INVESTIGATION ON CERTAIN ASPECTS OF FIXATION OF NITROGEN BY LEGUMES AND NON-LEGUMES.

Thesis presented by

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Summary of Thesis presented by Thomas P. Ferguson, B.Sc. entit
"Investigations on Certain Aspects of Fixation of Nitrogen by
Legumes and Non-Legumes".

Section I of the thesis has the sub-title "The Effect of Oxygen Supply on the Functioning of the Root Nodules of Red Clover". In this part of the work the effect of oxygen supply on the growth and nitrogen accumulation of nodulated red clove plants in nitrogen-free rooting medium and of non-nodulated plants supplied with combined nitrogen has been investigated b two different techniques.

In the first technique the root systems only were exposed to adjusted oxygen supply. The plants were grown in water culture, and oxygen /nitrogen gas mixtures containing 21, 12, and 1 per cent oxygen respectively were bubbled through the culture solution of different series of plants. Protection against ingress of atmospheric oxygen was provided.

In the second technique the clover plants were grown in sand culture with the plants wholly enclosed within 5-litre aspirator bottles. The gas space in these bottles was kept filled with the gas mixtures listed in the preceding paragraph except that 1 per cent carbon dioxide was now added to provide for photosynthesis.

The reactions of the plants to variation in oxygen supply were in general similar in both types of experiments. With nodulated plants growth at 12 per cent oxygen was significantl superior to that at 21 per cent. Reduction to 5 per cent led to a curtailment of growth, the same being true when oxygen wa further lowered to 1 per cent. With the non-nodulated plants growth at 21 and 12 per cent oxygen was essentially similar, but was reduced at 5 per cent and again at 1 per cent. The extent of the response to different oxygen levels was, howeve much less marked in the whole-plant enclosure experiment and this is considered to be due to the limitations necessarily imposed on growth by the experimental arrangement.

By graphical means it is deduced from these experiments that the optimal oxygen level for the growth of nodulated clov plants is approximately 10 per cent, and that for non-nodulate plants approximately 15 per cent. It is assumed that the differences in response of the two types of plants to variatio in oxygen supply is due to effects exerted in the nodules, so that clover nodules appear to function most effectively at a reduced oxygen level and that in fact nitrogen fixation is favoured by the prevalence of some degree of anaerobiosis in t nodule.

In the root-system enclosure experiments the number of nodules formed per plant was essentially similar at 21, 12 and 5 per cent oxygen, but was much reduced at 1 per cent. This last result could have been wholly due to the poor root growt at 1 per cent oxygen. It is concluded that the process of no initiation has a greater tolerance towards oxygen supply thar the nodule has in its later stage as a nitrogen-fixing organ.

Section II of the thesis has the sub-title "Observations the Formation and Significance of the Root Nodules of Alnus glutinosa (L.) Gaertn". Here the conclusion reached by previ investigators that nodulated alder plants can fix atmospheric nitrogen and display vigorous growth in water culture free of combined nitrogen is confirmed. Also evidence is advanced to show that the fixation of nitrogen actually takes place withi the nodulated plant and probably within the nodule. There is reason to doubt that fixation is associated with nodulated al trees in the field, and this fixation may be of considerable ecological and general importance.

It has been shown that in water culture nodule formatior occurred over the pH range 4.2 to 7.0 but most freely betweer 5.4 and 7.0. No nodules developed at pH 3.3. At favourable nodules visible to the naked eye developed as soon as 10 days after inoculation. The optimum pH for the growth of nodulate plants in solution free of combined nitrogen lies between pH 4.2 and 5.4.

By growing non-nodulated alder plants supplied with comb nitrogen in water culture it has been shown that the failure of the plants to develop nodules at pH 3.3 was due to the fact th although the host plant can tolerate such a low pH level the nodule organism cannot. It has also been shown that non-nodulated plants supplied with ammonium sulphate made more vigorous growth than those supplied with sodium nitrate.

In experiments with both nodulated plants and plants supplied with combined nitrogen in which the culture solution in certain cases was aerated it was found that a high oxygen supply had a beneficial effect on the growth of the former but had no effect on the latter. It is concluded from this finding that nodules have a higher oxygen requirement than roots.

CONTENTS OF THESIS.

ACKNOWLEDGMENTS.

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PREFACE.

The first part of this thesis deals with one particular example selected from the best known group of symbiotic nitrogen fixing systems, namely nodulated leguminous plants. The existing literature relating to leguminous nitrogen fixation is already very compendious, but the interest thus demonstrated has been chiefly on the part of agricultural bacteriologists. Relatively rarely have leguminous plants been subjected to investigations by plant physiologists, and the result of this is that information is defective in aspects calling for a plant physiological approac Thus it will be seen in the first section of the thesis that the important question of the effect of oxygen supply on growth of nodulated leguminous plants has been subject to critical study on only two previous occasions.

In the second part of the thesis a study is made of the root nodules of a non-leguminous plant, the Alder. In the minds of most people "symbiotic nitrogen fixation" and "leguminous nitroge fixation" have been almost synonymous despite the fact that, as will appear from the appropriate review of the literature, in the case of Alder strong evidence for fixation of nitrogen under cultural conditions has existed for many years. The preoccupatio with legumes arises of course from the immediate agricultural importance of the fixation associated with them. But from an ecological standpoint fixation of nitrogen by non-legume genera such as Alnus, Myrica, and Hippophaë is probably of greater interest than the leguminous type, because of the greater range of habitats favoured by the former plants as a group, while the results of pollen analysis indicate that some of these plants were prevalent in earlier times. It is therefore appropriate that the significance of non-leguminous root nodules should receive more attention than in the past.

SECTION I OF THESIS.

The Effect of Oxygen Supply on the Functioning of Root Nodules of Red Clover.

CONTENTS OF SECTION I.

INTRODUCTION.

Although the course of the fixation process in the legume nodule is still problematical there are several *a priori* considerations which might form a basis for an expectation that a plentiful oxygen supply will be of particular importance to nodulated plants. Thus the possibility exists that oxygen may be directly utilised in the fixation process. Again it is likely that the fixation mechanism is integrated with the respiratory processes of the bacteria or of the nodule cell, being perhaps dependent on the latter for metabolic hydrogen or for energy in the form of high-energy phosphate bonds. Undoubtedly the role of oxygen in respiration is of great importance and so for the above reasons it is clearly conceivable that oxygen supply will be of particular importance to nodules. In addition there is possibly a higher concentration of living and metabolically active material in root nodules than in the other parts of the plant and for this reason also, oxygen supply may be particularly critical.

Closer examination of these arguments shows that none is particularly strong. Thus no recent scheme which has been devised to explain the various steps in the fixation mechanism has considered the possibility of oxygen being directly utilised. Also, even if respiration is closely integrated with the fixation mechanism, it seems unlikely that this process will have to be greatly increased, with a resulting high oxygen demand, as fixation in nodulated leguminous plants is a relatively slow process. Finally, it is considered by several investigators that the bacteroids in the nodule are in an inactive state and would therefore be unlikely to require a plentiful supply of oxygen.

It is thus clear that no particular answer to the question of the importance of oxygen to the function of leguminous root nodules can be foreseen, and only by actual experimentation can further information be acquired. In this study the main object has been to determine whether the legume nodule in its development and functioning has in fact any distinctive oxygen requirement differing from the rest of the plant and especially from the roots. This is clearly a question of both practical and theoretical importance. Thus it bears directly on the nature of the fixation process, while at the same time increased knowledge of the oxygen requirements of legume nodules is likely to contribute to the more effective cultivation of leguminous crops, or to the explanation of already observed preferences of such crops in respect of cultural procedure and soil type.

Previous observations have been made which bear either directly or indirectly on this problem. Thornton (1930) observed that in the case of nodulated lucerne plants grown under aseptic conditions in agar culture, nitrogen fixation did not occur so long as the nodules were embedded in the medium, but where nodules developed on the surface of the agar or were exposed to the air by the shrinkage or cracking of the medium, increase in growth and darkening of the leaves took place. He concluded from these results that the inadequate air supply within the agar was responsible for the nodules being unable to function in a normal manner. Very similar results were obtained by Virtanen and von Hausen (1935). They observed that nodulated peas grown in agar culture exhibited better growth when exposure of the nodules took place under the above-mentioned conditions and they arrived at the same conclusion as Thornton. Jensen (unpublished work communicated to the author) confirmed this observation with lucerne plants but also found that under similar conditions of growth, nodulated clover plants (i.e. the subject of the present investigation) did not react in the same way, since fixation was not delayed until exposure of the nodules to the atmosphere.

Golding (1903) and Virtanen and von Hausen (1935) observed that in nodulated pea plants grown in water culture, fixation was poor when all the nodules were submerged in the solution, but exposure of the nodules to the air effected by withdrawing part of the solution from the container resulted in good growth by the plants.

Virtanen and von Hausen (1936) studied the effect of forced aeration on the growth of peas in water culture. In inoculated cultures, with nitrogen-free culture solution and in the absence of aeration, abundant nodules were formed but remained relatively small, while with aeration the nodules were larger and the amount of nitrogen fixed rose from 10 mg. to 73 mg. per plant and the dry weight was also considerably increased. The experiment included a single culture of peas supplied with an excess of ammonium sulphate, where satisfactory growth was made without aeration. From these results it was concluded that the oxygen content of the culture solution was adequate without aeration for root but not for nodule development and function, and that the nodule has a relatively high oxygen requirement. In these experiments the non-aerated plants were not subjected to any particular level of oxygen, but merely to the gradually increasing deficiency resulting from the continued growth and respiration of the root system. As no determinations of the amount of dissolved oxygen in the culture solution were made, it is impossible to say under what conditions of reduced oxygen supply these plants were actually grown. These investigators also showed that nodulation was completely prevented by the passage of a stream of gaseous nitrogen through the solution.

Bond (1951), using a technique which gave closer control over the oxygen content of the culture solution than that employed by Virtanen and von Hausen, grew Soya bean plants with their root systems in culture solution which was kept in equilibrium with oxygen /nitrogen gas mixtures containing 21, 12 and 5 per cent oxygen respectively. He observed that in the case of nodulated plants grown in nitrogen-free culture solution, reduction of the oxygen content of the medium resulted in a progressive reduction in dry weight and in the fixation of nitrogen. A somewhat similar but smaller effect was observed with non-nodulated plants supplied with ammonium-nitrogen, but no reduction occurred when nitrate-nitrogen was employed.

Research bearing either directly or indirectly on the respiration of root nodules of leguminous plants has also been carried out by several investigators, and obviously investigation of this type are also likely to throw light on oxygen requirement Reinau (1927), while studying the evolution of carbon dioxide from the surface of soils in which different crop plants were growing, observed that the rate of carbon dioxide production was usually substantially greater in the cases of soils carrying legumes, than from soils in which other plants were growing. The following figures are quoted from Reinau (gm. carbon dioxide per square metre of soil surface per hour).

Clover	0.558.	Mustard	0.218.
Seradella	0.305.	Rye	0.285.

Similar results were obtained by Hasse and Kirchmeyer (1927) who also measured rate of carbon dioxide production from soils bearing various crops (gm. carbon dioxide per square metre per day).

Bare Ground	1.6 - 4.8.
Rye	5.4 - 10.2.
Potatoes	11.8 - 12.7.
Lucerne	9.0 - 21.0.

The explanation of these results may be that the root nodules of legumes are centres of active katabolism and that this activity caused the differences in carbon dioxide production which were recorded. Other explanations are however possible. Starkey (1931) showed that the rhizosphere of leguminous plants has a bacterial population nearly five times as large as that of non-legumes. Thus this increase in the evolution of carbon dioxide from soils carrying legumes could have been brought about by the respiration of these plants plus that of the large number of bacteria. Furthermore, soils evolve varying amounts of carbon dioxide according to their physical structures and this fact tends to complicate the interpretation of results obtained in the field.

Rippel and Krause (1934), by measuring the disappearance of carbohydrates from the leaves of plants, demonstrated that although the destruction of carbohydrates by non-nodulated pea roots was of a similar order to that of the roots of non-leguminous plants, this carbohydrate demand by the root system was much higher when root nodules were present on this legume. Such a finding points to pea nodules having a high rate of respiration and therefore perhaps also a high oxygen requirement.

Allison, Ludwig, Hoover and Minor (1940), using a Warburg apparatus, measured the rate of respiration of detached nodules and fragments of roots of a number of leguminous plants. Their experimental findings showed that the nodule is potentially a more rapidly respiring organ than the root, but that in air, nodule and root respiration were very similar. They considered that this was brought about by the internal supply of oxygen within the nodule limiting the respiration to a value which happened to be near that of the roots. However in an atmosphere of pure oxygen, the rate of nodule respiration increased and became more than twice that of roots, indicating that nodules have the capacity for more rapid respiration than roots, provided that oxygen can be supplied to the inner cells. These authors realised, however, that although the use of detached organs for respiration observations allowed for precise measurements of gaseous exchange, the probability exists that under such conditions the process of fixation may cease in the nodule. At the same time, as respiration of both roots and nodules remained fairly constant during a period of several hours, detachment appeared to have no progressive effect on the process of respiration.

Meanwhile Bond (1941), using a technique which involved the use of intact plants of Soya bean in aerated water culture, observed that the amount of carbon dioxide produced per unit dry weight of nodule tissue was approximately three times that for root tissue. Confirmation of this finding was made by Asprey and Bond (1941) when they showed that the intake of oxygen per unit dry weight of detached nodules was two to three times that of detached roots, with corresponding differences in respect of carbon dioxide production. However although definite differences exist in the findings of Allison, Ludwig, Hoover and Minor on the one hand and Asprey and Bond on the other, both sets of results suggest that a curtailment of oxygen below the air value will tend to affect the respiration and perhaps other activities in the nodule at an earlier stage than in roots. On the other hand, a very rapid rate of nodule respiration may not be necessary for maximum fixation and may lead to wastage of the plant's food supply.

Wilson and Fred (1937), whose work will receive detailed consideration later in the thesis, investigated the importance of oxygen to red clover. Their plants were grown in sand and were wholly enclosed in 2- or sometimes 10-litre bottles. The atmosphere within these bottles was adjusted to contain different partial pressures of oxygen, ranging from 0.6 atmospheres to 0.012 atmospheres of oxygen (or 60 per cent to 1.2 per cent oxygen, assuming a total pressure of 1 atmosphere). They found that nodulated plants in a nitrogen-free medium responded in a similar manner to the various oxygen tensions as did the non-nodulated plants receiving ammonium nitrate. Between 0.4 and 0.05 atmospheres the formation of dry weight and the assimilation of free or combined nitrogen were independent of the oxygen tension in the atmosphere, but below 0.05 atmospheres, assimilation of nitrogen and the formation of dry weight decrease for both types of plants in a similar manner, owing to a general slowing up in the metabolism of the entire plant. They concluded from their results that there was no evidence of any special oxygen requirement by nodulated clover plants.

Thus we see that there have been only two investigations in which the growth and development of legumes at carefully controlled oxygen levels have been studied, namely, that of Wilson and Fred just mentioned, and that of Bond (p. 11). In the experiments now to be described, an attempt has been made to ascertain whether the differences in results between Wilson and Fred on the one hand, using clover with the whole plant exposed to a selected oxygen tension, and Bond on the other, using Soya bean with the root system only at adjusted oxygen tension, were due to the use of a different technique or to the use of a different legume. Here both techniques have been used and the former authors' test plant, namely red clover, employed owing to its adaptability to the two experimental methods.

The principle common to both techniques is that the respons of (a) nodulated plants in solution free of combined nitrogen and (b) non-nodulated plants supplied with ample combined nitrogen, to exposure to different oxygen levels are compared. If it is assumed that the root system (excluding the nodules) and shoots of plants (a) have similar oxygen requirements to those of plants (b), then any differences in response to oxygen treatment can be attributed to effects exerted in the nodules.

Root-System Enclosure Technique.

M E T H O D S.

Two experiments have been carried out by this technique. Ordinary commercial Montgomery red clover seed was used in the initial experiment, but in order to reduce plant-to-plant variation S123 Red Clover seed kindly supplied by the Welsh Plant Breeding Station, Aberystwyth, was employed in the second experiment. Seeds of approximately similar size were surface sterilised and sown in troughs of autoclaved sand. At the stage when the second true leaf was unfolding, usually after about 14 days' growth, uniform seedlings were transplanted into water culture in large test-tubes, with one plant per tube. These tubes had a capacity of 200 cc. and were 20 cm. in height, with a diameter of 3.5 cm. and were fitted at the mouth with a three-holed rubber stopper (see Figs. 1 and 2). The largest hole contained a rubber inset with a second hole through which the root system of the seedling was introduced and the hypocotyl sealed in by means of "plasticine". This rubber inset was incorporated into the apparatus owing to the fact that at the time of transplanting the clover seedlings were very small, the hypocotyl being approximately only ½ inch long. Without such an inset the root system of the seedling would not have

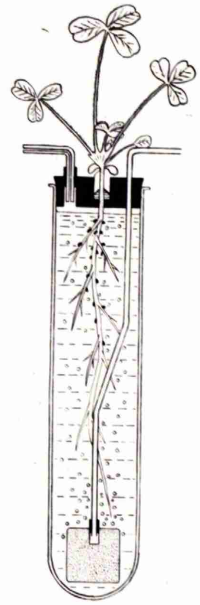


Fig. 1. Details of culture tube (x $\frac{1}{4}$).
For description see page 17.

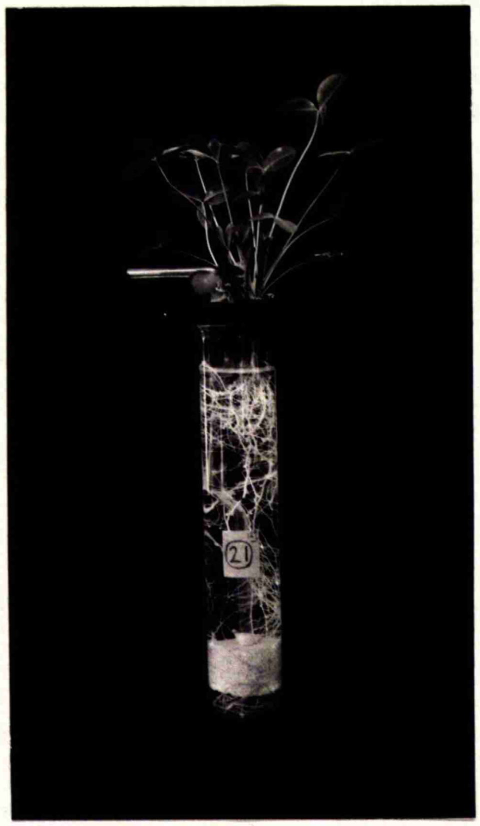


Fig. 2. A single culture tube with clover plant (x $\frac{1}{4}$)
(Inlet tube hidden behind outlet tube).

been properly immersed in the culture solution in the test-tube owing to the thickness of the rubber stopper and there would also have been a greater chance of damaging the seedling during the sealing-in process owing to the lack of working space round the hypocotyl. A second hole in the main stopper was closed by a small stopper bearing the gas outlet which consisted of capillary tubing of 1 mm. bore. Access could be gained to the culture solution by removing this stopper, in order that the solution might be renewed or samples withdrawn. The third hole allowed for the entry of the gas inlet tube which extended to the bottom of the culture tube and had a cubical porous aerator of 2.5 cm. edge fitted on the end.

Crone's nitrogen-free solution, made up according to the original formula except for the replacement of KNO_3 by KCl, was the basic solution employed in the experiments and was prepared as follows :-

KCl.....................0.75 gm.

$CaSO_4.2H_2O$.............0.50 gm.

$MgSO_4.7H_2O$.............0.50 gm.

$Ca_3(PO_4)_2$..............0. 25 gm.

$Fe_3(PO_4)_2.8H_2O$.........0.25 gm.

Distilled Water........1 litre.

One cubic centimetre of Hoagland's A toZ solution, with molybdenum added, was supplied to each litre of the Crone's solution. The solution was autoclaved before use and the culture tubes, their fittings, and all glass tubing used in connection with the removal and replacement of culture solution in the tubes were sterilised against the nodule organism. The culture tubes were placed in wooden racks each containing 6 tubes which were enclosed in black paper to exclude daylight from the root system in the solution.

The day following transplanting, the passage of the different oxygen /nitrogen gas mixtures was commenced and continued throughout the experiments, the gases being scrubbed through culture solution and dried over calcium chloride before entry into the tubes. A few hours after the passage of the gas mixture had been started, certain tubes were inoculated with an effective strain of the clover organism (Strain 49 - originally supplied by Rothamstead Experimental Station) and also in the initial stages of both experiments with these inoculated plants, 0.1 mg. of combined nitrogen was added to the culture solution in each tube in order to give the plants a little assistance during the period of nitrogen starvation before fixation commenced. In the case of the initial experiment in which nodulated plants and plants supplied with combined nitrogen as ammonium nitrate were grown at the same time, the latter plants received no combined nitrogen until visible nodules had developed on the former, but in the second experiment the combined nitrogen plants were supplied with ammonium sulphate from the beginning of the experiment as in this case no attempt was being made to keep the plants at approximately the same level of growth (see later). A small number of control tubes, uninoculated and with no combined nitrogen added, was included in order to confirm that under the cultural conditions employed the plants received no combined nitrogen from unintended sources.

In the first experiment three levels of oxygen were maintained in the culture tubes, but in the second this number was increased to four. The highest level was obtained by passing air (21 per cent oxygen) through one series of tubes, and the other three levels by passing oxygen /nitrogen mixtures containing 12, 5 and 1 per cent oxygen respectively through the other series of tubes. Thus the variation in oxygen content was accompanied by differences in nitrogen content of the gas mixture, but this is unlikely to be of any consequence since Wilson (1936) has shown that the fixation of nitrogen by nodulated clover plants is independent of the amount of nitrogen present as long as it exceeds 0.1 to 0.2 atmospheres (10 to 20 per cent). The air was supplied by a compressor pump (Edwards Type IV Compressor) for 20 hours out of each 24 hours (7 a.m. to 3 a.m.). The pump compressed filtered greenhouse air and included an oil baffle.

In addition the airstream before being supplied to the plants was passed through a 2-litre bottle tightly packed with cotton wool, as a further precaution against oil contamination. Actually no trace of oil was found in this filter bottle even after several months' use except immediately around the point of entrance of the airstream. The oxygen /nitrogen mixtures were obtained from the British Oxygen Co. Ltd and were supplied continuously. In the early stages of growth slow rates of gas flow sufficed to keep the solution in equilibrium with the gas mixture as shown by the determinations of dissolved oxygen (see below), but in the later stages it was necessary to increase the rate of flow in order to keep the solution as near as possible to the desired oxygen level. This increase in flow to about 45 cc. of gas per minute per tube was required when the roots and nodules were well developed.

Determinations of dissolved oxygen were made by the Winkler method, using a 1 cc. syringe pipette and an Agla micrometer pipette as described by Fox and Wingfield (1938). By fitting suitable lengths of narrow capillary tubing to the syringe pipette, samples could be withdrawn from midway down the culture tubes or from the bottom. The results of the determinations have been expressed as a percentage of the oxygen content of water in equilibrium with air at the temperature prevailing at the time of the determination, the data of Winkler (1889) being utilised in the calculation (see Appendix I). The effect of atmospheric pressure on dissolved oxygen has been ignored since it is very small within the normal range of barometric pressure. It is recognised that this method of expression conceals variations in absolute content of dissolved oxygen in the culture solution due to temperature changes. Thus the oxygen content of water in equilibrium with air at 760 mm. pressure varies from 6.6 cc. (at N.T.P.) at 18°C to 5.5 cc. at a temperature of 28°C, this being roughly the range of temperature variation in the greenhouse during the summer months. Thus it follows that on warmer days the oxygen supply in the culture solution is somewhat lower than on cooler days. This cannot be avoided unless it were possible to grow the plants at constant temperature. This consideration applies particularly to the root enclosure experiment where the culture vessel was open to the atmosphere. In the whole-plant enclosure method, a rise in temperature would lead to increased pressure of the gas contents of the aspirator bottle, which would in some degree compensate for the lower solubility at higher temperatures. By expressing the results of the determinations of dissolved oxygen in this way, it has been possible to seperate the temperature effect from other effects, e.g. uptake of oxygen by the plants, and also to present the results in more compact fashion.

The culture tubes were tested daily for gas-tightness by placing a drop of water at the exit of the gas outlet capillary tube. If an air bubble was formed this was taken as evidence that there were no leaks in or around the rubber stopper but where necessary the plasticine was adjusted around the hypocotyl. Also the culture solution was changed at fortnightly intervals. So far as possible the pH of the culture solution was maintained at 6.3 which is both the pH of unadjusted Crone's solution and also a suitable pH for the growth of nodulated clover plants or those supplied with combined nitrogen. Nodulated plants produce only slight lowering in the pH of the solution and this was corrected daily where necessary by suitable addition of N /10 NaOH. In the case of the plants supplied with combined nitrogen this operation had to be carried out every 12 hours at later stages.

In the first experiment 9 inoculated plants and 9 plants supplied with combined nitrogen were initially set up at each gas level. This experiment was carried out during the winter of 1950-51 and was conducted under artificial illumination, provided by a battery of seven 80 W. fluorescent tubes (length 5 ft.) arranged in a similar manner to that described by Low (1948). The intensity of the artificial light was approximately

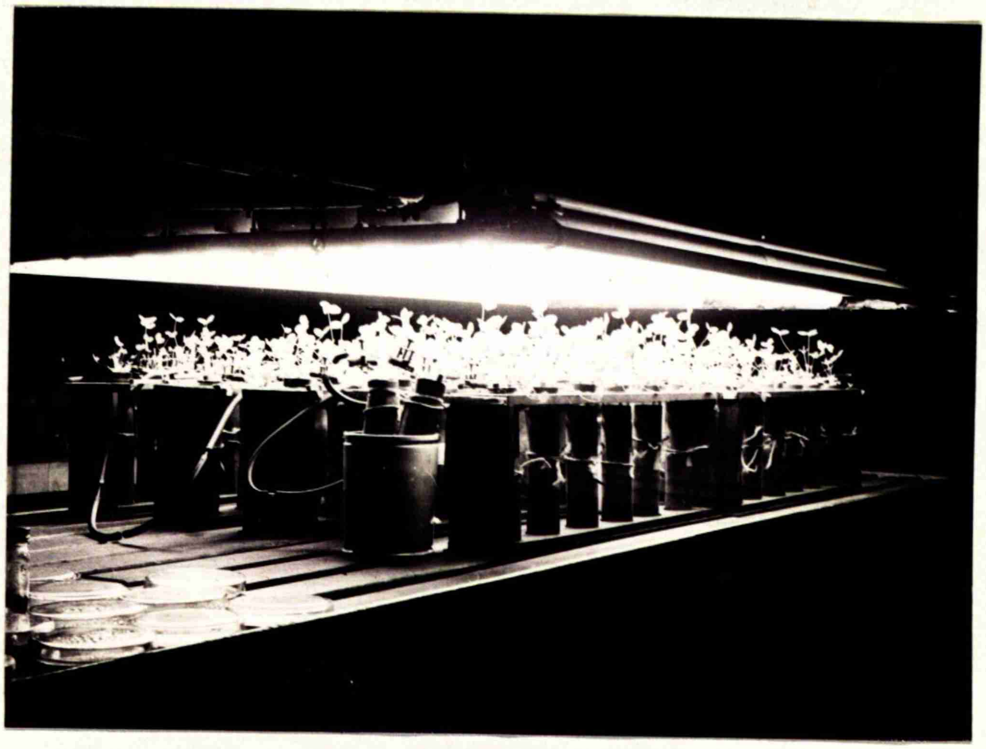


Fig. 3. Layout of experiment with fluorescent lighting. Winter 1950-51.

