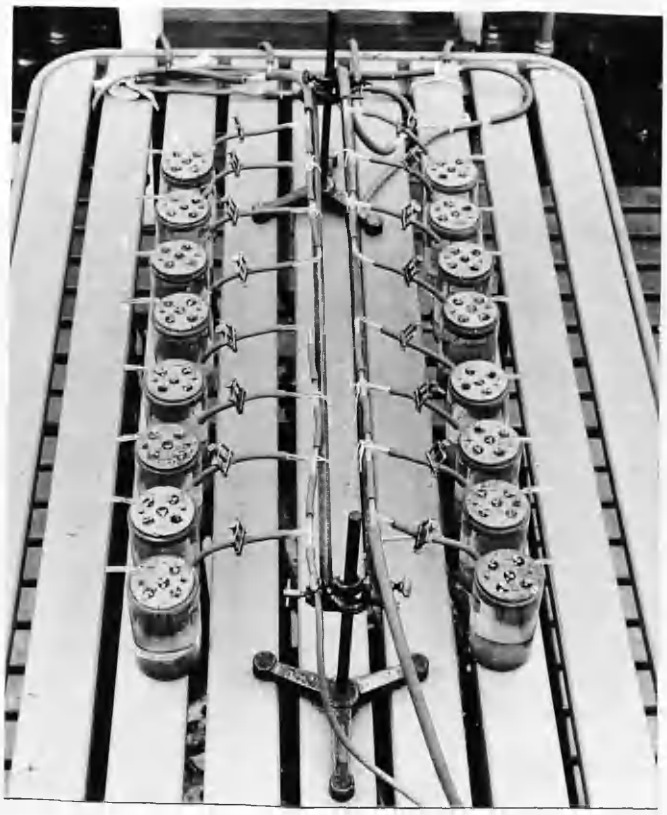
on those plants required to be non-nodulated, was performed at an early stage so that by the time the shoots had attained the necessary height the roots were already relatively well nodulated.

Plant containers for the gas-flow period.

For the nodule initiation experiment, wide glass jars (8 x 13 cm.) holding 400 ml. of culture solution were fitted with waxed cork stoppers into which five seedlings could be inserted. The seedlings were then sealed in with 'Plasticine' around the hypocotyl. A gas inlet capillary tube was provided to each jar. Small uniform bubbles of the gas mixtures could then be released at the foot of each jar through the obliquely ground end of a length of very fine capillary attached to the gas inlet tube. Another short length of capillary tube provided a gas outlet. Such jars and their general arrangement in the greenhouse may be seen in Plate 22. A black wrapper was tied around each jar, this only being removed to check the rate of bubbling and the level of the culture solution.

In the experiment on the further growth of already-nodulated plants, the plants were set up individually in large test-tubes (20 x 3.5 cm.) each of which held some 200 ml. of culture solution. The tubes were closed with three-holed rubber stoppers, one hole being for

PLATE 22.



Alder nodule initiation experiment showing arrangement of culture jars with gas-flow lines fitted.

the plant, a second for the gas exit tube - a short length of glass capillary - and the third for the gas inlet tube. The latter led to a cubical earthenware diffuser block at the foot of the test-tube. The tubes were placed in racks of six and attached to a gas manifold. Part of the general arrangement of such an experiment in the greenhouse is shown in Plate 23. Similar tubes were used by Ferguson and Bond (1954) for their root enclosure experiment on red clover.

The gas mixtures employed.

Oxygen-nitrogen mixtures containing 21, 12, 5 and 1% oxygen respectively, were supplied from 165 cu.ft. cylinders obtained from the British Oxygen Company. The actual oxygen levels in the cylinder gases were checked by means of the Haldane gas analysis apparatus, the results obtained being shown below:-

Desired % oxygen.	Actual % oxygen content. (Mean of six separate gas samples) 1954 cylinder	1955 cylinder
21	20.8	20.9
12	9.7	11.9
5	5.1	5.1
1	1.1	1.7

Slight departures from the desired oxygen percentages were recorded, the most serious being in the 1954 cylinder supposedly containing 12% oxygen where the

PLATE 23.

Part of the greenhouse layout of the Alder growth experiment Part II, non-nodulated plants, showing arrangement of gas-flow lines on racks of culture tubes.

percentage was found to be actually 9.7. With this exception the departures from the desired figures are unimportant and do not call for changes in designation.

After leaving the cylinders the gases were scrubbed by passing through bottles of culture solution and then dried through calcium chloride towers. Subsequently the gases were passed into manifolds and then into the culture vessels. A manometer was included in each circuit as a guide to the pressure obtaining. Gas flow into the actual culture vessels was regulated by screw clips on the rubber tubing.

Subsequent management of gas-flow cultures.

Gas tightness of the systems was checked every two days by applying a drop of water to the end of the gas exit tube fitted to each plant container. Formation and bursting of a bubble was taken to indicate that gas was not leaking out by an easier route in any other part of the container in question.

The rates of gas flow were suitably adjusted to keep the culture solution as nearly as possible at the appropriate equilibrium value as regards dissolved oxygen (data on this aspect will be given later). The Winkler process was used to determine dissolved oxygen, employing the 1 c.c. syringe pipette described by Fox and Wingfield (1938).

In all gas-flow experiments the complications which would have arisen in the gas supply lines prevented randomisation of jars and tubes. However, a fortnightly change was made in the relative position of each group of plants.

The culture solution was maintained at pH 6.3 during the experiments (except in one case detailed later) and frequent adjustments were made by the addition of acid or alkali to the plant containers as required. Solution changes were made at three-weekly intervals.

Harvest procedure.

In the nodule initiation experiment the nodules on each plant were counted under a binocular dissecting-microscope. Counts were made of the actual number of infection points (i.e., the points on the root system actually invaded by the nodule organism and from which branched nodules would eventually develop as the plant matured), and of the number of nodule lobes formed at each.

In the experiment on the further growth of already nodulated plants, dry weights of individual plants were obtained by drying them overnight in an oven at 95°C. and nitrogen estimations were made by Kjeldahl analysis.

Statistical analysis of various data was carried out and is presented with the results.

EXPERIMENTAL RESULTS

(a) NODULE INITIATION EXPERIMENT.

For this experiment Alder seed was sown on the 29th.June 1955 and seedlings at the one leaf stage transplanted directly into gas-flow jars on the 23rd. July. The plants were sealed into the stoppers on the 25th.July and the gas-flow started the following day.

An inoculum was prepared by grinding 2 gm. of nodules from greenhouse plants in 10 ml. distilled water and on the 27th.July, within ten minutes of its preparation, this inoculum was brushed on to the root systems of individual plants. For this operation the stoppers were momentarily removed from the jars and sealed in again immediately. During the period of inoculation the gas was allowed to flow rather more rapidly than when the experiment was sealed up and running normally. A second inoculation took place three days later but on this occasion 0.5 ml. of the inoculum was added to each jar by the removal of the gas exit tube. Thus there was little interference with oxygen balance in the solution in each jar.

Four jars were set up at each of four oxygen levels, viz., 21, 12, 5, 1% and, as already noted, the general arrangement of these jars in the greenhouse is shown in Plate 22.

At the higher oxygen levels nodule initials marked as red swellings on the roots were first noted ten days after inoculation. Some further days elapsed before nodules were detectable at 1% oxygen, and in these plants the nodules were white in colour and thus free of anthocyanin.

When the plants were harvested on the 30th August, some five weeks after inoculation, all were at the four-leaf stage with shoots approximately 1-2 cm. in height. Although, as will be noted below, there were notable differences in nodule development at the different oxygen levels, the commencement of fixation was too recent for these to be reflected in the growth of the plants.

The counts of infection points and of nodule lobes are presented in Tables 22 and 22a. Each lowering of the oxygen level is accompanied by a significant reduction in the number of infection points and lobes. The mean number of lobes per nodule cluster stays steady at approximately 1.5. The nodules were too immature to be detached and their weight obtained,

TABLE 22.

Effect of the oxygen supply to the root system on nodule initiation in Alder.

Percentage oxygen supplied	Mean no. per plant.		Relative change.	
	Infection points	Nodule lobes	Infection points	Nodule lobes
21	10.6	16.7	100	100
12	7.9	11.2	75	67
5	5.2	8.5	49	51
1	2.2	3.6	21	21

The number of plants harvested at the 21, 12, 5 and 1% oxygen levels was 18, 20, 17 and 19 respectively.

