" SOME ASPECTS OF BACTERIAL SYMBIOSIS ".

— being a Thesis presented by

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The research work was carried out during the years 1936-1940, during the first of which the author held a Carnegie Research Scholarship, working at the Department of Botany of the University of Glasgow, by courtesy of the Regius Professor of Botany, Dr. John Walton. During the years 1937-1940, the research was continued both at the above department and at the Department of Botany and Bacteriology, Royal Technical College, Glasgow.

The author wishes to express his appreciation to Dr. James P. Todd, Professor of Pharmacy at the Royal Technical College, and to his colleague, Dr. Blodwen Lloyd of this Department for their co-operation and friendly interest during the course of the work.

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GENERAL INTRODUCTION.

The great value of leguminous crops in enhancing soil fertility has been known for many centuries, and the practical and economic benefits to be derived therefrom have been exploited to the full in many countries. The scientific basis of these observations, however, has been a subject of study for a much shorter period, and workers on the special physiology of the Leguminosae and their associated root nodule bacteria have still many problems to solve.

The development of this study on a scientific basis received a great impetus when Hellriegel, at the beginning of the present century, connected the ability of the legume to contribute to the nitrogenous content of the soil with the presence of the root nodules. Beijerinck and others, at the end of the last century, attempted to prove that the bacteria isolated from the root nodules were responsible for the increased nitrogen content of the legume plant; their essays to obtain nitrogen fixation in artificial culture, however, were inconclusive, and later workers have been unable to demonstrate this property outside of the legume tissues. It soon became apparent that the association of the bacterium with the legume was in the nature of a symbiosis; the organism was given the name of *Bacillus radicicola*, by which name it is generally known in this country, but the generic name of *Rhizobium* has been subsequently adopted by American systematists. An important discovery was made that the root nodule bacteria could be grouped into different strains which were somewhat specific in their infective powers. It was noted that a particular bacterial strain would infect only a certain host genus or a few closely related genera, and from such observations, the study of cross-inoculation groups and their commercial applications has arisen; a number of distinct Rhizobium species are now generally recognised.

Both the academic and agricultural problems arising out of the special conditions within the Leguminosae, together with an excellent review of the historical development of the work, are very adequately presented in the monograph by Fred, Baldwin and McCoy (1932) of the Department of Agricultural Bacteriology, University of Wisconsin. The workers of the Madison school have contributed a large amount of valuable information in the last few years. They have been concerned with various aspects of the problem, including cross-inoculation studies and host specificity, the carbohydrate/nitrogen ratio and its relation to nitrogen fixation, and, more recently, some work on the mechanism of symbiotic nitrogen fixation with reference to a number of factors which might influence the process.

Another centre of research on similar problems has been at Helsinki, where Professor A.I.Virtanen and his co-workers have studied exhaustively the question of excretion of nitrogenous compounds from the root nodules into the rooting medium. This problem has been further elaborated in its relations to the associated growth of non-legumes and the benefits derived by the latter in view of the nitrogenous legume excretion. The Helsinki workers have, until recently, obtained this excretion consistently in all their cultures; this phenomenon has proved somewhat elusive with other workers, and, during this year, Virtanen reports that excretion was absent in some of his most recent work. They have also contributed work of considerable value in their attempts to isolate the intermediate compounds found in the process of symbiotic nitrogen fixation, and have suggested a possible scheme for this complex reaction.

In Great Britain, two centres have also

been engaged on such work - at Rothamsted, a large amount of cytological research into the structure and development of the nodule has been pursued by Thornton and others. They have also carried out many plot experiments in connection with the associated growth of legumes and non-legumes, nitrogen manuring of legume crops and the study of grassland clovers inoculated with different bacterial strains. Extensive work on the artificial inoculation of lucerne and other commercial crops has also been done by them.

At Glasgow, Bond has been carrying out work on the special physiology of the root nodules with reference to the transfer of nitrogenous compounds from the bacterial cells to the host plant, the excretion of nitrogen and associated growth and the utilisation of plant carbohydrates by the associated bacteria.

In Australia, some work along similar lines has been done by Strong and Trumble at the Waite Institute, Adelaide.

Numerous other contributions to this literature have appeared and are discussed in the following reviews:-

FRED E.B., BALDWIN I.L., and McCOY E. 1932: Root Nodule Bacteria and Leguminous Plants. University of Wisconsin Studies in Science, No. 5.

NICOL H. 1934: The derivation of the nitrogen of crop plants with special reference to associated growth. Biological Reviews 9: 383-410.

———— 1936: The utilisation of atmospheric nitrogen by mixed crops. Month. Bull. Agric. Sci. and Pract. 6: 201-216; and 7: 242-256.

THORNTON H.G. 1936: The present state of our ignorance concerning the nodules of leguminous plants. Sci. Prog. 31: 236-249.

WILSON P.W. 1937 The Symbiotic Nitrogen-Fixation by the Leguminosae. Bot. Rev. 3: 365-399.

VIRTANEN A.I. 1938: Cattle Fodder and Human Nutrition. Cambridge 108 pp.

The work in this present thesis comprises four aspects of the study of bacterial symbiosis presented separately under four titles:-

I. Fixation of nitrogen by different strains of the Soya bean nodule organism when associated with certain varieties of the host plant.

II. The mechanism of transfer of fixed nitrogen from the nodule bacteria to the host plant.

III. The excretion of nitrogenous substances from leguminous root nodules into the rooting medium.

IV. An investigation of the growth of leguminous plants in water culture, with special reference to the effect of aeration on growth and fixation.

I.

Fixation of nitrogen by different strains of the Soya bean nodule organism when associated with certain varieties of the host plant.

INTRODUCTION

In earlier work on the physiology of the root nodule bacteria, it soon became apparent that not merely one general type of Rhizobium was responsible for the nodulation of legumes, but that a number of more or less specific strains existed. These were found to nodulate only one particular species or group of legumes, and, since the beginning of the century, a considerable amount of work on the problem of cross-inoculation groups has been done - see Fred, Baldwin and McCoy (1932).

The present investigation concerns a particular aspect of this problem - namely, the variation in effectiveness of different strains of bacteria when associated with varieties of the same legume host. An effective strain is generally considered to be one which will produce nodules with actively fixing bacteria, contributing substantially to the nitrogen symbiosis of the two partners; effectiveness is measured in terms of nitrogen fixed. A number of workers have noted both inter-specific and inter-varietal differences in the relative effectiveness of a number of strains, and the object of this work is to investigate the problem further with Soya bean varieties.

Perkins (1925), on a basis of counting nodules, found that a particular strain of the Soya bean organism became adapted specifically to a certain host variety. Briscoe and Andrews (1938), in soil cultures of Soya beans, obtained differences in the response of two host varieties associated with two strains of the nodule organism. Erdman and Wilkins (1928) carried out field tests on commercial bacterial cultures using Soya bean as the host legume. They reported varietal specificity in that some strains were better than others in the production of nodules, but that no one strain was best on all the varieties; this was taken to imply that the most efficient association of a strain was with a particular variety. Bjälfve (1933) confirmed these observations in the Vetch group.

The most recent contributions on this aspect of host plant specificity have come from the Wisconsin group, working with a series of strains associated with different species and varieties of Melilotus and Medicago [Wilson, Burton and V. S. Bond (1937); Burton and Wilson (1939)]. They found that between species of the same genus associated with different bacterial strains there was a more marked influence on the effectiveness than when varieties of the same species were the host plants. Their conclusions are, however, that varietal specificity does influence the symbiosis in certain combinations.

The possibility of factors, not inherent in either host or bacterial strain, influencing the effectiveness of the association has been suggested by Raju (1938). He carried out a considerable number of experiments on the Cowpea and Cicer groups and concluded that the intensity and duration of sunlight influenced the magnitude of a strain's effectiveness - relative to others under similar conditions - but were unable to affect materially its inherent capacity in nitrogen fixation. He also decided that the host plant profoundly influenced the fixation by the particular strain with which it was associated, but on only one occasion did he obtain any change in the efficiency ranking of a number of strains used with two varieties of Bengal gram. There were no such differences due to host influence in the case of two varieties of Red gram associated with the same strains.

Strong (1937) obtained variable effectiveness in different combinations of Clover varieties and Rh. trifolii strains. Certain strains will freely invade the host tissues but are not always effective in the fixation of nitrogen.

The present experiments are designed to investigate any possible host specificity between legumes of the closest taxonomic relationship - namely, between four varieties of the Soya bean associated with four strains of Rh. japonica. The work was carried out during two summers (1938 and 1939) of considerably different sunlight conditions, and this variation provided some data for the investigation of results such as those obtained by Raju and attributed by him to this factor. Since three of these host varieties have been found, in some degree, suitable for cultivation in this country, it was believed that the results might also have some direct practical significance.

The 1938 plants were grown, harvested and analysed by the present author; the 1939 experiment was set up by Dr. Bond who also carried out some of the analyses. Harvesting and the rest of the analyses were effected by the present author.

METHODS.

As stated, the observations were made during the years 1938 and 1939 on sand cultures of Soya beans [Glycine hispida (Moench.) Maximowiez], with a strict control over the general cultural conditions.

The nitrogen-free culture solution used was that of Virtanen (1935),supplied to 12% of the weight of dry sand; further small quantities of this solution were added at intervals of 7-14 days, and, in addition, sterile distilled water was added more frequently to replace moisture lost in transpiration. This was carried out by weighing the pot and adding the water or solution to the appropriate fraction of the sand weight. A microelement solution - Loomis and Shull (1937) - was also added to the sand along with the culture solution so that each pot received 10 cc. of this solution at each change. The culture vessels were glazed, earthenware pots, containing 3·6 Kg. of a coarse quartz sand *, buffered to pH 6·5 with 7 gm. $CaCO_3$; the pots of sand were sterilised at 120°C for 3 hours before use.

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* For mechanical analysis, see Bond and Boyes (1939), p. 914.

The seeds used were as follows:-

1. Manchu - presented by the U.S. Department of Agriculture.
2. Brown C - presented by the National Institute of Agricultural Botany, Cambridge.
3. Green Jap - purchased from Messrs. Fordson
   Black O
   Estates, Boreham, Essex.

The bacterial strains were obtained from the Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis., U.S.A. These were Strains Nos. 505, 9, 17 and 507; of these, the first three are considered to be "effective", although in different degrees, and No. 507 "ineffective" with respect to their nitrogen fixation ability.

In order to isolate the different bacterial strains and to prevent undesired cross-infection during the life of the plants, a framework lined with Windolite screening divided the pots inoculated with one particular strain from their neighbours (see p.16a Photograph I); uninoculated pots, grown during both years in one of these compartments, were not nodulated by accidental infection at anytime throughout the experiments. It was thus apparent that the strains were effectively segregated in this manner; there was one exception in the 1938 plants [Manchu - Strain 507], when unexpectedly large amounts of nitrogen were obtained in two pots out of three (see Table I, p.19).

PHOTOGRAPH I.

CULTURE FRAME.

The compartments are separated from each other by Windolite screening but are open at the top and have removable fronts for access to the plants.

The method of seed selection was by weight; a number of random samples (50-150) were taken for weighing from the stock, a suitably narrow weight range with a negligible nitrogen variation selected, and the requisite number of seeds for the experiment taken. These were surface - sterilised by flaming absolute alcohol on the testa and sown in a trough of sterile, watered sand in a slight excess over the actual requirements for the pots. When the radicles were 1-2 in. long, the healthiest seedlings were removed and transplanted into the sand of the culture pots, and inoculated by pouring approximately 5 cc. of a suspension of the appropriate bacterial strain on to the roots of each plant. During the growth of the legumes, the testas and cotyledons were removed when shrivelled, and, together with dead leaves shed, were preserved in formalin vapour for analysis with the rest of the plant material,(in 1939, the cotyledons were not collected).

The plants were grown under glass at an average day temperature of 65°-75°C, Soya bean having proved to be an excellent subject for such treatment. There was, however, a considerable deterioration in the amounts of sunlight available for the plants in 1939, and the general growth reflected this change. The pots within each compartment were periodically moved round in order to equalise the effect of mutual shading by the plants.

Each variety was harvested separately, all plants of one variety being harvested together at a stage in which partly ripe pods were present. The complete harvest of the crop was spread over 2-3 weeks, the plants being 100-130 days old. They were carefully removed from their pots, washed free of adhering sand, cut up and dried in an oven at 95°C until constant in weight. The dried material was quickly powdered in a mill and stored in tightly stoppered bottles in an airtight tin.

Duplicate or triplicate samples [0·4-0·5 gm.] of dried material for analysis from each pot were weighed out in a constant minimum time to obviate moisture absorption by the powdered tissues. The estimation of total organic nitrogen was carried out by using Ranker's (1925) modification of the official Kjeldahl method, and tests indicated that an accurate recovery of nitrogen was obtainable by this process. The reagents used were of AnalaR brand and the values for the standard solutions were checked at regular intervals; allowance for the blank estimations on the reagents alone was made in the calculations.

RESULTS

TABLE I DRY WEIGHT and NITROGEN FIXATION.

Figures for 1 pot; 4 plants per pot in 1938, 5 plants in 1939.

