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SOME ASPECTS OF SYMBIOTIC FIXATION OF NITROGEN.

Thesis presented by

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C O N T E N T S O F T H E S I S.

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

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

A great volume of literature is printed each year on symbiotic nitrogen fixation. Most of the published work has been concerned with the association of the root nodule bacteria with leguminous plants but in recent years some interesting work has been done on the symbiotic associations of non-legumes.

The association of the root nodule bacteria with leguminous plants has captured the imagination of many scientists during the past eighty years and today workers with varied interests devote their attention to this group. Botanists, Bacteriologists, Geneticists, Agronomists and Chemists all find this association a fruitful subject for study.

Botanists and Bacteriologists are interested in how this delicate equilibrium between plant and bacteria is maintained. Normally both the bacteria, which belong to the group Rhizobium, and the leguminous plant benefit from the association, the legume supplying the bacteria with carbohydrate and the bacteria, fixing nitrogen from the atmosphere, supplying the plant with nitrogenous compounds. However an upset of the equilibrium often results in the bacteria living parasitically on the plant.

Legumes have an important place in any system of agriculture, not only in Britain but throughout the world.

There is small need to stress the importance of leguminous plants to humanity since one of the basic ideas of modern agriculture is to find the best way to include them in the crop rotation. This of course has been stressed for a long time, but with the ever increasing world population there is an increasing demand for protein. It has been said that nations go to war for protein and the history of our century bears this out. In Europe a succession of dictators in several countries have turned covetous eyes on other countries where rich crops could be grown - especially crops of soya beans and other legumes with their high protein content.

The Natural Order Leguminosae is a very large order, distributed over most parts of the earth's surface, but is most abundant in warm temperate climates. It is well represented in Britain. It contains 10,782 species described as in 487 genera. Not all of these have been examined to see if they have nodules. Allen & Allen (1947) found that 887 species contained in 167 genera are reported as nodulated and 77 species of 17 genera as lacking in nodules.

The sub-order Papilionaceae is of supreme importance to the farmer and to mankind in general. It includes some of the most important fodder crops, many of the plants have a profound effect on other fodder plants and a number of seeds are utilised for human consumption. This is due to the plants being rich in

nitrogenous compounds and mineral constituents, particularly calcium and phosphates, and because they usually help to replenish the nitrogen in the soil.

To show how important this group of plants is in many of the world's major problems the following cases are worthy of notice:-

1. Permanent pasture. The establishment of permanent pasture at home and abroad was greatly advanced with the realisation of the part played by wild white clover, which is rich in protein, calcium and phosphate and provides nourishment to the grasses and humus to the soil. An ideal pasture contains 30% wild white clover and 70% grass with perennial ryegrass dominant. One of the virtues of wild white clover is its ability, aided by basic slag and potash fertilisers, to increase the production of high-class protein. It has been said that wild white clover has done more to increase milk production in recent years and to prevent a further reduction in the meat ration than any other single factor..

2. Marginal land. The problem of increasing the fertility of marginal land to make it more productive and so carry more sheep and cattle is one of the main agricultural problems in this country. Stapledon (1944) writes, "The cornerstone of land improvement in all parts of the world is the leguminous plant". After it has been established it is possible to effect startling grass-land

improvement, and this in its turn is the first step towards arable land. The problem of improvement then, he says, is the problem of finding the appropriate legume. In Britain he considers grassland improvement depends on wild white clover. In New Zealand hill pastures have been greatly improved by the use of yellow trefoil.

3. Dust Bowl in America. This problem of worn out land has persisted for a long time, contrary to what one would expect from our go-ahead cousins in America. They are now coming to the conclusion that the solution is a grass rotation, and many agronomists consider that the most important factor in the maintenance of the grass cover is the establishment of the appropriate legume.

4. Legumes in Australia. In Australia there are indications that the nitrogen in the wheat belt soils, originally some of the most fertile soils in the country, is being depleted. The Rural Bank of New South Wales has sponsored a study of the distribution of natural legume species and the possibilities of their development. Purchase, Vincent & Ward (1949) carried out this work and report that few legumes survive the cultivation practices of the district, so that very little contribution could be made to the soil nitrogen by them. They suggest planned sowing of legumes in the rotation since at present natural pastures are seldom introduced in the cropping system. They think that after re-establishment

sufficient nitrogen for one or two wheat crops might be fixed in two years by the legumes in an average natural pasture.