Table 22a.

Summary of analysis of variance on the number of infection points formed on Alder seedlings after five weeks growth at varying oxygen levels.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	720	3	240
Within groups	368	70	5

Variance Ratio = 4.8
which is significant at P = 0.01

Minimum differences required between means for significance.

P = 0.05.

Comparison (O_2 levels)	Difference (in no.) Required	Observed
21 and 12	1.5	2.7
12 and 5	1.5	2.7
5 and 1	1.5	3.0

but there is no doubt that the amount of nodule tissue per plant fell with diminishing oxygen in much the same proportion as the number of infection points.

The experiment thus demonstrates that curtailment of oxygen supply severely reduces the extent of infection of the roots by the nodule organism (or at any rate, infection followed by nodule initiation), so that under such conditions the plant is poorly nodulated.

(b) THE EFFECT OF OXYGEN SUPPLY ON THE FURTHER GROWTH OF ALREADY NODULATED ALDER PLANTS WITH PARALLEL STUDY OF NON-NODULATED PLANTS.

This experiment comprised two parts; the first was conducted in 1954 using nodulated plants, and the second in 1955 using non-nodulated plants supplied with combined nitrogen. It was hoped that by a comparison of the two parts the specific effect of oxygen tension on nodule development and function might be detected.

Part 1. Nodulated plants.

Alder seedlings from a sowing of the 27th. March 1954 were transplanted into jars of nitrogen-free Crone's solution on the 7th. May and inoculated two days later with an inoculum prepared by grinding

15 gm. of field nodules in 100 ml.distilled water. The first nodules appeared 2 - 3 weeks after inoculation.

Four weeks after transplanting the pH of the culture solution was lowered from its initial value of 6.3 to 5.5, this being the first step in lowering the pH to the range found by Ferguson and Bond (1953) to be most suitable for Alder growth subsequent to nodulation. At this stage also a small amount of combined nitrogen was supplied to each jar at the rate of 2 mg. ammonium-nitrogen per litre. This was a temporary measure to sustain the plants until fixation became really active, and the solution was changed back to the nitrogen-free form two weeks later. The pH was then lowered to 5.0, the value maintained throughout the duration of the experiment.

Transfer to gas-flow culture tubes took place on the 14th.June and gas flow was commenced on the 18th.June. Eighteen nodulated plants were set up at each of four oxygen levels viz., 21, 10, 5 and 1%. At this time the plants were approximately 2 cm. in shoot height and bore 4-5 true leaves. Several small clusters of nodules were present on each plant. A harvest of twelve such plants showed them to have a mean dry weight of 35 mg. and a mean total nitrogen content of 0.44 mg.. These values

were later used in calculating the plant increments made during the period of the actual experiment.

Two comparable non-nodulated plants were also set up at each oxygen level as controls to confirm that there was no trace of free ammonia in the gas mixtures which might provide a source of combined nitrogen to the plants in culture. It was soon evident that these plants were receiving no nitrogen and they eventually died off after showing no further development.

In the course of the experiment determinations of dissolved oxygen in the culture solution showed that the oxygen levels remained fairly close to the equilibrium values appropriate to the different gas streams. The values obtained are given in Table 23.

A difference in growth between plants at the different oxygen levels became noticeable after two weeks of gas-flow. Plants at the lower oxygen levels tended to lag behind in growth and to show yellowing of the leaves. With the passage of time these differences became accentuated, and at harvest commencing on the 14th.August, after eight weeks of gas-flow culture, there were pronounced differences in size, expecially between the plants at the two extreme oxygen levels. Typical plants at harvest

TABLE 23.

Results of determinations of dissolved oxygen in culture tubes. These results are expressed as a percentage of the oxygen content of water in equilibrium with air at the temperature prevailing at the time of the determination.

% oxygen supplied	Expected oxygen content of soln.	Values observed. Days of gas-flow. 12	26
21	100	93	92
10	48	43	43
5	24	21	17
1	5	3	3

Each result is the mean value of three determinations. Samples were drawn in each case from the centre of the culture tubes.

are shown in Plates 24, 25 and 26.

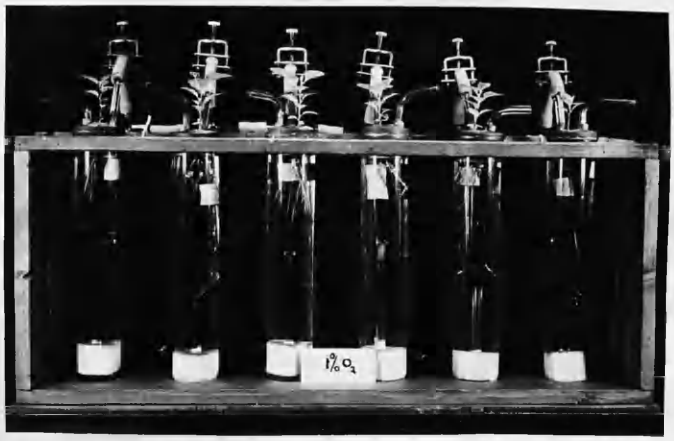
In Table 24 the increases in size, dry weight, and nitrogen content shown by the four groups of plants during the period of gas-flow are presented, the relevant statistical treatment being summarised in Table 24a. It is clear that as the oxygen supply was curtailed, growth, in all its attributes, fell also. Actually the difference in dry weight increase between the 10% and 5% oxygen levels fails to attain significance, but there is little doubt that this is because these oxygen levels were closer together than had been intended, and the general tendency is for growth to fall continuously. The dry weight increment (whole plant) at 1% oxygen is seen to be less than a quarter of that at 21%, and that in total nitrogen, indicative of the amount of nitrogen fixed, less than a fifth.

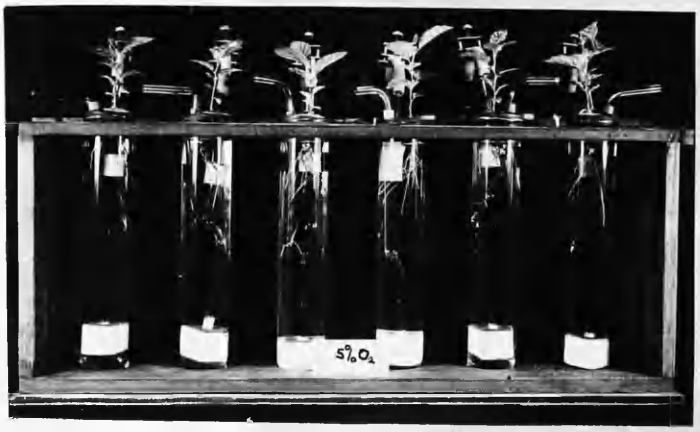
Owing to the necessity of harvesting the large number of plants involved as quickly as possible, so that the data might be comparable, it was not possible to count the nodule clusters. It may however be assumed that the increase in nodule dry weight during the experiment was due chiefly to the further growth of nodule clusters present at the commencement. It is obvious from the data in

PLATE 24.

Alder growth experiment.
Part I, nodulated plants.

Typical rack of plants from each oxygen level (1, 5, 10 and 21% O_2).