300 foot candles at plant level and the light was supplied for 16 hours (7 a.m. to 11 p.m.) each day (see Fig. 3). In the second experiment the number of plants was increased to 18 in the case of the inoculated plants and 16 with the plants receiving combined nitrogen, the experiment being performed in two parts (Part I - inoculated plants : Part II - combined nitrogen plants). This division of the experiment was carried out owing to limited greenhouse space and the amount of available equipment. Fig. 4 shows the layout of this second experiment which was performed in normal daylight.

The experiments were continued for a period of 8 - 11 weeks. Owing to the extremely complicated arrangement of gas manifolds that would have been necessary, randomisation of the tubes was not attempted. The lighting of the bench on which the experiments were conducted was very uniform in the case of the experiment in natural light, and in addition the arrangement of the various racks of plant tubes was frequently changed. With artificial lighting the light was less uniform but the tubes were changed weekly rather than every fortnight. At the termination of the experiments, nodules were counted, dry weights were determined by heating the plant material at 95°C and total nitrogen by the Kjeldahl method, the plants being treated singly in these operations.

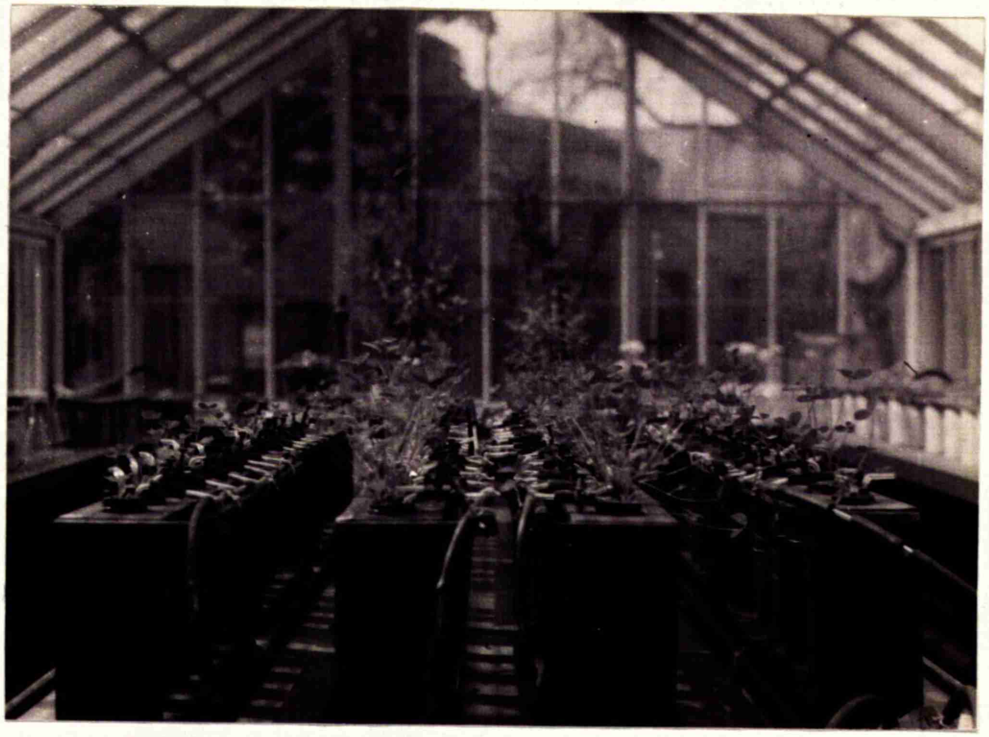


Fig. 4. General view of an experiment in progress.
Summer 1951.

EXPERIMENTAL RESULTS.

The data for the two experiments that have been carried out will be presented separately, except that the results of the determinations of dissolved oxygen of both experiments can conveniently be considered together.

The results of the determinations of dissolved oxygen present in the culture solution at the midway level (8 cm. from surface of solution) are summarised for the two experiments in Tables 1 and 2. As indicated already 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. The anticipated values for dissolved oxygen from oxygen /nitrogen mixtures containing 21, 12, 5 and 1 per cent oxygen are 100, 57, 24 and 5 respectively and we see from these Tables that in the early stages of the experiments the values obtained were very near to those anticipated. However as the experiment progressed and the plants increased in size with accompanying increase in root respiration, it was found impossible to maintain the oxygen levels quite up to the desired figures without an inconveniently high rate of gas-flows so that in the later stages the oxygen levels were appreciablly below the intended values.

Table 1.

Results of Determinations of Dissolved Oxygen, 1950-51 Experiment. All Samples taken at Depth of 8 cm. from Surface of Solution.

	Type of Culture.	Days from Transplanting into Culture Tubes.										
		2	30	31	36	37	43	44	57	58	64	65
Air	Nod.	98*	95	-	98	-	95	-	92	-	84	-
	Non-Nod.†	98	-	98	-	93	-	88	-	86	-	84
12% O_2	Nod.	55	49	-	49	-	47	-	46	-	43	-
	Non-Nod.†	54	-	49	-	47	-	45	-	44	-	35
5% O_2	Nod.	23	20	-	20	-	20	-	19	-	19	-
	Non-Nod.†	23	-	20	-	21	-	16	-	15	-	14

*Each figure indicates mean value of three determinations.

†Plants supplied with ammonium nitrate.

Table 2.

Results of Determinations of Dissolved Oxygen, 1951 Experiment. All Samples taken at Depth of 8 cm. from Surface of Solution.

	Type of Culture.	Days from Transplanting into Culture Tubes.									
		2	7	15	16	22	29	36	43	49	57
Air	Nod.	99*	-	99	-	97	96	95	96	-	96
	Non-Nod.†	98	97	-	95	-	-	-	-	89	-
12 % O_2	Nod.	54	-	51	-	52	51	49	43	-	43
	Non-Nod.†	53	50	-	49	-	-	-	-	44	-
5% O_2	Nod.	24	-	18	-	17	15	20	15	-	16
	Non-Nod.†	24	20	-	19	±	-	-	-	15	-
1% O_2	Nod.	5	-	5	-	3	2	3	3	-	3
	Non-Nod.†	5	5	-	3	-	-	-	-	2	-

*Each figure indicates mean value of three determinations.

†Plants supplied with ammonium sulphate

On a few occasions samples for oxygen determination were withdrawn from near the bottom of the culture tubes as well as from the midway level. Such double determinations showed that in a given tube the oxygen content was uniform throughout the culture solution in that tube and confirmed that no back entry of atmospheric oxygen was taking place, the latter also being indicated by the fact that the percentage oxygen in a given tube never exceeded the expected value.

Growth Data for First Experiment (Nov. 21, 1950 - Feb. 5, 1951).

In this experiment which, as noted, was performed under artificial illumination of the type already described, both inoculated plants and plants supplied with combined nitrogen were grown at the same time. In the case of the inoculated plants nodules visible to the naked eye appeared two weeks after inoculation and there were definite indications of nitrogen fixation approximately four weeks after inoculation. With the combined nitrogen plants the addition of ammonium nitrate was delayed for a time in order to compensate for the initial nitrogen starvation period experienced by the nodulated plants. However once nitrogen fixation commenced in the latter plants the non-nodulated ones received 10 mg. combined nitrogen per tube with further additions at regular intervals so that ultimately the culture solution had the same nitrogen content as normal Crone's solution.

pH adjustment of the solution for both types of plants was commenced in the fourth week of the experiment and while in the case of the nodulated plants maximum falls in pH value to 6.0 were recorded during the 24 hour period between adjustments, falls to 5.5 frequently occurred with the combined nitrogen plants and here (as already noted) the pH of the solution had to be tested every 12 hours and adjusted where necessary.

Shortly after fixation commenced differences in growth between nodulated plants at different levels of oxygen became apparent. The plants receiving 12 per cent oxygen were, rather surprisingly, distinctly superior to those at 21 per cent, and were also superior to those at 5 per cent. Such a condition continued until the termination of the experiment. The mean data obtained at the harvest of these nodulated plants are presented in Table 3 where it is shown that the dry weight and total nitrogen content of the plants at the 12 per cent level of oxygen were greater than those at 21 and 5 per cent oxygen. The statistical treatment* also presented in this Table shows that the apparent superiority of the 12 per cent oxygen plants over those at 21 per cent attained significance, but that the minimum difference required for significance was not quite

*A specimen analysis of variance is shown in Appendix II.

Table 3.

Mean Data (per Plant) obtained at Harvest of Nodulated Plants of First Experiment.

% Oxygen in Gas Supplied.	No. of Nodules.	Dry Weight (mg.)				Total Nitrogen (mg.)
		Nodules	Roots	Tops	Total	
21	119	8	47	138	193	5.01
12	240	12	91	207	310	9.82
5	195	8	52	148	208	6.54

Nine plants were initially set up at each oxygen level. For number of plants harvested see below.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison	No. of Plants Harvested	Dry Weight (mg.)	Total Nitrogen (mg.)
21% and 12%	8 and 9	117 (obsvd. 116)	3.72 (4.81)
21% and 5%	8 and 8	120 (14)	3.83 (1.53)
12% and 5%	9 and 8	117 (102)	3.72 (3.28)

reached on comparison of the plants grown at 12 and 5 per cent oxygen. It should be noted that in this experiment the differences between means required for significance were high, this being due to the large plant-to-plant variation, consequent no doubt on the use of commercial seed, and also to the rather small number of replicates. Typical groups of nodulated plants of this experiment are shown in Figs. 5 - 7.

In the case of the non-nodulated plants supplied with ammonium nitrate, a relationship between oxygen tension and the growth of the plants also developed at an early stage in the experiment but here such differences in growth were not so pronounced as with the nodulated plants. The plants at 12 per cent oxygen were on the whole very slightly superior to those at 21 per cent oxygen and growth at these two oxygen levels appeared slightly superior to that at the 5 per cent level. Although these visual observations were borne out by the mean dry weight figures obtained at harvest and presented in Table 4, the statistical treatment shows that the minimum differences required for significance had not been obtained on comparison of the growth of the plants at any of the oxygen levels. It should however be noted that the differences between means for 12 and 5 per cent were much nearer significance than those brtween 21 and 12 per cent oxygen. Typical groups of plants

Fig. 5. Nodulated plants of first experiment. 21% oxygen (x 1/8)

Fig. 6. Nodulated plants of first experiment. 12% oxygen (x 1/8)

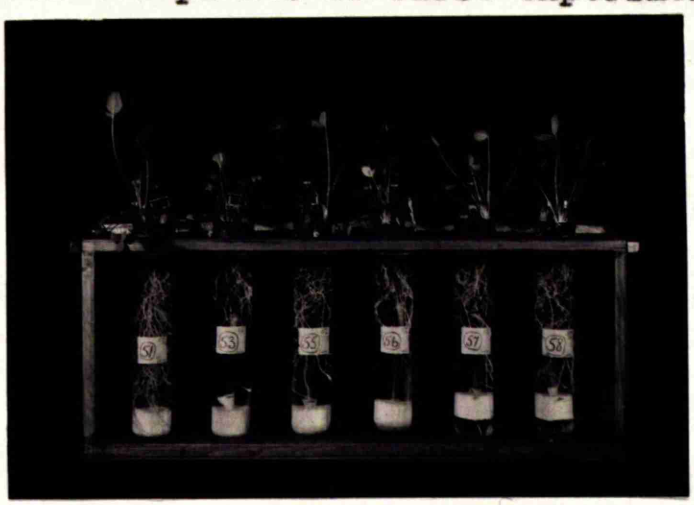


Fig. 7. Nodulated plants of first experiment. 5% oxygen (x 1/8).

Table 4.

Mean Data (per Plant) obtained at Harvest of Non-Nodulated Plants receiving Ammonium Nitrate of First Experiment.

% Oxygen in Gas Supplied.	No. of Plants Harvested.	Dry Weight (mg.)			Total Nitrogen (mg.)
		Roots	Tops	Total	
21	9	248	599	847	31.21
12	9	236	684	920	35.13
5	9	168	472	640	23.04

These plants received a total of 72.5 mg. nitrogen per tube during the experiment. Nine plants were initially set up at each oxygen level.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison	Dry Weight (mg.)	Total Nitrogen (mg.)
21% and 12%	392 (obsvd. 73)	16.32 (3.92)
21% and 5%	392 (207)	16.32 (8.17)
12% and 5%	392 (280)	16.32 (12.09)

from the three oxygen tensions are shown in Figs. 8 - 10.

The conclusions that may be drawn from this first experiment are of limited nature, firstly because only three levels of oxygen were employed, and secondly because the relatively small number of replicate tubes combined with the large plant-to-plant variation resulted in large differences in growth being necessary for significance. It is, however, definite that nodulated and combined nitrogen plants differed in their relative response to 21 per cent oxygen as compared with 12 per cent. The growth of nodulated plants at 12 per cent was clearly superior to that at 21 per cent, while the corresponding small difference with combined nitrogen plants was far below significance. A further curtailment of oxygen from 12 per cent to 5 per cent produced a similar response with both types of plants, viz., an apparent reduction in growth which just failed to attain statistical significance.

It may be noted that in this experiment the combined nitrogen plants made much more growth than those dependent on nodule nitrogen, due, it seems, to the fluorescent light employed being less suited to the needs of nodulated plants. Thus at the 12 per cent level the mean dry weight of the combined nitrogen plants was three times that of nodulated plants. The possibility may suggest itself that the absence of a clear optimum in growth

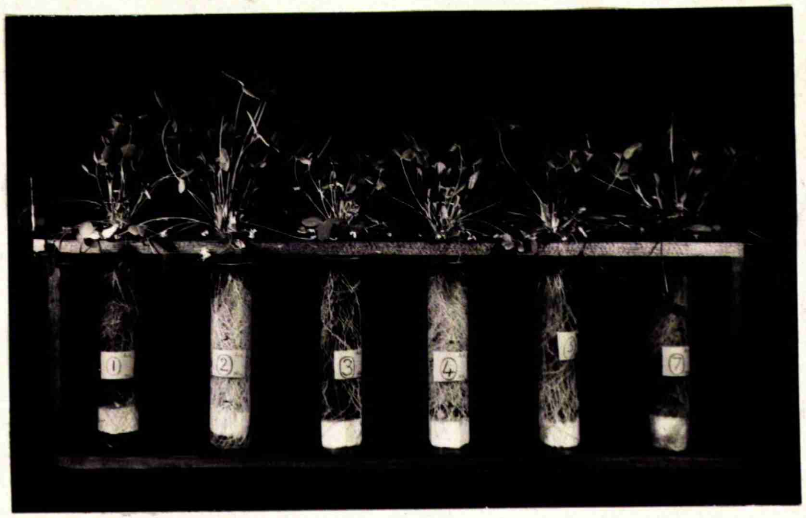


Fig. 8. Non-nodulated plants of first experiment supplied with ammonium nitrate. 21% oxygen (x 1/8).

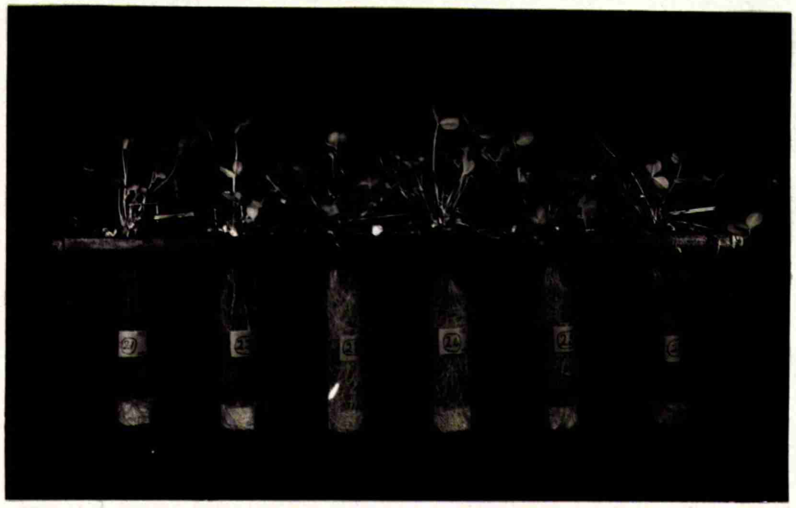


Fig. 9. Non-nodulated plants of first experiment supplied with ammonium nitrate. 12% oxygen (x 1/8).

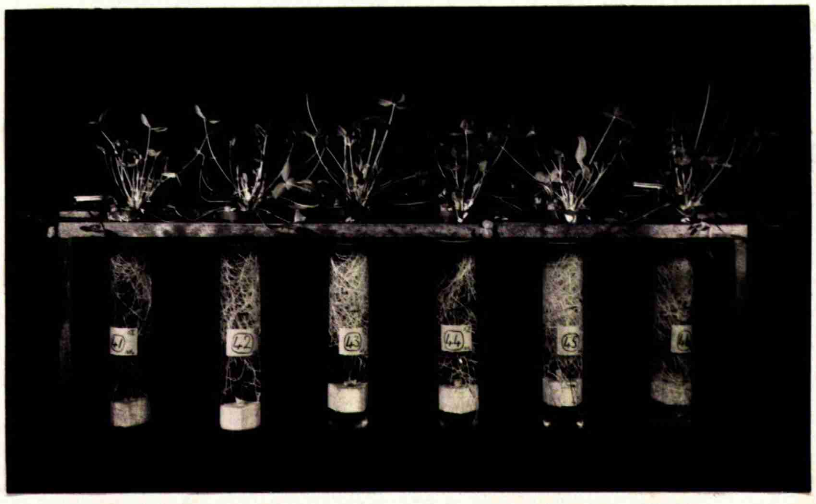


Fig. 10. Non-nodulated plants of first experiment supplied with ammonium nitrate. 5% oxygen (x 1/8)

at 12 per cent oxygen with the combined nitrogen plants might have been due to some limitations resulting from the general large size of the plants, e.g., limitation of root space. It is however important to notice that even in the earlier stages of growth, when no question of such limitation arose, there was never such a clear difference between the 21 per cent and 12 per cent plants as there was with the nodulated type.

Growth Data for Second Experiment (April 3 - June 4, and July 3 - August 25, 1951).

In this second experiment which was divided into two parts as already mentioned and performed under normal daylight, it was hoped to lower the differences required for significance by using a pure strain of red clover seed and by increasing the number of replicates. Also in this experiment four levels of oxygen, namely 21, 12, 5 and 1 per cent were employed, and owing to the findings of Gilbert and Shive (1945) that some plants can utilise the oxygen of the nitrate ion under conditions of reduced supply of free oxygen, ammonium sulphate was substituted for ammonium nitrate as the source of nitrogen to the uninoculated plants.

In the case of Part I of the experiment, involving nodulated plants, nodulation and nitrogen fixation commenced after an interval similar to that in the first experiment and pH adjustmen was performed from the third week after transplanting into cultur solution. The springtime conditions in the greenhouse obviously suited these nodulated plants better than the winter conditions and strong plants were obtained at all but the 1 per cent level of oxygen. The effect of lowering the oxygen tension was similar to that obtained in the initial experiment but the differences in growth at the various levels were more pronounced and also the plants were more uniform at a given treatment. Again best growth (by eye judgment) was shown at 12 per cent oxygen, followed by 21 and 5 per cent, which exhibited rather similar growth, while at the additional level, namely 1 per cent oxygen, the plants were very small. Mean data obtained at the harvest of these plants are presented in Table 5. The statistical treatment shows that the attempt by the above mentioned methods to reduce the differences required for significance was in fact successful, so that the conclusions that may be drawn are correspondingly clearer than in the first experiment, though generally in keeping with the latter. Growth and fixation at 12 per cent oxygen are clearly superior to those at 21 and 5 per cent, and at 5 per cent are superior to those at 1 per cent, findings which may be summarised thus:-

$$21\% < 12\% > 5\% > 1\%$$

At the 1 per cent level of oxygen the nodules remained small and white and in these respects were comparable with those

Table 5.

Mean Data (per Plant) obtained at Harvest of Nodulated Plants of Second Experiment.

% Oxygen in Gas Supplied	No. of Nodules.	Dry Weight (mg.)				Total Nitrogen (mg.)
		Nodules	Roots	Tops	Total	
21	231	26	117	280	423	12.73
12	248	29	160	542	731	20.40
5	328	20	102	370	492	13.10
1	178	3	23	61	87	2.35

18 plants were initially set up at each oxygen level. For number of plants harvested see below.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison	No. of Plants Harvested	Dry Weight (mg.)	Total Nitrogen (mg.)
21% and 12%	17 and 18	144 (obsvd. 308)	3.96 (7.67)
21% and 5%	17 and 16	148 (69)	4.08 (0.37)
21% and 1%	17 and 17	145 (336)	4.01 (10.38)
12% and 5%	18 and 16	146 (239)	4.03 (7.30)
12% and 1%	18 and 17	144 (644)	3.96 (18.05)
5% and 1%	16 and 17	148 (405)	4.08 (10.75)

normally produced by an ineffective strain of the nodule organism. Only in the closing stages of the experiment could the presence of haemoglobin be detected within the nodules, and it never attained the intensity shown in nodules subjected to higher oxygen tensions. Photographs of typical plants of Part I of this experiment are shown in Figs. 11 - 16.

Part II of this experiment comprised uninoculated plants supplied with ammonium sulphate. Some difficulty was encountered in controlling pH with these plants. Although in the later stages of growth pH checking and adjustment was carried out every 12 hours, even in this relatively short interval falls from the desired value of pH 6.3 to 5.0 occurred. Thus these plants grew at a somewhat lower mean pH than the nodulated plants of Part I of this experiment, but it is unlikely that the difference was large enough to invalidate in any way comparisons between the two types of plants.

The mean data obtained at harvest of these plants are summarised in Table 6. The means for the plants grown at 12 per cent oxygen are slightly higher than those for 21 per cent, but the differences are a long way short of significance, providing a notable difference from the corresponding comparison in nodulated plants. Reduction of oxygen to 5 per cent led to a fully-significant curtailment of growth, and the same applied to

Fig. 11. Single nodulated plant. 12% oxygen (x 1/7).

Fig. 12. Nodulated plants of second experiment.
Left to right - 21, 12, 5 and 1 per cent oxygen (x 1/7).

Fig. 13. Nodulated plants of second experiment. 21% oxygen (x 1/8).

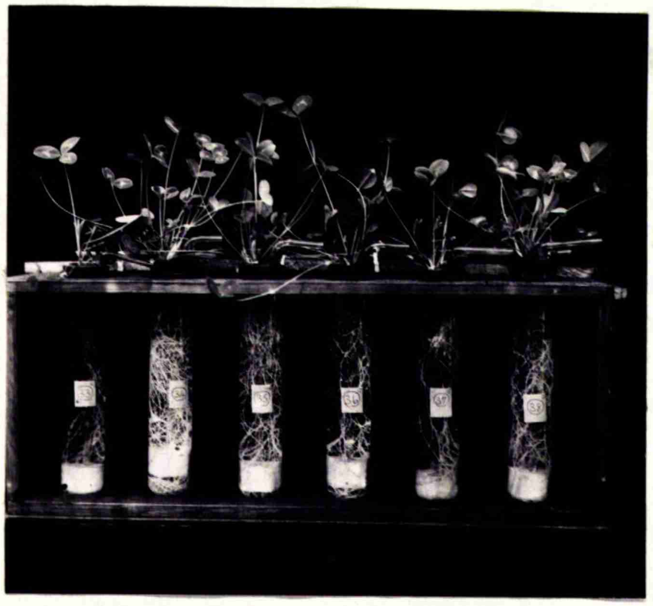


Fig. 14 Nodulated plants of second experiment. 12% oxygen (x 1/8).

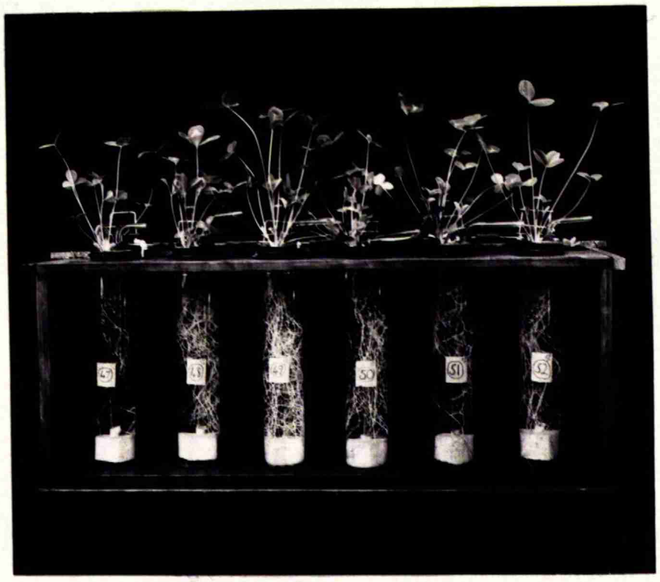


Fig. 15. Nodulated plants of second experiment. 5% oxygen (x 1/8).

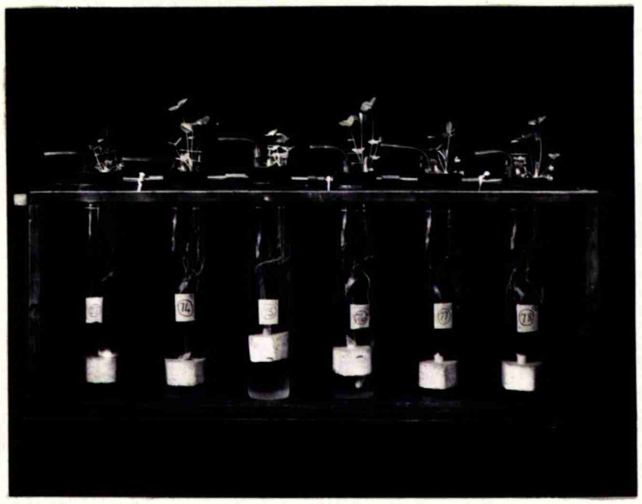


Fig. 16. Nodulated plants of second experiment. 1% oxygen (x 1/8)

Table 6.

Mean Data (per Plant) obtained at Harvest of Non-Nodulated Plants receiving Ammonium Sulphate of Second Experiment.

% Oxygen in Gas Supplied.	No. of Plants Harvested.	Dry Weight (mg.)			Total Nitrogen (mg.)
		Roots	Tops	Total	
21	14	217	476	693	18.57
12	16	278	512	790	21.45
5	14	173	388	561	16.50
1	11	49	119	168	5.22

These plants received a total of 60 mg. nitrogen per tube during the experiment. 16 plants were initially set up at each oxygen level.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison	No. of Plants Harvested	Dry Weight (mg.)	Total Nitrogen (mg.)
21% and 12%	14 and 16	184 (obsvd. 97)	4.20 (2.88)
21% and 5%	14 and 14	197 (132)	4.40 (2.02)
21% and 1%	14 and 11	213 (525)	4.88 (13.35)
12% and 5%	16 and 14	184 (229)	4.20 (4.95)
12% and 1%	16 and 11	197 (622)	4.40 (16.23)
5% and 1%	14 and 11	213 (393)	4.88 (11.28)

the further reduction to 1 per cent. These results may be summarised thus:-

$$21\% = 12\% > 5\% > 1\%$$

Photographs of typical plants from this part of the experiment are shown in Figs. 17 - 21.