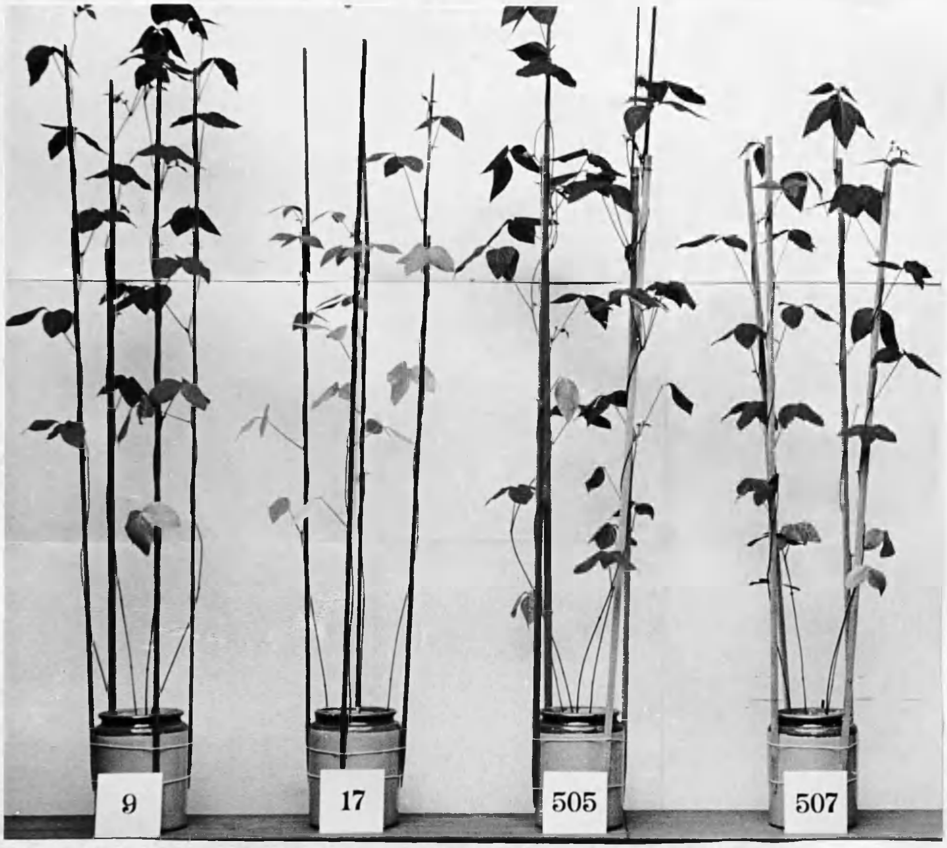
STRAIN		MANCHU (1938)		MANCHU (1939)		GREEN JAP (1939)		BLACK O (1939)		BROWN C (1938)	
		1.	2.	1.	2.	1.	2.	1.	2.	1.	2.
[505]	1.	16·98	264·5	13·66	202·4	7·70	131·9	11·35	192·2	7·31	169·8
	2.	15·56	237·2	14·40	230·2	9·86	158·0	13·70	231·4	8·09	181·9
	3.	15·72	232·6	13·18	189·7	7·43	110·0	13·45	244·4	6·21	136·0
	Ave.	16·09	244·8	13·75	207·4	8·33	133·3	12·83	222·7	7·20	162·6
[9]	1.	12·13	153·6	13·26	182·8	8·96	151·9	20·17	245·3	7·17	125·3
	2.	12·09	190·9	13·15	169·4	9·29	146·3	17·87	211·7	7·16	125·4
	3.	14·05	182·6	13·00	169·8	7·64	107·9	17·58	265·8	5·69	94·5
	Ave.	12·76	175·7	13·14	174·0	8·63	135·4	18·54	240;9	6·67	115·1
[17]	1.	10·51	46·9	10·03	61·5	9·41	125·4	13·81	157·4	6·32	69·1
	2.	7·60	37·5	11·46	70·5	8·96	107·0	12·41	154·8	5·61	61·0
	3.	6·30	31·2	8·61	53·2	7·48	84·9	17·32	164·5	5·71	49·5
	Ave.	8·14	38·5	10·03	61·7	8·62	105·8	14·51	158·9	5·88	59·9
507]	1.	12·50	[198·0]*	5·85	-1·4	7·41	7·4	6·58	-9·0	4·47	-0·2
	2.	10·71	[170·3]*	5·15	-7·4	6·03	2·5	6·28	-8·2	5·18	3·8
	3.	4·95	-0·7	5·90	-3·1	6·47	2·7	6·68	-8·5	5·32	1·0
	Ave.	4·95	-0·7	5·63	-4·0	6·64	4·2	6·15	-8·6	4·99	1·5

Column 1. - Dry Weights (gm.).

Column 2. - Nitrogen fixed (mg.); seed nitrogen subtracted.

* Cross infected - see text.

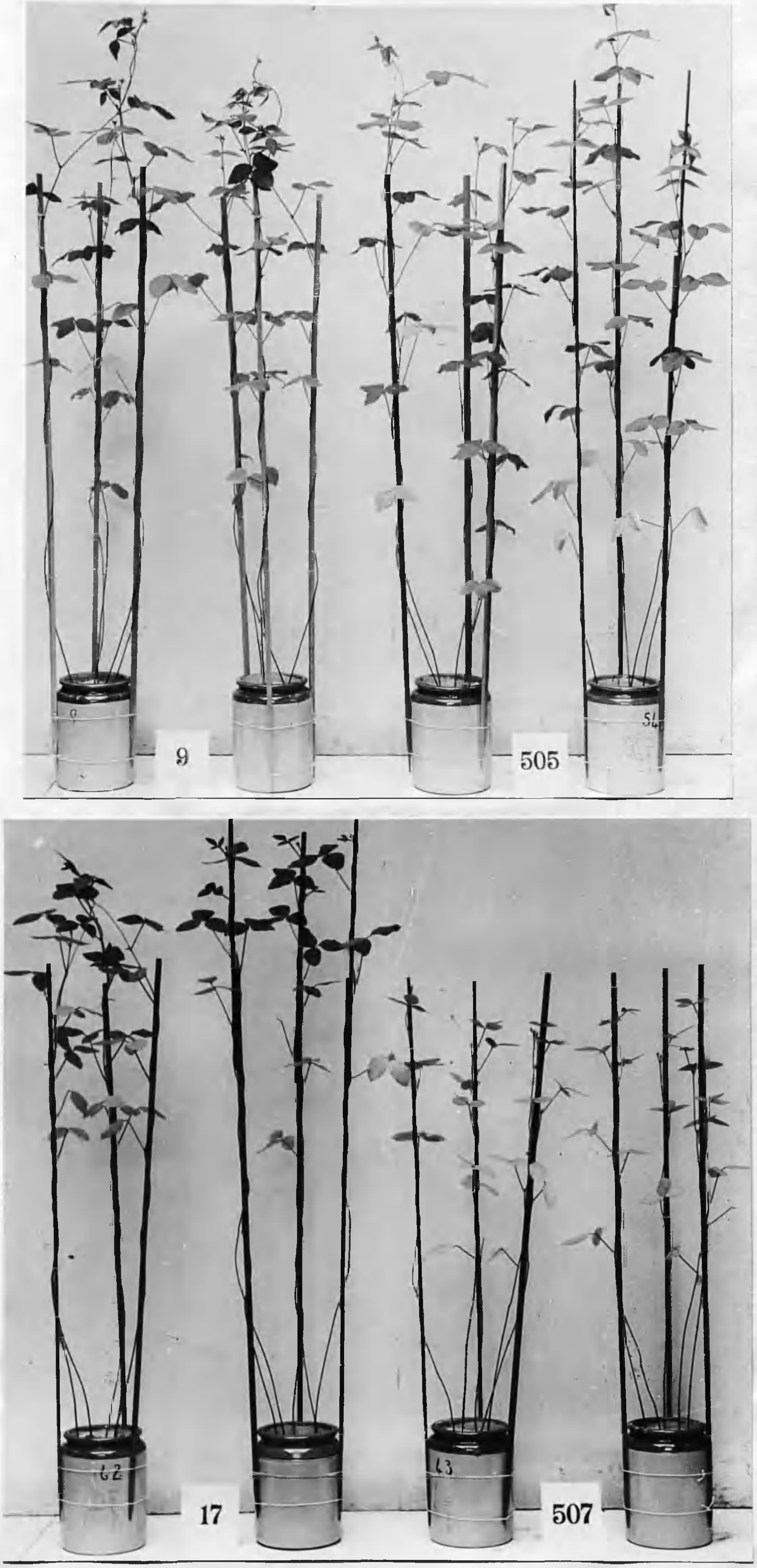
PHOTOGRAPH. II.



MANCHU 1938.

By oversight, the sample selected for strain 507 was one of the two cross-infected pots - the difference in growth appearance from all other examples of this strain associated with the different varieties bears out this suspicion (see Text, p.16).

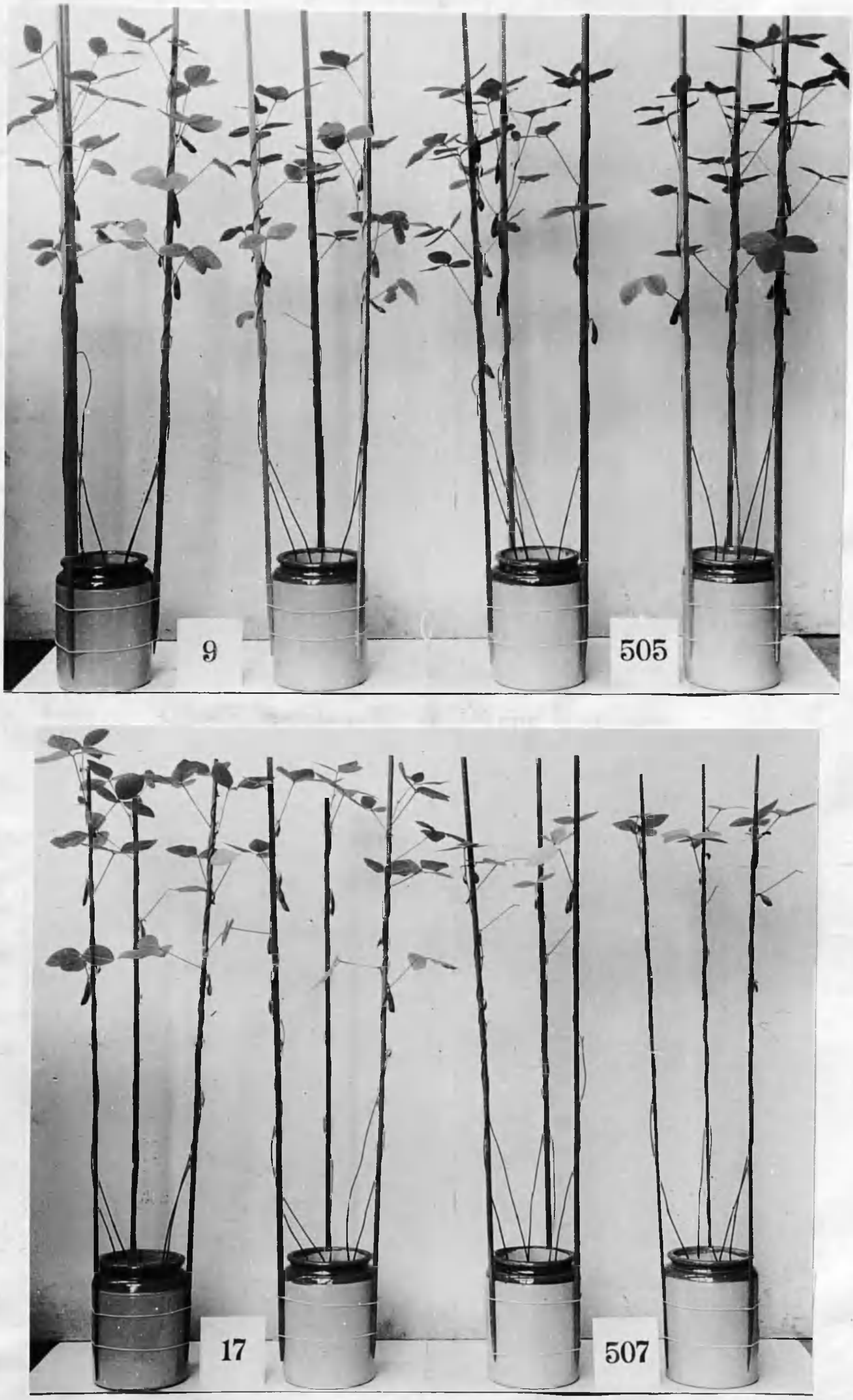
PHOTOGRAPH III.



MANCHU

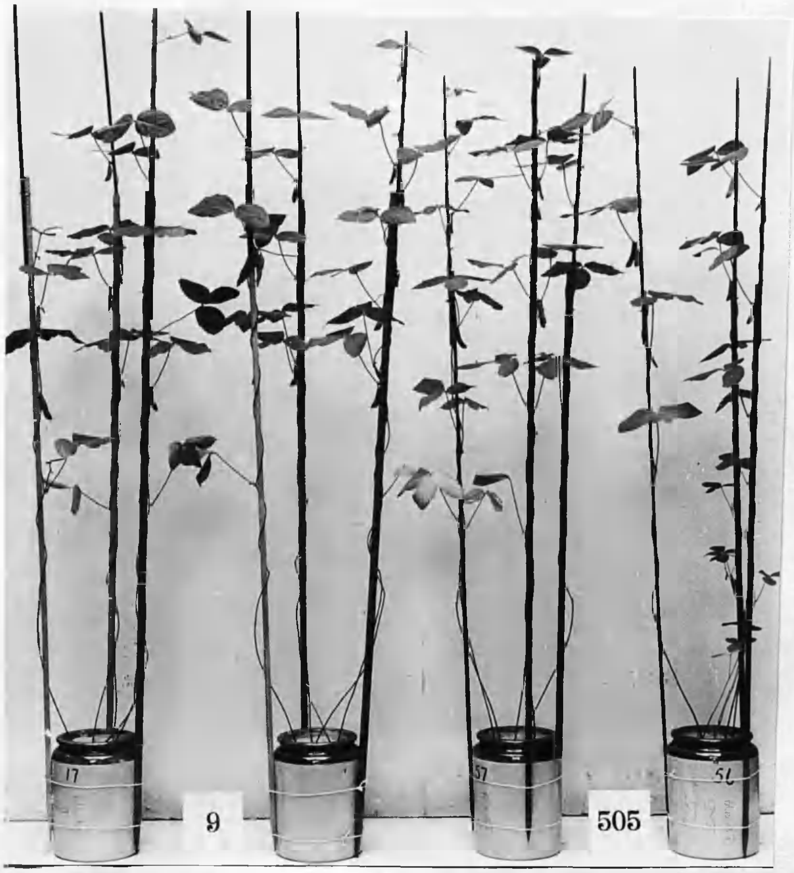
1939.