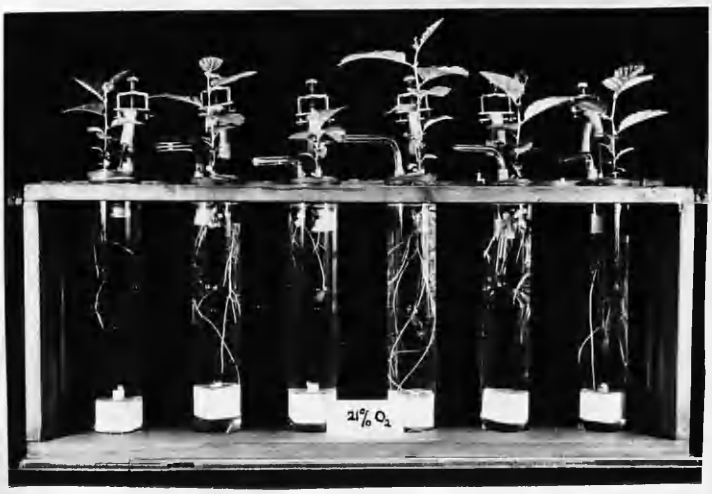
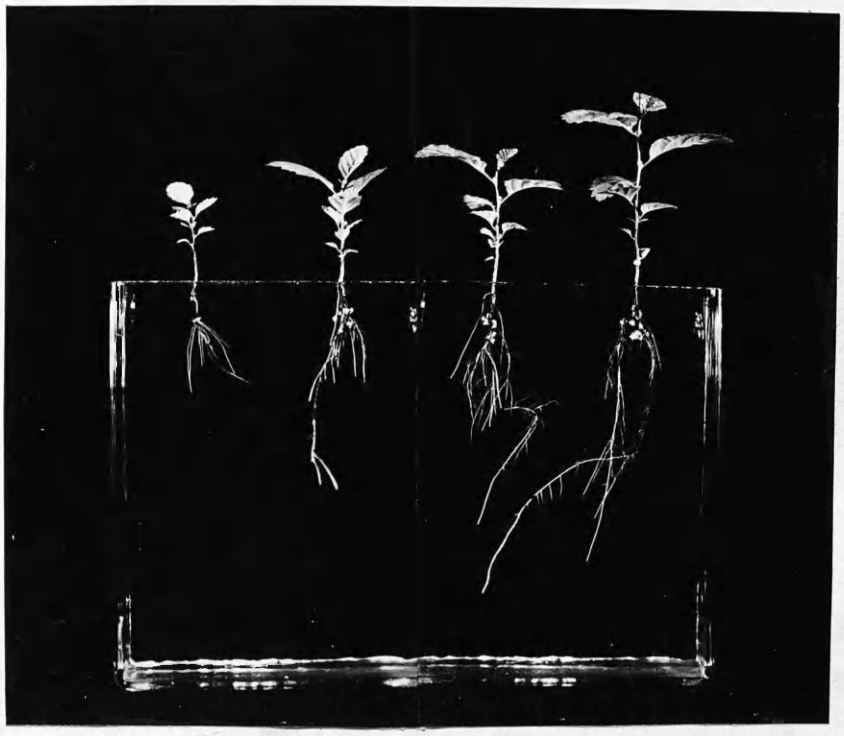


PLATE 25.

Alder growth experiment. Part I, nodulated plants. One typical tube from each oxygen level at close of period of gas-flow. From left to right, 1, 5, 10 and 21% oxygen has been supplied to the nodulated root systems.

PLATE 26.



Alder growth experiment. Part I, nodulated plants. Typical plants which were supplied with from left to right, 1, 5, 10 and 21% oxygen respectively.

X $\frac{1}{4}$.

TABLE 24.

Increases in height, dry weight and nitrogen content shown by nodulated Alder plants grown at different oxygen levels.

Percentage oxygen supplied	Number of plants	Shoot height (mm.)	Mg. dry weight		Nitrogen content (mg.)
			Nodules	Whole plant	
21	16	66	20	328	8.8
10	18	53	11	212	5.6
5	17	48	9	183	4.7
1	18	22	4	78	1.7

The values shown in the above table were obtained by subtracting from the data yielded at harvest after eight weeks of gas-flow the corresponding data for plants at the beginning of the gas-flow period. These were as follows:-

Shoot height = 20 mm..
Dry weight nodules (estimated) = 1 mg..
Whole plant dry weight = 35 mg..
Nitrogen content = 0.44 mg..

TABLE 24a

Summary of analysis of variance on whole plant dry weight increments the means of which are given in Table 24.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	535386	3	178462
Within groups	800492	65	12315

Variance Ratio = 14.5
which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (O_2 levels)	Difference (in mg.) Required	Observed
21 and 10	76	116
10 and 5	75	29
5 and 1	75	105

Table 24 that this growth was severely reduced at the lower oxygen levels.

In addition to the data provided in Table 24, it may be noted that the mean percentage nitrogen contents of the plants in order of decreasing oxygen supply were 2.53, 2.41, 2.34 and 1.83. These are consistent with the visual evidence of nitrogen deficiency at the lower oxygen levels already noted.

Further consideration of the results of the experiment will be postponed until the data for non-nodulated plants have been presented.

Part II. Non-nodulated plants.

The same general technique as used with nodulated plants was followed except that inoculation was omitted. Culture jars and tubes were sterilised before use to remove any possible trace of the Alder nodule organism. No plants became inadvertantly nodulated (and in all general handling of Alder, no plant not inoculated in the usual way has done so within the experience of the present author).

Seedlings from a sowing of the 31st.March 1955 were

transplanted into Crone's culture solution containing ammonium sulphate supplied at the rate of 50 mg. nitrogen per litre of solution. Plants were grown in this manner until they had attained a stature similar to those at the start of gas-flow in the nodulated plants experiment, that is of shoot height 2-3 cm., and bearing 4-5 true leaves. Thus the 1955 plants were transferred to tubes on the 3rd.June, the solution again containing 50 mg. ammonium nitrogen per litre. Fourteen plants were set up at each of the oxygen levels viz., 21, 12, 5 and 1%. Gas-flow was commenced on the 4th.June and continued until the 9th.July, some five weeks later. The nitrogen level in the culture solution was raised to 100 mg.per litre on the 4th.July to ensure that growth was not retarded in any way by shortage of nitrogen.

Plant growth in this experiment was very rapid and as already noted gas-flow culture was maintained for only five weeks by which time the plants were comparable in development to the nodulated plants similarly treated but for a longer period in 1954.

Determinations of dissolved oxygen were again made in the case of this experiment and the results obtained were similar to those already given for 1954. Larger root systems were formed in the 1955 plants

and it was found necessary late in the growing period to increase the gas-flow considerably to maintain the required oxygen levels.

Plants similar to those sealed into the gas-flow tubes at the commencement were found to have a mean dry weight of 25 mg. and this value was later used to determine dry weight increments accrued during the experimental period.

Typical plants at harvest are shown in Plate 27 and from this the similarity of development at the three higher oxygen levels may be noted. This similarity is further shown in the harvest data given in Table 25. The summary of the analysis of variance relative to the dry weights is given in Table 25a. It will be seen that the dry weight remained statistically unaffected by oxygen reduction until the final reduction from 5 to 1%. These plants thus display a markedly lower sensitiveness to curtailment of oxygen supply than do nodulated plants.

In general it may be said of these plants that reduction in development due to oxygen supply only becomes detectable at the lowest oxygen levels. A comparison between these and the nodulated plants will be made in the discussion to follow.

PLATE 27.

Typical plants of the Alder growth experiment. Part II, non-nodulated plants supplied with ample combined nitrogen. During growth their root systems were exposed to, from left to right, 1, 5, 12, 21% oxygen respectively.

X 1/6.

TABLE 25.

Increases in height and dry weight shown by non-nodulated Alders growing in the presence of combined nitrogen at different oxygen levels.

% oxygen supplied	Shoot height (mm..)	Whole plant dry wt.(mg.)
21	100	412
12	98	393
5	95	346
1	73	215

Fourteen plants were harvested at each oxygen level and the data shown above were obtained by subtracting from the data obtained at this harvest after five weeks of gas-flow the corresponding data for plants at the beginning of the gas-flow period. These were as follows:-

Shoot height = 25 mm..

Whole plant dry weight - 25 mg..

TABLE 25a.

Summary of analysis of variance on whole plant dry weight increments the means of which are given in Table 25.

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between groups	331479	3	110493
Within groups	354024	52	6808

Variance Ratio = 16.23

which is significant at P = 0.001

Minimum differences required between means for significance.

P = 0.05

Comparison (O_2 levels)	Difference (in mg.) Required	Observed
21 and 12	63	19
12 and 5	63	47
5 and 1	63	131

DISCUSSION

It has been shown in the nodule initiation experiment that a high oxygen level around the Alder root system favours infection by the nodule organism and thus leads to more abundant nodulation. Existing knowledge of the infection process in non-legumes is too meagre to permit of much further analysis of this finding. Fletcher (1955) attempted to elucidate the method of infection in Bog Myrtle, which is presumably similar to that in Alder, and demonstrated that an early effect of inoculation was a deformation of root hairs reminiscent of that occurring in legumes. Presumably this is preceded by a multiplication of the nodule organism in the rhizosphere under the influence of specific root secretions, with a production in the organism of special infective stages not present in the crushed nodule inoculum. The actual invasion of the root tissues by the organism could not be traced by Fletcher and the next undoubted event was the appearance of nodule initials in the pericycle of the parent root, suggesting along with other evidence, that the nodule represents a modified lateral root.