It should be noted that in this experiment the disparity between the general growth of the nodulated and the combined nitrogen plants was very small compared with that in the winter-time experiment. This was partly because growth conditions were now more favourable to the nodulated plants and also because the duration of the experiment with combined nitrogen plants was deliberately shortened somewhat.

In connection with these two experiments, 11 control tubes of non-nodulated plants which did not receive combined nitrogen were set up, with one or two culture tubes at each oxygen level. The growth of all these plants was negligible and at harvest it was found that the mean nitrogen content of these plants was 0.1 mg. and the mean dry weight 21 mg. Such figures confirm that under the experimental conditions employed the plants received no combined nitrogen from unintended sources, as the total nitrogen content of a clover seed is in the region of the above mentioned figure. They also show that the figures in the above Tables for nitrogen content of nodulated plants indicate

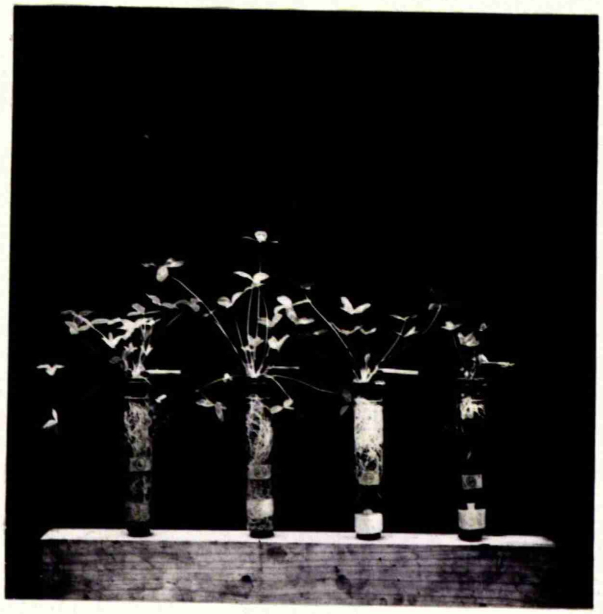


Fig. 17. Non-nodulated plants of second experiment supplied with ammonium sulphate. Left to right - 21, 12, 5 and 1 per cent oxygen (x 1/11).

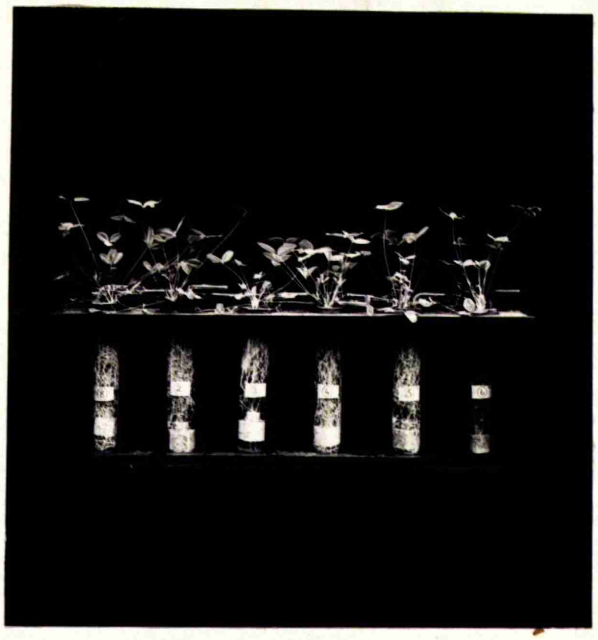


Fig. 18. Non-nodulated plants of second experiment supplied with ammonium sulphate. 21% oxygen (x 1/11).

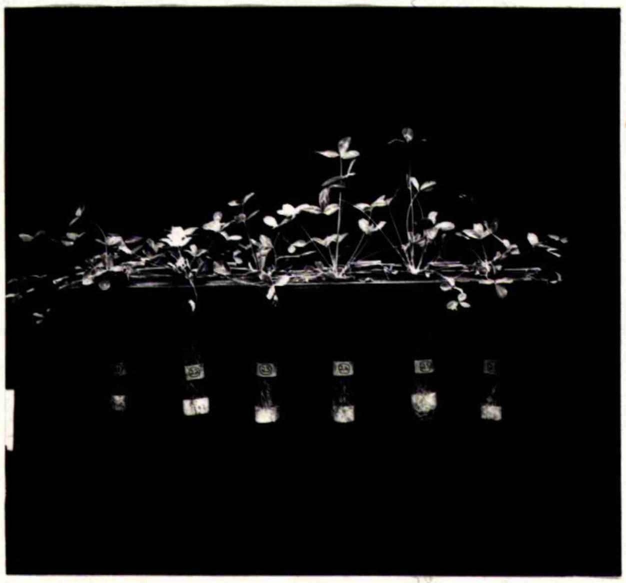


Fig. 19. Non-nodulated plants of second experiment supplied with ammonium sulphate. 12% oxygen (x 1/11).

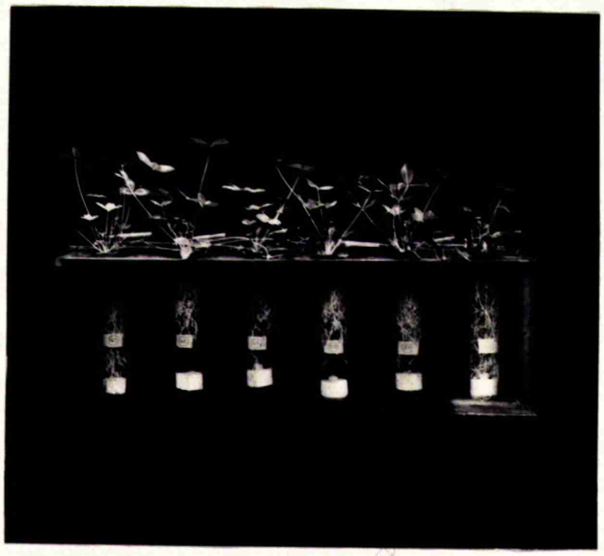


Fig. 20. Non-nodulated plants of second experiment supplied with ammonium sulphate. 5% oxygen (x 1/11).

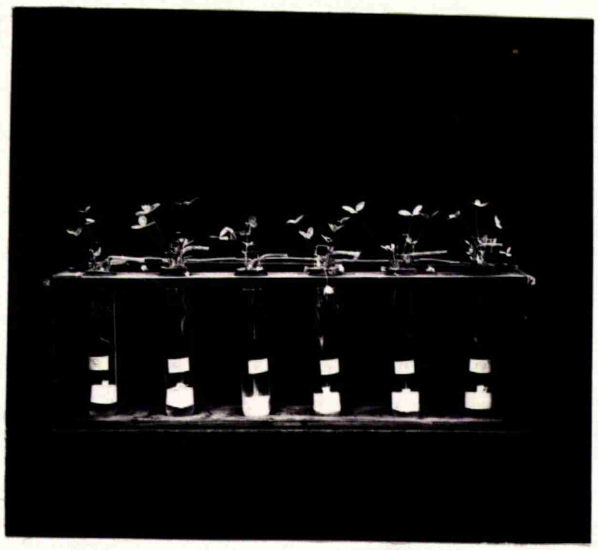


Fig. 21. Non-nodulated plants of second experiment supplied with ammonium sulphate. 1% oxygen (x 1/11)

accurately the amount of nitrogen fixed by these plants.

DISCUSSION.

When growing plants under the conditions employed in the present experiments, in which the root system alone was subjecte to various oxygen tensions while the shoot was exposed to the normal atmosphere of the greenhouse, one must, (as pointed out by Bond (1951)), consider the possibility of a downward diffusi of oxygen through the plant itself as a means of relieving the tension of oxygen imposed on the root system. Obviously if such a downward movement could readily occur the technique employed would have to be considered unsatisfactory as it would be impossible to control the oxygen supply to the root tissue by adjusting the oxygen level in the surrounding culture solution. Given such a downward movement of oxygen, then when a gas mixture with only 1 per cent oxygen was supplied to the rooting solution one would anticipate that a diffusion of oxygen out of the roots into the solution might occur under the influence of the differences in partial pressure. The determinations of dissolved oxygen present in the culture solution gave no indication of such an outward flow since at all times during the experiment the oxygen tensions were at or below the equilibrium point. Also comparison of growth, say between plants grown at 1 per cent oxygen and those at 12 per cent oxygen (see Fig. 22)

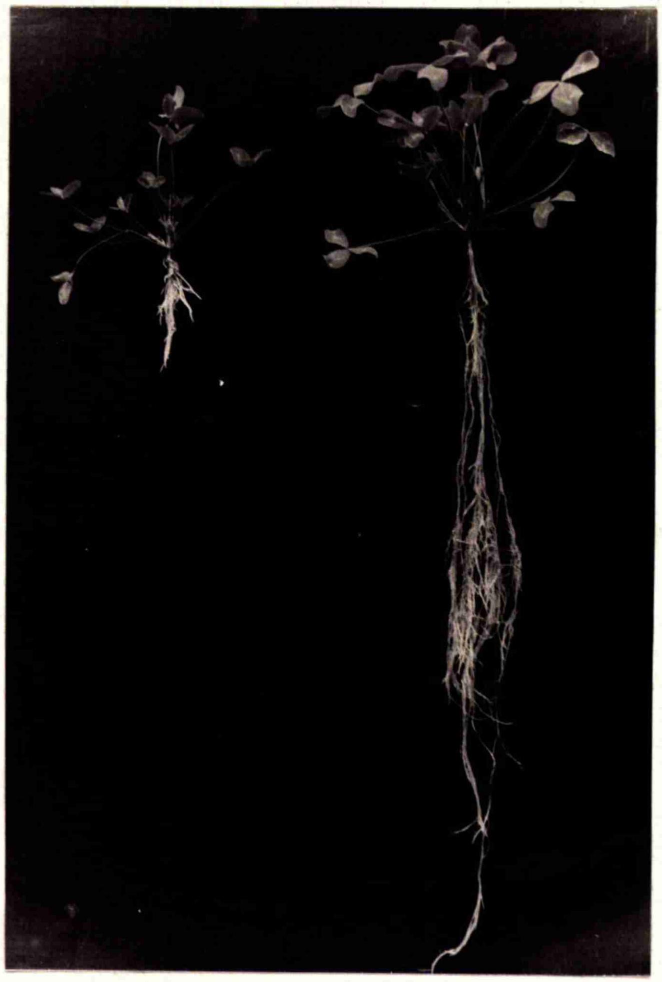


Fig. 22. Non-nodulated plants of second experiment supplied with ammonium sulphate. 1 per cent oxygen (left) and 12 per cent oxygen (right). (x 1/5).

make it abundantly clear that the gas stream was the major source of oxygen for the root system and that no appreciable movement of oxygen through the plants took place.

The results of these two experiments (particularly of the second one) may be summarised by saying that on comparison of plants grown with their roots in water culture in approximate equilibrium with oxygen /nitrogen gas mixtures containing 21, 12, 5 and 1 per cent oxygen respectively, it was found that with nodulated plants there was a significant increase in dry weight for the whole plant and its various parts when the oxygen tension was decreased from 21 to 12 per cent oxygen, followed by a reduction in dry weight as the oxygen level was lowered to 5 and then to 1 per cent oxygen. A similar position held for the total nitrogen content and therefore for the amount of atmospheric nitrogen fixed. In the case of the non-nodulated plants supplied with combined nitrogen, growth was essentially similar at both 21 and 12 per cent oxygen and this was followed by a decrease in growth as the tension was further lowered to 5 and then to 1 per cent oxygen. Although a reduction in growth took place with both types of plants as the oxygen tension was reduced below 12 per cent oxygen, it should be noted that the fall in the case of the nodulated plants was steeper than that exhibited by the plants receiving combined nitrogen owing to the marked increase in growth at 12 per cent oxygen by the former. From all these observations it can be stated that the nodulated plants reacted to the various oxygen tensions in a different manner from the plants supplied with combined nitrogen. A study of the relative dry weights and total nitrogen contents illustrates these points (see Table 7 and Graph 1).

It thus appears that when the oxygen tension is reduced to a value approximately half that of the air value, the activities of the nodulated root system are increased, whereas the non-nodulated root system supplied with combined nitrogen is only slightly affected and to a much smaller degree. Assuming that the oxygen requirements of the roots of both types of plants are of a similar order, we must conclude that the differences in growth were caused by the efficiency of the nodules being increased at an oxygen tension below the air value. Further consideration of this at first sight somewhat surprising though quite clearly established result will be postponed to the Discussion coming after the description of the whole-plant enclosure experiment. The comparison of these results for clover and those of Bond (1951) for Soya bean, using a similar technique, will also be postponed to the later Discussion.

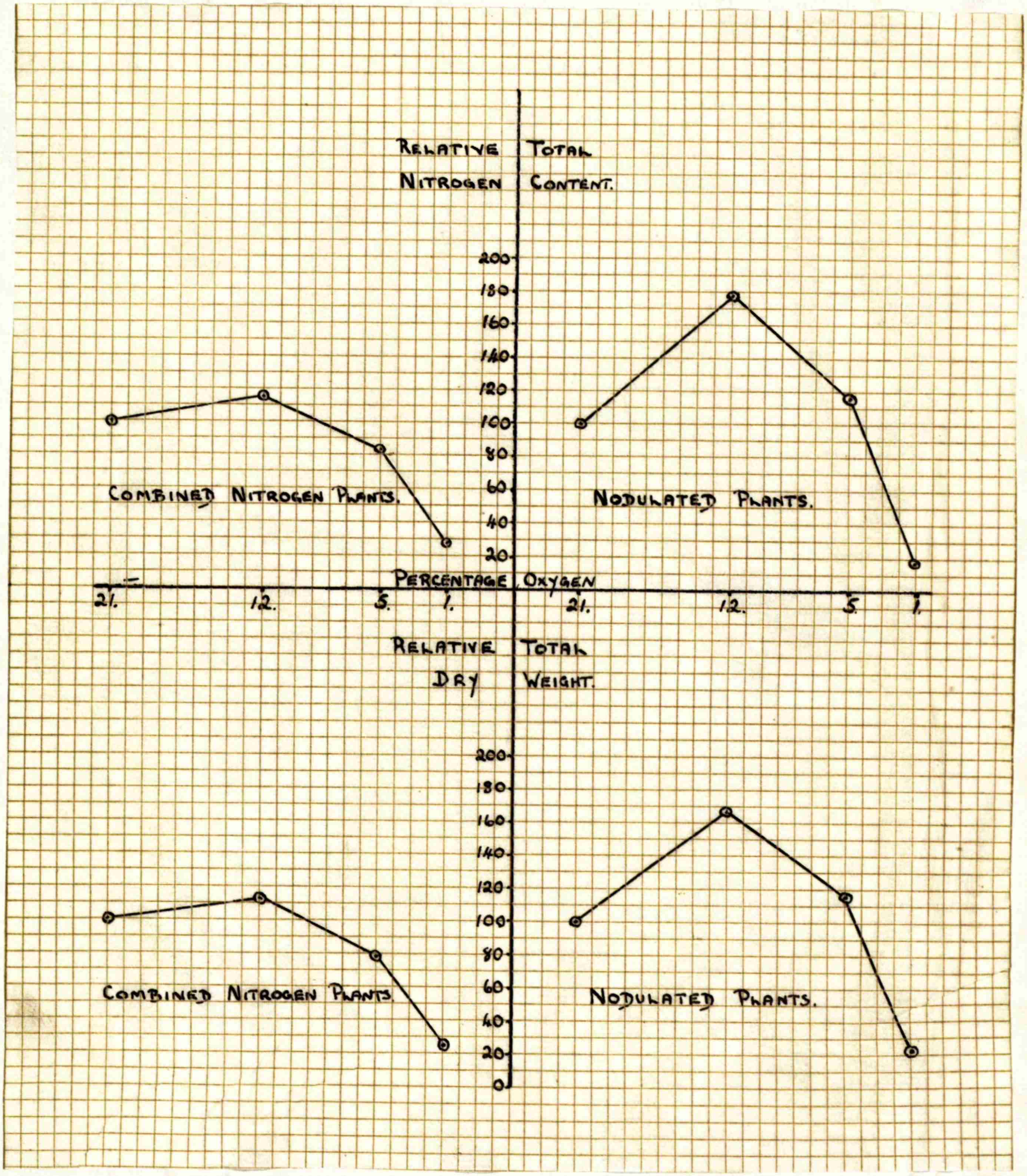
The data for number and dry weight of nodules at the different oxygen levels in the second experiment are reproduced

Table 7.

Relative Values calculated from the Data of the Two Experiments with Mean at 21 per cent taken as 100.

% Oxygen in Gas Supplied.	Nodulated		Non-Nodulated	
	Dry Weight.	N-Content.	Dry Weight.	N-Content.
21	100	100	100	100
12	167	178	112	114
5	113	117	79	82
1	21	18	24	28

The figures shown are the mean values of the two experiments.



Graph 1. Relative values calculated from data of the two experiments with mean at 21 per cent taken as 100.

separately in Table 8. It will be noted that according to the statistical treatment the number of nodules is similar at 21 and 12 per cent oxygen, but increases significantly at 5 per cent below which there is a sharp fall. It must be pointed out however that the high mean at 5 per cent was largely due to the effect of a small number of atypical plants bearing 400 - 495 nodules each, and forming a group quite distinct from the general run of the plants. For such a thing to happen is not an unusual experience in connection with the nodulation of legumes. Thus it is preferable to conclude that over the range 5 - 21 per cent oxygen the number of nodules formed is essentially unaffected. The only previous observations on this aspect are those of Bond (1951), who on Soya bean found nodule numbers again unchanged over the range 5 - 21 per cent oxygen. The reduction in nodule number at 1 per cent is undoubtedly due at least in part to the reduction in root growth (shown also in the combined nitrogen plants) and consequently in opportunities for infection. Bhaduri (1951) showed in Phaseolus that the number of nodules produced was dependent upon the extent of the root system. The dry weights of nodules per plant at different oxygen levels shows a close parallel to those of the plants themselves, so that the maximum is at 12 per cent. Since it has been concluded already that the nodules function best in the

Table 8.

Effect of Oxygen Tension on Initiation and Development of Nodules Inoculated Plants of Second Experiment.

% Oxygen in Gas Supplied.	Mean Number of Nodules per Plant.	Mean Dry Weight of Nodules (mg) per Plant.
21	231 (57 - 350)*	26
12	248 (153 - 394)	29
5	328 (179 - 495)	20
1	178 (64 - 300)	3

* Figures in parentheses indicate extreme values.

Statistical treatment shows that the difference between means for both nodule numbers and dry weight is significant in all cases except that between 21 and 12 per cent oxygen.

region of 12 per cent oxygen, it appears that there is a difference between the oxygen tolerance of the early stages in nodule development and that of the nodule as a functional unit in nitrogen fixation, the latter being higher.

Whole-Plant Enclosure Technique.

M E T H O D S.

It will be remembered that in this particular portion of the research a technique similar to that employed by Wilson and Fred (1937) was to be used in which the clover plants are grown rooted in sand and wholly enclosed in large aspirator bottles, the atmospheres within these being adjusted to contain different proportions of oxygen.

In the experiment now to be described the sand employed as the rooting medium was a washed quartz sand kindly supplied by the General Refractories Co. Ltd., and which was known from previous experimental work to contain no more than traces of combined nitrogen. The plants were grown in this sand in glass aspirator bottles with a capacity of 5 litres and 32.5 cm. in height, with diameters of 6 cm. and 17 cm. at the mouth and below the shoulders respectively. The sand in these bottles had a depth of 6 cm. Except for a preliminary period during which the bottles were closed by cotton wool plugs, they were fitted with rubber stoppers each of which had two holes, the gas inlet tube passing through one of these and thence extending almost to the surface of the sand. The gas outlet tube passed through the other hole (see Fig. 23). This outlet tube was connected by

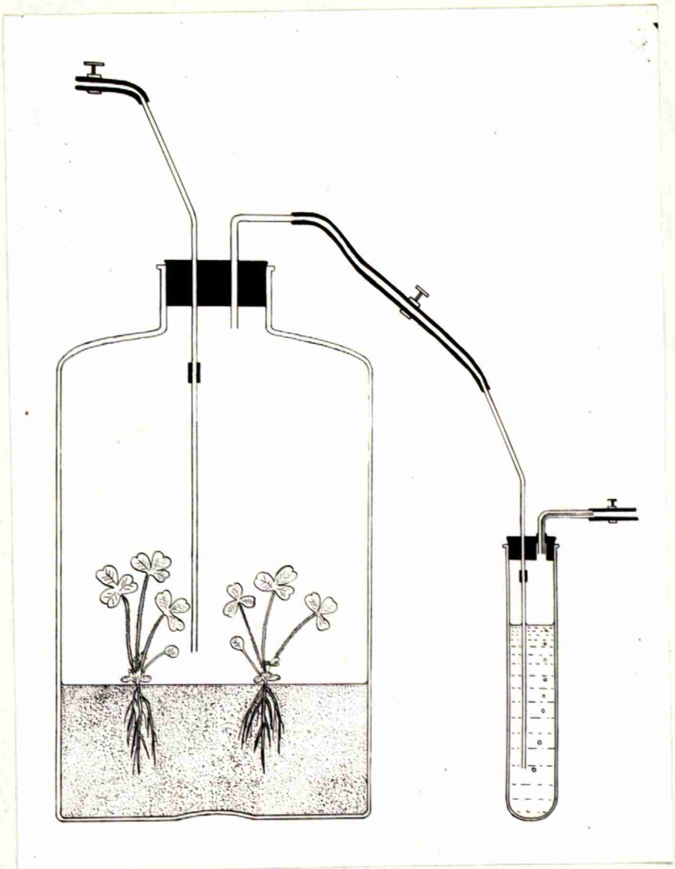


Fig. 23. Arrangement employed for whole- plant enclosure experiment. (x 1/5). For description see text.

means of thick-walled rubber tubing to a "tell-tale" which consisted of a test tube of height 15 cm. and 2.5 cm diameter, containing water and closed at the mouth with a rubber stopper bored with two holes. These holes again allowed for the passage of the gas inlet and outlet tubes, the former extending almost to the base of the test tube, while the latter consisted of a short length of capillary tubing of 1 mm. bore. By removal of this outlet tube, access could be gained for the purpose of withdrawal of samples of water from the "tell-tale".

By means of the "tell-tale" the rate of passage of gas through the 5-litre bottles could be judged and was controlled by a screw clip on the rubber tubing between the bottle and the "tell-tale". A short length of rubber tubing with a screw clip fitted was also attached to the capillary outlet of the "tell-tale" in order to allow of the closure of the outlet during the periods when the gas flows were turned off. Also by analysing the water in the "tell-tale" for dissolved oxygen (see later) the oxygen content of the atmosphere within the plant bottles could be ascertained.

Crone's nitrogen-free solution with the minor elements added was again used and was added to the sand in the proportion one sixth by weight. Both the sand and the nutrient solution were autoclaved before use and the bottles and their fittings sterilised against the nodule organism.

This experiment was divided into two parts carried out consecutively, Part I comprising nodulated clover plants and Part II non-nodulated plants supplied with combined nitrogen. S123 Red Clover seed, supplied by the Welsh Plant Breeding Station, Aberystwyth, was again used and selection and sterilisation of the seed were effected as before. In Part I of the experiment the seeds were inoculated with an effective strain of the clover organism (Strain 49) by dipping the seed into a suspension in sterile water of the nodule bacteria before sowing in the sand in the 5-litre bottles. The seeds were sown individually into small holes made in the sand with a sterilised glass rod by picking them up in the flame-sterilised loop of a long inoculating needle and then covering them over with sand, the sterilised glass rod being used in this final operation. In Part II of the experiment seed inoculation was omitted but 52 mg. of nitrogen per bottle as ammonium sulphate was in this case added with the Crone's solution. A further 14 mg. of nitrogen per bottle was added to the sand when these plants had reached the 3-leaf stage and just before the gas flows were turned on (see below). Ammonium sulphate was used rather than ammonium nitrate which was the source of nitrogen in the reported experiments of Wilson and Fred (loc. cit.) because of the view that the oxygen of the nitrate ion can be utilised by plants under conditions of reduced oxygen supply (see p. 33). A small number of control bottles, uninoculated and with no combined nitrogen added, was included in the experiment in order to ascertain the amount (if any) of nitrogen which the plants might be obtaining from the sand. In all cases 15 seeds were sown in each bottle.

The emergence of seedlings above the sand commenced four days after sowing and a few days later the number of plants in each bottle was reduced to 7 in the case of the nodulated and control plants, but to 6 in those receiving combined nitrogen since stronger growth was anticipated with these latter plants.

Up to this point the bottles were closed by cotton wool plugs, so that the atmosphere within the bottles consisted of ordinary air. This preliminary period of growth in air was provided firstly because there was a corresponding period in the previously described root enclosure technique, and secondly because Wilson and Fred included a similar preliminary period. After two weeks from seedling emergence (at a stage when the plants bore 3 leaves) the atmospheres within the bottles were adjusted to different oxygen levels. The rubber stoppers were fitted to the bottles and these connected to the gas manifolds. The gas mixtures were again obtained in compressed form from the British Oxygen Co. Ltd. (Medical Section) and resembled these employed in the root enclosure technique except that each mixture now contained 1 per cent carbon dioxide. The latter was added because with the system of intermittent gas flow adopted (see below) the growth of the plants would otherwise have been limited by the lack of carbon dioxide. Thus there were four oxygen /nitrogen / carbon dioxide mixtures, containing 21, 12, 5 and 1 per cent oxygen respectively, with 1 per cent carbon dioxide in all cases. The gas mixtures were scrubbed through culture solution and dried over calcium chloride before entering the plant bottles.

In view of the cost of the gas mixtures and of the large volume of the plant bottles relative to the amount of plant substance, it was decided that continuous flow would probably be unnecessary, at least during the early stages of growth. It may be noted that whereas in the root-system enclosure technique the effect that had to be guarded against was a lowering in oxygen tension owing to root respiration, with the present technique it is the possibility of an increase in oxygen level, due to photosynthesis, that has to be borne in mind. The results of oxygen determinations on the gaseous contents of the plant bottles (see below) confirmed that intermittent gas supply was satisfactory not only in the early stages of growth but also in the later stages. The initial displacement of the air originally present in the bottles required the passage of the gas mixtures at the customary rate of about 20 cc./minute for a period of about 6 hours (see later), after which each bottle was isolated by closing the three screw clips shown in Fig. 23. On subsequent days the gas mixtures were passed for a period of 2 hours, increased to 4 hours in later stages of growth. The order in which the screw clips were closed ensured that the atmosphere within the bottle was left at or very near to atmospheric pressure.

As indicated already, information on the oxygen content of the atmosphere within the plant bottles was obtained by means of determinations of dissolved oxygen present in samples of the water of the "tell-tale" tube. After a period of gas flow this water will be in equilibrium with the gas emerging from the plant bottle. With the system of intermittent gas flow indicated above the routine procedure was to commence the gas flow, and then after a period of about 30 minutes to withdraw a 1 cc. sample of the water in the "tell-tale" with the syringe pipette and determine its dissolved oxygen content by the method already described (Root-System Enclosure Technique - Methods). It was assumed that any change in the composition of the gas within a given bottle during the period of no-flow would be reflected by the oxygen data so obtained. The results of these oxygen determinations were again expressed as a percentage of the oxygen content of water in equilibrium with air at the temperature prevailing at the time of the determination.