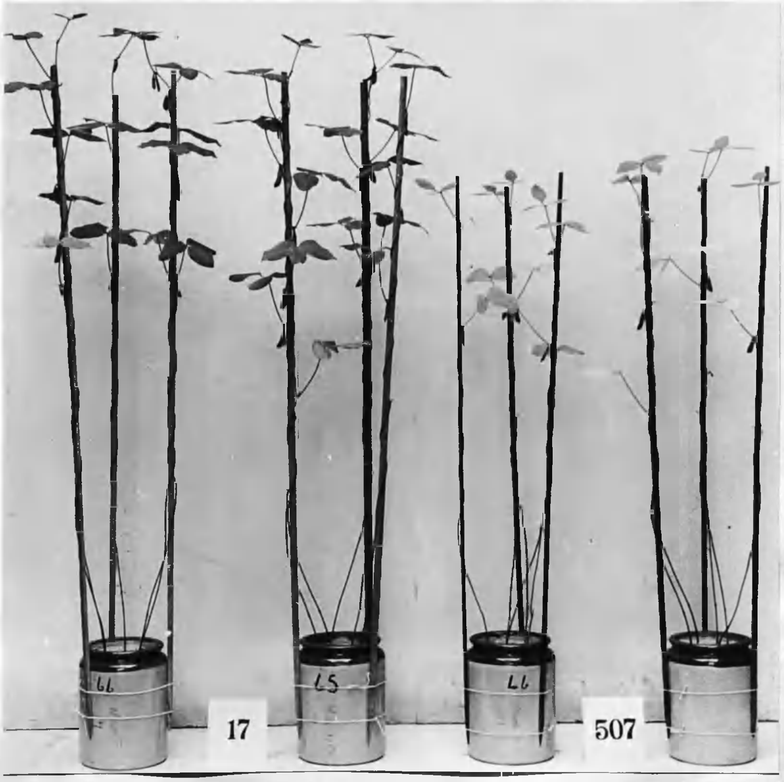
PHOTOGRAPH IV.



GREEN JAP 1939.

PHOTOGRAPH V.

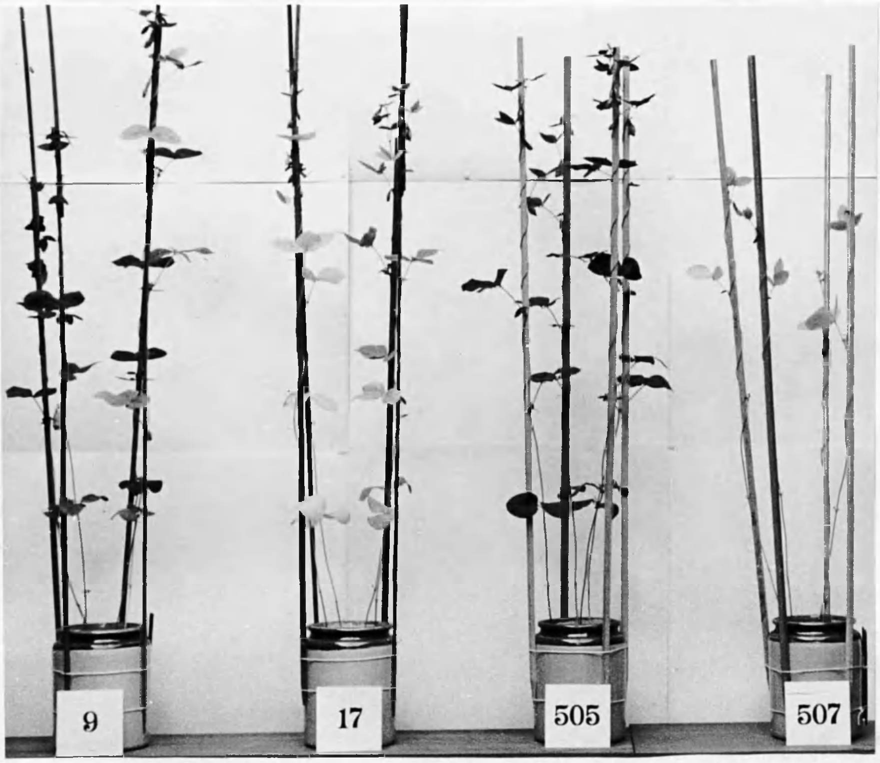




BLACK O

1939.

PHOTOGRAPH VI.



BROWN C 1938.

Three replicate pots were grown in each variety, and the growth periods were - 1938 - late May to early September and - 1939 - late April to late August. The strain combination, dry weight and nitrogen fixation data is presented in Table I, p.19.

The separation of the pots within the screened compartments was successful in preventing cross-infection except in the case of two pots - Nos. 1 and 2 of the Manchu-507 combination. It is noted that these plants produced much greater dry weight and nitrogen fixation figures than would be expected from the strain concerned, which was consistently ineffective with all other varieties.

Although there is some variation between the data for three pots within a combination, there are considerably greater differences between the pot groups. The effectiveness ranking of the strains in association with Manchu is 505, 9, 17 and lastly 507; this order is in agreement with the work of Ruf and Sarles (1937) under quite different conditions for three of these - 505, 9, and 507 - using Manchu as the host variety. During this work in the Madison greenhouses, they found also that 507 was ineffective in fixing nitrogen and obtained figures for it which were below uninoculated controls in dry weight and nitrogen content. Although no direct comparison between the dry weights and the nitrogen content of the uninoculated controls and the "507" plants of the present work is possible (the former were not harvested and analysed), the two series were very similar in appearance - light green, small leaves, spindly stems and of generally weak appearance, suggesting nitrogen starvation. This effect was also noticed by Raju (1936) in conditions of insufficient light, when his poor strains became parasitic on the host plant and actually removed carbohydrate material, thus reducing the dry weight of the legume.

The figures for dry weight and nitrogen fixation per pot in Table I p.19 are not directly comparable throughout, since the number of plants per pot varied in the two years.

DISCUSSION.

In making conclusions from the given data, certain incidental factors affecting the results must be examined. These might probably have an important influence on the final figures obtained under the conditions of the experiment.

Firstly, it must be borne in mind that the data are drawn from cultures grown during two years of considerably different environmental conditions. In 1938, the plants were grown one fewer per pot than in 1939, and, in the latter year, the climatic conditions were much poorer. This may partly account for the decreased nitrogen fixation in Manchu - 505 and 9 during 1939.

Perhaps a more important factor is the normal growth habit of the legume. Of the four varieties employed, Manchu was the largest grower, with Black O next; Green Jap and Brown C were both smaller types. Hence, it is to be expected that strains associated with Manchu would fix - other things being equal - a greater amount of nitrogen than with the other varieties. There is evidence, however, that other factors do affect these expected results. In order to assess the relative effectiveness of the different strains associated with a particular variety, a suitable control would be to grow uninoculated pots supplied with full requirements of culture solution, including nitrogen. This treatment would provide plants which are as fully standard as could be obtained under the particular cultural conditions - sunlight, growth periods, sowing conditions, variety growth habit, etc. - and would offer a point of contact between the different varieties for a comparison of relative effectiveness of the strains. In addition, such control plants would afford a basis of comparison between cultures of one variety grown under different conditions, e.g. Manchu cultures of 1938 and 1939.

In the absence of such standard plants, the basis actually employed has been to take the most consistently good strain - 505 - and to express the figures obtained from the remaining three strains in terms of it (see Table II p.24). Since, from inspection, strain 505 appeared to be comparably effective with the different hosts, then the relative figures for a given strain on different hosts are also comparable.

TABLE II.

RELATIVE FIXATION - the nitrogen fixation figures per plant are expressed as % of the most uniform strain 505.

STRAIN	MANCHU (1938)		MANCHU (1939)		GREEN JAP (1939)		BLACK O (1939)		BROWN C (1938)	
	1.	2.	1.	2.	1.	2.	1.	2.	1.	2.
505	61•2	100	41•5	100	26•7	100	44•5	100	40•7	100
9	43•9	72	34•8	84	27•1	102	48•2	108	28•8	71
17	9•6	16	12•3	30	21•2	79	31•8	71	15•0	37
507	-0•2	-0•3	-0•8	-2	0•8	3	-1•7	-4	0•4	1

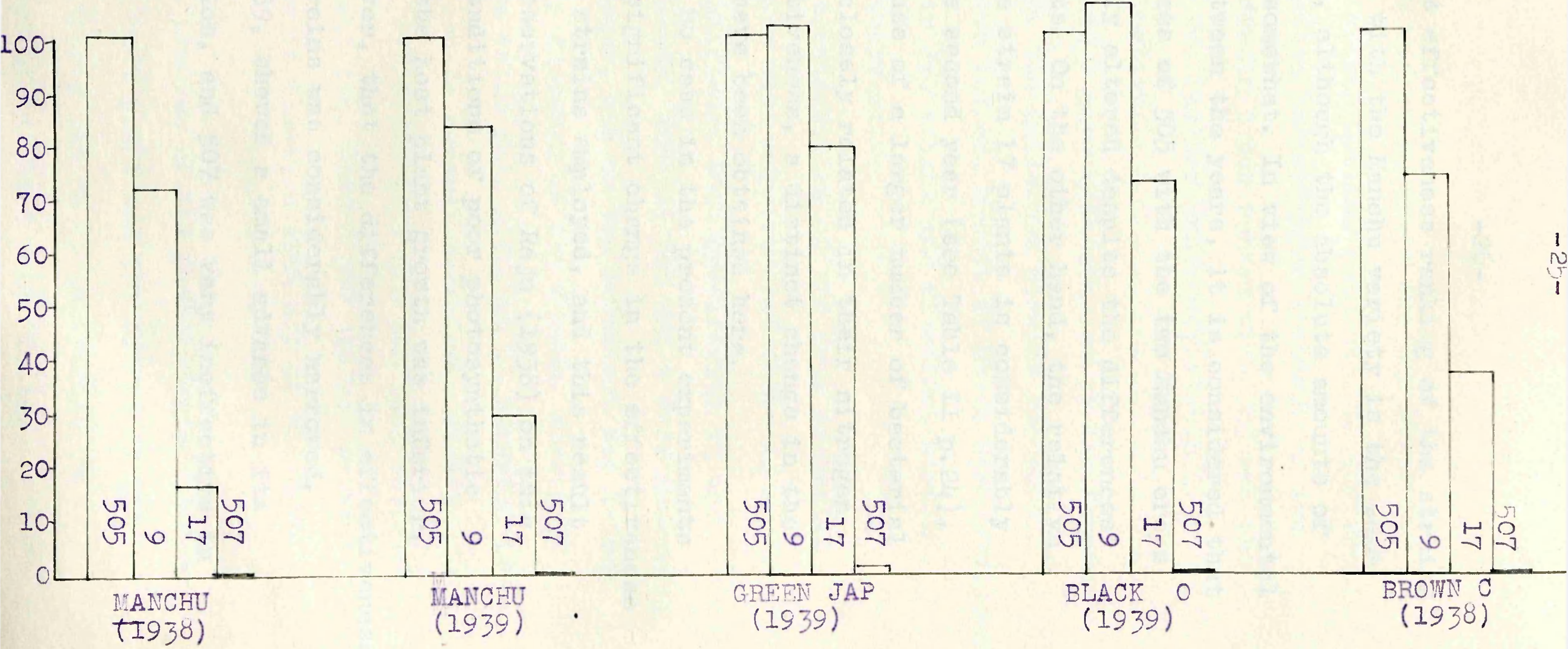
Column I. - Average nitrogen fixation (mg.) per plant from Table I.

Column 2. - Relative fixation index.

TEXT FIGURE I - DIAGRAM OF RELATIVE NITROGEN FIXATION.

(Strain 505 represented as 100 units

and strains 9, 17 and 507 expressed in terms of it).



The effectiveness ranking of the strains in association with the Manchu variety is the same for both years, although the absolute amounts of fixation vary somewhat. In view of the environmental differences between the years, it is considered that the effectiveness of 505 with the two Manchu crops is not radically altered despite the differences in absolute data. On the other hand, the relative position of the strain 17 plants is considerably improved in the second year (see Table II p.24), and, with the use of a larger number of bacterial strains, more closely related in their nitrogen fixation effectiveness, a distinct change in the ranking might have been obtained here.

In no case in the present experiments was there any significant change in the effectiveness ranking of the strains employed, and this result confirms the observations of Raju (1938) on this point; under conditions of poor photosynthetic activity when the host plant growth was inferior, he found, however, that the differences in effectiveness between the strains was considerably narrowed. Strain 9,in 1939, showed a small advance in its relative position, and 507 was very ineffective in both years.

The varieties Green Jap and Black O, with different growth habits, responded in different relative fashion to the nitrogen fixation of the four strains. Strain 9 now ranked with 505 and the position of 17 was further enhanced; strain 507 was still ineffective.

Brown C gave data similar to the Manchu variety of 1938. The different strains had almost the same relative values, except 17 which was considerably improved both in dry weight and fixation; strain 507 continued ineffective.

A consideration of the data presented in the two Tables will suggest that there is no great evidence of significant host specificity in the present experiments. Strains 505 and 9 were generally good in that order, but on two occasions the latter became slightly improved on the former. Strain 17 was of a medium value and, although it improved its relative position on several occasions, it was never quite so effective with all the host varieties as were the first two. Strain 507 did not prove to be of any value in fixation with any of the host varieties.

The problem of the Carbohydrate/Nitrogen balance of the host plant and its effect on the fixation of nitrogen may be of importance in determining a so-called host specificity.

Raju (1938) believes that the photosynthetic activities of certain host species or varieties may be below the optimum requirements of a particular strain for nitrogen fixation. A series of inoculated plants, assisted at the beginning of their growth by the addition of a small amount of combined nitrogen - to obviate the nitrogen starvation period of the earlier stages - and grown under good lighting conditions, should provide data which would elucidate Raju's proposition. It is of interest to note in this connection, that the dry weight and nitrogen fixation data change in parallel fashion with the different combinations of strain and host; Raju (loc.cit.), on the other hand, often found variations in this respect with Red Gram.

In the same paper, he has also suggested that certain strains may reach a "full" effectiveness at a lower state of host vigour than others - in this respect, some more rapidly growing legume varieties might reach this point ahead of other types and thus derive an initial advantage in nitrogen fixation. If such is the case, then the host variety influence may work in this manner. Manchu was always found to be a more rapid grower at the seedling stage than the British varieties - the increased amounts of nitrogen fixed when in association with strain 17 during the less favourable

1939 season, may be accounted for by some such reason as above.

The main conclusions of the experiments are that, although a limited amount of host specificity was indicated with certain strains - notably 17 - no one strain of mediocre effectiveness with one variety became outstandingly effective with another variety, at least under the cultural conditions described. It appears also that the British varieties are of similar affinities towards the Rhizobium strains as one American variety - Manchu, so that seeds of the former may, with confidence, be inoculated with strains of proved efficiency with the American type. The author is informed that Dr. Thornton arrived at the same conclusions from his experiments at Rothamsted and Woburn.

SUMMARY.

1. Sand cultures of four Soya bean varieties - Manchu (American), Green Jap, Black O, Brown C (last three British) - associated with four strains of Rhizobium japonica were grown under greenhouse conditions.