In the present experiments the curtailment of oxygen supply might restrict the multiplication of the organism either outside or inside the root, assuming the nodule organism to have a highly aerobic nature. It is unlikely that the critical effect of reduced oxygen supply was on root hair development, since the nodules tended to develop on the roots already existing prior to inoculation and to the establishment of the differential oxygen treatment. A more detailed study will be necessary before the pronounced oxygen effects now reported can be explained. Attention could then be given to the question whether the anthocyanin pigments which are associated with nodule initials in high but not in low oxygen conditions (see page 112) play any important part in nodule development.

In view of the present author's results it becomes probable that the beneficial effect of forced aeration on the nodulation of Alder plants observed by Ferguson and Bond (see page 103) and ascribed by them to a stirring effect, was in fact due to improved oxygen supply. Also, with reference to another aspect mentioned in the Introduction (page 101), nodulation in Alder is obviously a more highly aerobic process than in the case of the legumes Soya bean and Clover.

The results of the experiments relating to the effect of oxygen supply on the further growth of Alder plants will next be considered. It is quite clear that nodulated plants, dependent on nodule nitrogen for their growth, are much more sensitive to curtailment of oxygen supply than are non-nodulated plants supplied with combined nitrogen in amount ample for growth. This is evident from Table 26, where data on a relative basis are presented for the two types of Alder plant. A resemblance is shown to similar data previously obtained for legumes and datailed in Table 21.

The reduced growth eventually shown (below 5% oxygen) by the non-nodulated Alder plants is no doubt due to interference with the respiratory activities of the roots, which in turn could curtail salt uptake. With 5% or more of oxygen present these effects were insufficiently exerted for a significant reduction in growth to be produced. Since there is no reason to suppose that the roots themselves of nodulated plants differ materially in their oxygen requirements from those of non-nodulated plants, then it follows that the greater sensitiveness of nodulated plants to reduced oxygen supply is due to a relatively high oxygen requirement on the part of the nodules. That the nodules were, at reduced oxygen tension, giving

TABLE 26.

The relative development of nodulated and non-nodulated Alder plants, the latter supplied with combined nitrogen, when grown with their roots at varying oxygen tensions.

Values are based on dry weight and shoot height increments.

% oxygen supplied	Nodulated plants		Non-nodulated plants	
	Dry wt..	Shoot ht..	Dry wt..	Shoot ht..
21	100	100	100	100
12	-	-	95	98
10	65	80	-	-
5	56	73	84	95
1	24	33	52	73

inadequate support to the plant is indicated by the visual and analytical evidence of nitrogen deficiency in these plants, already noted.

The further analysis of this impairment in the provision of fixed nitrogen by the nodules to the plant at reduced oxygen supply is complicated by the fact that both formative and functional effects may be involved in this long-term experiment. The effect might be simply that the growth of nodules cannot take place adequately under conditions of reduced oxygen supply, so that the physical basis for fixation is not provided. On the other hand, the oxygen effect might be on the fixation process. If the earlier-developing nodules were unable to fix nitrogen at the necessary rate, then the growth of the plant and of later developing nodules would suffer. The nodule initiation study, which dealt with events mostly prior to the onset of appreciable fixation, indicated that oxygen supply is of special importance for nodule growth. Further consideration of this problem will be given following the presentation of the data on detached nodules to be given in Part IV of this Thesis.

Fixation is still operative at the lowest oxygen level supplied. In this case one must conclude that

conditions within the nodules must be almost totally anaerobic. Such fixation as does occur is possibly taking place in the surface layers of the nodule. Consideration of these plants in particular raises the question of the possible downward diffusion of oxygen through the interior of the plant. In the absence of any direct evidence on this, it may be concluded from the pronounced curtailment of growth demonstrated at low oxygen levels that such downward diffusion did not occur to any considerable extent.

The overall result of the foregoing experiments is that the oxygen factor is of considerable importance in the initiation, growth and/or function of Alder nodules. There is a general similarity to the effects observed in legumes by previous workers. It may be concluded that nodulation in Alder will be restricted in any environment where the oxygen concentration is lower than that in air.

S U M M A R Y

The effect of the supply of oxygen to the root systems of Alder plants has been studied in experiments in which oxygen-nitrogen gas mixtures of different composition were bubbled through the culture vessels in which the plants were growing in water culture.

In an experiment in which the root systems were inoculated with the nodule organism in the presence of different levels of oxygen, it has been shown that the the number of nodules is progressively reduced as the oxygen level is lowered from the normal 21%.

In a second experiment the effect of variation of oxygen supply on the growth of already nodulated plants and of non-nodulated plants, the latter supplied with combined nitrogen, was compared. The formwr proved to be considerably more sensitive to oxygen reduction. It is concluded that oxygen supply is of special importance in the growth and/or functioning of the Alder nodule.

P A R T IV.

Studies on respiration and fixation of nitrogen in detached non-legume root nodules.

PART IV.

C O N T E N T S

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

The two main metabolic activities of root nodules are undoubtedly respiration and nitrogen fixation. As noted previously, the discovery that by use of isotopic nitrogen the persistence of nitrogen fixation in nodules after detachment from the plant can be detected and measured, both in legumes and non-legumes, marks a big advance and facilitates the investigation of nodule metabolism very materially. Short-term experiments relating to the effect of various factors on fixation now become possible, whereas in the past such studies could only be made by means of long-term experiments of the type described in the previous part of this Thesis. The information yielded by such an experiment is useful and necessary but its analysis is complicated by the fact that growth as well as functional effects are involved.

In the work now to be described observations have been made on the respiratory characteristics of detached non-legume nodules and on the fixation of nitrogen shown by them. Some aspects of the work link up naturally with the previous section (Part III of Thesis).

In the case of legumes there is a limited amount of information about nodular respiration. Some authors have dealt with still-attached nodules. Thus Reinau (1927) found that the evolution of carbon dioxide from the surface of soil in which legumes were growing was usually higher than from soil carrying other plants, and he ascribed this to a particularly active respiration on the part of the nodules. Bond (1941) used a gas-flow method with baryta absorption towers to compare the respiration of nodulated and non-nodulated root systems of Soya bean at different stages in development, and concluded that respiration per unit dry weight was approximately three times as great in the nodules as in the roots. Extensive observations on the respiration of detached legume nodules and of portions of roots were reported by Allison, Ludwig, Hoover and Minor (1940) and Allison, Ludwig, Minor and Hoover, (1940). Their primary purpose was to "obtain fundamental information on the biochemical processes taking place in the nodule as a basis for a better understanding of the nitrogen fixing process occurring there". Their general finding was that the respiratory rates shown by nodules were of the same order as those of the corresponding roots when the material was

respiring in air. At the same time they concluded that nodules were potentially capable of more rapid respiration, since in an atmosphere of oxygen nodule respiration was more than doubled, whereas roots showed no increase as compared with air. These workers further observed that the respiratory quotient (R.Q.) was well above unity for nodules respiring in air, the range for soya bean nodules for example being 1.23 - 2.00. From their results they suggested that nodule respiration in air appeared to be limited by an internal lack of oxygen, that is, partially anaerobic conditions prevail within nodule tissues.

Apart from the article by Bond & MacConnell (1955), which incorporated some of the work now to be described, there appear to have been no previous publications referring to respiration of non-legume nodules.

Until the advent of the ^{15}N technique there was no clear evidence that any fixation of nitrogen continued in root nodules after detachment from the plant. It appeared that any persisting fixation was too small to be detected by the Kjeldahl process. Actually the earlier ^{15}N experiments of the Madison workers gave unsatisfactory results, but by carrying out the mass spectrometric analyses on the acid-soluble fraction only of the nodule nitrogen, Aprison & Burris (1952)

were able to report indubitable and consistent evidence of fixation in detached Soya bean nodules. Since then further data have been published from Madison, the latest in the article by Burris, Magee, & Bach (1955), in which studies of the effect of oxygen tension on fixation are described.