Part I of the experiment which comprised nodulated plants was continued for a period of 8 weeks and Part II with non-nodulated plants supplied with combined nitrogen for 7 weeks. In both cases the arrangement of the bottles was altered at frequent intervals in case there were slight inequalities of lighting on the table on which the bottles were arranged. The layout of this experiment is shown in Fig. 24. The full experiment extended over the period April - August, 1952, under natural light, and during warm sunny days it was found necessary to spray the bottles with water in order to prevent the temperature within the bottles rising to a degree which might have resulted in harm to the plants. In this way, together with the use of the usual roof screens, the temperature within the bottles was usually prevented from rising above 25° C, except on particularly hot days. At the termination of the experiment the plants were harvested individually and the dry weights ascertained by heating the plant material at 95° C. The total nitrogen content was determined by use of the Kjeldahl method, individual plants being analysed in the case of the nodulated and control plants, while with the non-nodulated plants supplied

Fig. 24. Layout of experiment - whole-plant enclosure technique. Approximately three weeks after sowing of seed.

with combined nitrogen all plants from a given oxygen level were bulked prior to Kjeldahl analysis.

EXPERIMENTAL RESULTS.

The results of the determinations of dissolved oxygen present in the water contained in the "tell-tales" for both parts of this experiment are shown in Tables 9 and 10. It may be recalled that except for the first day these oxygen determinations were made 30 minutes after the gas flows had been restarted. Any changes in the composition of the atmosphere within the bottles that had arisen during the periods of no-flow would, it was anticipated, be reflected in the oxygen data so obtained. The Tables show that the expected values for oxygen content (namely 100, 57, 24 and 5 for oxygen /nitrogen /carbon dioxide gas mixtures containing 21, 12, 5 and 1 per cent oxygen respectively) were initially obtained and that there was no suggestion of any significant deviation from these values throughout the whole growth period.

The oxygen data presented in Table 11 and Graph 2 are also of interest. They show successive readings obtained during the initial displacement of air from two 5-litre bottles by the 1 per cent oxygen mixture passing in at the usual slow rate (see p. 53) into the bottles. The results indicate that the

Table 9.

Results of Determinations of Dissolved Oxygen. Part I - Nodulated Plants.

% Oxygen in Gas Supplied.	Days from Commencement of Gas Flow.								
	1	10	12	14	21	27	31	36	42
21	98*	97	97	97	96	95	96	100	98
12	57	56	55	56	55	56	56	58	58
5	24	24	23	24	24	23	24	26	26
1	5	4	4	5	5	5	5	6	5

*Each figure indicates mean value of three determinations on different bottles.

Determinations on first day made 6 hours after commencement of gas flow.

Table 10.

Results of Determinations of Dissolved Oxygen. Part II - Non-Nodulated Plants.

% Oxygen in Gas Supplied.	Days from Commencement of Gas Flow.								
	1	3	7	10	17	24	31	38	40
21	99*	99	98	98	97	99	100	99	98
12	56	55	57	57	56	57	57	56	57
5	24	24	24	24	23	25	25	24	25
1	5	5	4	5	4	5	6	5	5

*Each figure indicates mean value of three determinations on different bottles.

Determinations on first day made 6 hours after commencement of gas flow.

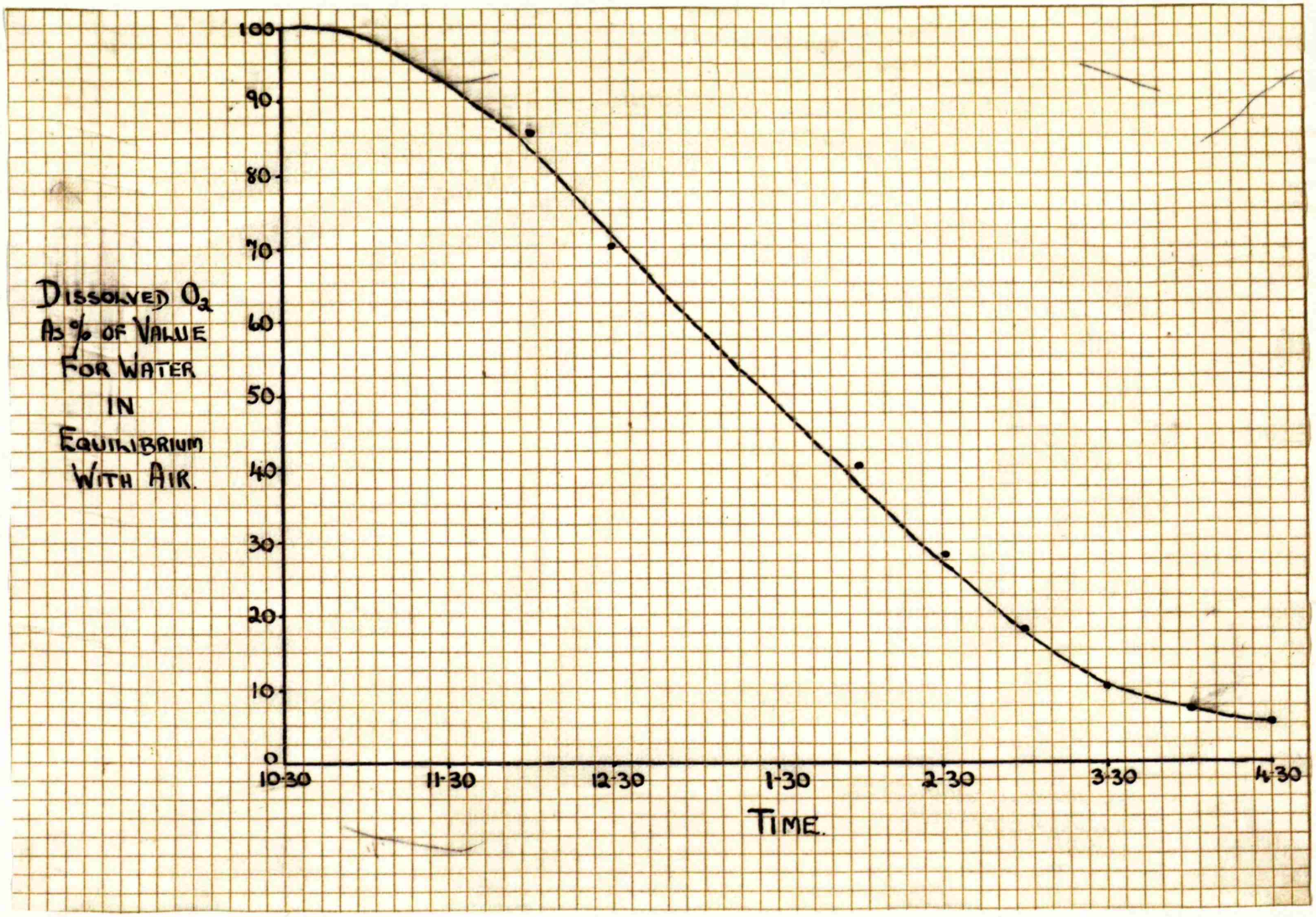
Table 11.

Series of Determinations of Dissolved Oxygen on 1 per cent Bottles at Commencement of Gas Flow.

Time.	Dissolved Oxygen as % of Value for Water in Equilibrium with Air.
10 - 30 a.m.*	----
12 noon.	85
12 - 30 p.m.	69
2 - 00 p.m.	41
2 - 30 p.m.	28
3 - 00 p.m.	18
3 - 30 p.m.	10
4 - 00 p.m.	7
4 - 30 p.m.	5

* Commencement of gas flow.

Readings were made alternately on two bottles being supplied with 1 per cent oxygen.



Graph 2. Series of determinations of dissolved oxygen on 1 per cent bottles at commencement of gas flow.

displacement of the original gaseous contents (in this instance air) of the bottles occurred in an orderly manner and that the gas at first displaced was uncontaminated with the entering gas. This confirms that the routine method of examining the water in the "tell-tale" 30 minutes after commencemnt of the gas flows is likely to give a reasonably accurate reflection of the percentage oxygen in the bottle prior to the commencement of gas flow.

As noted in "Methods", in connection with this experiment 4 control bottles, each containing 7 non-nodulated plants not supplied with combined nitrogen, were set up in order to ascertai the amount of combined nitrogen which might be available to the plants from the sand. At harvest it was found that these plants had an average dry weight of 6.5 mg. per plant and an average total nitrogen content of 0.2 mg. As the total nitrogen content of a clover seed is in the region of 0.1 mg., it is confirmed that the amount of combined nitrogen present in the sand was very small and also that the figures for total nitrogen content of nodulated plants to be presented below indicate correctly the amount of nitrogen fixed by these plants.

<u>Growth Data for Part I of Experiment - Nodulated Plants. Growth Period from April 4 to May 27, 1952.</u>

Six bottles, each containing 7 nodulated plants, were set up for each of the oxygen levels. The first indications of nitrogen fixation were observed approximately four weeks after sowing. At this stage the plants which had been inoculated with the nodule organism were larger than the controls and also the leaves of the former were dark green compared with the paler ones of the latter. After a further week differences became obvious between the growth of the plants at different oxygen levels. Best growth was already shown in an atmosphere of 12 per cent oxygen, followed by 5, 21 and 1 per cent oxygen in that order. This relationship between the growth at different oxygen levels, as assessed by eye judgment, persisted during the remainder of the experimental period.

Much the same picture is presented by the mean data obtained at harvest (see Table 12). In respect of both dry weight and nitrogen content the growth of plants at 12 per cent oxygen is seen to be superior to that at 21 per cent on the basis of mean data. The statistical treatment shows that the difference in mean total nitrogen (the latter being a measure of the amount of fixation) is fully significant, while the difference in dry weight is near enough to the required value to be considered significant. The relatively small differences between means at 12 and 5 per cent are a long way from significance, but those between 5 and 1 per cent are fully significant. The position

Table 12.

Mean Data (per Plant) obtained at Harvest of Nodulated Plants.

% Oxygen in Gas Supplied.	No. of Nodules.	No. of Leaves.	Length of Longest Petiole. (cm)	Total Dry Weight. (mg)	Total Nitrogen (mg)
21	38	9	11.5	109.5	3.1
12	45	11	14.7	134.5	4.0
5	54	11	13.1	129.3	3.9
1	56	15	7.4	97.9	2.9

Six 5-litre bottles with 7 plants in each were set up at each gas level.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison.	No. of Plants Harvested.	Total Dry Weight (mg.)	Total Nitrogen. (mg.)
21% and 12%	42 and 42	26.7 (obsvd. 25)	0.85 (0.9)
21% and 5%	42 and 41	27.0 (19.8)	0.86 (0.8)
21% and 1%	42 and 41	27.0 (11.6)	0.86 (0.2)
12% and 5%	42 and 41	27.0 (5.2)	0.86 (0.1)
12 %and 1%	42 and 41	27.0 (36.6)	0.86 (1.1)
5% and 1%	41 and 41	27.1 (31.4)	0.87 (1.0)

may be summarised thus :-

$$21\% < 12\% = 5\% > 1\%$$

It may further be noted that there is no significant difference between plants at 21 and 1 per cent oxygen. Both typical bottles and groups of nodulated plants from Part I of the experiment are shown in Figs. 25 and 26.

With further reference to the statistical treatment in Table 12, it may be noted that the differences required for significance are relatively as large as in the root-system enclosure experiment, despite the fact that 42 plants were grown at each oxygen level in the present experiment as compared with only 18 in the former. This indicates that additional factors conducive to plant-to-plant variation were operative in the present experiment. One such factor may have been the shading effect of the plants situated near the wall of a given plant bottle on those in more central positions. Another may have been that the enrichment of the gas supply with carbon dioxide would allow those plants which were genetically equipped for stronger growth to make fuller use of this faculty than was the case in the root-system enclosure experiment where the leaves were exposed to ordinary air only. In addition there was some suggestion of the operation of what may be termed a "bottle-factor", which led to the plants in one particular bottle being

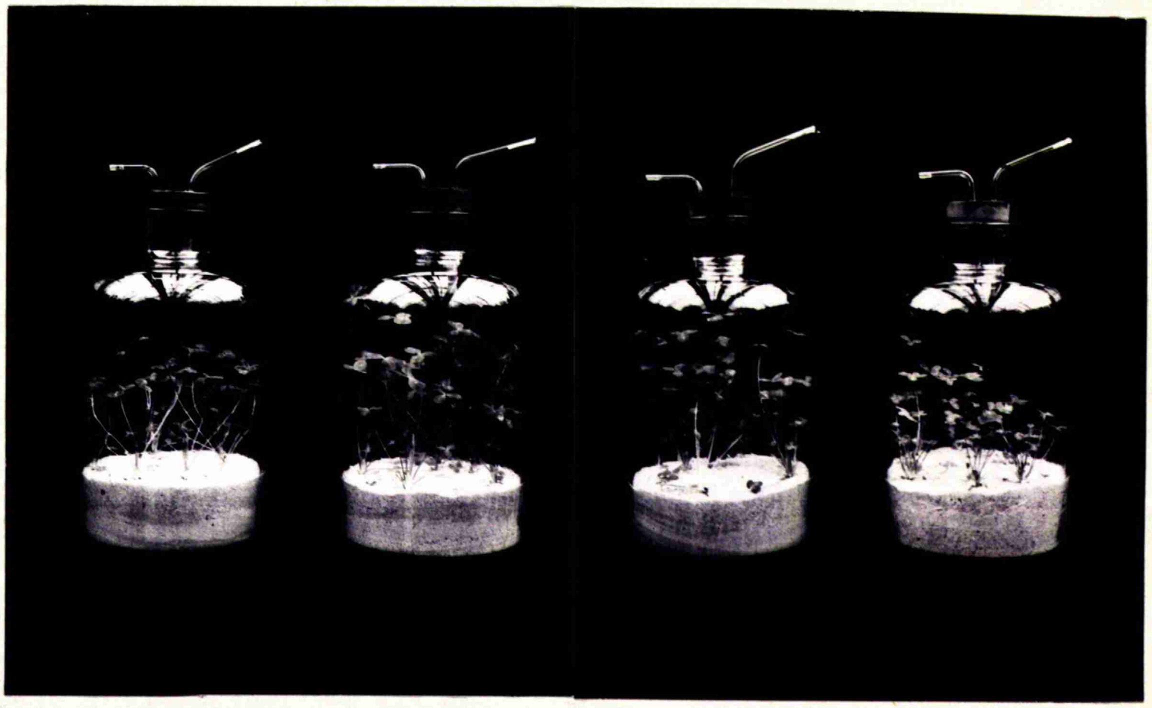


Fig. 25. Bottles of nodulated plants. Left to right - 21, 12, 5 and 1 per cent oxygen (x 1/8).

Fig. 26. Groups of nodulated plants (7 plants in each group Left to right - 21, 12, 5 and 1 per cent oxygen (x 1/7)

generally different in vigour of growth from those in the remaining bottles at the same oxygen level. The identity of thi factor (if in fact it existed) was not discovered. It is certain that deviations in the oxygen contents of individual bottles, due to leaks or other irregularities, were not responsible. It seems most likely that the effect was due to chance association in certain bottles of inherently relatively strong or weak growing plants.

It is also shown in Table 12 and in Figs. 25 and 26 that the growth form made by these nodulated plants varied according to the atmosphere in which they were grown. Those in 1 per cent oxygen developed into a stocky rosette type of plant with a large number of leaves but with short petioles, while those subjected to higher oxygen tensions tended to develop into a more upright plant with fewer leaves but with longer petioles and larger laminae.

Although the mean number of nodules produced at each of the four oxygen tensions increased from 38 to 56 as the oxygen level was reduced from 21 to 1 per cent oxygen (Table 12), statistical analysis shows that the minimum difference between means require for significance is not attained when the groups are compared, i.e. nodulation appears to have been unaffected by the variation in oxygen supply. This differs from the result obtained in the root enclosure experiments, since the number of nodules there was greatly reduced at 1 per cent oxygen. It should however be noted that in the present experiment the numbers of nodules were also very much smaller than in the root enclosure experiment This is no doubt a result of restricted growth of the wholly enclosed plants (see later), and it is possible that because of this restriction, plants at higher oxygen levels were unable to form as many nodules as they would otherwise have done.

An interesting phenomenon shown in the plants at 1 per cent oxygen was the first-order lateral roots which emerged from the sand at an angle of 5 - 20 degrees and projected into the atmosphere within the bottle (see Fig. 27 and 28). It is presume that aerotropism was responsible for this development. These roots after attaining a length in the atmosphere of 0.5 to 2.0 cm tended to curve downwards towards the sand again and grew along the surface without actually re-entering the rooting medium.

Growth Data for Part II of Experiment - Non-Nodulated Plants supplied with Ammonium Sulphate. Growth Period from July 12 to August 21, 1952.

Here again six bottles, each now with 6 plants, were set up at each oxygen level. In the case of these plants supplied with ammonium sulphate the differences in growth at the four oxygen tensions became apparent at approximately the same time as with

Fig. 27. Nodulated plants grown in 1 per cent oxygen, showing roots above the surface of the sand (x 1/2).

Fig. 28. As above, photograph from overhead (x 1/3).

the nodulated plants. Here the plants in 12 per cent oxygen appeared to be slightly superior to those in 21 per cent, followed by 5 and 1 per cent oxygen. Such observations were borne out at the time of harvest and the mean data obtained for these plants are presented in Table 13 along with the results of statistical analysis.

From the statistical treatment it is found that the difference between means required for significance was not obtained between dry weights of plants at 21 and 12 per cent oxygen, but that a significant reduction occurred when the tension was lowered from 12 to 5 and then to 1 per cent oxygen, i.e.

$$21\% = 12\% > 5\% > 1\%$$

Although the minimum difference between means required for significance is not quite reached on comparing the plants grown at 5 and 1 per cent oxygen, it is very nearly obtained, and taking into account the large difference between the mean total nitrogen contents of these two groups it can be considered that the groups are significantly different. It may also be noted that in this part of the experiment growth at 21 per cent oxygen is much greater than at 1 per cent. Typical bottles and groups of plants from Part II of the experiment are shown in Figs. 29 and 30.

Table 13.

Mean Data (per Plant) obtained at Harvest of Non-Nodulated Plants receiving Ammonium Sulphate.

% Oxygen in Gas Supplied.	No. of Leaves.	Length of Longest Petiole (cm.)	Total Dry Weight (mg.)	Total Nitrogen. (mg.)
21	11	16.5	137.1	5.1
12	12	18.2	148.9	5.6
5	14	12.8	112.3	4.7
1	15	9.2	86.6	3.6

Six 5-litre bottles with 6 plants in each were set up at each gas level. Each bottle received a total of 66 mg. nitrogen during the experiment.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison.	No. of Plants Harvested.	Total Dry Weight (mg.)
21% and 12%	35 and 35	29.6 (obsvd. 11.8)
21% and 5%	35 and 35	29.6 (24.8)
21% and 1%	35 and 36	29.2 (50.5)
12% and 5%	35 and 35	29.6 (36.6)
12% and 1%	35 and 36	29.2 (62.3)
5% and 1%	35 and 36	29.2 (25.7)

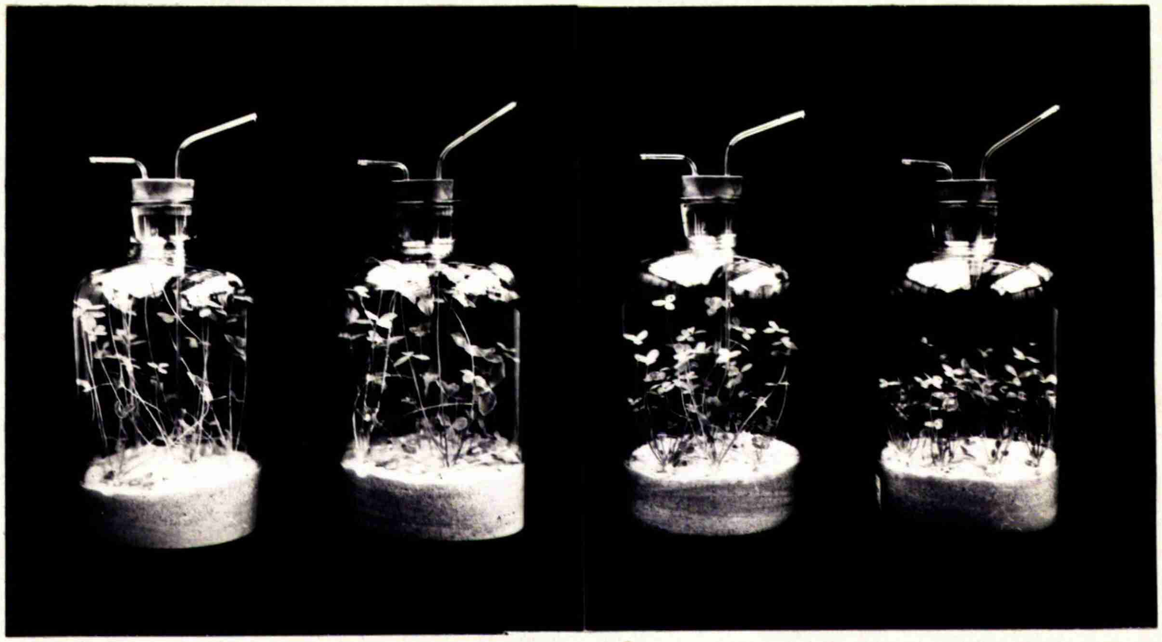


Fig. 29. Bottles of plants supplied with ammonium sulphate. Left to right - 21, 12, 5 and 1 per cent oxygen (x 1/8

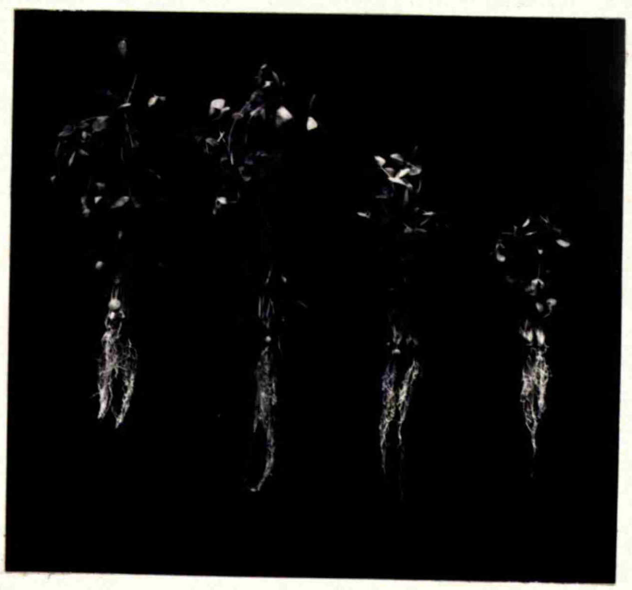


Fig. 30. Groups of plants supplied with ammonium sulphate (6 plants in each group). Left to right - 21, 12, 5 1 per cent oxygen (x 1/7).

In this part of the experiment there was again a certain amount of variation between bottles at any one of the four oxygen tensions and also the differences in growth form taken on by these plants at the various tensions were of a similar nature to those already described for the nodulated plants. Root emergence of the type already described for nodulated plants at 1 per cent oxygen also occurred at this tension with plants supplied with combined nitrogen, but here it was not so pronounced nor did it occur so frequently.

Discussion, including Comparison of Results obtained by Root-System Enclosure and Whole-Plant Enclosure Techniques, and General Conclusions.

A. Discussion of Results of Whole-Plant Enclosure Technique.

If attention is confined in the first instance to effects shown to be statistically significant, the findings emerging from this whole-plant enclosure experiment may be stated as follows. With nodulated plants, wholly dependent on nodule nitrogen, the dry weight of the plant was greater in the presence of 12 per cent oxygen than in that of 21 per cent. Plants supplied with 5 per cent oxygen grew as well as those with 12 per cent, but a reduction in dry weight was shown when the oxygen level was reduced to 1 per cent. With non-nodulated plants supplied with combined nitrogen, growth was similar at 21 and 12 per cent oxygen, this being followed by a progressive reduction in dry weight as the proportion of oxygen was decreased to 5 and then to 1 per cent.

These findings can be summarised in the following notation:-

Nodulated Plants.	Non-Nodulated Plants.
$21\% < 12\% = 5\% > 1\%$	$21\% = 12\% > 5\% > 1\%$

It is clear that these results provide no evidence that the nodulated plant, for optimal growth, has a higher oxygen requirement than the plant supplied with combined nitrogen.

In fact the opposite seems to be the case, since the highest level of oxygen (21 per cent) exerted a detrimental effect on the growth of nodulated plants. This same feature emerged, of course, from the root-system enclosure method.

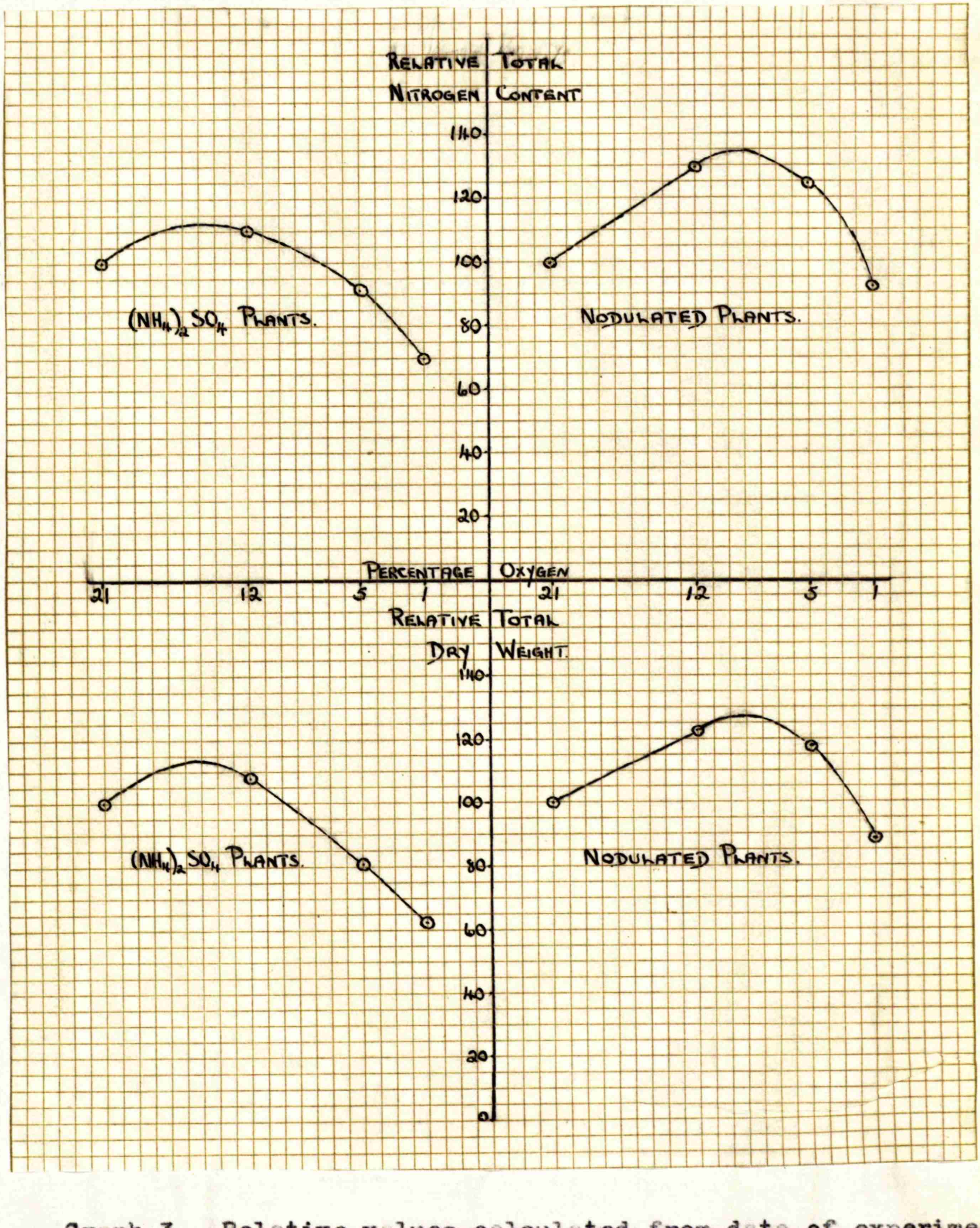
Further discussion will be facilitated if the data are re-calculated on to a relative basis and plotted in graphical form (see Table 14 and Graph 3) with smooth curves fitted to the actual points in the graph. As shown in the previous treatment the vertical distance between successive points in the graph does not in all cases attain statistical significance, but the general circumstances suggest that the various inflections of the curves may be accepted as indicating a genuine response to differences in oxygen level. On this basis it appears that with both nodulated and non-nodulated plants the optimal oxygen level for growth and nitrogen fixation or uptake lies below the air level, but to a different extent, the optimum for nodulated plants being approximately 10 per cent and that for non-nodulated plants in the region of 15 per cent. The further implications and explanation of these findings will be considered in a later section of this discussion.