2. Dry weight and nitrogen data were taken, the latter being expressed also in relative terms for the purposes of comparison within each group.

3. There was evidence for some variation in strain effectiveness when associated with different host varieties.

4. The strains which were most effective with the American variety were also effective with the British types.

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II.

The mechanism of transfer of fixed nitrogen from the nodule bacteria to the host plant.

INTRODUCTION.

The process by which the nitrogenous compounds, elaborated within the bacteria of the root nodules, are made available to the host plant is still incompletely elucidated.

Some advance in our knowledge of the chemical process involved and the nature of the intermediate compounds isolated during the fixation of nitrogen by symbiotic bacteria has been made by the work of Virtanen (1938) in Finland. He believes that the nitrogen is transferred in the form of amino-acids, mainly asparagine, but offers no explanation as to how it passes from the bacterial cell to the host plant. In this respect, several mechanisms have been postulated, e.g. Fred, Baldwin and McCoy (1932) p.188, " ..... (1) plant enzymes may attack the bacteria and change their complex nitrogenous substance into a form which may be assimilated by the plant; (2) soluble nitrogenous compounds may be excreted by the bacteria; (3) death and autolysis of the bacterial cells may liberate the nitrogenous compounds in forms available for plant nutrition; (4) a bacteriophage, capable of lysing the rhizobia may account for the production of soluble nitrogenous materials". It is interesting to note, however, that, after extensive work, Dangeard (1926), Milovidov (1928) and Thornton (1930) have all found that a cytological examination of the root nodule tissues shows little sign of any bacterial breakdown until the later stages in the life of the nodule are reached. Of the above suggestions, only (2) is independent of any bacterial disintegration process in the liberation of the nitrogenous compounds, and would appear to have good claims in explanation of the transfer.

In recent work by Bond (1936), an inspection of the plant nitrogen data showed obviously that the nitrogenous compounds became available at an early stage in the legume development. Bond (loc. cit.) appears to have been the first to study this transfer in a quantitative manner. From sand cultures of Soya beans at 3-4 weeks up to 16 weeks, he took regular samples and analysed the plants and nodules separately for total nitrogen. He was thus able to calculate the amounts of nitrogen fixed and transferred through successive harvests; from these observations, it appeared that there was a large amount (70-90%) of the nitrogen fixed in the nodules regularly transferred into the host plants. This transfer began shortly after the effective development of the nodules on the roots of the Soya beans. He concluded from the evidence obtained in the experiment that there was little delay in the liberation of these nitrogenous compounds into the host cytoplasm and that the transfer rate was "thus proportional to, and probably governed by, the prevailing rate of fixation".

This interpretation of the data presented in Bond's paper was criticised on various grounds by Wilson and Umbreit (1937). Their main contention is that his data does not allow a choice to be made from among the four mechanisms of nitrogen transfer already suggested (p.35), since they claim, although unsupportedly, that bacterial breakdown may be difficult to detect within the nodule tissues. They indicate that a lag of only several hours is necessary for enzymic or autolytic action on the bacterial cells to make their nitrogenous compounds available for absorption by the host plant. These observations would certainly bear a considerable weight if they were supported by the necessary cytological evidence - but it is well known that such is not forthcoming, and, therefore, the objection to Bond's interpretation loses much of its relevance. In view of the work of the cytologists and the quantitative results presented by Bond (1936), it is difficult to envisage any mechanism of transfer other than a passive exosmosis of nitrogenous compounds from the bacterial cells; such a process is well known in the secretion of exotoxins by many pathogenic bacteria.

Bond (1938) has replied to the American paper and says that "transfer by excretion requires no such destruction of bacteria, and is therefore, for the present, to be preferred to the other theories". An important difference between the Wisconsin and Glasgow experiments is that the latter plants received a regular high amount of nitrogen from the bacteria from the earliest stages, and that this rate continued more or less constant for the rest of the life of the legume. The Wisconsin plants did not show a high percentage transfer in the younger plants. Such differences in the findings of the two stations might contribute to the difference of opinion on the interpretation of the data.

The present experiment was set up to extend the work by Bond on the Soya bean to another leguminous plant. It was designed to study the quantitative relation between fixation of nitrogen and the transfer of fixation products from the bacteria and the nodules to the rest of the host plant - in this case, Pea (Pisum sativum L.).

METHODS.

The plants were grown in glazed earthenware pots containing 3·6 Kg. of coarse, quartz sand; this was treated, as previously described, by sterilising for 3 hrs. and adding Hiltner's culture solution to 12% of the weight.

Sutton's "Little Marvel" dwarf variety of Pea (Pisum sativum L.) was used; seeds of uniform weight were initially surface sterilised by flaming with absolute alcohol and were then allowed to imbibe water under sterile conditions on damp blotting paper. When the radicles had pierced the testas, the seeds were inoculated and sown in the pots ½" deep. The inoculum was prepared from the crushed nodules of a Pea plant; in order to secure as pure an inoculum as possible, the nodules were surface sterilised by immersion in a solution of mercuric chloride and hydrochloric acid for two minutes; the nodules were next rinsed in several changes of sterile distilled water, then in ammonium sulphide solution followed by many further changes of water. The nodules were then ground up in a sterile mortar and made into a suspension in 200 cc. of water; this preparation was used 1 cc. per seed for inoculation.

The plants were sown on July 10th., 1936, and grown during the late summer in a greenhouse, under conditions similar to those already described in the first section. The testas, cotyledons and falling leaves were retained over formalin vapour for analysis with the rest of the harvested plant material.

Samples, each comprising of four pots, were harvested at approximately 10 day intervals during the growth period. These were removed from their pots with care, the nodules detached, counted and weighed after 1 hr.'s drying at 95°C. The rest of the material was cut up, dried to constant weight at 95°C and powdered for analysis as before.

Analyses on the dried plant material were carried out in triplicate samples per pot, employing Ranker's (1925) modification of the salicylic acid Kjeldhal method. The nodules were estimated in bulk.

RESULTS.

The growth of the plants was only fairly satisfactory, due mainly to the fact that circumstances prevented the experiment being set up earlier in the growing season. The vegetative phase of the plants was, in consequence, shortened, and the amount of fixation lower than would otherwise have been obtained.

The data for dry weight, nitrogen content and nodule counts are presented in Table I.

From these figures, it is seen that there is no direct corellation between the numbers of nodules and the fixation efficiency, but it is to be noticed that the nodule weights increase steadily as the plants become older. At the same time, there is a general decrease with age in the percentage nitrogen in the nodules. In harvest No. 4, the nitrogen figure for the denodulated plants is similar to that of the previous harvest, indicating that a lag in fixation and therefore in the transfer of nitrogenous compounds from the nodules to the host tissues had occurred. The adverse weather conditions may have prevented fixation at this stage of the experiment, and there is probably also a sampling variation in this harvest.

TABLE I.

Data for dry weight and nitrogen content per pot of 4 plants; period of growth, July 10- Sept. 18, 1936.

Harvests	Age of plants (days)	Stage of developt.	No. of nods.	Dry wt. denod. pl. (gm.)	Nitr. cont. denod. pl. (mg.)		Nitr. cont. nodules. (mg.)	% of N Fixed	Total nitrogen (mg.)
1.	29	6 leaves	114	0·80	73·9 *		8·0		
			171	0·84					
			208	0·87	78·2				
			163	0·78					
Ave.			164	0·82	38·0	95 %	2·0	5·0	40·1
2.	41	8 leaves flowers	170	0·98	95·5		14·0		
			159	1·11					
			117	1·03	92·9				
			169	1·07					
Ave.			154	1·05	47·1	93 %	3·5	7·0	50·6
3.	51	10 leaves young pods	269	1·35	108·6		11·64 *		
			266	1·49					
			199	1·35	102·7		10·12		
			175	1·32					
Ave.			225	1·38	52·8	90·9	5·3	9·1	58·1
4.	60	10 leaves pods filling	182	1·37	103·8		8·35		
			133	1·49					
			223	1·40	107·0		9·96		
			255	1·73					
Ave.			198	1·50	52·7	92·0	4·6	8·0	57·3
5.	71	pods almost full	205	1·83	114·5		8·8		
			243	1·64					
			169	1·61	122·3		7·2		
			183	1·92					
Ave.			200	1·75	59·2	93·7	4·0	6·3	63·2

Average data per pot from 4 pot sample.

* Data from two combined pots.

The final harvest showed a slight improvement in these respects, although the metabolism of the symbiosis was distinctly slowed down in the later stages of the growing period.

DISCUSSION.

The results shown in this series of experiments confirm the data obtained by Bond (1936) with Soya bean. The interpretation placed upon the figures is that there was a high percentage of nitrogenous compounds elaborated within the bacterial cells transferred to the host cytoplasm, and that this passage was going on continuously during the development of the legume.

I support of this view, it is seen from Table I p.42 that the nodule tissues, including the bacterial cells, never at anytime contained more than a few milligrams of nitrogen, whereas the host plants steadily increased their nitrogen content during their growth; Table II p.45 shows that this regular transfer of nitrogen formed a considerable amount of that fixed. The ratio column in this Table confirms the high transfer obtained by Bond (loc. cit.).

TABLE II.

Amounts of nitrogen fixed and transferred from nodules into plants during successive periods of growth; seed nitrogen = 33 mg.; data derived from Table I.

Period from sowing (days) 1.	Fixation of nitrogen (mg.) 2.	Nitrogen transferred from nodules to plants (mg.) 3.	Ratio of nitrogen transferred to nitrogen fixed as 4.
0-29	7·5	6·4	85
29-41	10·5	9·1	86
41-51	7·5	5·7	76
60-71	5·9	6·5	110

Column 2 obtained by subtracting the total nitrogen figure of one harvest from the succeeding harvest (see Table I).

Column 3 obtained by subtracting the denodulated plants' nitrogen content figure of one harvest from the succeeding harvest (see Table I).

[During the period 51-60 days, there was no fixation of nitrogen and thus no figures are presented].

This interpretation conflicts with that of Wilson and Umbreit (1937) who derived their data from Soya bean experiments at Madison, continued over several years. They found that in the early stage of legume growth and nitrogen fixation, about 30-50% of the total nitrogen fixed was retained in the nodules. This conclusion is not supported by much experimental evidence, as their analyses began mainly after the "early" stage of the growth had been passed. It must be pointed out that, whereas they obtained a transfer ratio of 40-45% at 28-32 days, the present author obtained a ratio of 85% at 29 days. The Madison workers agree that there is a subsequent stage when " a fairly constant quantity of the nitrogen fixed is transferred from nodule to plant (80-90 per cent)". At the end of the development of the legume, they also note a period of variable nitrogen relations. On the basis of their data, they conclude that the rate of transfer is not a function of the bacterial cell so much as a "regulatory mechanism" determined by the host requirementssin nitrogen at any moment.

It appears obvious, however, that whatever construction is placed on the data obtained from such experiments, there must be relatively large amounts of nitrogenous compounds already available for transfer at a very early stage in the symbiosis. It is further contended that the present experiments show that, under these conditions at least, high rates of transfer do commence at a very early stage in the development of the plants. This is not to say, however, that the same applies necessarily under all conditions; the rate of utilisation of these nitrogenous compounds by the host plant will certainly affect the rate of removal from the nodules and this, in turn, the initial transfer from the bacterial cells.

The experiment demonstrates that the mechanism is able to provide for a rapid movement of a high proportion of the nitrogen fixed from an early stage in the legume development. This proposition would conform to the unsupported suggestion of Bond (1936) that the excretion of nitrogenous compounds from the bacterial cell might well be the expression of a respiratory mechanism, rather than the formation of amino-acids to be released by autolysis or enzyme action at the conclusion of the life of the bacterium. This passive movement of nitrogenous compounds from the bacterial cell to the host cytoplasm would then be a continuous phase in the inter-relations of the symbionts, and not a phenomenon dependent only on the host demands for a supply of nitrogen. Such a hypothesis may be correlated with the activities of nitrifying bacteria in the soil, where the formation of nitrites and nitrates in this case are generally regarded as a result of processes essentially respiratory in nature.

The question of nodule efficiency in respect of the amounts of nitrogen fixed - as measured in relation to the dry weight of the nodules - is of some interest. It is noted that there is a period of greatest efficiency towards the middle of the legume development; at the beginning and the end of the growth period, the nodules do not fix nearly as much nitrogen weight for weight. In the young nodules, it may well be that the infected cells are not yet completely filled with bacteria, or that the bacterial population has not begun to fix nitrogen under optimum conditions. It is, however, in the later stages that the first signs of bacterial decay are detected - this would naturally be reflected in a decreased nodule efficiency; the fixation of nitrogen in such nodules falls considerably, but it is significant that the transfer ratio still remains at a high figure [this is true generally of the last three harvests (Tables I and II) in which the total nitrogen figures are not considerably altered]. Thus, in the older nodule, the bacteria which are still fixing nitrogen are behaving in similar fashion to those of the most vigorous fixation stages. It would appear, then. that the digestion of bacteria is not a necessary preliminary to the release of nitrogenous compounds for the host plant's use.

SUMMARY.

1. Pot cultures of Peas were set up to investigate the process of transfer of nitrogenous compounds formed in the bacterial cells to the host cytoplasm.

2. Data is presented indicating that a large percentage of the nitrogen fixed in the nodules is regularly transferred to the host tissues, and that this supply of nitrogen to the higher symbiont becomes available shortly after the commencement of fixation.

3. A comparison of these results with those of other workers is made, and suggestions to account for the process are offered.

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III.

The excretion of nitrogenous substances from leguminous root nodules into the rooting medium.

INTRODUCTION.

The term "excretion" was first employed in the present connection by Lipman (1912); he observed a distinct benefit in the nitrogen content of certain non-legumes grown in association with legumes, which was not detected in pure cultures of the former. To account for this, he suggested a process whereby a certain amount of the nitrogen fixed in the nodules found its way into the surrounding rooting medium and was absorbed by the associated non-legume. This important observation, with all its practical implications, was largely laid aside until the matter came under the attention of Virtanen and his co-workers at Helsinki who have published a number of valuable papers during the last decade.

The occurrence of such a leakage of nitrogen into the rooting medium of a nodulated legume, and its subsequent absorption by any associated non-legumes, has been proved conclusively over a large number of pot culture experiments by the Finnish workers. They analysed the sand of legume cultures and found that a considerable amount of the nitrogen fixed had been excreted from the root systems. In their associated cultures, the non-leguminous "detector" plants were invariably richer in nitrogen than comparable controls grown by themselves. That this excretion took place from the nodules themselves, was demonstrated by Virtanen (1938) - a tube was inserted into a sterile, uninoculated sand culture, and the sand within it inoculated with a suitable bacillus strain, so that the only roots which developed nodules were those growing into the tube. The two different sands were analysed for total nitrogen and only that within the tube showed any increase. He concluded that the excretion was connected closely with the activities of the nodule bacteria and not merely a leakage from the roots themselves.