Meanwhile Bond (1955) had shown that fixation likewise continued in detached Alder nodules, and moreover to a considerably greater extent than in detached Soya bean nodules under similar conditions, so that significant enrichment was shown in analyses of the total nodule nitrogen. Thus the mean enrichment shown by samples of Alder nodules was 0.067 atom ^{15}N per cent., as compared with only 0.017 atom per cent. for samples of Soya Bean nodules. In experiments carried out after those of Bond, Virtanen, Moisio, Allison and Burris (1954) also reported fixation in detached Alder nodules and commented on its vigour. Bond & MacConnell (1955) showed that fixation similarly persists in detached Bog Myrtle nodules.

In the work now to be described the following aspects have been investigated;-

(A) the respiration rates in air of nodules and roots of different ages of Alder, Bog Myrtle and Sea Buckthorn

have been determined, by means of measurements of oxygen uptake, using a Warburg respirometer. Respiratory quotients were also determined.

(B) the effect of external oxygen concentration on the respiration of Alder nodules and roots and (in collaboration with Dr. G. Bond) on the effect of the same factor on fixation of nitrogen.

(C) a study of the effect of malonate on Alder nodule and root respiration.

METHODS

The measurement of respiration.

In all experiments the rate of respiration was measured by the uptake of oxygen. Only when respiratory quotients were required was the experimental technique modified to give also the carbon dioxide output.

Such measurements were made by means of the well known Warburg constant volume respirometer. The general principle of this instrument is that if, at constant temperature, one holds the volume of a gas constant, any change in the amount of gas can be measured by change in pressure. Thus living tissue respiring in air would give a recordable change in pressure due to oxygen uptake if the carbon dioxide released in respiration were removed, as is of course achieved in the Warburg technique by means of potassium hydroxide contained in the centre well of the special Warburg respiration flask. A flask of this type is attached to a manometer from the periodic reading of which the amount of oxygen that has been absorbed can be seen as a change in pressure. The manometer and flask are attached to a shaker apparatus so that the flask is immersed, and constantly moved in, a constant temperature

bath. The instrument used in the present investigations was fitted with a shaker panel on either side of the constant temperature bath and was capable of carrying a total of fourteen manometers and flasks (the volume of each Warburg flask being approximately 20 c.c.). Standard manometric techniques were employed in using the apparatus these being based on detailed descriptions on the use of the Warburg respirometer given by Umbreit, Burris, and Stauffer (1945) and Dixon (1951).

In general results are expressed in terms of QO_2, that is cu.mm. oxygen consumed per hour, per mg. dry weight.

For determinations of the Respiratory Quotient (R.Q.) the first method of Dickens and Simer as described by Dixon (1951) was employed. As the samples used could readily be obtained of the same weight within the suggested 10% limit, the method proposed for correction for unequal weights of tissue was not used.

In all experiments the basal medium in the respiration flasks consisted of 2 ml.of the nitrogen-free form of Crone's culture solution previously detailed. In the case of the malonate experiment this solution was again used except that potassium malonate was added

to give final strengths of 0.001 M, 0.01 M, 0.025 M, 0.05 M and 0.10 M malonate. Also in the latter case adjustments were made to the pH of the solution by the addition of sulphuric acid.

Measurements of oxygen uptake were made over four to five hour periods, changes in the manometer readings being recorded half-hourly. Under normal conditions the uptake remained relatively constant over this period.

Material for respiration measurements (i.e. whole nodules or portions of roots) was taken from greenhouse plants growing in nitrogen-free culture solution. The period between the removal of nodules from the plants and the transfer of weighed samples to the respiration flasks was from ten to fifteen minutes. To obtain the volume of a single sample (i.e. for one flask) a weighed quantity of the material was immersed in water in a 5 ml. measuring cylinder and its volume measured. By calculating a density figure the volume of smaller quantities of material could be established.

The nodules collected for each experiment were thoroughly mixed together, having been drawn from several plants, before being weighed into samples suitable for placing in the respiration flasks.

When roots were required, firm white parts were chosen which had an approximate diameter of 1-2 mm., and these were cut into centimetre lengths.

Although in the work to be described a distinction has been drawn between material taken from first and second year plants it may be noted that in actual fact the roots selected were probably all of current year's growth and the nodule lobes also were probably of recent formation though springing from older nodule growth.

The measurement of fixation by the use of ^{15}N.

Gaseous nitrogen containing excess ^{15}N was prepared, as described by Bond and Scott (1955), in a nitrometer by the action of sodium hypobromite on ammonium nitrate. containing 36.2 atom per cent. ^{15}N in the ammonium radical. The nitrogen was transferred to a gas burette and thoroughly shaken with 5 per cent. sulphuric acid as a precaution against the possible presence of traces of ammonia containing excess ^{15}N.

Specimen tubes were used as containers for nodule material, the nodules being placed in these with 0.5 ml. of culture solution. Several such tubes were fixed to a manifold built from capillary tubing, with connections

to a manometer, a filter pump and a gas burette containing the gas mixture appropriate to the experiment. The whole system was then evacuated and the prepared gas mixture containing excess ^{15}N admitted.

Suitably placed stopcocks made itpossible to detach the nodule containers from the manifold when the gas had been admitted and they were transferred to an incubator at 24°C. for a definite period.

The distillates from the Kjeldahl process, after total nitrogen had been estimated, were evaporated down to a small volume and thereafter assayed for ^{15}N content by the Chemistry Department of this University, using a Metropolitan-Vickers Mass Spectrometer II. Similar material not exposed to excess ^{15}N was also analysed to provide control samples.

EXPERIMENTAL RESULTS

A. The respiration in air of nodules and roots of Alnus, Myrica and Hippophaë.

Data for Alder are presented in Table 27. It will be observed that in respect of the QO_2 there is a large seasonal effect in the case of nodules from first year plants, values being considerably higher in summer than in autumn. In July the oxygen intake of nodules from first year plants is much greater than that of nodules from second year plants.

Nodules from first year plants respire two to three times as fast as roots from the same plants though in the case of material from second year plants the same relation is not consistently shown. The mean R.Q. values all approximate to unity.

The more limited results obtained with Bog Myrtle and Sea Buckthorn nodules and roots are given in Table 28. In the case of both species second year plants were used comparable with Alder of the same age examined in July 1954. As in the case of these particular Alder results

TABLE 27.

QO_2 and R.Q. values obtained for the nodules and roots (respiring in air) of first and second year Alder plants.

Values given are means based on from five to ten replicate samples. The minimum and maximum values in each series are given in brackets along with the mean figure.

QO_2.

Date	1st.year plants		2nd.year plants	
	Nodules	Roots	Nodules	Roots
July 1954	10.46 (7.58-12.81)	3.53 (2.82-4.18)	1.83 (1.37-2.27)	2.01 (0.95-2.36)
October 1954	3.14 (2.69-3.82)	-	2.72 (2.24-3.00)	1.03 (0.97-1.09)
September 1955	5.07 (3.91-5.84)	2.14 (2.06-2.26)	-	-
October 1955	2.80 (2.68-2.95)	-	1.37 (1.10-1.71)	-

R.Q.

Nodules from 1st.year plants (September 1955): 1.0 (0.9-1.0)
Nodules from 2nd.year plants (July 1954): 1.16 (1.04-1.25)
Roots from 2nd.year plants (July 1954): 0.92 (0.8-1.1)

TABLE 28.

The oxygen uptake and R.Q. of nodules and roots (respiring in air) taken from second year plants of Bog Myrtle and Sea Buckthorn.

July 1954.

Plant species	QO_2 (mean of 10)		R.Q. (mean of 4)	
	Nodules	Roots	Nodules	Roots
Bog Myrtle	2.90 (1.85-3.52)	3.32 (2.61-3.93)	1.03 (0.92-1.12)	1.17 (1.07-1.47)
Sea Buckthorn	4.51 (2.64-6.86)	3.66 (3.02-4.35)	0.98 (0.88-1.19)	1.00 (0.70-1.29)

The minimum and maximum values obtained in each series are given in brackets under the mean figures.

little difference was shown between nodule and root respiration though it is possible that first year nodules of both Bog Myrtle and Sea Buckthorn would show similar activity to young Alder nodules. The R.Q. values again approximate to unity. It will be observed on comparing the actual oxygen uptake of the nodules of the three species (as second year plants) that Sea Buckthorn shows the greatest activity.