The results obtained by the present author with the whole-plant enclosure technique will now be compared with those of Wilson and Fred (1937). First of all the method of expression

Table 14.

Relative Values calculated from Data of Experiment, with Mean at 21 per cent taken as 100.

% Oxygen in Gas Supplied.	Nodulated.		Non-Nodulated.	
	Dry Weight.	N-Content.	Dry Weight.	N-Content
21	100	100	100	100
12	123	130	108	110
5	118	125	82	92
1	89	93	63	70



Graph 3. Relative values calculated from data of experimen
with mean at 21 per cent taken as 100.

of the oxygen status of the different series of plants comprising the experiment must be considered. Obviously in investigating the effect of oxygen supply on plant growth, the critical consideration is the number of oxygen molecules per unit volume of gaseous phase surrounding the plant. In the present author's case the oxygen is indicated as a percentage by volume of the gas mixture supplied to the plants, while Wilson and Fred employ a partial pressure basis, no doubt because the technique adopted in their experiments resulted in the total gas pressure in the plant bottles varying from one experiment to another. Under such circumstances a percentage basis would obviously be unsatisfactory, as shown by an imaginary example in which two plant bottles may be supposed to be filled with a gas mixture containing 5 per cent oxygen, to a total pressure of 0.5 atmospheres in one case and 1 atmosphere in the other. The number of oxygen molecules per unit volume of gas phase in the second bottle will obviously be twice that in the first bottle, so that the plants within the two bottles will differ in their oxygen supply. If however a partial pressure of oxygen (pO_2) of 0.05 atmospheres is established within both bottles, but by addition of other gases (say nitrogen) the total pressure within one bottle is raised to 1 atmosphere and in the other to 2 atmospheres, the actual oxygen supply, in respect of number of oxygen molecules per unit volume of gas space, to plants within the two bottles will be similar.* In the present author's experiment, as noted, the total gas pressure within the plant bottles was constantly very close to 1 atmosphere and in view of this the percentage volumes so far employed can, for the purpose of comparison with Wilson and Fred's data, be transferred into terms of partial pressures as follows:-

1 per cent	=	0.01 atmospheres.	Given a total gas pressure of 1 atmosphere.
5 per cent	=	0.05 atmospheres.	
12 per cent	=	0.12 atmospheres.	
21 per cent	=	0.21 atmospheres.	

Wilson and Fred carried out many separate experiments with red clover at different times of the year, but they give no indication of the scale of their experiments, viz., the number of replicate bottles and the number of plants per bottle, so that the sum total of their experimental work is uncertain. In addition they consider that some of their experiments are

* It shouldbe noted, however, that in the diffusion of oxygen into the tissues of the plant, molecule impedance may take place owing to the fact that in the bottle where the total pressure was raised to 2 atmospheres, a greater number of nitrogen molecules will be present in the atmosphere.

unreliable owing to the fact that hydrogen was added to the atmospheres within the bottles in order to bring the pressure to 1 atmosphere. Subsequent experiments suggested that hydrogen exerts an inhibitory effect on fixation. However, on p. 506 of their paper, graphs considered to indicate the over-all findings are presented in respect of pO_2 not exceeding 0.2 atmospheres (see Fig. 31) and these (particularly the two referring to plants grown in the absence of hydrogen) will be used as the main basis of comparison.

With both nodulated and non-nodulated plants (the latter supplied with ammonium nitrate), growth as indicated by nitrogen content was considered to be unaffected by reduction in oxygen supply until a pO_2 of 0.05 atmospheres was reached (Fig. 31). Below this, growth was reduced. Their work also included experiments in which pO_2 was increased above that of normal air. At values above pO_2 0.4 growth of both nodulated and non-nodulated plabts was reduced. A general picture thus emerges from Wilson and Fred's work of growth being unaffected by changes in pO_2 over the range 0.05 - 0.4 atmospheres, while outside this range growth is reduced. In all these respects nodulated and non-nodulated plants behave similarly in their experiments.

While there is a general resemblance between the present author's results and those of Wilson and Fred, there are

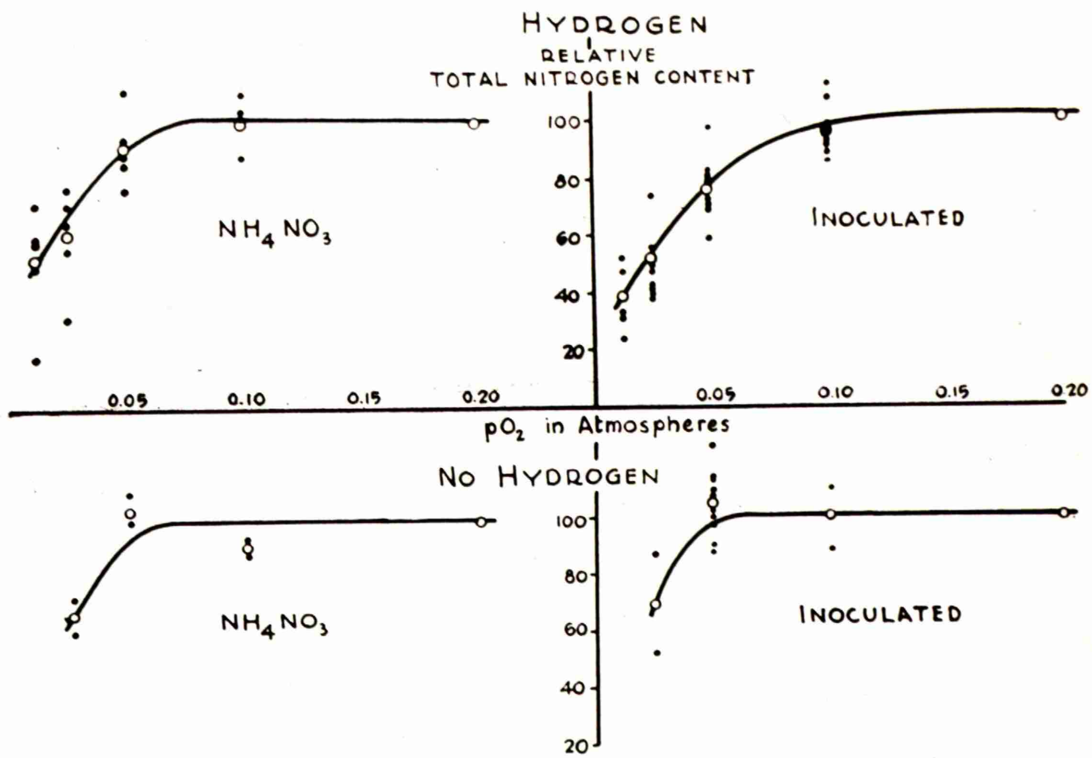


FIGURE 1

Effect of low partial pressures of oxygen on uptake of free and combined nitrogen in the presence and absence of hydrogen. (Full dots refer to individual experiments; circles to the mean of all experiments.)

Fig. 31. Graphs showing over-all findings of Wilson and Fred's experiments. Relative nitrogen contents are calculated fsom data of experiments with mean at pO_2 taken as 100. Where 0.1 atmospheres was the highest pO_2 used, then this nitrogen content is taken as the standard.

important points of difference. Thus

(1) The plants were not found by the present author to be insensitive to changes in oxygen supply over the range 0.05 - 0.21 atmospheres. In the case of the nodulated plants there is clear evidence of an optimum oxygen in the region of 0.10 atmospheres (= 10 per cent oxygen). Although with non-nodulated plants the evidence for a corresponding optimum is less certain, it is quite clear that a reduction from pO_2 0.12 (12 per cent) to 0.05 (5 per cent) resulted in less vigorous growth.

(2) Although the present author's results agree with those of Wilson and Fred in the indication provided that the nodulated plant has not a higher oxygen requirement than the non-nodulated type, they go beyond this and suggest that the reverse is the case, namely that the processes of growth and fixation are best suited by a lower oxygen level than that optimal for non-nodulated plants supplied with combined nitrogen.

In endeavouring to find the reason for these differences in results a handicap is imposed by the lack of experimental detail concerning Wilson and Fred's experiments. One noteworthy feature is that their plants were often smaller compared with those in the present experiment. Thus the mean dry weight per nodulated plant grown in air for all experiments detailed was 65 mg. compared with 109 mg. in the present experiment, although the growth periods were of the same order. This suggests that growth in Wilson and Fred's experiments was limited by some factor which may have been responsible for the failure of these experimenters to detect optimal oxygen levels. It is possible that limitation was due to low carbon dioxide supply. It is understood from a personal communication from Prof. Wilson that in his experiments 50 cc. of carbon dioxide was initially added to the atmospheres in the 10-litre plant bottles and that the assimilation of this gas through the action of photosynthesis was followed by the use of a pH indicator contained in a weak bicarbonate solution. When the original carbon dioxide had been exhausted, more was added. It seems possible that this method may have resulted in photosynthesis being limited at certain periods, although the initial concentration of carbon dioxide was high. It is noteworthy that on p. 504 Wilson and Fred specially mention that in certain experiments a decrease in oxygen from that in air to pO_2 0.1 atmospheres resulted in better growth and fixation of nitrogen, as is precisely the present author's experience. They were however inclined to regard this as an anomalous result associated with relatively high summer temperatures in the greenhouse. But it seems equally possible that this was a correct and typical result and that it was in other experiments where this improved growth was not shown that upsetting or limiting factors were operative.

B. Comparison of Results obtained by the Two Techniques.

It will be recollected that one of the main objects of this investigation was to determine whether the conclusions as to the importance of oxygen supply for nodule function in a given legume were the same irrespective of whether the root system only or the whole plant was exposed to different oxygen tensions. In the present section a comparison will be made of the results obtained by the author with the two techniques.

In the first place the findings based on statistical treatment will be brought together as follows, the position being exactly the same whether dry weight or nitrogen content is considered.

Root-System Enclosure.

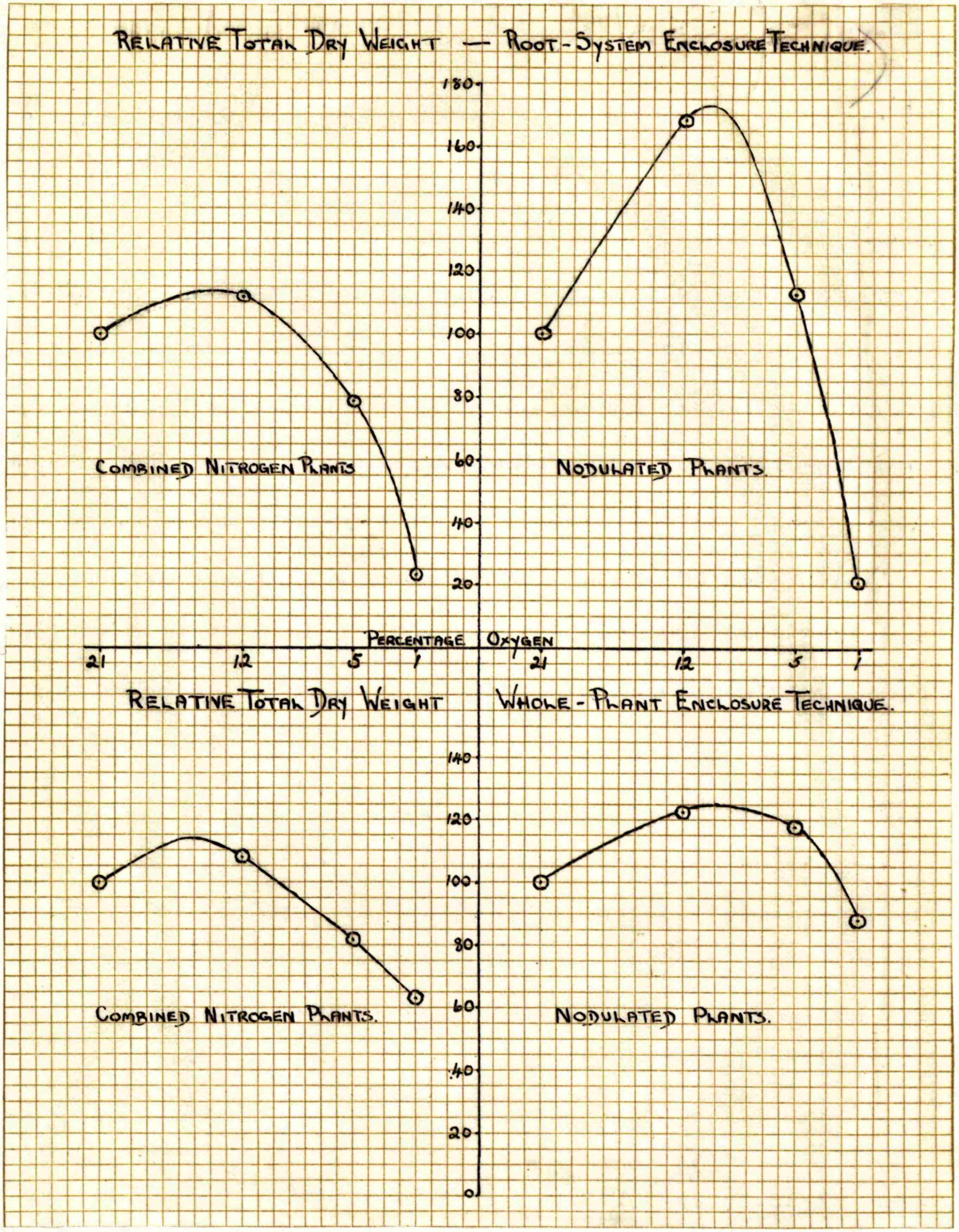
Nodulated Plants.	Combined Nitrogen Plants.
$21\% < 12\% > 5\% > 1\%$	$21\% = 12\% > 5\% > 1\%$

Whole-Plant Enclosure.

Nodulated Plants.	Combined Nitrogen Plants.
$21\% < 12\% = 5\% > 1\%$	$21\% = 12\% > 5\% > 1\%$

In addition the relative data (in this instance for dry weight) already presented in appropriate sections are assembled together in Graph 4.

Taking nodulated plants first, both techniques show growth



Graph 4. Comparison of relative values calculated from data of the two experimental techniques with mean at 21 per cent oxygen taken as 100.

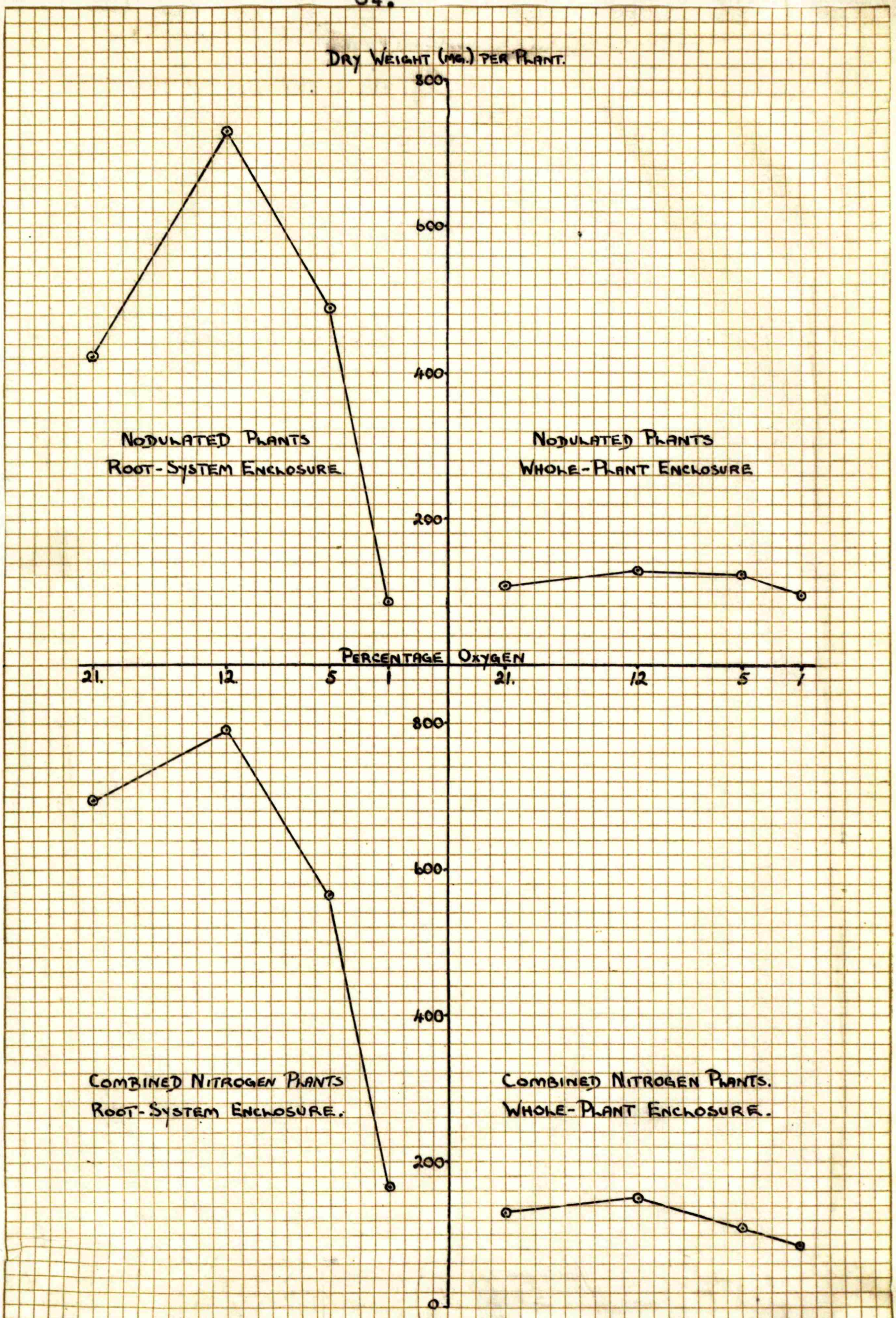
and nitrogen fixation to be significantly superior at 12 per cent oxygen to that in air, though the increase on the relative basis with the whole-plant enclosure technique is only about 1/3rd as big as that with the alternative technique (see Graph 4). In a similar way the effects of further reduction in oxygen supply were less pronounced in the whole-plant enclosure experiment. Thus there was no significant reduction in growth accompanying the reduction in oxygen from 12 to 5 per cent, contrasting with the effect with root enclosure, and while a further reduction of oxygen to 1 per cent produced a significant decrease in growth, the extent of this was much less than that in the root enclosure. It may also be noted that with whole-plant enclosure the dry weight of plants grown with 1 per cent oxygen was not significantly lower than that of plants grown at 21 per cent oxygen. However with both techniques an optimum growth point in the region of 10 per cent oxygen is implied by the graphs.

With non-nodulated plants supplied with combined nitrogen the sequence of significant differences is precisely the same by both techniques. Growth at 12 per cent oxygen is not significantly greater than at 21 per cent. Reduction of oxygen to 5 per cent and then to 1 per cent is accompanied by a reduction in growth. But it is again evident that responses to oxygen changes were less marked with the whole-plant enclosure method, as witness for example the much smaller difference between the plants grown at 1 per cent and those at 21 per cent as compared with the root-system enclosure method (see Graph 4). The results with both methods suggest that optimal growth by these plants would be in the region of 15 per cent oxygen.

The position is then that both techniques present a similar picture of the oxygen relationship of nodulated and non-nodulated plants, namely that the former have a lower optimal oxygen requirement than the latter, the actual optima indicated being similar by both methods. The oxygen effects are however less pronounced in the whole-plant enclosure method. If the performance at the most favourable oxygen level actually employed (12 per cent oxygen) is taken as a base line, thus the position is that the reaction to either increased or decreased oxygen from this base line are smaller than with the root-enclosure experiments.

In considering the explanation of this reduction in the effect of oxygen tension upon growth in the whole-plant enclosure method, it is important to note firstly that although the plants were of healthy appearance, the size attained by the plants under the conditions imposed by this technique was very much smaller than with corresponding plants under the conditions of root-system enclosure, except at the 1 per cent oxygen level. This is clearly brought out in Graph 5, where the mean absolute dry weights for plants grown by the two methods are compared. Thus plants in the whole-plant enclosure experiment supplied with 12 per cent oxygen attained less than one quarter of the dry weight of corresponding plants in the root-system enclosure experiment. On the other hand, at 1 per cent oxygen, nodulated plants grown wholly enclosed were of similar absolute dry weight to those grown by the alternative technique, while with combined nitrogen plants the disparity was smaller than at higher oxygen levels.

These data suggest that under conditions of total enclosure there is considerable limitation of growth due to factors inherent in the method, and moreover the degree of limitation increases the more the plants tend to grow. Thus the plants at 21, 12 and 5 per cent oxygen are prevented from showing their full reaction to increased oxygen by the increasing degree of limitation imposed upon them. There are several reasons why such limitation of growth is likely with the whole-plant enclosure system. Undoubtedly there is appreciable reduction in the intensity of the light available to the plants. All the light has of course to pass through the wall of the aspirator bottle, while the rubber stopper in the neck of the bottle interferes with light from overhead. Measurements with a light meter (equipped with a separate photo-electric cell which could be inserted into the



Graph 5. Comparison of mean absolute dry weights for plants grown by the two experimental techniques.

aspirator bottle) indicated that light entering the sides of the bottle was reduced by 1/3rd, and the light from above by half, compared with intensities outside the bottle. These observation were made on a dry bottle. During growth condensed moisture was usually present on the inside of the bottles, resulting in further reduction in light. In addition as growth occurs there will be increasing mutual shading by the plants within a given bottle. In the root enclosure method lighting is quite unobstructed except towards the end of the growth period, when some overlapping of marginal leaves occurs. There can be little doubt that the reduction in growth in the whole-plant enclosure method was largely attributable to curtailment in light intensity.

Another feature which may restrict growth is that the plant in the aspirator bottles are exposed to a saturated atmosphere for much of the time, as indicated by the condensation of moisture already noted. This is an unnatural condition for clover, and the restriction of transpiration entailed may retard salt uptake and transport and in this way affect growth adversely. This factor too might be of unequal incidence and bear more seriously on plants which were tending to grow more strongly, because the larger leaf area would result in some increase in transpiration and hence heighten the degree of saturation of the atmosphere.

Consideration must also be given to the difference in type of rooting medium in the alternative techniques, i.e. water culture versus sand culture. General experience in the field of soil-less culture of plants ("hydroponics") appears to point to a solid rooting medium being preferable, but this assessment is based on many considerations not all of which apply to the present experiments. It is however interesting to note that Allison and Shive (1923) in their laboratory scale experiments on the growth of Soya bean plants found that the plants grown in sand culture exhibited superior growth to those in culture solution although the rooting medium in both instances was aerated in a similar manner, e.g. by the passage of an air stream through the rooting medium or by the use of aerated drip cultures However the essential question in the present case is whether the imposition of a reduced level of oxygen (say 5 per cent) in the rooting medium is likely to handicap a plant in solution medium more or less than a plant in sand medium. In the former the gas phase round the root system is in the form of rapidly moving bubbles, while in sand culture it exists in the spaces between the sand particles, and here the oxygen absorbed by the roots must be replaced by unaided diffusion from the atmosphere within the aspirator bottle. In both systems there is of course also oxygen dissolved in the culture solution. It is difficult to evaluate the relative effectiveness of these two systems in permitting ready access of oxygen to the roots, without a more direct comparison being available. Thus significant data would no doubt be yielded by a root-system enclosure experiment in which sand was used for the rooting medium in place of culture solution. It is however, probably justifiable to conclude that the difference in rooting medium was not responsible to any material degree for the differences in results obtained by the two methods.

A final point concerns the oxygen tensions actually obtaining in the two sets of experiments. It was pointed out earlier that in the root enclosure experiments the rapid growth and respiration of the plants coupled with the rather small volume of solution surrounding the roots made it impracticable, in the later stages of growth, to maintain the dissolved oxygen at the intended levels. The deficiency was naturally most serious at low oxygen levels. Thus it is clear that plants intended to receive 1 per cent oxygen had in fact something like 0.5 per cent available in later stages of growth. With the total enclosure apparatus there was of course no parallel tendency for the oxygen content of the atmosphere above sand level to fall, since the predominating process was now photosynthesis. Actually the results of oxygen determinations and the general circumstance of the large volume of gas in the bottle relative to the amount of leaf tissue indicate that photosynthesis had (and could have had) no material effect on the gas phase, which may therefore be concluded to have been at all times at the intended oxygen content. But we have no information as to the oxygen content of the sand and there may well have been some reduction below that of the atmosphere above the sand, resulting in the roots at least being exposed to a level of oxygen somewhat below that intended, as in the root-system enclosure experiments.

The general conclusion is then that the less pronounced effects of variation in oxygen supply shown in the whole-plant enclosure experiment are due to the limitations on growth enforced by this method of growing the plants, the reduction in light intensity and perhaps the persistent high humidity of the atmosphere surrounding the plants being particularly important. If a choice had to be made between the two methods of investigating the oxygen relation of legume root nodules, the preference would certainly lie with the root enclosure method. Any characteristic responses of nodulated plants to oxygen supply are presumably due essentially to effects obtained in the nodule, so that there is no point in enclosing the whole plant, with the consequent imposition of artificial conditions and limitations of growth which this entails. This preference is, however, always dependent on the feasibility of maintaining the root system at a reduced level of oxygen supply while the shoot is exposed to the normal atmosphere, i.e. the possibility of internal diffusion of oxygen must always be borne in mind. In the present experiments with clover no evidence of such internal diffusion of oxygen has been obtained, as already noted.

C. General Discussion and Conclusions.

In this final section of the Discussion we have to consider the explanation and implications of the main findings of the author's experiments.

These are that under the varied conditions employed, for nodulated clover plants certainly, and for non-nodulated plants possibly, the optimum oxygen level for growth and assimilation of free or combined nitrogen is below that of normal air. As shown in previous sections, there is good evidence that for nodulated plants about 10 per cent oxygen is optimal, the corresponding figure for non-nodulated plants being perhaps in the region of 15 per cent, though the evidence for the existence of this optimum is weaker.