The word excretion suggests an active process, but it is generally agreed that any passage of chemical substances from the bacterial cell and nodule is of a passive nature. At the same time, it is difficult to find a word which adequately describes the process envisaged, and it has now passed into the vocabulary of the literature on symbiotic nitrogen fixation. It is to be noted that the same term had previously been used in reference to a movement of fixed nitrogen from the bacterial cells to the host cytoplasm, not necessarily followed by movement into the soil.

The work already quoted (Virtanen et al. 1935. 1936, 1937, 1938) indicates that the legume (usually Pea) may excrete as much as 80% of the total nitrogen fixed into the rooting medium; when non-legumes were grown in association with the legume plants, the excretion tended to become even greater, so that the former acted as a stimulus to the process. Virtanen has suggested a number of possible factors influencing the phenomenon and has described carefully the conditions under which his experiments were set up. Attempts at a repetition of his work by other experimenters, however, have generally resulted in failure to obtain excretion, although Wilson (Wilson and Burton 1938), working at Helsinki, found some evidence of it in certain of his Pea cultures. In this paper, they review critically a group of experiments carried out at Helsinki and at Madison; in the latter place, cultures of a Finnish Pea and bacterial strain varied considerably in their excretion (see also Wilson and Wyss 1937). In addition, they found that plants grown under varying climatic conditions and with different amounts of total nitrogen fixed also varied in this respect.

A number of other workers have reported negative findings. Ludwig and Allison (1937) failed to get any excretion with associated cultures of different legumes including Pea and Soya bean and a variety of cereals. Trumble and Strong (1937), Strong and Trumble (1939) and Shapter (1939), working at the Waite Institute, Adelaide, Australia, obtained negative results in both pot culture and plot tests for excretion. Engel and Roberg (1935), working on Clovers and non-leguminous, nodulated types such as Alder, Hippophaë and Eleagnus, found no excretion. Variable results were obtained by Thornton and Nicol (1934) and Nowotnowna (1937) at Rothamsted using lucerne, clover and peas as the legumes and barley and ryegrass as the detectors. This work included plot experiments and some evidence of benefit to the non-legumes was shown but the main interest concerned the effect of nitrogenous manuring on the associated growth relations. Nicol (1934, 1935) has reviewed the literature on associated growth to that date, and Wilson (1937) has collated later information in his review.

The present author set up experiments in 1937 designed to detect excretion of nitrogen from the nodules of Pea, as an extension of observations on the passage of fixed nitrogen from the nodules to the host plants (Part II). At that time, the only observations on excretion available were those of Virtanen and Thornton and Nicol, in addition to Lipman's work. Some of the results of the present experiments have already been described briefly in a publication by Bond and Boyes (1939).

METHODS.

Sand culture methods were again employed in these experiments using glazed pots of two sizes containing 3·6 Kg. and 1·8 Kg. respectively. The original nitrogen content of the sand was determined and was found to be 22 mg. per 3·6 Kg. In certain cases, which depended on sand analyses, the sand was ignited before autoclaving to reduce the original nitrogen; this treatment resulted in a figure of 4·5 mg. per 3·6 Kg. of sand. The sand was buffered, as before, with the addition of $CaCO_3$, and the culture solutions used were (a) Rothansted, (b) Hiltner's, both without nitrogen.

The plants were grown during the summer months of 1937 and were under glass in a temperate greenhouse, as in previous experiments.

The cultures consisted both of legumes alone and legumes associated with non-legumes or uninoculated legumes. The former were Peas (Pisum sativum L.) "Gradus", and Broad beans (Vicia Faba L.) "Monarch Longpod"; in the mixed cultures, Barley (Hordeum vulgare L.) "Spratt Archer" and French beans (Phaseolus vulgaris L.) were added. Sterilisation of the seed was carried out by immersion in absolute alcohol, followed by a 0·2% solution of corrosive sublimate and subsequent washing in several changes of sterile, distilled water. After imbibing for 2-3 days on damp blotting paper, the seeds were sown when the radicles had pierced the testas; as usual, a small excess was sown in each pot and the seedlings thinned out later to the requisite number. Inoculation of the Peas was carried out at sowing, using an effective Finnish bacterial strain HX, supplied by Prof. A.I. Virtanen. The Broad beans were inoculated in similar fashion, using a bacterial strain which had been isolated locally.

Harvesting of all the pots was effected (a) in the pure cultures, at various intervals during growth, (b) in the mixed cultures, at the end of the experimental period when the plants were mature. The plants were carefully removed from the sand and the latter was sifted for root fragments. After drying and mixing, four samples of 200 gm. of sand were taken from each pot; this was extracted with a known volume of water which was removed through a Büchner filter. The extract was concentrated to a suitable volume for combustion and distillation as for the plant material, using the Kjeldhal method. Root washings were also taken and, after evaporation, were analysed in the usual way.

The plant material analyses were based on triplicate samples per pot in the first three harvests of the pure cultures, and, subsequently, all analyses were carried out by bulk combustion in which the whole plant was placed in a flask and combusted directly.

RESULTS.

TABLE I.

Based on 1 pot samples; 1·8 Kg. of sand, original nitrogen content 2.3 mg. per pot; Rothamsted culture solution; "Gradus" Peas, 3 per pot, nitrogen content of 3 seeds = 42 mg.; Bacillus strain HX; period of growth, March 13th.- July 27th., 1937.

Harvests	Age of plants (days)	Stage of development (length and nod. per pl.)	Total nitrogen (mg.)	Sand nitrogen increase * (mg.)
Inoculated				
1.	36	5 leaves	36·6	1·6
2.	50	40 cm. 12 n.	46·2	1·2
3.	85	85 cm. 19 n.	64·4	8·7
4.	108	85 cm. 25 n.	61·9	5·7
5.	126	moribund with sec. shoots	66·8	6·7
Uninoculated				
1.	74	72 cm. sec. shoots	39·4	7·7
2.	130	–	38·0	6·1

* Obtained by subtracting original sand nitrogen figure from total sand nitrogen after experiment.

TABLE II.

Based on 1 pot samples; 1·8 Kg. of sand, original nitrogen content 2·3 mg. per pot; Hiltner's culture solution; "Gradus" Peas, 3 per pot, nitrogen content of 3 seeds = 44 mg.; Bacillus strain HX; period of growth, May 29th.- August 28th., 1937.

Harvests	Age of plants (days)	Total nitrogen (mg.)	Sand nitrogen increase * (mg.)
Inoculated			
1.	45	53·9	4·4
2.	54	65·0	3·6
3.	67	79·2	9·3
4.	91	96·5	10·5
Uninoculated			
1.	67	37·0	7·6
2:	67	37·3	8·5
3.	67	38·6	4·7
Ave.	67	37·6	6·9

* Obtained by subtracting original sand nitrogen figure from total sand nitrogen after experiment.

The figures presented in the first two Tables relate to pure legume crops. In the first case, the plants were grown under glass continuously, but in the second, the pots were placed outside on a sunny verandah for several hours per day during their development. This difference of treatment is reflected in the nitrogen fixation of the two series - in Table II there is a much better fixation which increased to a higher level at an earlier stage than in the first crop; this remained for some time at a lower maximum of nitrogen fixed. The uninoculated control plants, in both cases, are very similar in nitrogen content.

The sand analyses gave results which were consistently very low in respect of nitrogen content. The increase of nitrogen in the sand, above that already present at the beginning of the experiment, never exceeded a few milligrams, in contradistinction to the results of Virtanen. An estimation of the amount of nitrogen in the sand derived from remaining root fragments has been made by Bond (1938) and may account for some of this increase. There is a general tendency for this increase to be greatest as the plants become older, i.e. as the root systems become more extensive with age. This increase, however, is not regular, and is found, as would be expected from this explanation, in the uninoculated pots also.

TABLE III.

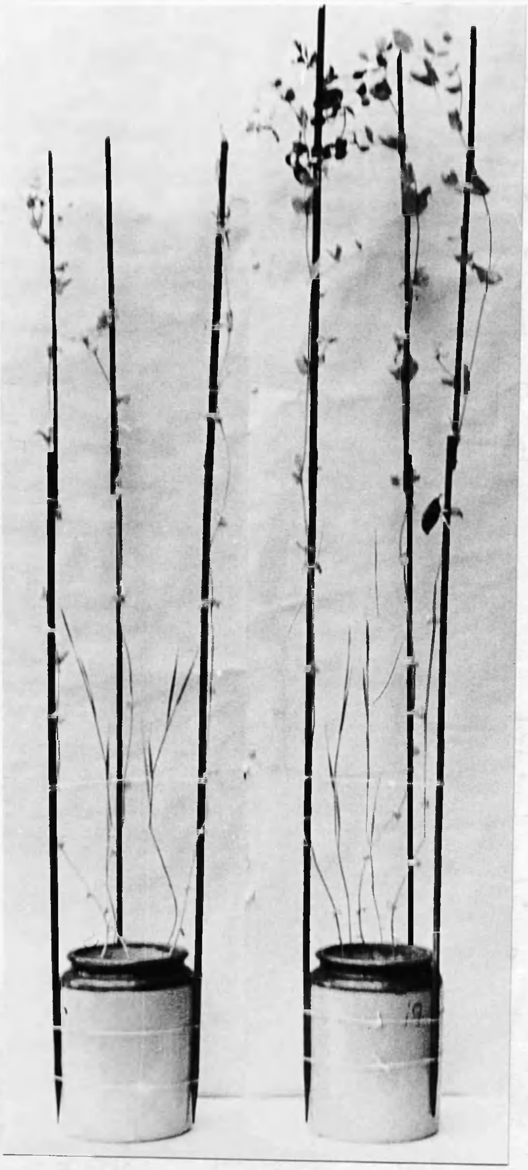
Based on 1 pot samples; 3·6 Kg. of sand, original nitrogen content 20 mg. per pot; Rothamsted culture solution; "Gradus" Peas, 3 per pot, nitrogen content of 3 seeds = 42 mg.; "Spratt Archer" Barley, 2 per pot, nitrogen content of 2 grains = 1·8 mg.: Bacillus strain HX; period of growth, March 18th.- July 26th.,1937.

Harvests	Age of plants (days)	Total legume nitrogen (mg.)	Nitrogen cont. of assocd. pl. (mg.)	Nitrogen incr. of assocd. pl.* (mg.)
Inoculated				
1.	133	72·0	2·9	1·1
2.	133	61·5	3·4	1·6
3.	133	similar	3·4	1·6
4·	133	similar	3·2	1·4
5·	79°	101·7	2·9	1·1
Uninoculated				
1.	133	36·4	3·1	1·3
2·	133	similar	2·7	0·9

* Seed nitrogen subtracted from previous column.

° Period of growth, June 5th.- August 23rd., 1937.

PHOTOGRAPH I.



PEAS and BARLEY

Mixed cultures of a legume and a non-legume, the latter being employed for the detection of excretion.

Left - uninoculated control Peas.

Right - inoculated Peas.

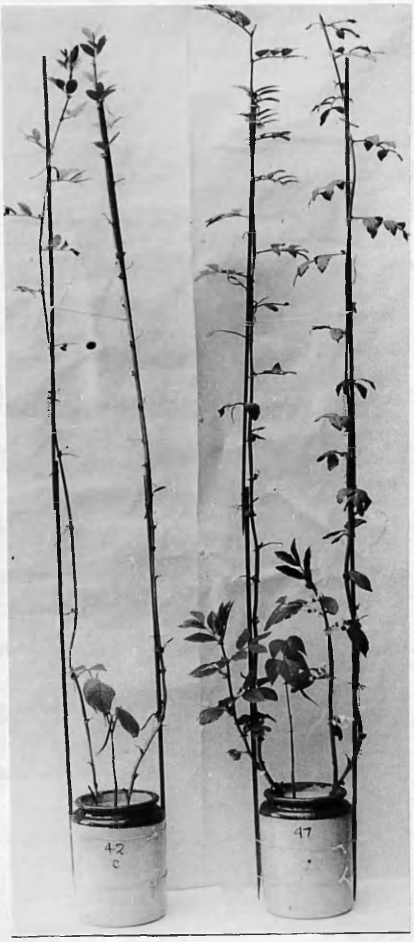
Note the similarity of the Barley development in both pots.

TABLE IV.

Based on 1 pot samples; 3·6 Kg. of sand, original nitrogen content 20 mg. per pot; Hiltner's culture solution; "Longpod" Broad bean, 2 per pot, nitrogen content of 2 deeds = 150 mg.; "Canadian Wonder" French bean, 1 per pot, nitrogen content of 1 seed = 17 mg.; Bacillus strain isolated from other Broad beans; periods of growth, Broad bean,- April 6th.- July 31st., French bean - April 27th.- July 31st., 1937.

Pot No.	Dry wt. (gm.)	Nitrogen fixed by Broad bean (mg.)	Nitrogen content of French bean (mg.)
Inoculated			
1.	15·4	312	16.
2.	15·2	305	17
3.	14·0	275	17
4.	similar	similar	19
Uninoculated			
1.	8·4	0	18
2.	similar	0	19
3.	similar	0	19

PHOTOGRAPH II.



BROAD BEAN and FRENCH BEAN.

Mixed cultures of legumes of different cross-inoculation groups. The Broad beans in the right hand pot were inoculated but not the French beans; the left hand pot shows uninoculated control Broad beans.

Tables III and IV are derived from mixed cultures of legumes and non-legumes. In the second case, the pots were prepared and the seeds sown by the present author; the cultures were thereafter taken over by Dr. Bond for harvesting and analysis.

In the first experiment, the Barley plants were grown in association with the Peas to act as "detector" plants for any possible excretion of nitrogen; this might be indicated by an increase in their nitrogen content. This increase was not obtained in any pot and the Barley plants varied little from their counterparts in the control pots, both in appearance and nitrogen content. In every case there was a slight nitrogen increase which cannot be attributed to an excretion process as envisaged by Virtanen.