B. The effect of external oxygen supply on the respiration of detached nodules and roots of Alder and on the fixation of nitrogen by similar detached nodules.

(1) Respiration experiments.

By use of a manifold arrangement three manometers and their attached flasks were treated as a unit when altering the atmosphere around the nodules. Appropriate oxygen-nitrogen gas mixtures were driven in by displacement after evacuation of the manifold, manometers and flasks. A second evacuation followed and a second sample of the gas mixture admitted. The pressure within the flasks and manometers was adjusted to atmospheric before they were attached to the shaker of the Warburg. The oxygen-nitrogen mixtures were obtained from the

same cylinders as used in the gas-flow growth experiments (see Part III of Thesis). When oxygen values greater than 21% were required appropriate mixtures of air and oxygen were prepared.

The experiments on the effect of oxygen on nodule and root respiration were performed in late September and early October in two successive years. In all tests reported three replicate samples were set up and the QO_2 values to be quoted are each the mean of three such samples. The results are given in Table 29 together with statistical treatment. Significant falls in oxygen uptake occur when the oxygen concentration is lowered below that in air in the case of both nodules and roots taken from second year plants (September 1954). The fall in oxygen uptake by nodules is much steeper than in roots. Significance is not however so quickly attained in the case of nodules from first year plants (September 1955) where greater variation between individual samples was found, but it is probable that the oxygen relation is essentially identical with that shown in the first experiment.

In the case of oxygen levels greater than 21% (experiment of October 1955) the data suggest that oxygen uptake by nodules increases up to 40% external oxygen and thereafter declines, but here again, owing to

TABLE 29.

The oxygen uptake of detached nodules and roots of Alder in varying external oxygen concentrations.

Each QO_2 given is the mean of three values.

	Date	Oxygen supplied (per cent).	QO_2 of nodules	QO_2 of roots
(a)	September 1954	21	2.72	1.03
		10	1.72	0.80
		5	1.14	0.64
		1	0.35	0.33
(b)	September 1955	21	4.24	-
		12	3.66	-
		5	2.46	-
		1	0.87	-
(c)	October 1955	100	3.23	3.56
		40	3.84	-
		30	3.31	-
		21	2.86	2.14

(a) = Material from second year plants.

(b) and (c) = Material from first year plants.

Differences required between means for significance at P = 0.05 from analyses of variance are as follows:-

(a) 0.43 for nodules; 0.08 for roots.

(b) 1.50 for nodules.

(c) 1.23 for nodules; 0.21 for roots.

variation from sample to sample, full statistical significance is not attained. In the case of the root samples in this experiment respiration at 100% oxygen is significantly higher than at 21%.

The above data are expressed on a relative basis in Table 30.

The possibility of material changes taking place in the percentage of oxygen supplied during the course of an experiment was examined. From calculations on oxygen uptake during a four-hour period the following estimates of the final oxygen concentration in the respiration flasks and manometers were obtained:-

Initial % O_2	Final % O_2
21	20.46
10	9.50
5	4.75
1	0.94

From these figures it may be seen that the fall in the percentage oxygen is negligible during such short-term experiments.

(2) Fixation experiments.

Two experiments were conducted on the effect of oxygen concentration on the fixation of nitrogen by

TABLE 30.

Relative oxygen uptake of Alder nodules and roots in varying external oxygen comcentrations.

(Rate of uptake in air taken as 100).

	Percentage oxygen supplied	Oxygen uptake (relative)	
		Nodules	Roots
(1)	21	100	100
	10	63	78
	5	42	61
	1	13	32
(2)	100	113	166
	40	134	-
	30	116	-
	21	100	100
	12	86	-
	5	58	-
	1	21	-

(1) = Material from second year plants.

(2) = Material from first year plants.

detached Alder nodules. The results obtained in the first of these experiments are presented in Table 31, this being the experiment to which reference was made by Bond and MacConnell (1955). It will be seen that as the initial oxygen level is reduced from 21% the amount of fixation falls, though the differences between means do not all attain the magnitude required for significance. This experiment was carried out before argon or helium gas for use as a 'filler' was available so that in order to economise in the use of the expensive isotopic nitrogen rather small containers had to be used. Thus the actual amount of oxygen supplied to the nodules, on the absolute basis, was small and the mean percentage of oxygen during even the first few hours in which the bulk of the fixation occurs was undoubtedly somewhat lower than the initial level because of respiratory consumption. While this experiment indicates that oxygen supply is undoubtedly a factor in fixation it cannot be concluded from this experiment that given a steady supply of 12% of oxygen, fixation would be less than at 21% of oxygen.

Table 31 also shows results for nodules exposed to initially 40% oxygen. This part of the experiment was carried out four days after the main part so that caution must be observed in comparing them in view of the possible effect of weather conditions immediately

TABLE 31.

First experiment on the effect of oxygen on fixation by detached Alder nodules.*

Experiment performed in early August 1954.

	% oxygen initially supplied				
	40	21	12	5	1
^{15}N content atom % of total nodule nitrogen	0.397	0.535	0.469	0.469	0.379
	0.364	0.500	0.444	0.436	0.428
	0.376	0.525	0.485	0.376	0.383
	0.457	0.493	0.420	0.422	0.385
Means	0.399	0.513	0.455	0.426	0.394
Increase over normal figure of 0.372 atom per cent.	0.027	0.141	0.083	0.054	0.022

* Four composite samples (0.6 gm.) of nodules from first and second year plants were set up at each oxygen level in specimen tubes (14 ml.) afterwards charged at one atmosphere with oxygen-nitrogen mixtures containing 20 atom per cent.15N. The nodules remained in the tubes for 24 hours at 21°C. and were then assayed for 15N content.

Minimum difference required (from analysis of variance) between means for significance (at P = 0.05) = 0.043 atom %.

prior to the detachment of the nodules on the subsequent fixation. It does appear however, that at this initial high level of oxygen fixation is largely suppressed.

Data for a second experiment are provided in Table 32. With argon now available a higher ratio of gas volume to nodule sample was provided and moreover in view of new information now available on the time relation of the fixation it was possible to reduce the period of the experiment from 24 to 4 hours, so that the depletion of the oxygen in the tubes was very much smaller than in the first experiment.

The isotope content of the nitrogen supplied was increased from 20 to 36 atom per cent., and the use of only 10% of nitrogen was permitted following the results of other experiments. Differences in fixation between duplicate samples tend to be large but it clearly cannot be stated from this experiment that reduction of oxygen supply has any effect on fixation until the initial level falls below 5%. Thus the continuous fall obtained in the first experiment must be provisionally ascribed to the rapid depletion of the external oxygen supply.

TABLE 32.

Second experiment on the effect of oxygen on fixation by detached Alder nodules.*

Experiment performed in late August 1955.

	% oxygen initially supplied			
	21	12	5	1
^{15}N content atom % of total nodule nitrogen	0.601	0.632	0.488	0.381
	0.455	0.442	0.544	0.384
Means	0.528	0.538	0.516	0.383
Increase over normal figure of 0.372 atom per cent..	0.156	0.166	0.144	0.011

* Two samples (0.6 gm.) of nodules from first year plants were set up at each oxygen level in specimen tubes (25 ml.) afterwards charged with gas mixtures containing the proportion of oxygen indicated above, 10 % nitrogen (with 36 atom per cent.15N) and argon to bring the pressure to one atmosphere. The nodules remained in the tubes for 4 hours at 23°C. and were then assayed for 15N content.

C. The effect of malonate on the respiration of the nodules and roots of Alder.

The function of malonate as an inhibitor of oxidatory respiration in living tissues is well known. It is commonly used to test for the operation of the tricarboxylic acid cycle in a given tissue, for malonate is generally regarded as a specific inhibitor for succinic dehydrogenase, thus causing an accumulation of succinic acid. In comparison with animal tissues the use of malonate in plant studies has been somewhat limited. James (1953a) has summarised the evidence relative to its use, in mildly acid solutions, on plants and points out that the specificity of this inhibitor has not been much investigated in plant tissues. Though the inhibition is normally reversible by adjustment of the succinic acid concentration, James (1953b) has indicated that the respiration of some cells is inhibited irreversibly by moderate doses of malonate and the site of this irreversible inhibition has not yet been identified.