As has been indicated earlier, in view of the previous literature it is surprising, at least on first sight, that either type of clover plant should show a preference for an oxygen level below that of air, and still more so that the optimum for nodulated plants should be lower than that for combined nitrogen plants. The circumstances under which these results were obtained have naturally been very carefully scrutinised with a view to detecting any source of error. One possibility that obviously had to be considered was that the air supplied to the plants at the 21 per cent level contained some harmful impurity absent from the other gas mixtures. This applies particularly to the root enclosure experiments, because there the air stream was supplied by an electric pump compressing greenhouse air, while the other gas mixtures were from cylinders. As stated in the Methods section, the air, in common with the other gas streams, was scrubbed in culture solution before being supplied to the plants, the argument being that it was specially desirable that impurities (if any) soluble in the culture solution should be removed ; in addition the air stream was thoroughly filtered through cotton wool before reaching the plants. It is, however, possible to imagine that some contaminant in the air stream was incompletely removed. But there are many reasons for rejecting this possibility. Thus the plants at 21 per cent level were in no way unhealthy or showing signs of root inhibition, but merely somewhat smaller than those at the 12 per cent level. Also, since the reduction of growth at 21 per cent compared with that at 12 per cent was much greater with nodulated than with non-nodulated plants it would be necessary for a postulated impurity to be specifically injurious to nodulated plants, and it is hard to imagine what type of substance it could be. Again it is relevant to mention that in other experiments, alder, Soya bean, pea and tomato have been grown excellently when supplied with air from the same compressor. However, the most conclusive evidence is that in the total enclosure method all the gas mixtures (as noted) were obtained from cylinders supplied by the British Oxygen Company (Medical Section), and the depressing effect of 21 per cent, as compared with 12 per cent oxygen was still shown. The same applies to the circumstance that again in the root enclosure experiments, the air stream was supplied for only 20 out of each 24 hours. It therefore seems highly probable that the effect in question represents a genuine response to oxygen supply.

In attempting to explain these findings, it is pertinent first to enquire first whether there is any parallel in the literature for a preference by an aerobic species for an oxygen level below 21 per cent. Insofar as this preference is peculiar to nodulated clover plants and to the nodules themselves, comparison with previous work relating to non-nodulated and mostly non-leguminous plants are largely irrelevant. It will, however, be recalled that non-nodulated clover plants showed some evidence of a preference for reduced oxygen supply. The number of previous investigations in which the growth of plants has been compared at different, carefully-controlled oxygen levels maintained in these cases in respect of the root systems, is not large. The general position has been reviewed by Conway (1940), Russell (1950), and Page and Bodman (1951). In many species root growth (and presumably whole plant growth) are adversely affected by oxygen levels below 9 - 12 per cent, though some species grow well at still lower oxygen tensions, while others again show an adverse effect if oxygen is reduced below 21 per cent. The reduction in growth observed in clover by the present author at 5 and 1 per cent oxygen levels is obviously in keeping with these previous findings. In addition there is of course a considerable amount of literature relating to the effects of reduced oxygen supply on individual activities of root tissues. Thus Steward, Berry and Broyer (1936) showed that excised potato roots exhibited a reduction in respiration and the absorption of potassium and bromide ions when the oxygen supply was reduced below 14 per cent. Hoagland and Broyer (1936), using excised barley roots, also found that respiration and the absorption of potassium decreased below 15 per cent oxygen, while absorption of bromide and nitrate was not affected until a level of 8 per cent oxygen was reached. Vlamis and Davis (1944) who worked with excised roots of tomato, rice and barley, found that there was a sharp reduction in the accumulation of potassium and bromide once the oxygen supply level was reduced to between 3 and 5 per cent oxygen. Once again a close correlation existed between respiration and the accumulation of these ions as the former was not greatly reduced until the oxygen tension was lowered to the above values.

In none of these previous papers is there any hint of a definite optimal oxygen concentration for plant growth or for root activities. But there is a suggestion of this in the experiments of Gilbert and Shive (1942). They grew Soya bean (non-nodulated), tomato and oats with the roots supplied with 0, 10, 20 and 40 per cent oxygen. With tomato and oats the best growth was shown at 40 per cent oxygen, but in the case of Soya bean chlorosis and crinkling in the leaves occurred at 20 and 40 per cent oxygen, i.e. growth was better at 10 per cent than at 20 per cent. These effects were ascribed to oxygen toxicity at the higher levels, and the leaf symptoms disappeared when the oxygen supplied to these plants was reduced. That one out of three species showed this evidence of a harmful effect of higher oxygen levels suggests that the susceptibility might be found to be fairly common were more species tested by the carefully controlled technique of these authors, though the precise nature of the symptoms might vary.

However, as has been mentioned above, the preference for a reduced oxygen tension observed by the present author is largely peculiar to nodulated clover plants dependent on nodule nitrogen, and is presumably due to effects obtaining in the nodules. The oxygen relations of the latter may be different from those of the normal parts of the plant, so that parallel behaviour in other organs or plants is not necessarily to be expected. Moreover it appears that the oxygen relations of nodules and nodulated plants vary between different legumes, as is indicated when the present author's results are compared with those of Bond (1951). It will be recalled that one of the objects of the present investigation was to determine whether the divergence in results between the work of Bond and of Wilson and Fred was due to the use of different legumes or of different techniques. Bond grew Soya beans by a root enclosure technique to which that employed by the present author was very similar. The origin of the air and gas streams in the two experiments was the same. Bond found that nodulated plants showed a progressive reduction in growth and nitrogen fixation as the oxygen tension was reduced from the air value to 12 per cent and then to 5 per cent, while non-nodulated plants supplied with combined nitrogen showed a much smaller response. He concluded that the Soya bean nodule is more sensitive to reduction in external oxygen level than are the roots. The new findings for clover are in marked contrast to those for Soya bean, so that it must be concluded that nodules and nodulated plants of different legume species vary greatly in their oxygen relations.

When the literature is closely studied it becomes clear that there is a basis for anticipating such differences between different legume nodules. From their studies of the respiratory characteristics of various legume root nodules, Allison, Ludwig, Hoover and Minor (1940) drew the important conclusion that anaerobic or partly anaerobic conditions exist within the nodules of most legumes, so that it is under such conditions that the fixation of nitrogen occurs. This was based on the findings that the respiratory quotient of detached nodules lying in air were frequently in excess of unity, but became very close to unity when the oxygen tension was increased, while the over-all rate of respiration showed a marked increase. Allison et al made no simultaneous measurements of nitrogen fixation, and as a matter of fact most investigators have been unable to detect fixation in excised nodules, but had it been possible for their experiments to be done on still-attached nodules the higher rate and more aerobic type of respiration induced by increased oxygen tension might not have been attended by greater fixation, and the reverse might have been the case. In other words the fixation mechanism may require some degree of anaerobiosis, perhaps because of competition between nitrogen and oxygen for hydrogen atoms.

The limited oxygen supply within the legume nodule no doubt arises as a result of the low surface area of the nodule relative to volume, of the frequently rather feebly developed air space system, and of the presence of physiological barriers to diffusion in the form of endodermal layers. A study of the distribution and functional effect of the latter was made by Frazer (1942), and she showed that the legume nodules studied by her were of two types. Nodules of spherical shape, of which those of Soya bean are examples, and showed an endodermis in the peripheral region which completely enclosed the bacterial tissue, except for a few points where gaps were present. In cylindrical nodules, such as those of clover, an endodermal layer was present in the older basal region of the nodule, but was absent from the apical meristematic region. Tests with dyes showed that the endodermis effectively prevented diffusion of water-soluble substances, and it is reasonable to assume that this would also apply to dissolved oxygen. Thus from Frazer's work there are grounds for believing that, not only on account of its greater size, the Soya bean nodule is less efficiently aerated than the clover nodule. It is significant to note that Allison et al (cited above), from comparisons of respiratory quotients, themselves concluded that oxygen penetrates Soya bean nodules less rapidly than it does the clover type of nodule.

A provisional explanation of the difference in response of nodulated Soya bean and clover plants to reduction in oxygen level from that prevailing in air is now possible, if it is assumed that for maximum effectiveness in fixation a certain degree of anaerobiosis is necessary within the nodule. No data for the composition of the gas within the air-spaces of any legume nodule are available, so that there is no direct evidence as to the actual degree of anaerobiosis. The present author's results with clover suggest that not more than 10 per cent of oxygen is required in the nodule. The argument then runs that in order to maintain such an optimal level within the nodule, a higher external level of oxygen is required in the case of Soya bean than in that of clover, for reasons given in the previous paragraph.

It is recognised that this hypothesis that legume fixation of nitrogen is best-suited by partly anaerobic conditions would have to be supported by further evidence before it could be finally accepted. For example, it would be of interest to extend the investigation of oxygen relations to other legumes, such as Phaseolus, with spherical nodules, and Pisum and Vicia, where the nodules are of the cylindrical type.

It remains to consider the significance of the present results in relation to the growth of red clover under field conditions. Russell (1950) and also Page and Bodman (1951) review data on the composition of the soil atmosphere, and it is quite clear that in the upper layers of a good quality agricultural soil a level of oxygen considerably in excess of 10 per cent is likely to prevail for most of the time. Great caution is of course necessary in applying the results of greenhouse experiments, carried out in artificial environment, to field conditions, but it does seem possible that in the upper strata clover nodules are exposed to supra-optimal oxygen tensions. It must however be remembered that red clover is by nature a deep rooting species, as evidenced by the observations of Weaver (1926), who showed that root development was still luxurious at a depth of 2 - 3 feet, while the tap root descended to a depth of 9 feet. In any case it cannot in general be argued that the conditions provided by a soil of good quality are necessarily optimal in all respects. This is clearly not so with regard to oxygen, since Gilbert and Shive (1942) and other authors have shown that some species grow best at oxygen tensions in excess of 21 per cent.

S U M M A R Y.

(To Section I of Thesis)

1. The effect of oxygen supply on the growth and nitrogen accumulation of nodulated red clover plants in nitrogen-free rooting medium and of non-nodulated plants supplied with combined nitrogen has been investigated by two different techniques.

2. In the first technique the root systems only were exposed to adjusted oxygen supply. The plants were grown in water culture, and oxygen /nitrogen gas mixtures containing 21, 12, 5 and 1 per cent oxygen respectively were bubbled through the culture solution of different series of plants. Protection against ingress of atmospheric oxygen was provided.

3. In the second technique the clover plants were grown in sand culture with the plants wholly enclosed within 5-litre aspirator bottles. The gas space in these bottles was kept filled with the gas mixtures listed in the preceding paragraph, except that 1 per cent of carbon dioxide was now added to provide for photosynthesis.

4. The growth of the plants and the assimilation of free or combined nitrogen at the different oxygen levels was assessed by visual inspection, but mainly on the basis of dry weight and total nitrogen data obtained at harvest

after several weeks' growth.

5. The reaction of the plants to variation in oxygen supply were in general similar in both types of experiment. With nodulated plants growth at 12 per cent oxygen was significantly superior to that at 21 per cent. Reduction to 5 per cent oxygen led to a curtailment of growth, the same being true when oxygen was further lowered to 1 per cent. With the non-nodulated plants growth at 21 and 12 per cent oxygen was essentially similar, but was reduced at 5 per cent and again at 1 per cent.

6. The extent of the responses to different oxygen levels was, however, much less marked in the whole-plant enclosure experiment, and this is considered to be due to the limitations necessarily imposed on growth by the experimenta arrangement.

7. By graphical means it is deduced from these experiments that the optimal oxygen level for the growth of nodulated clover plants is approximately 10 per cent, and that for non-nodulated plants approximately 15 per cent. These findings differ from those obtained in a previous investigation by other workers also using red clover and the whole-plant enclosure method, and reasons for the difference are discussed.

8. It is assumed that the differences in response of nodulated and non-nodulated clover plants to variations in oxygen supply observed by the present author are due to effects exerted in the nodule, so that the clover nodule appears to function most effectively at a reduced oxygen level. In this respect clover nodules appear to differ from those of Soya bean, the latter having been found by a previous investigator to function best at 21 per cent oxygen.

9. Reference is made to experimental and structural evidence from previous investigations indicating that a condition of oxygen deficiency exists in root nodules of legumes grown under normal conditions. In order to explain the results now obtained with clover the provisional assumption is made that nitrogen fixation is actually favoured by the prevalence of some degree of anaerobiosis in the nodule. There are good indications that the ventilation of the clover nodule is superior to that of the Soya bean nodule, hence in order to secure a particular state of anaerobiosis in the nodule a higher external level of oxygen will be required for Soya bean than for clover.

10. In the root-system enclosure experiments the number of nodules formed per plant was essentially similar at 21, 12, and 5 per cent oxygen, but was much reduced at 1 per cen

This last result could have been wholly due to the poor root growth at 1 per cent oxygen. It is concluded that the process of nodule initiation has a greater tolerance towards oxygen supply than the nodule has in its later stage as a nitrogen-fixing organ.

APPENDIX I.

Specimen Determination of Dissolved Oxygen.

Determination of the Amount of Dissolved Oxygen present in a Sample of Solution from a Culture Tube of Root-System Enclosure Experiment (21 per cent Oxygen passed).

Standard Data.

The following figures are used in the calculation.

Sodium Thiosulphate made up to be N/50 was standardised against N/50 Potassium Iodate and was found to be exactly N/50.

Now 1 cc. N Thiosulphate ≡ 8 mg. or 5.6 cc. oxygen at N.T.P.

∴ 1 cc. N/50 Thiosulphate ≡ 0.02 x 5.6 = 0.112 cc. oxygen at N.T.P.

Volume of barrel of syringe pipette = 1.3432 cc.

Volume of dead space and capillary extension = 0.096 cc.

1 cc. of reagents used in determination contains 0.0034 cc. of oxygen. Since 0.26 cc reagents are actually used in a determination the volume of oxygen introduced in this way = 0.00088 cc.

Method.

The method is as employed by Fox and Wingfield (1938) except in the following respect. They filled the dead space and the capillary with manganous chloride solution (the first of the reagents used in the Winkler process). Our experience shows that some of this reagent leaks out of the pipette into the plant culture tube from which a sample of culture solution is being withdrawn. In view of this, the procedure has been to fill the dead space and capillary extension with water of known oxygen content and to introduce the $MnCl_2$ subsequently by manipulation of the head screw, as is done anyway to draw in the remaining reagents.

Actual Determination.

Agla titration on extruded contents from syringe pipette, after completion of Winkler reaction = 0.0875 cc. thiosulphate.

∴ cc. oxygen in sample = (0.0875 x 0.112) - (oxygen introduced in reagents) - (oxygen present in water used for filling dead space and capillary)

= (0.0875 x 0.112) - [(0.00088) + (0.096 x 0.00605)]

= 0.008345

cc. oxygen in 10 cc sample = $\frac{0.008345 \times 10}{1.3432}$

= 0.06212.

Temperature of culture solution from which sample taken = 19 C. According to Winkler's data this would have contained, if in full equilibrium with air, 0.0648 cc. of oxygen (ignoring the slight effect of the salts present in the culture solution on dissolved oxygen content).

Therefore oxygen present as percentage of full equilibrium value = $\frac{0.06212 \times 100}{0.0648}$

= 96%

APPENDIX II.

Specimen Analysis of Variance.

(The data used are the dry weights of the nodulated plants of the second root-system enclosure experiment).

Plant No.	21% Dry Wt. (mg)	21% (Dry Wt)² (mg)²	12% Dry Wt. (mg)	12% (Dry Wt.)² (mg)²	5% Dry Wt. (mg)	5% (Dry Wt)² (mg)²	1% Dry Wt (mg)	1% (Dry Wt (mg)²
1	265	70225	1097	1203409	467	218089	361	123201
2	389	151321	635	403225	585	342225	51	2601
3	733	537289	374	139876	295	87025	70	4900
4	389	151321	788	620944	382	145924	40	1600
5	229	52441	1236	1527696	767	588289	74	5476
6	690	476100	1030	1060900	689	474721	73	5329
7	122	14884	381	145161	500	250000	50	2500
8	185	34225	564	318096	494	244036	112	12544
9	523	273529	630	396900	844	712336	63	3969
10	454	206116	100	10000	506	256036	53	2809
11	285	81225	559	312481	595	354025	94	8836
12	567	321489	693	480249	496	246016	47	2209
13	629	395641	380	144400	316	99856	106	11236
14	461	212521	1295	1677025	292	85264	42	1764
15	322	103684	771	594441	210	44100	104	10816
16	436	190096	916	839056	436	190096	70	4900
17	495	245025	687	471969			79	6241
18			1021	1042441				
Total	7074	3517132	13157	11388269	7874	4338038	1479	21093
Mean	422		731		492		87	

The analysis proceeds by obtaining the following terms:-

(1). Square the individual dry weights from each oxygen level and add totals.

$$3517132 + 11388269 + 4338038 + 210931 = \underline{19454370},$$

(2). Square the sums of the dry weights at each oxygen level, divide by the number of individuals at each level and sum these terms.

$$\frac{7074^2}{17} + \frac{13157^2}{18} + \frac{7874^2}{16} + \frac{1479^2}{17} = 2943616 + 9617036 + 3874992 + 128673.$$

$$= \underline{16564317}.$$

(3). Square the grand total of all dry weights from the four oxygen levels and divide by the total number of individual

$$\frac{(7074 + 13157 + 7874 + 1479)^2}{68} = \frac{29584^2}{68}$$

$$= \underline{12870780}.$$

Source of Variance.	Sums of Squares.	Degrees of Freedom.	Mean Square
Between Groups	(2) - (3) = <u>3693537</u>	3	1231179
Within Groups	(1) - (2) = <u>2890053</u>	64	45157

The degrees of freedom are derived as follows:-

(a) Between Groups : one less than the number of oxygen levels.

(b) Within Groups : each oxygen level contributes one less than the number of individuals at that level.

Testing the Between Groups Mean Square in the above analysis it gives a Variance Ratio of the following value.

$$\frac{1231179}{45157} = 27.3 \text{ for degrees of freedom } n_1 = 3,\ n_2 = 64.$$

This is considerably more significant than the 5 per cent level of significance (i.e. 2.8) and so significance exists in the amount of dry weight produced at the four oxygen levels.

<u>Calculation of the difference required between means for significance.</u>

Number of individuals at 21 and 12 per cent oxygen is 17 and 18 respectively.

21% and 12% $\sqrt{45,157} \times t_{64}0.05 \times \sqrt{1/17 + 1/18}$

$= 212.6 \times 2 \times \sqrt{35/306}$

$= 212.6 \times 2 \times 0.338$

$= 144$ mg.

Actual difference between means equals 309 mg., therefore dry weight at 12 per cent is significantly greater than that at 21 per cent oxygen

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SECTION II OF THESIS.

Observations on the Formation and Significance of the Root Nodules of *Alnus glutinosa* (L.) Gaertn.

CONTENTS OF SECTION II.

GENERAL INTRODUCTION.

Although more than 50 years have elapsed since Hiltner (1896) concluded from his experimental work that the nodulated Alder plant, like the nodulated leguminous plant, has the power to assimilate the free nitrogen of the atmosphere, this finding has attracted little attention or further study so that to-day our knowledge of the development and significance of the root nodules of this plant has only been increased to a slight degree

Hiltner (loc. cit.) showed that nodulated Alder plants grown in both nitrogen-free sand and culture solution exhibited healthy growth while those which were grown under similar conditions but were not inoculated with the nodule organism and thus remained free of nodules, died through a deficiency of nitrogen. It was from this observation that he concluded that Alders when possessing root nodules can fix atmospheric nitrogen in a manner comperable to legumes.

These findings were restated and slightly amplified by Nobbe and Hiltner (1904) in an illustrated paper in which the growth of nodulated and non-nodulated plants in nitrogen-free culture solution was compared. Once again it was shown that in such a medium, healthy growth could only be attained by plants possessing nodules. Some of their plants were grown to an age of 8 years by which time they had a height of two metres.

Möller (1912) confirmed the experimental findings of Hiltner. In his work analyses of the nitrogen content of the plants were carried out and he was able to show that large quantities of nitrogen (5.6 gm. per plant) had been accumulated after two years' growth.

Krebber (1932) repeated Hiltner's experiments but apart from deciding from a survay of the pH of the habitats of Alders to grow his plants in nutrient solution of pH 5.7, he simply verified that fixation of nitrogen appeared to be associated with nodulated plants.

Roberg (1934) who continued Krebber's experiments and Plotho (1941) also put forwad evidence for nitrogen fixation by nodulated Alders without providing much new information.

Virtanen and Saastamoinen (1933 and 1936) provided further evidence of fixation and in addition studied the effect of pH upon growth. Starting with plants which had already grown for two seasons under uniform conditions in sand culture, the pH of the rooting medium was adjusted to different values over the range 4 to 7 and the growth of the plants studied for a further two seasons. They concluded from this experiment that pH 6 was the most favourable for growth by nodulated plants. Non-nodulated plants supplied with ammonium nitrate were also included in these experiments and although the data for these plants were incomplete, the pH response appeared to be similar to that of nodulated plants. However the conclusions reached by these authors must be regarded as tentative, since only one or two plants were grown at each pH level and statistical analyses could not be carried out.

These same authors also expressed the view, without any experimental backing, that nodulated Alder plants grown in nitrogen-free culture solution required a plentiful supply of oxygen to the root nodules before satisfactory fixation and growth can be obtained and they pointed to the fact that in water culture the nodules tend to develop near the surface of the solution. This opinion was also partly based on the findings of Virtanen and von Hausen (1935) that in the case of nodulated pea plants, fixation was poor when the nodules were submerged in the culture solution but increased on their exposure to the atmosphere. Virtanen and Saastamoinen because of this view grew their Alder plants in sand culture and in an experiment to be reported later an attempt has been made to decide if the theory of these investigators is valid.

A further reference to the nitrogen fixing powers of nodulated Alder plants is made by Virtanen and Miettinen (1952). Here it is noted that Virtanen and von Hausen grew a nodulated plant in nitrogen-free sand culture for two years by which time the plant had attained a height of 261 cm. and a total nitrogen content of about 6.5 gm.

In another recent paper, Hawker and Fraymouth (1951) suggested that uncertainty still exists as to whether the endophyte in the root nodules of Alder is able to bring about the process of nitrogen fixation and they consider that the matter is still in doubt as some investigators have been able to obtain no positive evidence for its occurrence. A study of the literature shows however that there is strong experimental backing for the view that fixation does take place in association with nodulated plants and no paper relating to negative results is known to the present author. Even if such a paper does exist, the strong support on the side of fixation cannot be overlooked.

In this paper Hawker and Fraymouth refer, though without giving any details, to preliminary experiments of their own in which both nodulated and non-nodulated seedlings of Alder were grown in garden soil. They found that both types of plants grew with equal vigour and therefore considered this as evidence of the non-occurrence of fixation. But under the experimental conditions described no valid conclusions as to the occurrence of fixation can be drawn, since there could have been no control over the amount of combined nitrogen supplied to both types of plants and to the non-nodulated controls in particular. Such an experiment would have to be repeated using a rooting medium allowing for control over the access of combined nitrogen before it could be taken as evidence that nitrogen fixation is not associated with nodulated Alder plants. In view of Hawker and Fraymouth's paper the further evidence for fixation obtained in the present research will be presented in greater detail than would have otherwise been thought necessary.

In the present studies the following aspects have been considered:-

1. The effect of pH on nodulation and subsequent growth by nodulated Alder plants in water culture; parallel experiments with non-nodulated plants supplied with combined nitrogen have been included in certain instances in order to distinguish effects which are specific for nodulated plants.

2. The effect of aeration of the culture solution on nodule development and function.

Effect of pH on Nodulation and Growth.

M E T H O D S.

Seed collected from the field in the autumn of the previous year and stored at laboratory temperature was sown in the early spring in trays of horticultural peat. Neither the seeds nor the peat was sterilised before use. Germination commenced about two weeks after sowing and these nodule-free seedlings were transplanted into water culture when they were about 1.5 cm. high and when two to three leaves had formed. In this work glazed earthenware jars of 2-litre capacity were used for the water cultures, each jar being covered with a waxed teak square bored with seven holes. Each hole was fitted with a rubber inset containing a smaller hole through which the roots of the seedlings were introduced and the plant supported in the hole by means of a small rubber wedge.

Nitrogen-free Crone's solution with the minor elements added was the basic solution employed in the experiments (for formula see Section I). The pH of this solution was 6.3 and one series of plants was grown at this level. For the other series the solution was adjusted to pH 7.0, 5.4, 4.2 and 3.3 respectively by addition of suitable amounts of either sodium hydroxide or sulphuric acid.

The culture solution in the jars was changed at intervals of approximately three weeks and as far as possible the pH of the solution was maintained at the required level by frequent adjustment. The pH in the jars containing nodulated plants showed little changes in the initial stages of the experiment but later it became necessary to re-adjust the pH every day. In the case of the plants being supplied with either nitrate or ammonium nitrogen very rapid changes in pH occurred and daily adjustment was commenced at a much earlier stage.

Certain of the plants were inoculated with the nodule organism 24 hours after transplanting into water culture, by applying to each root system several drops of a suspension in water of pulverised nodules from field material. In the 1951 experiment, the inoculum was made up by pulverising 12 gm. of nodule material in 100 cc. distilled water but in 1952 its strength was increased by using 20 gm. of nodules with 100 cc. of water. A small amount of an autoclaved suspension, similar to that applied to the above plants, was added to the control jars in which no nodules were desired and where there was going to be no addition of combined nitrogen.

Most of the plants were grown for one season only and were harvested when leaf fall was about to commence. The plants were harvested individually, dry weights being obtained by heating at 95° C. and total nitrogen by the Kjeldahl method.

A total of fourteen plants have been allowed to continue into their second year of growth and were transplanted into either 4- or 8-litre jars at the beginning of the second season.

EXPERIMENTAL RESULTS.

1. Observations during 1st year of development.

Two experiments have been carried out relating to pH effect the main one in 1951 and a subsidiary one in 1952. The former consisted initially of 63 jars containing inoculated plants in nitrogen-free solution and also uninoculated plants, some of which were in nitrogen-free solution while others were supplied with combined nitrogen in the form of sodium nitrate or ammonium sulphate. The second experiment comprised initially 25 jars containing only inoculated plants in nitrogen-free solution. In both experiments plants were set up at pH 3.3, 4.2, 5.4, 6.3 and 7.0. No forced aeration was given to the culture solution in these experiments.

Inoculated Plants.

In the case of the inoculated plants, nodules became visibl to the naked eye from 10 days to 3 weeks after inoculation with a suspension of the nodule organism and at this stage formed white swellings on the roots. This differs from the findings of Krebber (1932) who observed red colouration in young nodules, but it has however been noted in Glasgow that the nodules tend to develop this red pigment when the root system is subjected to a certain amount of daylight. Bond (1951) made similar observations in his work on *Myrica gale* where the colour was shown to be due to the presence of anthocyanin. At the time of nodule formation the plants had formed 3 - 4 leaves and were 2 to 3 cm. in height (see Fig. 32), but Bond (unpublished work, communicated to the author) has shown that by earlier inoculation, nodules can be produced at a stage when a single leaf only has been formed.

In both the 1951 and 1952 experiments the nodules induced by inoculation carried out as indicated above were almost entirely confined to the upper portion of the root system near the base of the hypocotyl (see Fig. 33), but those in the 1951 experiment which were formed as the result of the second inoculation (see footnote to Table 16) which took place one month later, were scattered over a greater region of the root system and were well-submerged in the culture solution (see Fig. 34). These findings suggest that nodule formation is affected by the stage of development of the root system at the time of inoculation and in particular, perhaps, by the distribution of the root hairs.