The Broad bean - French bean experiment confirms the results of the previous mixed cultures. Although the French bean belongs to a different cross-inoculation group from the Broad bean, its roots were nodulated by the Broad bean bacillus strain. It was apparent, however, that the nodules were ineffective since no nitrogen was fixed. If any excretion had taken place, it was felt that a related legume of a different group might more easily absorb the particular nitrogenous forms released.

Whilst the Broad beans fixed relatively large amounts of nitrogen, none of this found its way to the French beans. Some of the latter, grown along with uninoculated Broad bean control plants, contained as much nitrogen as those in the inoculated pots.

DISCUSSION.

The results obtained from the experiments described in this section of the research confirm the lack of excretion of nitrogenous compounds into the rooting medium, characteristic of the Glasgow work. These negative findings receive support by other work at different stations already described and reviewed in Wilson's paper (1937).

A study of the different legumes reported to have given excretion, indicates that they belong mainly to the so-called "cool weather" types (Ludwig and Allison 1937). The Pea has given the most consistently positive results, and Virtanen (1938b) has also succeeded in obtaining the excretion effect in Clovers, Vetches and Alder. Thornton and Nicol (1934) report excretion with lucerne; Lipman (1912) used the Soya bean in his pioneer experiments but got only small amounts of excreted compounds. Wilson and Wyss (1937), however, obtained quite good excretion (40% of the total fixation) with Manchu Soya bean. In all the Glasgow work, none of the legumes employed

(Soya bean, Pea and Broad bean) have given any significant indication of excretion, and, in view of these variable reports, it would appear that the factor or factors controlling this phenomenon must be sought elsewhere than in the type of legume employed.

There are two further biological factors which may be concerned in the conditioning of excretion - bacillus strain and associated non-legumes.

In the first case, the bacillus strains employed in these experiments were known to be efficient in nitrogen fixation, and the Finnish strain HX, under Virtanen's conditions, has been used in cultures which have shown a vigorous excretion. It may be objected that the combination of British Pea varieties with a Finnish bacillus strain is not a suitable arrangement for excretion, since Virtanen (1938a) considers that the host variety and the associated strain are important factors. In further work at Glasgow (Bond and Boyes 1939), however, employing the Finnish Peas "Torsdag" and "Concordia" associated with the good strain HX, no evidence of excretion was forthcoming.

The possibility that a non-leguminous plant grown in association with a legume might stimulate the latter's excretion, has already been mentioned. Thornton and Nicol (1934) found that rye-grass grown along with lucerne contained twice as much nitrogen than when grown alone; Virtanen and others (1937b, 1938b) have provided a considerable body of data in support of this proposition. In mixed sand cultures of Peas and Barley, they found that from 20-75% of the excreted nitrogen found its way into the non-legume plants, and that the total excreted nitrogen - in the sand and absorbed by the non-legumes - was much greater than in a pure legume culture. The extent of this excretion was further influenced by the ratio of non-legumes to legumes; as this increased, the excreted amounts arose until the legumes actually suffered from the effects of nitrogen starvation although their total fixation of nitrogen had been stimulated by the presence of the non-legumes.

This kind of result was never obtained in the present work; in no case was there any suggestion of such excretion or that the associated non-legumes had benefit~~t~~ed by increased nitrogen content. From the figures obtained in the analyses of the Barley (Table III) and French bean (Table IV) associated plants, it was apparent that they had derived no additional nitrogen from any source during their growth. Their development was poor, although the root systems were well branched and obviously able to absorb the supplied salts of the culture solution; the roots of the two types of plants were closely connected, but, nevertheless, no nitrogenous benefit was forthcoming for the non-legumes.

The fixation of nitrogen in the Peas (Table III) was somewhat lower than normal for such cultures, and it may be suggested that the legumes required all the fixed nitrogen for their own metabolism so that the non-legume plants had perforce to do without any assistance. This contention is disposed of in view of Virtanen's finding that the associated plants stimulated the legume metabolism in the excretion of nitrogen - this was not at all a feature of the present work, possibly because the non-legumes were not suitable for this stimulative function.

Such biological factors, by themselves, are probably not the controlling factors in the occurrence of excretion, and a number of purely physical conditions, not inherent to the plant systems, have also been suggested.

The necessity of a solid medium of high adsorptive properties has been stressed by Virtanen (1935,1936,1937a), and he also considers that the air content of the medium is important. An increase in the air content - usually by the use of a finer grained sand - was found to increase the excretion of nitrogen. A comparison of the physical properties of the Finnish sand and the coarse quartz sand (Bond and Boyes 1939) used in these experiments shows that the former was considerably finer and contained a certain proportion (5%) of silt and colloidal material which would increase its adsorptive properties over the latter. On the other hand, Peas and other legumes grown by Dr. Bond (Bond and Boyes loc. cit.) in his "Superfine red sand" which was considerably finer than the Finnish quartz, did not excrete any nitrogen. Virtanen (1935, 1936) obtained no excretion in water cultures of Peas but found no difficulty in inducing nodulation and good fixation of nitrogen in cultures which were aerated - this problem is fully dealt with in another section - so that a distinct relation between excretion and fixation is not suggested. He believes that the nodules must be in contact with solid materials before excretion will take place.

In view of the object of these present experiments, the sand in which the plants had grown was analysed with great care. It was dried, washed, and filtered under pressure, using methods very similar to those of Virtanen (1937), but in no case did the nitrogen content of the sand exceed its original figure before use in culture by more than a few milligrams. An interesting confirmation of this lack of excretion was obtained in the examination of the washings of the roots taken at harvests. The amount of nitrogen derived from such likely extractions was always negligible so that no figures from this source are presented.

It is thus seen that, in this important matter of sand analyses, the present results are consistently at variance with those claiming positive evidence of excretion into the rooting medium.

In a very recent communication, Virtanen (1940) has suggested that the porosity of the culture pots - indirectly related to the air content of the medium - influences the process of excretion. He found that, generally, the pots which were most porous were also most efficient in stimulating excretion. The pots used by the author were all of glazed earthenware and, in this respect, differed fundamentally from those of Virtanen's latest work; this arrangement would undoubtedly interfere with the exchange of air between the atmosphere and the rooting medium through the porous pot wall, and might well have been considered among the conditioning factors, but for the fact that Virtanen himself has obtained excellent excretion using glass flasks and Wolff bottles as containers.

Another physical feature connected with the culture system and pointed out by Virtanen (1937) is the leaching effect of watering the plants. The water might dissolve any soluble excreted nitrogen and wash this down to the more absorptive regions of the root systemes where it would be reabsorbed. In pure cultures, this argument might explain the absence of excreted nitrogen in the sand, but, on the other hand, the roots of associated non-legumes would also absorb this nitrogen and a resultant increase in nitrogen content would be found. This was absent in the present experiments.

Finally, the quantity of light falling on such legume cultures has recently been studied in relation to fixation. Ludwig and Allison (1937) obtained varying responses to different light intensities and daily duration, without being able to make any definite conclusions. Wilson and Burton (1938) discuss the different arrangements of the Helsinki and Madison greenhouses in view of the possible effect of this factor on excretion. These workers obtained Pea plants, grown under very good conditions of sunlight, which were comparable to plants grown in the field. The Peas fixed large amounts of nitrogen but did not invariably show excretion, so that they do not consider lighting conditions to be an important factor. A detailed comparison of the sunlight conditions obtaining between the Helsinki and

Glasgow stations has been made in the paper by Bond and Boyes (1939). It is to be noted that the latter station receives much less direct sunshine than the former and that the daylength is also somewhat shorter, nevertheless, satisfactory growth and fixation of the same order as the Helsinki plants has been obtained, although invariably negative in respect of excretion. Artificial illumination of legume crops has also been employed - Virtanen (1937a) reports excellent excretion on various occasions in winter crops grown under 1000 W. lamps suspended above the culture pots. In unpublished work, mentioned by permission of Dr. Bond, a repetition of these experiments at Glasgow, under identical conditions, has yielded only further negative results in the matter of excretion.

From a consideration of these physical factors and the critical evidence of a number of experiments, it is again clear that they cannot be wholly responsible for the occurrence or absence of excretion, and the solution to the varied results obtained may be found in the proper combination of a number of these factors - both biological and physical.

The general growth of the legumes throughout the present series of experiments was never outstandingly vigorous, probably because circumstances necessitated the cultures being kept under glass most of the time, a preferable procedure for Peas being to place them out of doors as much as possible. Virtanen (1937B), however, obtained an excretion of 80% of the total nitrogen from Peas which had fixed relatively little. Wilson and Burton (1938) obtained negative results in respect of excretion under conditions of good and bad growth of the legume. It would appear that there is no fundamental connection between fixation and excretion.

The regular small increases of nitrogen found in the sand after harvests have still to be accounted for. This may be due entirely to root fragments left behind in the sand which was carefully hand-sifted before analysis, but, undoubtedly, some little organic matter of this nature remained. In addition, a certain amount of nitrogen may have reached the rooting medium from any legume rootlets which were bruised by the friction of the sand particles, thereby facilitating a leakage from the vascular system.

The whole question of the structure of the nodule and the possibility of an escape of nitrogenous compounds as envisaged by Virtanen, arises now. It is generally believed that the developing nodule is soon surrounded by an impermeable suberised layer outside the vascular connections;

this layer in the younger stages is incomplete at the distal end of the nodule,but, in the mature structure, closes up forming anatomically an organ which is less likely to provide a means of excretion than would an ordinary root - yet Virtanen has shown (see p.55) that excretion is a function of the nodule. In the older nodules, tissue necrosis is well known and it might be expected that a nitrogen leakage would occur from such structures; the excretion, as observed by recent workers however, occurs from an early stage in the life of the nodule, and that in apperciable amounts.

The general conclusions to be drawn from these experiments are that, under the Glasgow conditions of culture and environment, there is no evidence of an excretion of nitrogen from the nodules of Peas and Broad beans into the rooting medium. In mixed crops, there is no nitrogenous benefit derived by the non-legume from its association with legume plants. It is submitted that the whole question of the agricultural application of such theoretical hypotheses cannot be supported by much experimental work, at least in this country.

SUMMARY.

1. Pure cultures of Peas and mixed cultures of Peas and Barley and Broad beans and French beans were set up to investigate the reported excretion of nitrogenous compounds from the nodules of certain legumes into the rooting medium.

2. Analyses of plant material and the rooting sand derived from these cultures did not support the findings of some other workers - notably Virtanen at Helsinki - in which an increase in the nitrogen of the rooting medium and the associated detector plants was attributed to an excretion from the legume nodules.

3. A discussion of different factors - both biological and physical - which might influence excretion is made. Tests, under conditions which had given positive results with other workers, were consistently negative in the present work.

4. It is concluded that, as far as the present experiments are concerned, there is no evidence for such an excretion from the nodules of the legumes employed.

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IV.

An investigation of the growth of leguminous plants in water culture, with special reference to the effect of aeration on fixation and growth.

INTRODUCTION.

In the study of the physiological relations of the plant, the use of the water culture method rather than of solid rooting media, is often found to be advantageous. The elucidation of the special physiology of nodulated leguminous plants carried out in this laboratory has been concerned mainly with plants grown in sand culture, but it became desirable to investigate the circumstances under which satisfactory growth of nodulated legumes - especially Soya bean - in nitrogen-free water culture is possible.

A number of earlier investigators have attempted the culture of leguminous plants under such conditions, throwing some light on the problem. Nobbe and Hiltner (1899) concluded that nodulation could take place in water cultures, but that the nodules were of "little or no benefit to the plant". Golding (1903), who obtained moderately good growth of Peas in water culture, considered also that the nodules were of little value under these conditions and found that aeration of such nodulated plants did not benefit them in respect of growth and fixation. He grew unaerated plants which were twice as great as corresponding aerated cultures in dry weight and nitrogen content, but offers no explanation for this effect. In another series of experiments which received bubbling of the roots with pure oxygen, there was not much improvement and continued poor fixation of nitrogen. In a third series of nodulated Pea plants, in which the diffusion of oxygen from the air into the culture solution was obstructed by a layer of oil, he obtained no nitrogen fixation. He also grew a number of uninoculated control plants to which combined nitrogen as NH_4NO_3 was supplied - in all these cases, the plants were much superior to their inoculated counterparts. In some of the uninoculated jars, the roots were exposed to the air for certain periods, whilst the rest remained immersed continuously in the culture solution. The latter had a higher dry weight and nitrogen content than the exposed series.

Prucha (1915), working with Pea, obtained no stimulative effect on the number or size of the nodules when the roots were aerated. Nodules developed as much as 30 cm. below the surface of the soil extract solution which he used; apparently sufficient air for the purpose of nodulation was available at that depth. Under these conditions, he also found that nodules developed as long as new root tissue was formed but he made no observations on nitrogen fixation. Wilson (1917) has suggested that nodulation in water culture is a variable feature connected with the particular concentration and combination of salts used. He set up a large number of small, short term experiments and recorded only the occurrence or absence of nodules in each case.

Virtanen and von Hausen (1935, 1936) set up experiments with Peas in sand and water cultures, and found that efficiency in nitrogen fixation depended largely on the air supply to the nodules. In unaerated water cultures, where the nodules were completely submerged and nodulation was very poor, the fixation was very small by their standards, but similar to that obtained by Golding (1903) with comparable plants. As progressively increasing amounts of air became available to the legume roots by means of aeration or lowering the level of the culture solution, the nitrogen fixation increased proportionately. In the last instance, best fixation was obtained only when all the nodules were out of the solution and only the absorptive tips of the root systems were submerged. When the solution was covered with a layer of oil, or when a stream of nitrogen was bubbled through, no nodulation took place. Except in the last case, these results are precisely opposite to those obtained by Golding (loc. cit.).

From this experimental evidence, the Finnish workers concluded that "oxygen is indispensable for the formation and function of the nodules".

In this connection, the experiments of Wilson and Fred (1937) are of interest. They worked with sand cultures of red clover which were placed in a specially controlled atmosphere of which the pO_2 could be regulated. They found that both inoculated and uninoculated (with nitrate supplied) plants gave substantially the same response to variations in this factor - at an oxygen pressure of more than 0·4 atm., the fixation or uptake of nitrogen decreased markedly; at such higher pO_2 values, they believe that an increased respiration takes place, with a consequent depletion of carbohydrate reserve. Such plants were "pale green in color, spindly and smooth, whereas those grown at a more normal pO_2 were dark green, thrifty and pubescent". They concluded that their experiments showed that any hypothesis involving the use of molecular oxygen in nitrogen fixation was not tenable on the basis of their results, since they found no differences between the response of the inoculated and uninoculated series. They consider that molecular oxygen is only of indirect influence in so far as it affects the carbon metabolism of the plant, which conclusions are also at variance with those of Virtanen and von Hausen (1935, 1936).