In the present investigation the effect of potassium malonate on the respiration of the detached nodules and roots of Alder has been studied.

A summary of the results obtained is presented in Table 33 from where it may be seen that a maximum

TABLE 33.

The effect of potassium malonate supplied at various concentrations on the respiration of Alder nodules and roots in air.

Control QO_2 values are given for each particular experiment and % inhibition of respiration is calculated on this value. The actual QO_2 values quoted are those obtained in the fourth hour of the various experiments and each is the mean of three replicate samples.

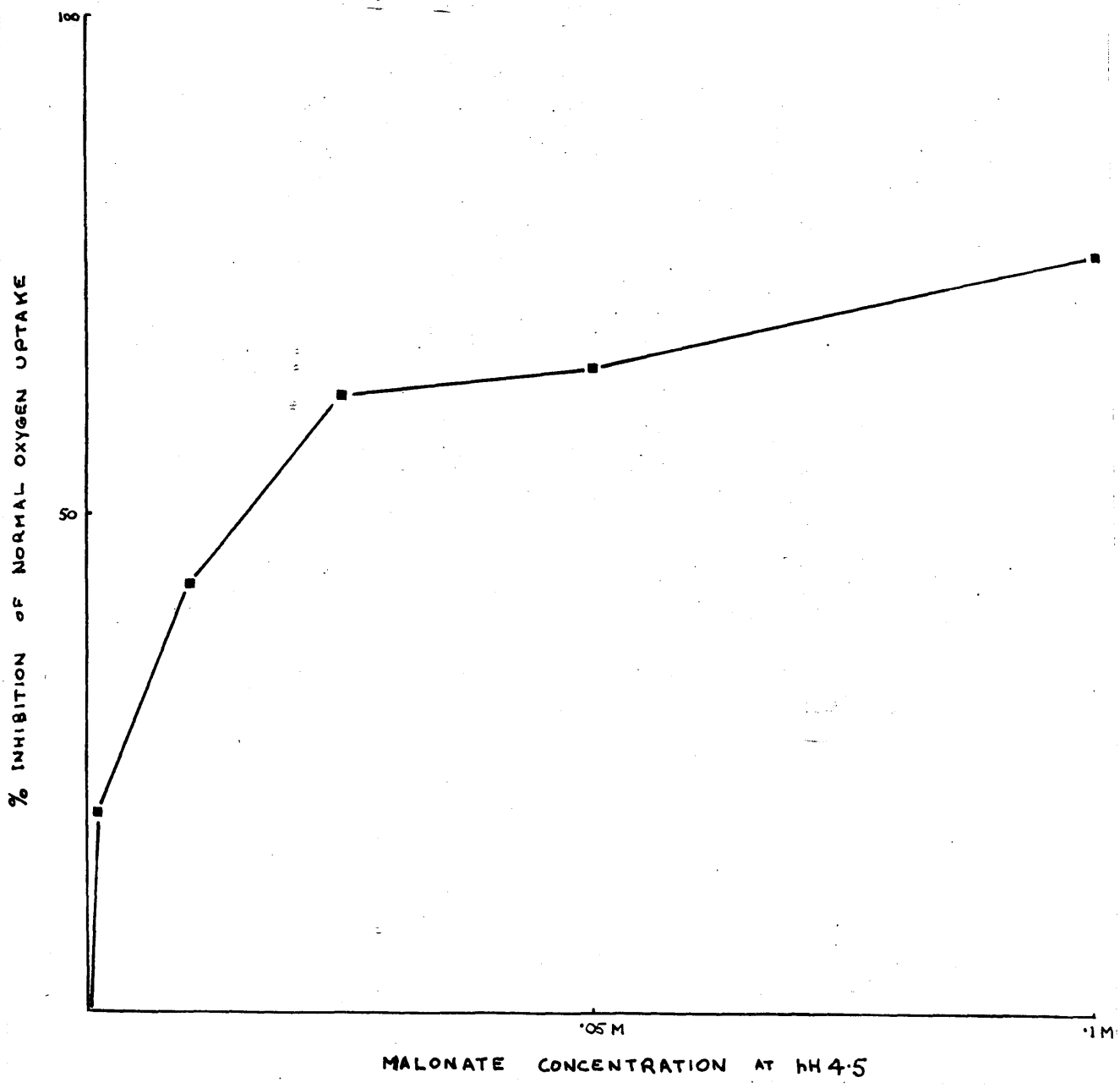
Material	Control QO_2	Malonate concentration	pH	QO_2 in malonate	% inhibition
Nodules	6.40	.025M	6.5	5.36	16
Nodules	5.19	.05M	4.5	1.81	65
		.01M	"	2.97	43
		.001M	"	4.14	20
Nodules	9.85	.025M	4.5	3.79	62
Nodules	4.99	.1M	4.5	1.16	77
Roots	3.66	.05M	4.5	1.14	69
		.1M	"	0.72	80

inhibition of nodule respiration of 77% was obtained with 0.1 M malonate at pH 4.5. Progressively less inhibition with decrease in malonate strength occurred and this effect is shown graphically in Figure 2. Minimum inhibition of 16% was obtained with 0.025 M malonate at pH 6.5 though the same concentration gave an inhibition of 62% when supplied at pH 4.5. This is in keeping with the observations on different material made by other workers.

The mean of five R.Q. values obtained in the presence of 0.05 M malonate was 0.9, indicating that anaerobic carbon dioxide was not produced.

Inhibition in the presence of malonate took over an hour to become really marked. For example in one case where at the end of the first hour the QO_2 of the control sample was 6.57, that of a sample in 0.05 M malonate was 5.20 (the samples in question being of Alder nodules from first year plants). At the end of the second hour the QO_2 for the control was 6.58 while that in the malonate had fallen to 2.94. Thereafter the QO_2 of the sample in malonate continued to fall but to a less marked extent while the control value remained comparatively steady.

FIGURE 2.

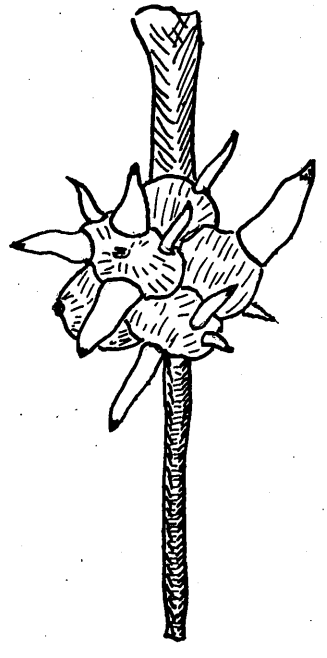


The effect of malonate on the respiration of detached Alder nodules.

DISCUSSION

Since both respiration and nitrogen fixation involve gas exchange with the atmosphere, the ventilation arrangements within root nodules is obviously of some importance in these processes. The Alder nodule is enclosed from an early stage by a superficial layer of cork, but lenticels are present as in the older roots of the plants. Owing to a hypertrophy of the complementary tissue under conditions of water culture these nodule lenticels are very conspicuous, especially in submerged nodules (Figure 3). Although as judged by the vigorous growth of Alder plants in water culture the lenticels function efficiently, the precise manner of their functioning cannot be stated definitely. Presumably the spaces between the cells of complementary tissue remain gas-filled and the exchange of gas with the culture solution occurs there. Bog Myrtle nodules also are enclosed in cork and here the lenticels appear to be replaced functionally by the nodule roots. A contrast is found in the Sea Buckthorn nodule where only the basal part of the nodule is covered by cork. The apical part remains devoid of cork, is white in colour and is doubtless the region of gaseous exchange.

FIGURE 3.



Alder nodule cluster showing lenticel outgrowths from the nodule lobes. This is typical of submerged nodules, the nodules growing above the solution having smaller lenticels.

X 4.

Drawing supplied by courtesy of Dr. T.P. Ferguson.

Of the three species mentioned Sea Buckthorn would appear to be the most suitably adapted for rapid gas exchange when fairly mature nodules are being considered. This is partly borne out by the fact already noted that these nodules (from second year plants) were more active in oxygen uptake than nodules from plants of the same age of Alder and Bog Myrtle.

Respiration of nodules and roots in air.