The simple nodules which arose as a result of inoculation soon started to branch and eventually developed into nodules clusters of the type found in the field. From the nodules flattened conical projections developed and these were found to consist of hypertrophied complementary tissue from the lenticels (see Fig. 35). The presence of lenticels on the

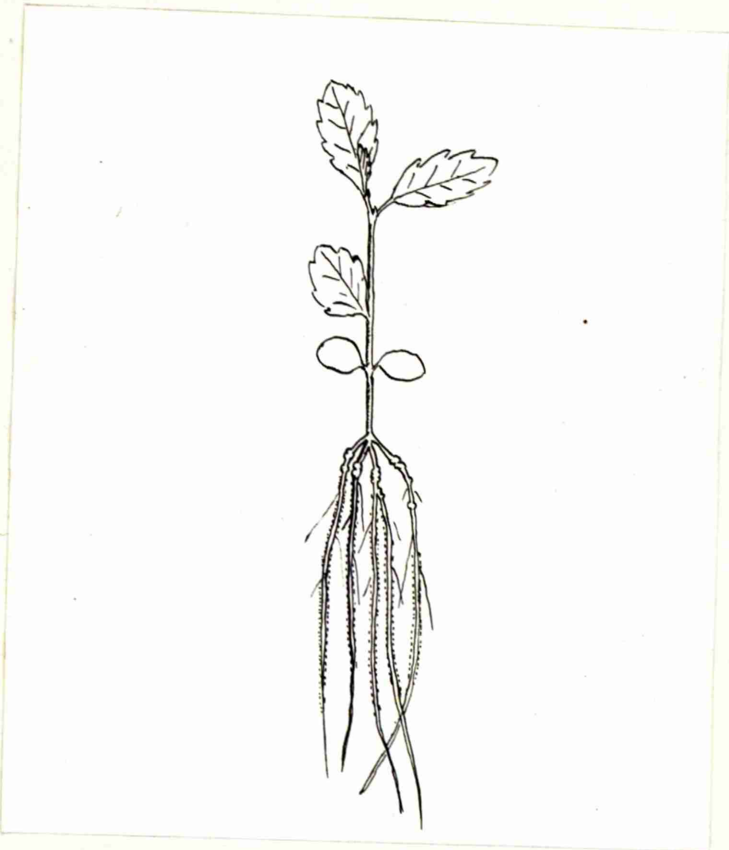

Fig. 32. Alder plant with nodules, 3 weeks after inoculation. The nodules first became visible 18 days after inoculation (natural size).

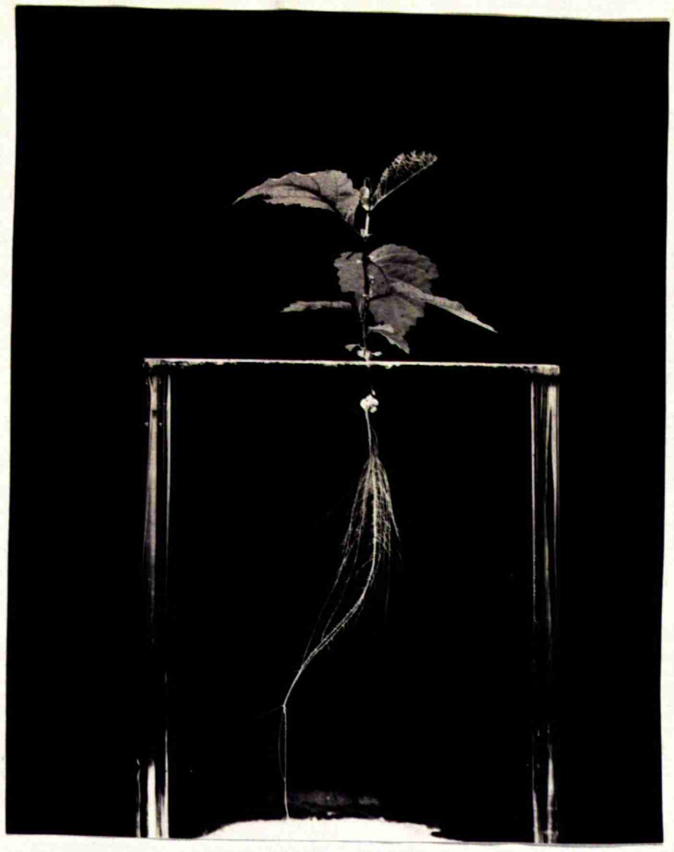

Fig. 33. Nodules situated near base of stem. Plant after 14 weeks' growth in water culture. (x ¼).

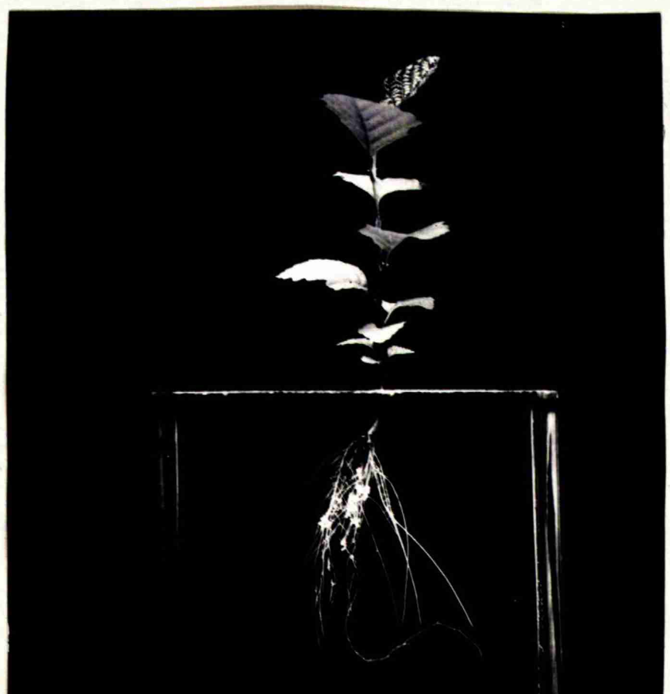

Fig. 34. Nodules scattered over root system. Plant after 19 weeks' growth in water culture. (x ¼).

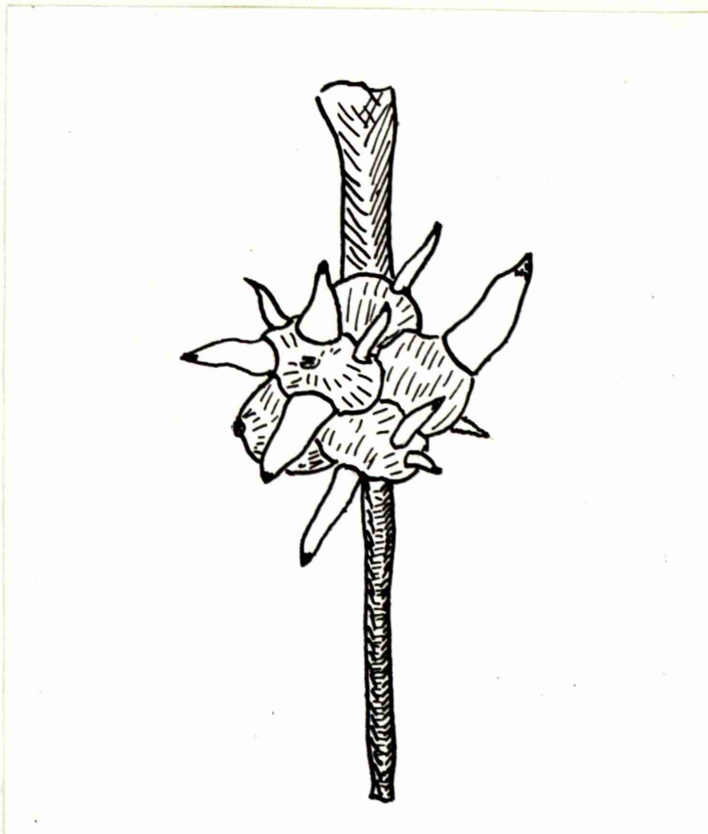

Fig. 35. Nodule cluster showing flattened conical projections which consisted of hypertrophied complementary tissue of the lenticels. (x 4).

nodules in field material was noted by Borm (1931) but the development of hypertrophied complementary tissue appears to be a feature of growth in water culture.

As shown in Table 15, the pH of the culture solution considerably affected the development of the nodules. In both experiments nodulation failed to take place at the most acid solution employed, namely pH 3.3 and these plants eventually became moribund and died. The number of plants nodulating at pH 4.2 was smaller than at higher pH levels, though the disparity was less marked in the 1952 experiment. In 1951 pH 7.0 appeared to be the most favourable for nodulation, but taking the two experiments together the three higher pH's appear equally favourable. The higher proportion of plants nodulating at all pH levels (except the lowest) in the 1952 experiment is attributed to the use of a stronger inoculum (see Methods).

Two weeks after nodulation there were definite indications of nitrogen fixation since the nodulated plants were now beginning to show stronger top and root growth than the uninoculated control plants in nitrogen-free solution. This superiority in growth by the nodulated plants became still more noticeable as the experiment proceeded. Because of the strong growth it became necessary to reduce the number of plants to

Table 15.

Effect of pH of Culture Solution on Nodulation.

pH of Culture Solution.	Number of Plants forming Nodules.	
	1951 Expt.	1952 Expt.
3.3	0/35	0/35
4.2	7/35	25/35
5.4	23/35	31/35
6.3	22/35	35/35
7.0	28/35	34/35

The total number of plants inoculated at each pH level was 35.

3 per jar. This change was made after three months' growth in the 1951 experiment, and after two months in 1952. Also in the latter year the total number of plants at each pH level was reduced to 18 at this stage.

As the plants increased in size it became necessary (as already noted) to re-adjust the pH of the culture solution in the jars every day because through root action falls of 0.3 to 0.4 of a unit were being recorded every 24 hours.

The mean data obtained at the harvest of the nodulated plants of the 1951 experiment along with the statistical treatment are presented in Tables 16 and 17. The plants were grown for a period of 6 to 7 months and the data are sub-divided into two groups for the reason stated in Table 16. From Table 16 it is seen that in both groups of plants the highest mean figure for height, dry weight, and nitrogen content was obtained at pH 5.4. Large plant-to-plant variation was exhibited at each pH level and statistical treatment (see Table 17) shows that the superiority in growth at pH 5.4 over pH 4.2 fails to attain significance, that there is no significance between growth at pH 6.3 and pH 7.0, and that the superiority in growth at pH 5.4 over that at the higher pH levels is significant in nearly all respects, i.e.

$$\text{pH } 7.0 = \text{pH } 6.3 < \text{pH } 5.4 = \text{pH } 4.2.$$

Table 16.

Mean Data (per Plant) obtained at Harvest of Nodulated Plants grown in Nitrogen-Free Solution. 1951 Experiment.

	pH of Culture Solution.	No. of Plants harvested.	Ht. of Shoot. (cm.)	Dry Weight (gm.)			Total Nitroge (mg.)
				Nods.	Tops & Roots	Total	
First Group	3.3	0	-	-	-	-	-
	4.2	2	38	0.152	10.60	10.75	153
	5.4	8	52	0.370	16.80	17.17	301
	6.3	5	41	0.231	9.21	9.44	170
	7.0	13	38	0.246	11.68	11.93	232
Second Group	3.3	0	-	-	-	-	-
	4.2	5	21	0.155	3.58	3.74	86
	5.4	15	23	0.209	4.29	4.50	100
	6.3	17	11	0.062	1.43	1.49	34
	7.0	15	11	0.066	1.48	1.55	37

8-week-old seedlings, 1.5 cm. high, were transplanted into water culture on 11th April 1951, with 35 seedlings at each pH. The plants of the first group formed nodules in response to the original inoculation and were harvested 15th October 1951. The plants of the second group nodulated after a re-inoculation on 18th May and were harvested 13th November 1951.

Table 17.

Minimum Difference between Means in Table 16 required for Significance at P = 0.05 from Analysis of Variance.

	Comparison	Ht. of Shoot. (cm.)	Total Dry Weight. (gm.)	Total Nitrogen. (mg.)
First Group	pH 5.4 & 6.3	12 (obsvd. 11)	5.87 (7.73)	112 (131)
	5.4 & 7.0	9 (14)	4.62 (5.24)	89 (68)
	6.3 & 7.0	11 (3)	5.36 (2.49)	102 (63)
Second Group	4.2 & 5.4	4 (2)	1.29 (0.76)	30 (14)
	5.4 & 6.3	3 (12)	0.91 (3.01)	21 (66)
	6.3 & 7.0	3 (0)	0.91 (0.05)	21 (3)

Typical plants from both the first and second groups are shown in Figs. 36 to 39.

The data obtained at the harvest of the 1952 experiment are shown in Table 18. In this experiment more plants were availabl for harvest than in either group of the previous experiment, and correspondingly more information was yielded. The greatest mean height was shown at pH 5.4, and the greatest dry weight at pH 4.2 but statistical treatment shows that actually there was no significant difference between these two pH's, both being equally favourable. Growth was significantly inferior at higher pH's, i.e. pH 7.0 < pH 6.3 < pH 5.4 = pH 4.2

Typical plants from this 1952 experiment are shown in Figs. 40 and 41.

From the results obtained from both these experiments it would appear that the optimum point for growth of nodulated Alder plants lies between pH 5.4 and 4.2.

Control Plants.

The mean data obtained at the harvest of the uninoculated control plants in nitrogen-free solution of the 1951 experiment are shown in Table 19. As these plants were able to make only negligible growth it is concluded that under the experimental conditions employed combined nitrogen was not made available to the plants from unintended sources. Therefore practically all

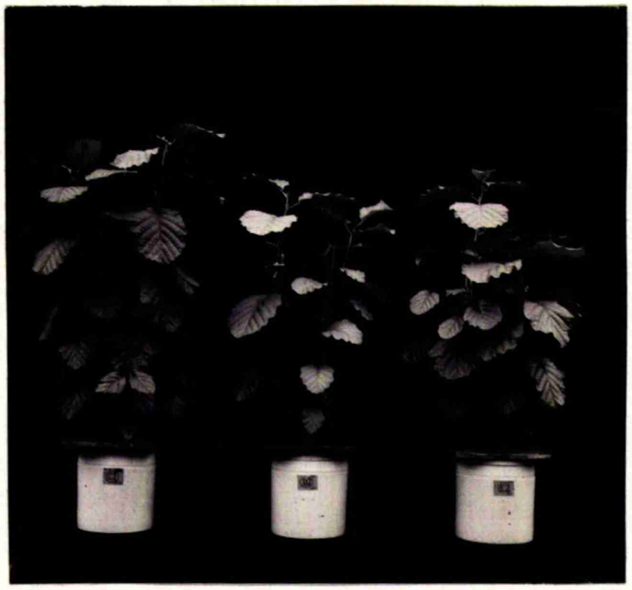

Fig. 36. Nodulated plants - first group of 1951 experiment. Left to right - pH 5.4, 6.3 and 7.0. After 5 months' growth in water culture. (x 1/16).

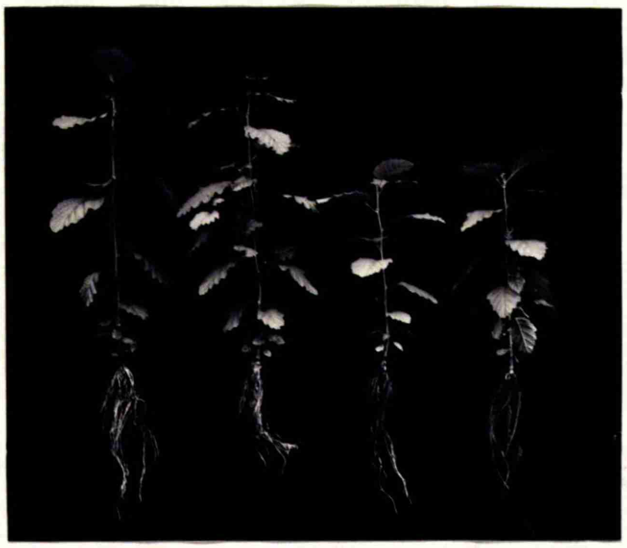

Fig. 37. Nodulated plants - first group of 1951 experiment. Left to right - pH 4.2, 5.4, 6.3 and 7.0. After 5 months' growth in water culture. (x 1/10).

Fig. 38. Nodulated plants - second group of 1951 experiment. Left to right - pH 4.2, 5.4, 6.3 and 7.0. After 6 months' growth in water culture. (x 1/16).

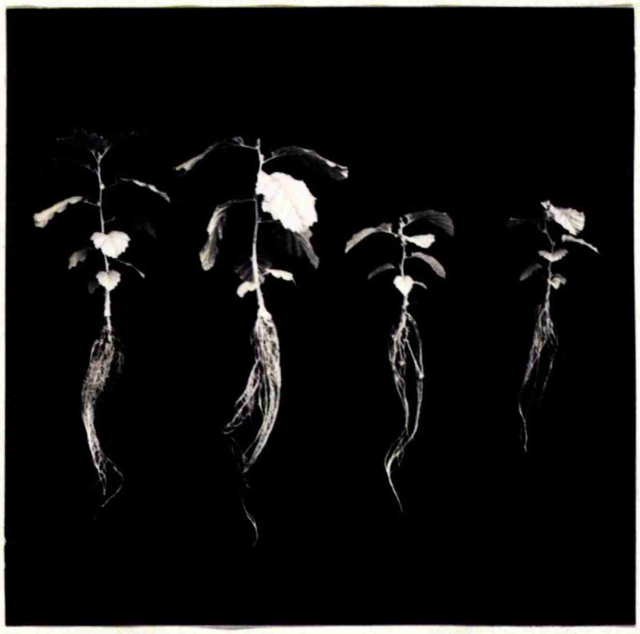

Fig. 39. Nodulated plants - second group of 1951 experiment. Left to right - pH 4.2, 5.4, 6.3 and 7.0. After 6 months' growth in water culture. (x 1/12).

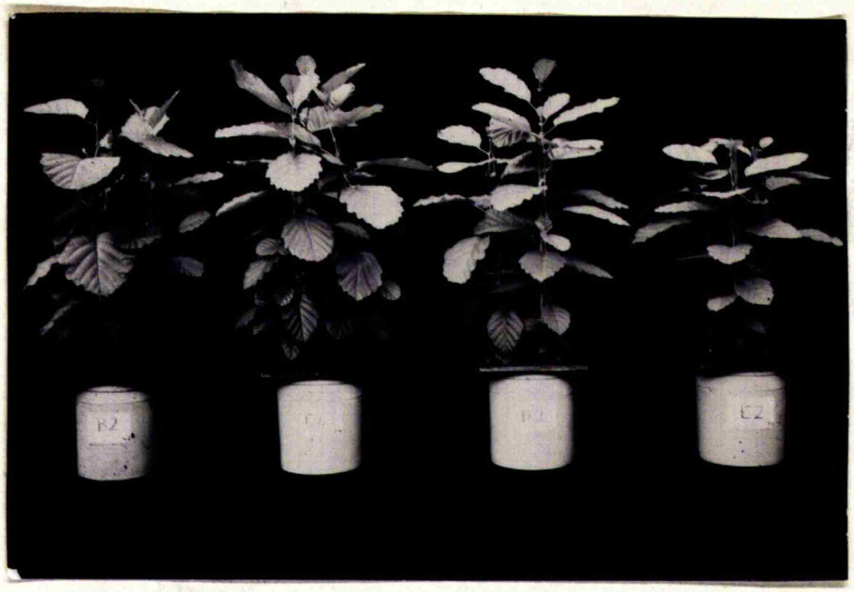

Fig. 40. Nodulated plants of 1952 experiment. Left to righ
- pH 4.2, 5.4, 6.3 and 7.0. After 5 months' growth
in culture solution. (x 1/15).

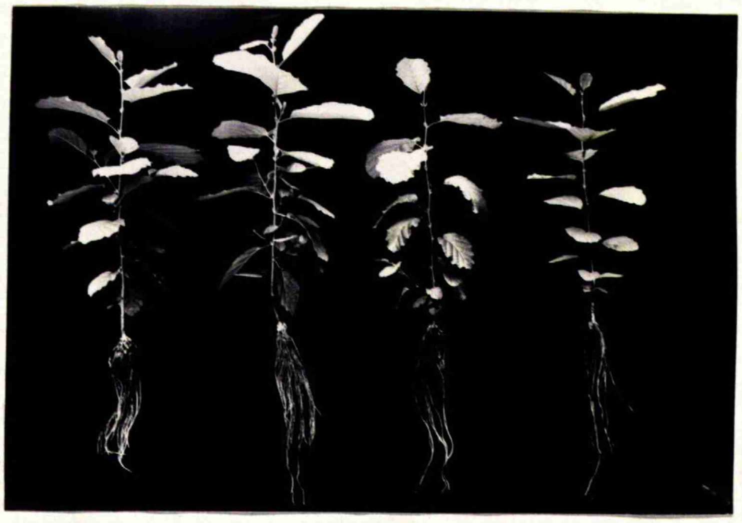

Fig. 41. Nodulated plants of 1952 experiment. Left to right
- pH 4.2, 5.4, 6.3 and 7.0. After 5 months' growt
in culture solution. (x 1/15).

Table 18.

Mean Data (per Plant) obtained at Harvest of Nodulated Plants grown in Nitrogen-Free Solution. 1952 Experiment.

pH of Culture Solution.	No. of Plants harvested.	Ht. of Shoot. (cm.)	Dry Weight (gm.)			
			Nods.	Roots.	Tops.	Total
3.3	0	-	-	-	-	-
4.2	18	49	0.221	0.97	5.56	6.75
5.4	18	52	0.222	0.83	5.36	6.41
6.3	18	43	0.159	0.59	3.92	4.67
7.0	18	37	0.133	0.56	2.72	3.41

8-week-old seedlings, 1.5 cm. high, were transplanted into water culture on 10th April 1952, with 35 seedlings at each pH level. On 2nd June 1952 this number was reduced to 18 plants per pH level and the plants were harvested on 3rd September 1952.

Minimum Difference between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison	Ht. of Shoot. (cm.)	Total Dry Weight. (gm.)
pH 4.2 & 5.4	5 (obsvd. 3)	1.05 (0.34)
4.2 & 6.3	5 (6)	1.05 (2.08)
4.2 & 7.0	5 (12)	1.05 (3.34)
5.4 & 6.3	5 (9)	1.05 (1.75)
5.4 & 7.0	5 (15)	1.05 (2.00)
6.3 & 7.0	5 (6)	1.05 (1.26)

Table 19.

Mean Data (per Plant) obtained at Harvest of Non-Nodulated Control Plants in Nitrogen-Free Solution.

pH of Culture Solution.	No. of Plants harvested.	Ht. of Shoot. (cm.)	Dry Weight. (mg.)	Total Nitrogen. (mg.)
3.3	5	2.5	18	0.36
4.2	5	2.5	19	0.41
5.4	10	2.5	18	0.36
6.3	7	2.5	21	0.40
7.0	9	2.5	16	0.33

These plants were transplanted into water culture on the same date as the inoculated plants of the 1951 experiment (see Table 16). At the two lowest pH levels 7 plants were set up, with 14 at remaining levels, but some of the plants died at an early stage and were discarded. Few of the remaining plants survived the full period of the experiment and they were harvested at intermediate dates as they became moribund.

the nitrogen accumulated in the nodulated plants is concluded to be the result of fixation. It will be seen from Table 16 that at pH 5.4 a mean of 301 mg. nitrogen per plant was fixed during a growth period of 6 months. Fig. 42 compares typical control and nodulated plants.

No control plants were grown during the 1952 experiment because in the previous year it had been shown that the experimental arrangement employed was satisfactory in respect of control of combined nitrogen.

Combined-Nitrogen Plants.

Let us now turn our attention to the uninoculated plants of the 1951 experiment which were supplied with combined nitrogen in the form of either sodium nitrate or ammonium sulphate. The first addition of combined nitrogen was made one week after transplanting into culture solution when the equivalent of 50 mg. nitrogen was added to each jar. These plants, as they did not have to undergo a period of nitrogen starvation, immediately went ahead in growth compared with the nodulated plants which had to await the commencement of nitrogen fixation (see Fig. 43). This superiority in growth however disappeared as the experiment progressed (see Fig. 44) and at the time of harvest the best of the nodulated plants were superior to those supplied with combined nitrogen (see Fig. 45).

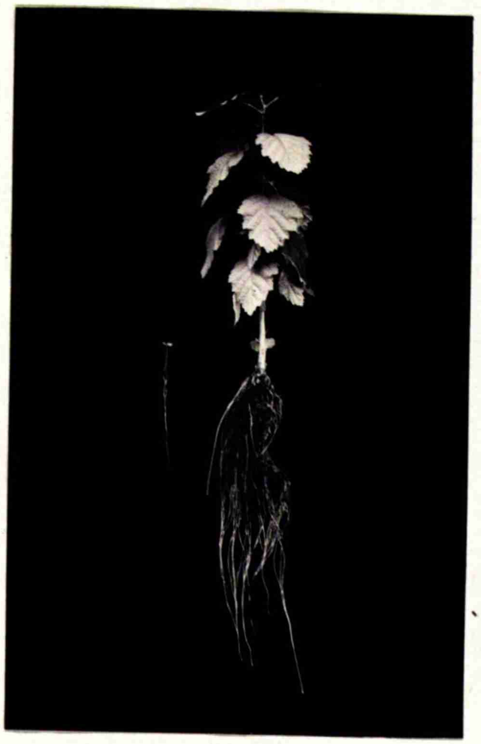

Fig. 42. Control plant (left) and nodulated plant (right) after 30 weeks' growth in nitrogen-free culture solution at pH 7.0. Plants from 1951 experiment, first group. (x 1/12).

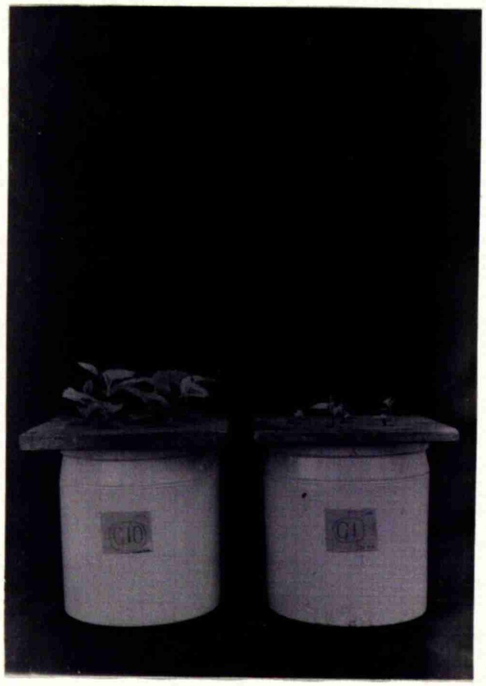

Fig. 43. Nodulated plants (right) and plants supplied with sodium nitrate (left) after 7 weeks' growth in culture solution at pH 5.4. (x 1/8).

Fig. 44. Nodulated plants (left) and plants supplied with sodium nitrate (right), after 16 weeks' growth in culture solution at pH 5.4. (x 1/10).