Various workers, using techniques other than water culture methods, have considered that oxygen is necessary for the functioning of the nodules. Thornton (1930) found that lucerne and clover seedlings with their nodules embedded in agar did not fix much nitrogen; at a later stage, when the agar cracked and the nodules had access to oxygen, the functioning and fixation was greatly improved. Feher and Bokor (1926) had already made this observation in respect of the nodules of other leguminous types.

The effect of the pH of the culture solution on the growth and fixation of the Soya bean has been studied by Bryan (1922) who found that there was an optimum narrow range about pH 6·5. In addition, he found that the limits for inoculation were pH 4·6 and pH 8; for the growth of Soya beans, pH 3·9 and pH 9·6; he also observed that the legume stabilises the culture solution at a favourable pH value. Loo (1928) believes that the pH factor is even more important than aeration on the legume development.

The present experiments were designed to investigate the possibility of securing satisfactory growth of nodulated Soya bean plants in nitrogen-free water culture, with special reference to the effect of air

The cultures of nodulated plants comprised two groups. In the first, a steady stream of air was passed through the aqueous rooting medium during the growth of the plants, while in the second group, no air was bubbled through the medium, though diffusion of air from the external atmosphere into the culture solution was possible. Observations were made on the growth and nitrogen fixation of these two groups of plants.

In addition, corresponding series of uninoculated control plants were grown under the same conditions in order to discover whether the aeration effect was related generally to the growth of the legumes, or whether it had some specific influence on the functioning of the nodules. These controls were supplied with a certain amount of nitrate nitrogen to balance the benefit derived from nitrogen fixation in the inoculated series.

During the summer of 1940, Dr. Bond carried out two sets of similar experiments to confirm the results obtained by the present author during 1938 and 1939. The data for one of these 1940 cultures is presented later.

METHODS.

Large, wide-mouthed, glass jars of approximately 3 litres capacity were used, and were initially sterilised by washing with alcohol followed by sterile distilled water. These were fitted, in 1938, with waxed, sterile shives, and, in 1939 and 1940, with flanged, teak caps; the covers were slotted to receive three seedlings, and bored to admit an aeration tube and a siphon tube which facilitated the replacement of the culture solution without exposing the roots by removal of the top. The jars were covered on the outside with opaque paper to prevent the growth of algae. Aeration was carried out by the use of glass tubes attached to porous stone diffusion blocks (1938 and 1940) or by simple tapering of the tubes (1939). These dipped deeply into the culture solution and were all connected outside the jars to a common air supply; the air was drawn from the greehouse and was thus relatively pure. It was filtered through cotton wool plugs to prevent infection of the control plants. An adequate air current was obtained from a small air pump operated by a water turbine; this arrangement gave a reliable and steady air flow during the first two years of the experiments.

In 1940, a better pump was installed and the aeration for that year was very much improved - this was reflected in the growth of the cultures.

The culture solution used was Bryan's (1922) modification of Crone's solution without nitrogen; owing to the large quantities required, this could not be sterilised, but care was taken to prevent infection by using water direct from the still. A microelement solution (see p.15) was added at the rate of 10 cc. per jar. The solutions were changed at intervals of 3-4 weeks in 1938 and 1939, and fortnightly in 1940 by siphoning off the old and replacing with the new. The water level was maintained by the addition of sterile distilled water as required, so that the root systems were kept entirely submerged.

An adequate number of Manchu Soya bean seeds of uniform weight were surface sterilised by flaming in alcohol and germinated in moist, sterile sand; when the radicles were 1-2 inches long, the seedlings were washed free of adhering sand, wrapped in a cotton wool plug and fitted into the slots of the covers. Inoculation was carried out as desired by delivering about 5 cc. of the inoculum - made from a culture of the Wisconsin strain 3 - through the plugged siphon hole of the cover.

As noted, the uninoculated control plants were supplied with combined nitrogen in the form of sodium nitrate; this was added in excess of the plants' estimated requirements so that there should be no deficiency at any time during the growing period. Each jar of three plants received aliquot portions at intervals to a total of 650 mg. of nitrogen in this way.

The pH of the culture solution, at the beginning of the experiments, was 6·6-6·7.

RESULTS.

TABLE I

1938 data; figures for 1 pot samples; 3 litres of culture solution; Manchu Soya beans, 3 per jar; bacillus strain Wisc.9; period of growth - June 16th.- October 3rd., 1938.

		INOCULATED		UNINOCULATED *	
	Jar No.	Dry wt. (gm.)	Nitrogen fixed (mg.)	Dry wt. (gm.)	Nitrogen content (mg.)
AERATED	1.	6·22	133·0	12·73	342·2
	2.	6·24	158·2	14·85	331·8
	3.	5·68	110·4	16·37	399·7
	Ave.	6·05	133·9	14·65	357·9
UNAERATED	1.	3·09	31·7	22·98	557·9
	2.	3·44	34·0	24·76	491·7
	3.	3·43	32·3	11·72	292·2 °
	Ave.	3·32	33·0	19·82	447·3

[In both nitrogen columns, the seed nitrogen has been subtracted].

* These received 650 mg. of nitrogen during the growth period, added in the form of a solution of $NaNO_3$ in sterile, distilled water.

° Seedlings suffered from leaf rolling and yellowing in the early satges and the plants never fully recovered - see text.

Observations on the 1938 plants at 1 month showed that the uninoculated plants were ahead in vigour and size as compared with the inoculated; the formers' leaves were invariably very large and the root systems were well branched. Differences between aerated and unaerated jars were not at all marked at this stage.

Further inspection at 2 months showed a still greater gap in the growth and appearance of the two series. The uninoculated jars now contained very large robust plants whilst the inoculated were much behind these in size and were considerably inferior to comparable plants in sand culture. Aeration, however, was now beginning to benefit the inoculated plants, those receiving this treatment being slightly ahead of the unaerated group. Nodulation was also better in the former group, although the nodules were still small and scattered.

The final observations, at approximately 3 months, showed the inoculated series to be of medium growth with small, light green leaves; there were a number of small pods, and the root system was fairly good with satisfactory nodulation. There was a marked difference between the aerated and unaerated plants of this series; the former were more robust and larger leaved than the latter and the nodules were mainly grouped at the surface and around the tap root, which arrangement is generally believed to indicate a symbiosis efficient for nitrogen fixation. These differences are reflected in the dry weight and nitrogen fixation figures in Table I (p.96); the aerated plants are shown to be twice as heavy and to have fixed four times as much nitrogen as their unaerated counterparts.

The uninoculated plants continued to be far superior to the inoculated series. The relevant data show a remarkable growth for greenhouse plants, and it is concluded that, under the conditions prevailing, the growth of the nodulated plants was limited by the supply of nitrogen from the nodules. There is an interesting difference found in the nitrate series in comparison with the inoculated plants, in that the unaerated jars were considerably better than the aerated. This result is further dealt with in the Discussion.

The variation between jars was quite small in view of the difficulty of many workers in obtaining a close correspondence in cultures of nodulated legume plants, but the differences between each category in the present experiment are well enough marked. In jar No. 3 of the uninoculated unaerated series, a considerable difference in dry weight and nitrogen content was obtained. The plants in this jar were obviously abnormal, and during the earlier stages ehibited a marked yellowing of the leaves, possibly due to some toxic material left behind in the jar after sterilisation.

In 1939, the plants (Table II p.100) followed a similar course of development but were generally inferior in quality to the 1938 cultures. This was due to a number of factors amongst which the growth period was important. During 1938, the plants were grown through a good period of sunshine and the 1940 cultures also benefitted in this way. The 1939 period of growth was both shorter and climatically poorer than the other two years; in addition, fungal attack on the uninoculated seedlings necessitated a new set up about 1 month later than the others with a resultant inferior development.

Again, in most of the 1939 jars, considerable difficulty was experienced in keeping the cultures free from a mixed bacterial and fungal contamination which formed a scum round the root systems and seriously affected the healthy development of the plants. As noted, numerous replacements were necessary, and, in general, the cultural conditions were unsatisfactory. The rapidly growing,uninoculated plants showed this deterioration of conditions the most markedly and the data from them was from a half to a third less than in 1938. The inoculated, aerated

TABLE II

1939 data; figures for 1 pot samples; 3 litres of culture solution; Manchu Soya beans, 3 per jar; Bacillus strain Wisc. 9; period of growth,

(a) inoculated - June 26th.- Sept. 23rd., 1939,

(b) uninoculated - July 20th.- Sept. 23rd., 1939.

		INOCULATED		UNINOCULATED *	
	Jar No.	Dry wt. (gm.)	Nitrogen fixed (mg.)	Dry wt. (gm.)	Nitrogen content (mg.)
AERATED	1.	3·30	26·6	10·81	249·3
	2.	3·10	22·8	7·14	158·8
	3.	3·68	28·6	7·30	195·2
	Ave.	3·36	26·0	8·42	201·1
UNAERATED	1.	4·19	52·1	16·68	272·8
	2.	3·42	36·3	13·53	261·4
	3·	3·97	38·3	15·79	318·2
	Ave.	3·86	42·2	15·33	284·1

[In both nitrogen columns, the seed nitrogen has been subtracted].

* These received 650 mg. of nitrogen during the growth period, added in the form of a solution of $NaNO_3$ in sterile, distilled water.

PHOTOGRAPH I I

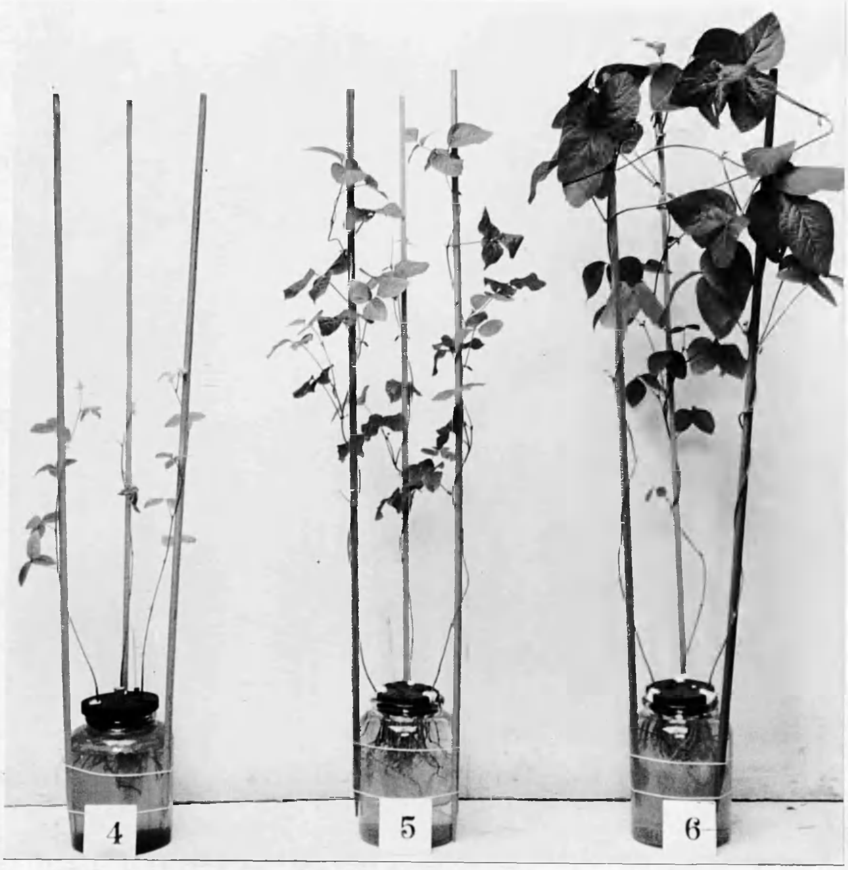
INOCULATED SERIES 1939

1. Oil covered (see text p. 103).

2. Aerated.

3. Unaerated.

PHOTOGRAPH III



UNINOCULATED SERIES 1939

4. Oil covered (see text p. 103).
5. Aerated.
6. Unaerated.

plants of 1939 were very poor and it is suggested that the adverse effects of culture contamination together with unsatisfactory climatic conditions offset the aeration benefit derived under more normal circumstances in 1938 and 1940; it is seen that the quality of these plants is below that of the unaerated group (Table II).

There is , again, a close approximation between replicate jars (except jar No. 1 of the uninoculated, aerated series), suggesting that the results obtained were not fortuitous but are accounted for by different conditions of culture.

Another group of plants were grown during 1939 under a third set of conditions. Three inoculated and three uninoculated jars were set up with a layer of liquid paraffin covering the surface of the culture solution to a depth of half an inch. This treatment was calculated to exclude the free access of air to the rooting systems and the nodules. In order to ensure that some growth would actually take place, the jars were aerated by hand pumping for a short time daily. These conditions resulted in extremely poor vegetative growth and no observed benefit from any possible nitrogen fixation or assimilation. Nodulation was very inadequate and, in the uninoculated series, the added nitrate was of no great value to the plants. The root systems, however, were of moderately good size despite the inferior development of the tops (Photographs II and III). The cultures were badly attacked by the contaminants discussed before and were discarded before harvesting time.

The 1940 plants of Dr. Bond (Table III) were grown during the best part of the season and received solution changes at more frequent intervals than in the previous years. This treatment resulted in the best crop of plants yet obtained under conditions of water culture in the Glasgow greenhouses, (Photograph IV). The nitrate plants were from five to six feet in height and both aerated and unaerated groups were similar - in this respect they varied from the 1938 and 1939 counterparts. The nodulated plants werealso of very good quality and of a higher dry weight than corresponding plants of the two previous crops. The aerated group contained the larger plants with bright green leaves and good nodulation - in this respect, they confirm the findings of the 1938 cultures.

TABLE III.

1940 data; figures for 1 pot samples; 3 litres of culture solution; Manchu Soya beans, 3 per jar; bacillus strain Wisc. 9; period of growth, May 15th. - August 10th., 1940.