It is well known that for most higher plants the QO_2 of their tissues lies below 2. The QO_2 for the nodules of Alder plants in their first year attained a level of 10.46 in July which is very high and indicative of intensive metabolic activity comparable with that of liver or brain tissue in animals. Of course it must be remembered that a substantial part of the nodule respiration is doubtless attributable to the endophyte - a micro-organism. According to the data available legume nodules show lower rates of respiration. Thus Allison, Ludwig, Hoover and Minor (1940) obtained QO_2 values averaging about 2.2 for various legume nodules respiring in air. Asprey and Bond (1941) obtained somewhat higher values, viz., 2.31 to 6.25, in the particular case of Soya bean nodules. Oxygen uptake by other symbiotic tissues has been examined; for example Harley, McCready and Brierley (1953) studying phosphate

uptake by excised mycorrhizal roots of Beech, quote figures for oxygen uptake by these same organs equivalent to QO_2 values of approximately 3.0.

The data presented here show that in Alder, nodule respiration has fallen considerably by the time September of October is reached and also that nodules from second year plants are considerably less active than those from first year plants. This is doubtless because the proportion of material active in respiration is smaller in the older nodules.

The comparison of nodule and root respiration showed that in July the QO_2 for nodules was three times that for roots in the case of first year plants. By September the disparity was slightly reduced. In comparison with material from second year plants the relation was more variable but an element of uncertainty comes in here as to the actual age and comparability of the material used. The great superiority of nodule over root respiration in young Alder plants is in marked contrast to the findings of Allison, Ludwig, Minor and Hoover (1940) that in various legumes the QO_2's of nodules and roots were similar. Bond (1941) on the other hand in the particular case of Soya bean, reported that nodule respiration was approximately three times that of roots.

The above remarks refer to oxygen intake on the dry weight basis. On the fresh weight basis the disparity between nodule and root respiration is greatly increased. Thus in the July 1954 comparison (first year plants) nodule respiration is now seven times that of roots (the oxygen intake of nodules as cu.mm. per mg. fresh weight per hour having a mean value of 1.24 as compared with 0,17 for roots). This is not surprising, since a large part of the fresh weight of the root is accounted for by the water of the vacuolar sap.

The R.Q. values for nodules of the three non-legumes lie in the range 0.98-1.16 (taking the mean values for each experiment), suggesting that the substrate for the respiration is carbohydrate, and that the respiration is fully aerobic. This indicates a difference from the legume nodules, in respect of which somewhat higher R.Q. values have been reported (Allison, Ludwig, Hoover and Minor, 1940; Asprey and Bond, 1941), leading to the view that some degree of anaerobiosis exists in legume nodules lying in air. Bearing in mind also the more rapid respiration shown in the Alder nodule it must be concluded that ventilation arrangements are much superior compared with those of the legume nodule. It should be noted that the R.Q. will include any intake of oxygen for the fixation process. The

fact that the values obtained are so close to those typical of normal respiration indicates that there is no appreciable separate use of oxygen in fixation. This finding applies however, only to detached nodules where the rate of fixation is somewhat lower than in attached nodules.

The results of the study of the effect of malonate on nodule and root respiration in Alder are in keeping with those of previous workers on other tissues (James, 1953a) and warrant the tentative conclusion that some form of Kreb's cycle is operative in the respiration of both organs.

The effect of oxygen supply on respiration.

When respiring in oxygen concentrations lower than that in air, Alder nodules and roots have shown the retardation in oxygen uptake typical of the majority of plant tissues under such circumstances (James 1953a). This retardation is however more marked in nodules than in roots. In oxygen concentrations greater than that in air the data reported provide some indication, though not conclusively, that nodule respiration at first increases and is thereafter depressed. These aspects were investigated by Allison, Ludwig, Hoover and Minor (1940) for legumes. They found that starting from 7% of oxygen supplied, respiration

rose continuously though at a diminishing rate until 100% oxygen was reached, where the respiration was rather more than twice that shown in air. It is clear that the response of Alder nodules to high oxygen supply is much less marked than in legumes, this being consistent with the conclusion reached above that ventilation is superior in Alder nodules.

Respiration and fixation.

The process of fixation is possibly dependent on that of respiration for energy and metabolites such as organic acids. The closeness of a link between the two processes can obviously be tested by varying conditions in such a way that the rate of respiration is altered from its normal value, the effect of this on fixation being then measured.

This approach has led to important conclusions on other aspects of cell activities, e.g., the connection between respiration and ion-uptake. Obvious methods of varying the rate of respiration are to adjust the oxygen supply or to introduce respiratory inhibitors such as malonate. Owing to long delays experienced in the examination of samples for ^{15}N content, it has only been possible to include in the present Thesis data obtained by varying the oxygen supply. As has been shown, the data suggest that in Alder the link

between respiration and fixation is not at any rate so close that a reduction in the rate of the former process is immediately accompanied by a reduction in fixation. Not until the external oxygen supply falls below 5%, a level at which respiration has fallen to about half its normal value, is fixation curtailed (this statement refers to the second fixation experiment). As regards the effects of external oxygen supply greater than 21% the data reported provide some evidence of a parallel response in that both respiration and fixation increase to a maximum and are then depressed, but further experiment will be necessary before this can be regarded as established.

The results recently reported by Burris, Magee and Bach (1955) are of interest in the present connection. They studied the effect of oxygen supply on the fixation of ^{15}N by sliced Soya bean nodules. Maximum fixation (actual increase in ^{15}N content was 0.3 atom per cent. of the acid soluble fraction of the nodule nitrogen) was experienced at 0.5 atmospheres oxygen, above and below which fixation fell rather sharply. As noted already there seems to be in Alder also a point beyond which further increase in oxygen supply restricted fixation, but in other respects the oxygen relation of fixation in Soya bean nodules appears to differ

from that in Alder. Burris et al.(1955) interpreted their results to indicate a close relation between respiration and fixation, but since they present no data on the respiration of their material at different oxygen tensions this conclusion seems scarcely justified.

Bearing of present results on Alder growth experiment.

The observations presented in this part of the Thesis on the respiration of nodules and roots of Alder and on the effect of oxygen supply on that process provide an explanation of the results of the growth experiment reported in Part III of the Thesis. There it was shown that nodulated plants exhibit a greater curtailment of growth when the level of oxygen supplied to their root systems is progressively reduced than do non-nodulated plants provided with combined nitrogen. The respiration data show that the oxygen intake in air is much higher in nodules of first year plants than in roots, and that nodule respiration is more rapidly curtailed than root respiration when the oxygen supply is lowered. Granted that the rapid rate of respiration shown by nodules is essential to their proper development and functioning, then it is to be expected that a curtailment of respiration enforced by a diminished oxygen supply will seriously interfere with the growth of plants dependent on their nodules.

SUMMARY

(1) The respiration of detached nodules and root portions of Alder, Bog Myrtle and Sea Buckthorn has been studied by means of a Warburg respirometer.

(2) The QO_2 for young Alder nodules respiring in air attains the relatively very high value of 10.46 in July and is then three times that for root portions of comparable age.

(3) QO_2 values for Alder nodules from second year plants and for comparable nodules of Bog Myrtle and Sea Buckthorn are lower and lie in the range 1.4 - 4.5. Root respiration is similar.

(4) Values obtained for respiratory quotients of nodules and roots are all close to unity indicating that a carbohydrate substrate is employed and that aerobic conditions prevail within the nodules. These R.Q. values also suggest that there is no significant direct utilisation of oxygen in fixation.

(5) A reduction in the oxygen supply below the air level results in retarded oxygen uptake by the nodules and roots of Alder. The fall in oxygen uptake by nodules is much steeper than in roots. In oxygen concentrations greater than that in air there is some indication that nodule respiration at first increases and is thereafter

depressed.

(6) By means of isotopic nitrogen, fixation by detached Alder nodules was shown to be curtailed when the external oxygen was reduced, though the more critical of two experiments suggested that the reduction of oxygen supply has no effect on fixation until the level falls below 5%. Thus in Alder the link between respiration and fixation is not so close that a reduction in the rate of the former process is immediately accompanied by a reduction in fixation.

(7) Malonate was shown to produce partial inhibition in the respiration of Alder nodules and roots suggesting that some form of Kreb's cycle is operative in the respiration of both organs.

(8) The respiration studies provide an explanation of the results obtained in the Alder growth experiment (described in Part III of Thesis).

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