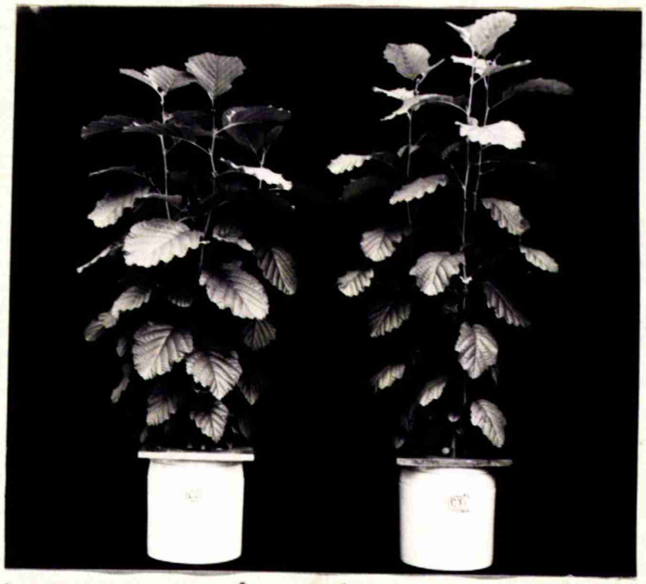

Fig. 45. Nodulated plants (left) and plants supplied with sodium nitrate (right), after 21 weeks' growth in culture solution (pH 5.4). These are the same plants as in Fig. 44. (x 1/13).

In the case of the plants receiving sodium nitrate, daily adjustment of the pH of the solution was again necessary owing to the increase in the pH value, particularly at the three lowest levels. Thus an increase of as much as 0.7 was shown in a period of 24 hours by plants of the pH 3.3 series. These nitrate plants were harvested after 5 months' growth in water culture and the data obtained at harvest and the results of statistical analysis are presented in Table 20. At no time throughout the experiment was there any marked difference in growth between the five groups of plants except in respect of height. From the Table it is seen that although the dry weight at pH 5.4 is greater than at the other pH levels, the differences do not reach significance. However in respect of height of shoot, that at pH 5.4 is significantly greater than at 3.3 and 7.0. Fig. 46 and 47 show typical jars and individual plants from the five pH levels.

Too few plants supplied with ammonium sulphate were set up (namely, one jar of 7 plants, later reduced to 3 plants) at each pH level to carry out statistical analysis, but the general result of the experiment was that vigorous growth was attained at all pH levels with the exception of pH 3.3 where it was inferior and marked stunting of the root system was exhibited. It should however be remembered that the pH of these

Fig. 46. Typical jars of plants supplied with sodium nitrate, after 21 weeks' growth in culture solution. pH from left to right - 3.3, 4.2, 5.4, 6.3 and 7.0. (x 1/16)

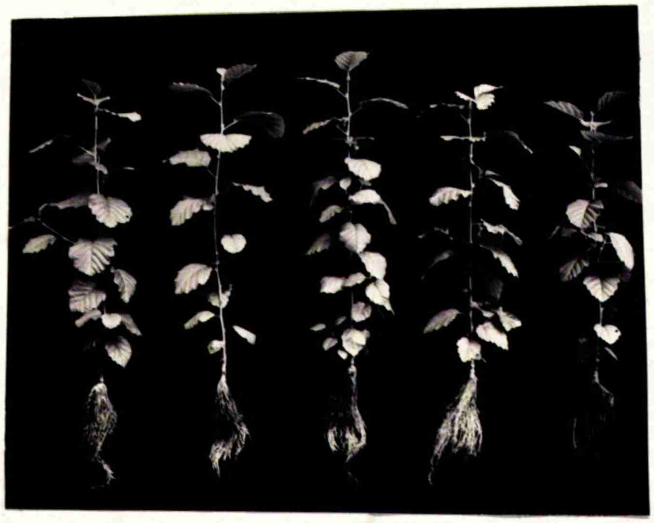

Fig. 47. Typical plants supplied with sodium nitrate, after 21 weeks' growth in culture solution. pH from left to right - 3.3, 4.2, 5.4, 6.3 and 7.0. (x 1/11).

Table 20.

Mean Data (per Plant) obtained at Harvest of Non-Nodulated Plants supplied with Nitrate-Nitrogen.

pH of culture Solution.	No. of Plants harvested.	Ht. of Shoot. (cm.)	Dry Weight (gm.)	Total Nitrogen. (mg.)
3.3	15	54	12.82	163
4.2	15	57	12.53	166
5.4	15	60	13.34	164
6.3	15	55	11.86	163
7.0	15	51	11.09	163

8-week-old seedlings, 1.5 cm. high, were transplanted into water culture on 11th April 1951. There were initially 5 jars each with 7 plants at each pH, but in July this number was reduced to 3 per jar. The plants were harvested 15th Sept. 1951. During the growth period a total of 275 mg. nitrate-nitrogen per plant was added to the culture solution.

Minimum Differences between Means required for Significance at P = 0.05 from Analysis of Variance.

Comparison	Ht. of Shoot. (cm.)	Total Dry Weight. (gm.)
pH 3.3 & 5.4	5 (obsvd. 6)	2.44 (0.52)
4.2 & 7.0	5 (6)	2.44 (1.44)
5.4 & 7.0	5 (9)	2.44 (1.25)

cultures being supplied with ammonium sulphate tended to fall and although daily re-adjustment was carried out, these plants nominally grown at pH 3.3 were exposed for at least short periods to a level as low as 2.8. In the early stages of the experiment these ammonium plants were slightly larger than those receiving sodium nitrate (see Fig. 48) and this condition continued throughout the period of growth. Typical plants at time of harvest are shown in Fig. 49.

2. Observations in 2nd year of growth.

An experiment has been performed in which nodulated plants were allowed to grow for a second season in water culture in order to study further the effect of pH on growth. Owing to the large amount of space required by plants in their second year, only two plants could be included at each pH. Ten nodulated Alder plants which had been grown under uniform conditions in water culture at pH 6.3 throughout their first season (1951), were transplanted in March 1952 into individual glazed earthenware jars of 4-litre capacity. These jars were covered with waxed teak squares with a hole bored in the centre through which the roots of the plants were introduced. As stated, two plants were grown at each of the five pH levels used in the pH experiments already reported. Owing to the fact that some well-submerged nodules were present on the plants it was

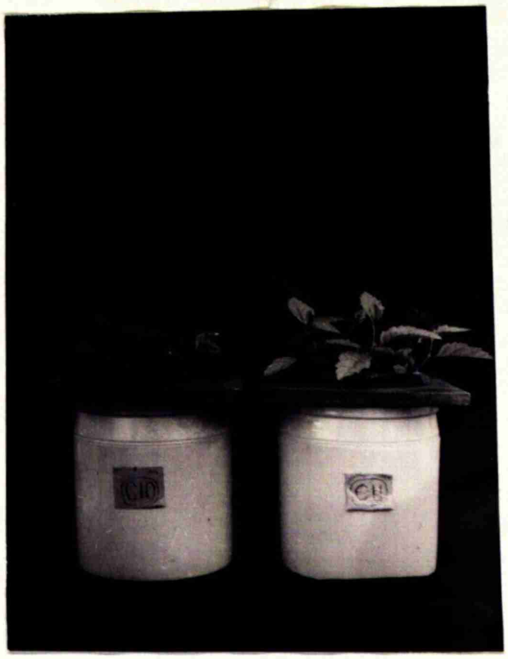

Fig. 48. Plants receiving sodium nitrate (left) and ammonium sulphate (right), after 7 weeks' growth in culture solution at pH 5.4. (x 1/8).

Fig. 49. Plants receiving ammonium sulphate, after 21 weeks' growth in culture solution at pH 6.3 (left) and 7.0 (right). (x 1/16).

decided to provide forced aeration, this being supplied by a compressor pump for 20 hours out of each 24 hours (7 a.m. to 3 a.m.).

The plants at the beginning of the second season were not uniform in respect of fresh weight and height of shoot and a selection was made at the time of transplanting into the various solutions so that the mean height for the two plants at each pH level was approximately similar (see Table 21).

Growth was continued until July 1952 when the plants were harvested and the height of main shoot, total length of side shoots and fresh weight were noted. The data obtained at this harvest are presented in Table 22, and show (right hand end column) that the greatest relative increase in fresh weight was obtained at pH 4.2. The greatest increase in main shoot height occurs at pH 4.2 and pH 6.3 while in respect of total length of side shoots the highest figure is obtained at pH 5.4. These figures indicate that growth was better at pH's 4.2, 5.4 and 6.3 than at pH 3.3 and 7.0 but because of the restricted number of plants in the experiment a more definite conclusion cannot be made.

It is however noteworthy that already-nodulated Alder plants can grow at pH 3.3, a pH which was found to be completely unfavourable for infection and the initiation of nodules. Plant

Table 21.

Details of Nodulated Plants at Beginning of Second Season's Growt

Plant No.	Ht. of Shoot. (cm.)	Fresh Weight. (gm.)
1	10	8.7
2	9	13.5
3	11	7.6
4	10	8.9
5	9	10.0
6	13	8.1
7	9	10.5
8	15	12.3
9	9	9.1
10	12	8.7

These plants were grown for a period of seven months in water culture at pH 6.3 and overwintered in a heated greenhouse. They were transplanted into 4-litre jars at the beginning of the second season.

Table 22.

Data obtained at Harvest of Nodulated Plants after Two Seasons' Growth in Culture Solution

pH of Soln.	Plant No.	Ht of Main Shoot. (cm.)	Increase in Height of Main Shoot. (cm.)	Mean.	Total Length of Side Shoots. (cm.)	Mean.	Increase in Length of all Shoots. (cm.)	Mean.	Total Fresh Wt. (gm.)	Final Fresh W / Initial Fresh W	Mean
3.3	1	63	53	61	172	160	225	221	161	18.5	14.4
	2	77	68		148		216		138	10.2	
4.2	3	76	65	95	202	475	267	570	332	41.0	43.3
	4	134	124		748		872		405	45.5	
5.4	5	101	92	82	406	633	498	715	291	29.1	34.6
	6	84	71		860		931		325	40.1	
6.3	7	87	77	95	502	585	579	680	277	26.4	30.0
	8	127	112		668		780		413	33.6	
7.0	9.	70	61	81	323	349	384	430	171	18.8	25.4
	10	112	100		376		476		278	32.0	

grown at such a pH were however unhealthy and exhibited marked stunting of the roots. Another interesting feature of this experiment is the very rapid growth made by these plants in their second season both with regard to height of shoot and fresh weight. Plant No. 4 at pH 4.2 showed an increase in shoot height from 10 cm. to 134 cm. and an increase in fresh weight from 8.9 gm. to 405 gm. all in a matter of approximately five months.

Effect of Aeration of Rooting Medium on Nodulation and Growth.

M E T H O D S.

Nodule-free Alder seedlings which had been grown in trays of horticultural peat were again transplanted into water culture when 2 to 3 leaves had formed and when they had attained a height of approximately 1.5 cm. For this work glass jars of capacity 2½-litres were used. These jars had a height of 21 cm. a diameter of 16 cm. narrowing to 9 cm. at the neck and were closed at the mouth by cork stoppers which contained 5 holes. Each hole was fitted with a rubber inset containing a smaller hole through which the roots of the seedlings were introduced and the plants supported by a small rubber wedge.

Nitrogen-free Crone's solution with the minor elements added was again the basic solution employed and the pH in all the jars was maintained at 6.3 throughout the experiment. Inoculation of the plants in certain of the culture jars was carried out 24 hours after transplanting, using the same method as already described, while the remaining plants were supplied with ammonium-nitrogen.

Half of the cultures of each type were given forced aeration which was supplied continuously by a compressor pump. No attempt was made to ensure that the cultures were air-tight and in non-aerated jars free gaseous diffusion could take place between the external atmosphere and the air space below the stopper. Determinations of dissolved oxygen present in the culture solution were performed, using the same method as in Section I.

At the termination of the experiment the plants were harvested individually as before.

EXPERIMENTAL RESULTS.

This experiment consisted of 16 jars, 10 of which contained inoculated plants in nitrogen-free solution and the remaining 6 uninoculated plants supplied with combined nitrogen in the form of ammonium sulphate. Half of the jars of each type received forced aeration, as noted.

Nodules which were visible to the naked eye developed 14 days after inoculation with a suspension of the nodule organism. In the case of the aerated jars, nodules developed at various depths in the solution, whereas in the non-aerated cultures they were mostly confined to a region near the surface of the solutio These aerated plants also showed signs of nitrogen fixation at an earlier stage than the corresponding non-aerated plants and grew more vigorously throughout the experiment (see Figs. 50 - 5 The mean data obtained at the harvest of these nodulated plants along with the results of statistical analysis are presented in Table 23 and it is shown that as a result of aeration, the mean dry weight and nitrogen content of the nodulated plants were almost doubled.

Throughout the experiment there was no appreciable differen in growth between the aerated and non-aerated plants supplied with combined nitrogen. This was confirmed by the harvest data, since the small differences between means are not significant (see Table 23).

Fig. 50. Nodulated plants of aeration experiment after 17 weeks' growth in nitrogen-free solution. Aerated on left and non-aerated on right. (x 1/10).

Fig. 51. Jars containing nodulated plants of aeration experiment after 17 weeks' growth in nitrogen-free solution. Non-aerated on left and aerated on right. (x 1/10).

Table 23.

Mean Data (per Plant) obtained at Harvest of Aeration Experiment

Type.	No. of Plants harvested.	Ht. of Shoot. (cm.)	Dry Weight (gm.) Nodules	Top & Roots	Total	Total Nitrogen (mg.)
Nodulated Non-Aerated.	20	11	0.059	1.48	1.539	36
Nodulated Aerated.	18	16	0.126	3.03	3.156	73
Non-Nodulated Non-Aerated,	14	23	-	-	3.16	75
Non-Nodulated Aerated.	13	19	-	-	2.81	73

The growth-period in water culture of nodulated plants extended from 18th May to 27th October 1951 and for non-nodulated plants from 13th July to 23rd October 1951. To these latter plants a total of 200 mg. combined nitrogen as ammonium sulphate was supplied per plant.

The application of the "t" test for the dry weight data in the above Table showed that at P = 0.05 the mean for the nodulated aerated plants is significantly greater than for the corresponding non-aerated plants. There is no significant difference between the two groups of non-nodulated plants. The same position holds as regards height of shoot.

Typical plants and jars of plants are shown in Figs. 52 - 53.

The results of the determinations of dissolved oxygen present in the culture solution are expressed in the same manner as before (see Section I) and are presented in Table 24. The first determinations on the culture solution in which nodulated plants were growing were made 8 weeks after the commencement of the experiment and at this stage the oxygen content in the non-aerated jars had fallen only slightly below that in the aerated cultures. However as the experiment progressed the value in the former dropped to half that in the latter. With the plants supplied with ammonium sulphate growth was more rapid, causing an earlier fall in the oxygen content of the solution in the non-aerated jars.

Fig. 52. Non-nodulated plants of aeration experiment, supplied with combined nitrogen, after 9 weeks' growth in water culture. Non-aerated on left and aerated on right. (x 1/9).

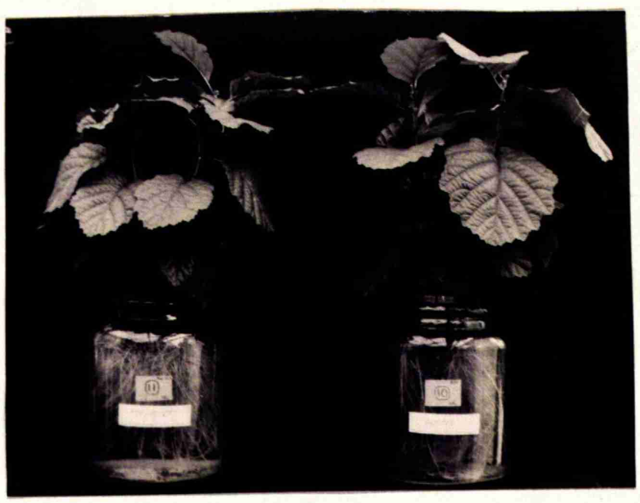

Fig. 53. Jars containing non-nodulated plants of aeration experiment, supplied with combined nitrogen, after 9 weeks' growth in culture solution. Aerated on left and non-aerated on right. (x 1/10).

Table 24.

Results of Determinations of Dissolved Oxygen in Culture Jars of Aeration Experiment.

Type of Culture.	Oxygen Content of Culture Solution. July 12.	Aug. 23.	Oct. 5.
Nodulated Non-Aerated.	81	53	50
Nodulated Aerated.	94	96	97
Non-Nodulated Non-Aerated.	-	51	47
Non-Nodulated Aerated.	-	93	96

See text for method of expression of oxygen data. Each value shown is the mean of four determinations from seperate jars, the samples being withdrawn from midway down the jars.

GENERAL DISCUSSION.

Evidence for Fixation of Nitrogen.

The experimental results presented above are in complete agreement with the findings of earlier investigators that nodulated Alder plants can exhibit vigorous growth in a rooting medium to which no combined nitrogen has been added and confirm that the fixation of atmospheric nitrogen is associated with nodulated Alders under the experimental conditions employed. Although the majority of the present experiments deal with the growth of plants in their first year, ten nodulated plants (as already noted) were grown for longer periods in nitrogen-free solution in connection with a pH experiment, and in addition four other plants were grown on for two full seasons at pH 5.4 to permit of observations on growth and nitrogen fixation. One of the latter plants was grown from seed sown in April 1950 and harvested in October 1951. At the time of harvest this plant had attained a height of 112 cm. while the total dry weight and nitrogen content were 219 gm. and 2.53 gm. respectively (see Fig. 54). From Table 22 it has been seen that even larger plants have been grown and there is no reason to doubt that they had a greater nitrogen content than this particular plant.

Owing to the fact that no investigator has as yet been

Fig. 54. Nodulated Alder plant after 17 months' growth in nitrogen-free culture solution. (x 1/14).

successful in the isolation of the organism from the root nodules of Alder and growing it in pure culture, it is impossible to grow nodulated plants under aseptic conditions of the type employed with legumes. Therefore although the evidence for fixation associated with nodulated Alder plants under the conditions of the experiments appears to be most conclusive, the theoretical possibility might still be thought to exist that a free-living nitrogen fixing organism was introduced with the inoculum and that fixation by this organism was responsible for the growth of nodulated plants. Bond (1951) referred to this point in respect of experiments with nodulated plants of *Myrica gale*, but in the light of more recent work it is considered that this possibility of fixation being due to the presence of a free-living nitrogen fixer can now be dismissed for the following reasons.

1. In the reported experiments only those plants which developed nodules after inoculation were able to make satisfactory growth. Occasional inoculated plants on which nodules failed to form were limited in their growth and behaved in a similar manner to the plants of the control jars.

2. Uninoculated Myrica plants grown with their roots in the same jar of nitrogen-free culture solution as nodulated

Alder plants, made no growth.

3. The removal of the nodules from an Alder plant grown in nitrogen-free culture solution resulted in the plant exhibiting all the symptoms of nitrogen starvation (chloros of leaves, scorching of leaf margin and cessation of growth in a very short time (see Fig. 55). After a further interval new nodules developed and the nitrogen deficiency symptoms started to disappear.

If a free-living nitrogen fixing organism was present in the culture solution one would expect the products of fixation to become available to all the plants in a given jar, but from the above experiments it is seen that no evidence of this is forthcoming. From item 3 and also from the fact that the nodule dry matter contains a relatively high percentage nitrogen content - 3.58 per cent compared with 1.79 per cent for the rest of the plant (mean figures for all pH levels in 1951 experiment) - it appears that the fixation of nitrogen is dependent upon the presence of nodules and that it occurs in the nodules themselves.

Alder trees growing in the field normally possess nodules and there is no reason for doubting that fixation of atmospheric nitrogen occurs under conditions such as these, but it will be a matter of considerable difficulty to actually prove that

Fig. 55. Left - Normal nodulated Alder plant.
Right - Plant from which nodules have been removed with consequent development of symptoms of nitrogen deficiency (chlorosis, reduced leaf size). (x 1/12).

N.B. The Panchromatic plate has failed to register the full colour difference between the two plants.

fixation occurs in the field. According to figures kindly provided by the Forestry Commission there are 24,500 acres of Alder woods in Great Britain, a total which only includes woods of 5 acres and above and takes no account of the narrow belts of trees along the sides of rivers and streams. If fixation by these trees is proportional to that obtained for plants grown under the conditions of the above experiments it will be realised that Alder must contribute considerably to the total amount of biologically-fixed nitrogen in this country.

Hawker and Fraymouth (loc. cit.) in their experiments with Alder found that both naturally and artificially inoculated seedlings did not develop nodules on the root system until the plant had reached a considerable size and they concluded that such a finding pointed to the fact that Alder, unlike nodulated legumes, was less dependent on or independent of the nodule organism. From the experiments reported above it has been shown that nodule formation in Alder after artificial inoculation takes place at an early stage in seedling development and is in every way comprable with leguminous plants. Also in nitrogen-free solution the Alder plant is completely dependent on nitrogen fixed by the nodules for growth.

<u>Effect of pH on nodulation and growth.</u>

From the experimental results presented it is seen that the infection of the plant and the subsequent formation of nodules as a result of inoculation with a suspension of the nodule organism takes place over the pH range 4.2 to 7.0. From the 1951 experiment it appeared that the initiation of nodules occurred most freely over the range pH 5.4 and 7.0 and probably best at pH 7.0, but in 1952 it was found that although initiation was still best over the range pH 5.4 to 7.0 there was little or no variation between the three pH's that were employed in this range. In both experiments although nodule development occurred at pH 4.2, the success of infection and initiation was less than at pH 5.4, 6.3 and 7.0. At pH 3.3 the inoculated plants in both years completely failed to develop nodules and eventually became moribund and died whereas those supplied with nitrate-nitrogen were able to make satisfactory growth. Such a finding must be taken as an indication that the nodule organism is incapable of withstanding such acid conditions, and that death was not due to the plant being incapable of growth at such a pH level but rather to nitrogen starvation.

From the ecological viewpoint and also in connection with forestry this information points to conditions under which Alder plants may be expected to become established and such information may also be of importance in connection with the identification and isolation of the nodule organism. In the past, by the use of cytological methods and attempted isolation, no generally-accepted conclusion has been made as to the identity of the endophyte (for review of literature see Hawker and Fraymouth, 1951) and it is possible that the study of the physiology of the nodulated plant may assist materially in the ultimate identification of this organism.

At this stage it is of interest to compare the acid tolerance of the endophyte in the Alder nodule with that of Myrica gale and also certain leguminous plants. Bond (1951) showed that Myrica, grown in nitrogen-free culture solution, could form nodules at pH 3.3, therefore showing that the endophyte responsible for the development of the nodules on this plant exhibits slightly greater tolerance to acid conditions than Alder. However in the case of both clover and lucerne, Jensen (1943) has shown that nodulation fails at pH values below 4.4 and 5.1 respectively. From these findings it can be stated that the endophyte of the non-leguminous nitrogen-fixing Angiosperms which have been investigated at Glasgow are both capable of bringing about the development of nodules at lower pH values than the species of Rhizobium which cause nodulation on the above leguminous forage plants.

From the above pH experiments it has been found that the pH requirement for the initiation of the nodules is higher than that for the subsequent growth of nodulated plants, since the most favourable pH for both the growth of the plants and fixation of nitrogen was in the range pH 4.2 - 5.4 while that for nodule formation lay between pH 5.4 - 7.0. Such results do not agree with the earlier investigations performed by Virtanen and Saastamoinen (loc. cit.) who, as already stated, worked with older plants and found that in nitrogen-free sand culture, nodulated plants exhibited best growth at pH 6 as compared with that at pH 5 and pH 7. This slight divergence in results was however probably due either to the use of sand culture in place of water culture or to the fact that the above mentioned authors had very few plants at each of the pH levels employed.

Effect of aeration of culture solution.

Virtanen and Saastamoinen (loc. cit.) in their experiment with nodulated Alder plants grown in non-aerated nitrogen-free culture solution noted that under such conditions of growth nodules formed as a result of inoculation normally developed on that region of the root system which was near the surface of the solution. They considered that such an observation indicated that the nodules of Alder have a relatively high oxygen requirement and in further experimental work employed sand culture so that this requirement might be met.

In the aeration experiment reported above it has been shown that in the development of nodulated Alder plants aeration of the culture solution is beneficial, but a closer study of the data shows that such a fiding cannot be taken on its face value as a definite indication of a high oxygen requirement on the part of the nodule. In this experiment the benefits of aeration were shown during a very early stage, as at the end of the first month more and also submerged nodules were present and better growth exhibited in the case of the plants in the aerated jars compared with those which were not being subjected to forced aeration. From the oxygen data (see Table 24) it is shown that at this early stage there was little variation in the oxygen content of the solutions in the two types of jars and that the values were not lowered owing to the respiration of the root systems until a later date. It may be that the infection process or the multiplication of the organism are very highly aerobic but it appears likely that the increase in nodulation was caused by the stirring effect of the solution brought about by the passage of the air stream and the resulting dispersal of the endophyte over the newly formed parts of the roots. Also, as noted already, submerged nodules will develop under conditions of non-aeration if inoculation is effected at a late stage, where the oxygen content of the solution would not be high.

It does appear highly probable that in the later stages of this experiment the higher oxygen content of the solution resulting from forced aeration allowed these submerged nodules to function in an effective manner. Although from Table 23 it is seen that in the case of both aerated and non-aerated jars the relationship between nodule dry matter and nitrogen fixation is of a similar order, it has been shown that when submerged nodules are present without aeration (1951 pH experiment - 2nd Group) their effectiveness in the fixation of nitrogen is reduced. Thus it is possible to compare in Table 16 the fixation of nitrogen by plants with nodules localised near the surface of the solution and those with nodules submerged in the solution. The fixation per unit dry weight of nodule tissue is of a higher order in the first group than in the second. Thus at pH 5.4 it is 814mg. per gm. nodule tissue in the first group compared with 478 mg. per gm. in the second.

Such experimental results point to a higher oxygen requirement for nodule tissue than for root tissue since in the case of the plants supplied with combined nitrogen the oxygen supply was adequate for efficient functioning of the roots without aeration. However before a definite decision could be made as to the precise oxygen requirements of Alder nodules a technique giving greater control over the oxygen supply would have to be used.

SUMMARY.

(To Section II of Thesis)

1. The conclusion reached by previous investigators that nodulated Alder plants can fix atmospheric nitrogen and display vigorous growth in water culture free of combined nitrogen is confirmed.

2. Evidence is advanced to show that the fixation of atmospheric nitrogen actually takes place within the nodulated plant and probably within the nodule.

3. There is no reason to doubt that fixation is associated with nodulated Alder trees in the field, and this fixation may be of considerable ecological and general importance.

4. Experiments have been performed in which inoculated Alder plants were grown in water culture in order to investigate the effect of the pH of the culture solution on (a) the initiation of nodules by the organism and (b) the subsequent growth of the plants and the fixation of atmospheric nitrogen

5. It has been shown that nodule formation occurred over the pH range 4.2 to 7.0 but most freely between 5.4 and 7.0. No nodules developed at pH 3.3. At favourable pH nodules visible to the naked eye developed as soon as 10 days after inoculation.

6. The optimum pH for the growth of nodulated plants in solution free of combined nitrogen lies between pH 4.2 and 5.4.

7. By growing non-nodulated Alder plants supplied with combined nitrogen in water culture it has been shown that the failure of the plants to develop nodules at pH 3.3 was due to the fact that although the host plant can tolerate such a low pH level the nodule organism cannot.

8. Non-nodulated plants supplied with ammonium sulphate made more vigorous growth than those supplied with sodium nitrate.

9. In experiments with both nodulated plants and plants supplied with combined nitrogen in which the culture solution in certain cases was aerated it was found that a high oxygen supply had a beneficial effect on the growth of the former but had no effect on the latter.

10. The benefit of forced aeration on nodulated plants was thought to be due (a) to the stirring effect of the air-stream and (b) to the influence of an increased oxygen supply on the functioning of the nodules, especially of the ones well-submerged in the culture solution. It is concluded that nodules have a higher oxygen requirement than roots.

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