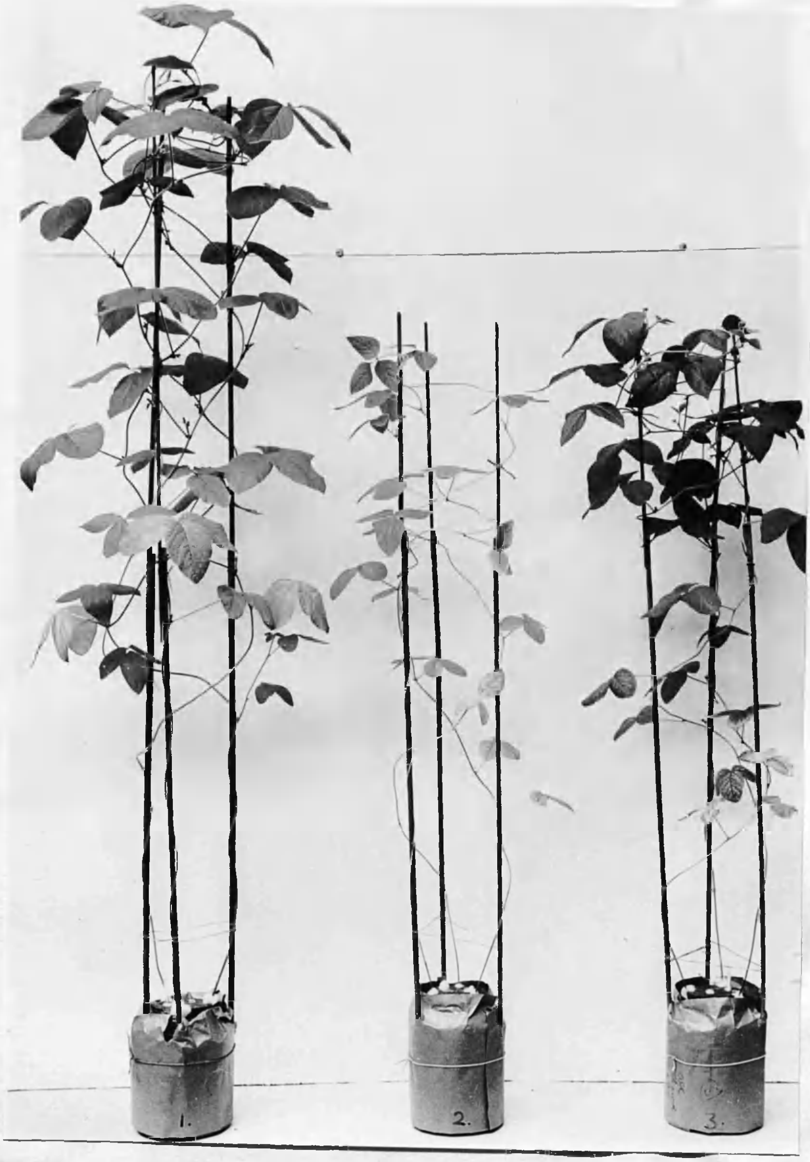
		INOCULATED	UNINOCULATED °
	Jar No.	Dry wt. (gm.)	Dry wt. (gm.)
AERATED	1.	12·57	30·22
	2.	12·54	30·27
	3.	8·39 *	23·72
	Ave.	11·17	28·07
UNAERATED	1.	7·23	27·99
	2.	7·95	27·38
	3.	5·17	–
	Ave.	6·78	27·69

* Originally a "nitrate" jar, but solution changed and inoculation effected on June 3rd. to replace a faulty original inoculated jar. In appearance, the plants of this jar showed marked benefit from aeration although they never caught up with Nos. 1 and 2.

° These received 650 mg. of nitrogen during the growth period, added in the form of a solution of $NaNO_3$ in sterile, distilled water.

PHOTOGRAPH

IV.



WATER CULTURES 1940

1. Nitrate jar.
2. Inoculated, unaerated jar.
3. Inoculated, aerated jar.

DISCUSSION.

Observations on three years of water cultures of nodulated Soya beans indicate that aeration has a pronounced effect beneficial to the nitrogen fixation and general growth of the plants.

In 1938, the dry weight and nitrogen fixation figures of the aerated plants (Table I p.96) were considerably more than those of the corresponding unaerated jars; the 1939 plants made disappointing growth for reasons stated elsewhere (p.99) and did not confirm the 1938 figures for the aerated group; the two sets of 1940 plants both confirm markedly the 1938 cultures - the dry weight figures for the first 1940 crop are given in Table III (p.105), and the second crop, not yet harvested, show aeration benefit in the inoculated series by observation. From general appearance, the nodulated plants of both of these latter crops are richer than the unaerated in nitrogen, so that even greater differences will be shown when the fixation figures become available.

These results are in accordance with the findings of Virtanen and von Hausen (1935, 1936) who obtained very marked improvement in their aerated cultures of nodulated Peas. Golding (1905) came to completely different conclusions concerning the effect of aeration on the mechanism of fixation in water cultures of Peas, and obtained results more comparable with those relating to the uninoculated plants of the present work. The matter rests there at the moment and further work is needed to elucidate the problem, especially concerning the effect of the constituent gases in the aerating current. A series of experiments in which the plants were aerated with a pure stream of oxygen and nitrogen together with a closer control of the access of air through the top of the culture jar might throw some light on this problem.

Under the present conditions of culture, all jars, irrespective of the aeration process, presumably received about 130 mg. of dissolved oxygen and 60 mg. of dissolvednitrogen at each change of the culture solution (assuming that the solution was in equilibrium with the atmosphere). Air will also diffuse through the wool plugs of the tops. Thus, in both aerated and unaerated jars, the amounts of nitrogen available in this way for the fixation process, were in excess of the quantities of nitrogen actually fixed during the development of the plants. With regard to the oxygen concentration, it is obviously desirable to know what precise differences existed in the content of this gas in the solutions within aerated and unaerated jars. It is unfortunate that circumstances did not allow of investigation of this point in the earlier experiments; a few estimations of oxygen by the Winkler method were carried out by Dr. R.F. Jones on jars of the 1939 cultures. These were made in the early stages of development when the root systems were small and did not fill the jars with a vigorous growth; only small differences in oxygen content were revealed at this stage. It seems obvious, however, that in the later stages when the jars became filled with a dense growth of roots, that considerable differences in oxygen content existed between the aerated and unaerated jars.

It may also be that the beneficial effect of aeration on inoculated plants is due to the removal of by-products such as CO_2 and root acids which might have a toxic effect on the fixation process. The mere agitation of the solution by the aerating apparatus, resulting in the replacing of respiratory by-products with air, may be more important than any role played by oxygen itself in the chemistry of fixation.

The type of nodulation in both the aerated and unaerated inoculated groups was of the "efficient" kind, indicating that the organism associated with the legume was potentially a good nitrogen fixer. However, only within the aerated group was this good fixation realised and it is suggested that the aeration of the root systems provided conditions more favourable for this process. The unaerated plants may have depleted their oxygen reserves during nodule formation to a level which could be of little subsequent assistance to the bacteria. It is to be noted that, in contrast to Virtanen and von Hausen's (1936) experiences with immersed Pea root systems, nodulation of such roots in the present work was invariably obtained.

The unsuccessful oil covered cultures of Soya beans produced plants of a very inferior type (Photographs II and III). This result was also obtained by Golding (1903) but Free (1917) grew buckwheat plants successfully in sealed water cultures. Further experiments along these lines, but not using oil which tends to clog the root systems and prevent normal growth, would throw light on the dependence of the roots on a constant supply of oxygen. It is of interest to note that nodules were actually developed on the roots of the present author's plants but these were apparently unable to assist in the growth of the legumes - it may be that the oil impeded the access of the bacteris to what oxygen and nitrogen were available in the culture solution.

The nitrate series of plants presented some interesting problems in the nutrition of uninoculated Soya beans in water culture.

Firstly, they derived such benefit from their easily assimilated nitrogen source as to make them far superior to their nodulated counterparts. This feature has already been noted in a previous section and the appearance of such plants is shown in Photographs III and IV (pp. 102 and 106).

Secondly, aeration of the root systems had a different effect from that in the inoculated jars. The marked increase in dry weight and nitrogen content of the latter group was not apparent in the nitrate series. In 1938 and 1939, the unaerated plants were actually superior in these respects to the corresponding aerated nitrate cultures, but this difference was not seen in the 1940 experiment.

It is generally assumed that aeration of water cultures is a process beneficial to the growth and development of the plants concerned. As suggested, the benefit may be connected with increased respiration and removal of toxic substances from the root systems, but it is noted that the value of aeration is disputed by Free (1917) who found that the degree of aeration did not materially affect the growth of his plants - those receiving slow aeration, oxygen bubbling or nitrogen bubbling were not significantly better than his open-access, unaerated controls or even his sealed cultures from which all air was cut off. Allison and Shive (1923) worked with uninoculated Soya beans in soil and sand culture and submitteddata for dry weights but not for nitrogen content. With similar plants in water culture, including a supply of combined nitrogen as $Ca(NO_3)_2$, they found that aeration of the culture solution did not produce plants which were better than corresponding unaerated cultures. The main difference in the aerated group was a better development of the root system; this was shown "not so much by the higher dry weight of the root substance produced as by the development of a very efficient absorbing system". Clark and Shive (1932) obtained similar results with aeration of Tomato roots in water culture. Loehwing (1934), similarly, but using soil cultures of Soya beans, found that roots growing under anaerobic conditions were devoid of root hairs, and that the CO_2 concentration may become toxic; abundant aeration increased root branching and root hair production together with an improvement in the growth of the tops. In the present work, there was no improvement in the development and dry weight of the aerated which, on the contrary, were inferior in branching and absorptive capacity. It was consistently noted, during the three years' cultures, that the root systems of the unaerated plants were better (not so marked in 1940) than their aerated counterparts. The absorptive capacity of the secondary rootlets was very great and they filled the culture jar in the later stages of development. These observations were more obvious in the nodulated series; the nitrate plants were almost uniformly large and such differences were not so easily detected.

It would appear, then, generally, that in Soya bean water cultures, aeration exerts some inhibiting effect on the development of the root systems as compared with unaerated plants under similar conditions.

This inhibition may be due to a number of factors such as the possible toxicity of the air stream. The air was taken from the greehhouse in which samll amounts of coal gas might be present from an adjoining laboratory; this air source was latterly scrubbed through unglazed porcelain chips and filtered through cotton wool plugs before passing into the culture solution. This treatment did not have any effect on the development of the root systems. Alternatively, the mechanical agitation of the roots and the stirring up of solid particles of undissolved salts which coated the rootlets, may also have contributed to this inhibiting effect.

A further factor which probably exerts quite an appreciable effect on the culture of legumes in water is the pH of the solution. A preliminary test on the freshly made up Crone's solution gave a pH of 6·6-6·7, but, at the end of a month's growth, this had lowered to pH 4·4 in the nodulated cultures. The uninoculated jars remained longer at the original value, but at the end of two months, these had also lowered to the figure for the inoculated group. Aeration apparently affected only the rate of lowering of the pH; the cultures thereafter stabilised their pH at about 4·4.

These observations are somewhat at variance with those of Bryan (1922) who found that, in solutions of optimum pH for the Soya bean, the pH tended to remain steady at that figure during the growth of the plants. He, however, renewed his solutions daily and the cumulative effect of root acids may have been minimised in this way. Bryan does not give any figures for nitrogen fixation in his plants which were grown without aeration; in one series, the dry weights are given and these are comparable with the unaerated, inoculated plants in the present work. In photographic appearance, they appear to be more robust and healthy than plants of similar dry weight in the present work.

It is now necessary to account for the large differences in size and development between the nodulated and nitrate series of plants.

This is probably to be explained on the ground that the more easily assimilated nitrate source of nitrogen is of immediate value to the uninoculated seedlings, whereas, it was only after several weeks' growth that the benefit from fixation of nitrogen became apparent in the inoculated plants. It is suggested that the energy required for the development of nodules, in the one case, depleted the carbohydrate reserves of the seedlings - this resulting in a lag period of several weeks during which the bacteria in the nodules established themselves and began to transfer nitrogenous compounds to the host. A light nitrate manuring at this stage in the development of legumes in the field is known to be of considerable value and may possibly be explained on this hypothesis. On the other hand, the nitrate control plants, avoiding this initial expenditure of energy on nodulation, had a very favourable carbohydrate/nitrogen ration which resulted in the development of robust plants. There was no lag period observed in this case and the seedlings grew rapidly without interruption.

The effect of combined nitrogen on the growth of legumes has been discussed in terms of the carbohydrate/nitrogen ration by Wilson and his colleagues at Madison. Umbreit and Fred (1936) have suggested that, in plants of low carbohydrate content, combined forms of nitrogen are the most easily assimilable source, and that comparable inoculated plants with nitrogen derived from the fixation process, are inferior in dry weight and nitrogen content. Such an observation is applicable to the present work - the seedlings of both series started with the same carbohydrate reserves from the seeds, but in the inoculated plants the observed lag was obviously impeding the formation of new tissues, so that these legumes never overtook the nitrate control plants.

The difference in habit observed between the two series was also found by Wilson and Fred (1937) under different conditions. It is already noted that they obtained inferior plants at higher pO_2 values, and good plants at more normal oxygen tensions; they believe that, in the former case, an increased respiration is stimulated with a consequent depletion of carbohydrate reserves, resulting in poor vegetative development. A similar state of affairs probably exists in the case of the young inoculated seedling grown in nitrogen-free culture solution and without an added supply of combined nitrogen.

In conclusion, it appears to be clear that the unaerated, uninoculated plants, supplied with combined nitrogen, obtained adequate oxygen for the purposes of root development and vigorous growth, and the nodules of the inoculated plants require a higher supply of oxygen to sustain comparable growth.

Aeration of these latter cultures supplied this need in some measure and increased dry weight and fixation resulted.

Nevertheless, a comparison of the nitrate control plants and the nodulated plants show that the supply of fixed nitrogen from the nodules is definitely the limiting factor in the growth. An estimation and comparison of the respiration of root and nodule tissues should thrown light on this particular point.

SUMMARY.

1. Experiments are set up to investigate the growth and nodulation of Soya beans in water culture, and experimental methods for their culture under aerated and unaerated conditions are described.

2. A record of the growth progress of both inoculated and uninoculated plants is given, indicating the large differences in vegetative development between the two series. These differences are discussed in terms of the carbohydrate/nitrogen rationof the host legume and the special oxygen demands of the nodules.

3. Evidence for the beneficial effect of aeration on the functioning of the nodules and the general growth of the inoculated plants is shown together with a review of the variable results of other workers. Aeration is demonstrated to have little effect on the growth of the root systems of the nitrate plants.

4. Comments on the influence of the pH of the culture solution, the growth of legumes in oil covered jars and the occurrence and type of nodulation in water cultures are made.

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