Some of the more important legumes with their uses can now be considered. Red and alsike clover are grown for their fodder value in short term pastures or alone. Vetches and lupins are used as green manure. They will grow on very poor sandy soil. In the poor soils of Central Germany lupins and ryegrass are grown in alternate years. This rotation was practised long before the function of the nodule was known. The lupins are fed steamed or along with oats to cattle. Kidney vetch and black medick grow well on poor calcareous soils where other plants would give a scanty crop. Trefoils are not very productive but are usually included in permanent grass mixtures for lighter soils because of their good quality and permanence. Lucerne is not a common crop in Scotland because even with the appropriate bacteria in the soil the temperature is a few degrees below its normal temperature range. In the South of England however lucerne is a valuable crop for animal feeding, giving three to four cuts of fodder every season. Lucerne, introduced originally from Spain, is the foundation of beef production in the Argentine and, of course, it is the alfalfa of America, one of the commonest leguminous crops grown there. Peas are grown for human consumption and for silage. Sainfoin is a valuable

fodder for growth on dry barren calcareous soils. It is a high-yielding crop and resists frost better than lucerne. Field beans are advantageous for animal feeding and in Scotland a great deal of attention is being paid to methods of improving the yield. Kidney and other beans are grown for human consumption and there is also a large number of varieties imported from abroad for cattle feeding. Fenugreek is a common flavouring material. Ground nuts are rich in oil and rate as a valuable export of some of Britain's smaller colonies. Soya beans are the second largest oil crop in America. As well as oil they contain more protein than an equivalent weight of best beef. Soya beans have been the basic foodstuff for thousands of years of the peoples of the Far East. A country which could grow sufficient soya beans could exist for a long time without dairy produce and meat. If it was possible to grow soya beans in large quantities in Britain and popularise their use our meat problems might be solved.

Some excellent reviews of the literature on symbiotic nitrogen fixation have been published. The earlier work is reviewed by Fred, Baldwin & McCoy (1932) and more recent work by Wilson (1940) and Allen & Allen (1950). There are also extensive summaries of work on specialised topics. In the last few years no very important advances in this study have been made comparable to the isolation of the root nodule organism, the

establishment of cross-inoculation groups and the observation that the bacteria differ in their nitrogen fixing powers. However a number of advances in certain aspects have been made which merit some consideration here.

1. Identification and function of the pigments in leguminous root nodules. Although the presence of the red pigment in the root nodules was noted soon after the organism was first isolated it is only recently that it has been investigated thoroughly. Pietz (1938) considered the red colour to be due to dopa (dihydroxyphenylalanine), a substance which occurs in other organs of legumes, and also in non-legumes. It is thought to be involved in the oxidation of tyrosine to melanin by the enzyme tyrosinase. This finding appears now to have been incorrect, since Kubo (1939) isolated the red pigment from soya bean nodules and identified it as a homoprotein analagous to haemoglobin. Virtanen (1945) and Keilin & Wang (1945) confirmed Kubo's results in identifying the pigment as haemoglobin. Virtanen also showed that it appears to be essential for nitrogen fixation since he noted that it is present only in effective nodules.

Two other pigments in the nodule have been identified, a green pigment and a brown one. Virtanen & Laine (1946) isolated and identified them as choleglobin and methaemoglobin respectively. They found that the brown

pigment seemed to be in equilibrium with the red pigment. In the root nodules of vigourously growing legumes on bright days and the haemoglobin content is high compared to the methaemoglobin. On cloudy days the brown colour deepens in the nodules as the methaemoglobin content increases. Keilin & Smith (1947) however could find no sign of methaemoglobin in the nodules which they examined. They suggest that the brown colour is due to an oxidation product of phenolic oxidases similar to that found in damaged plant tissues. Virtanen & Laine found that the green pigment is different from the brown pigment since after it has been formed no further fixation takes place.

The precise function of haemoglobin remains obscure. It scarcely seems possible that it acts as a carrier of oxygen, as in animals, since there is no provision for circulation in the nodule. Virtanen & Laine suggest that nitrogen fixation involves a cyclical change in the valency of the haemoglobin iron. Keilin & Smith think that it is unlikely that the fixation reaction could be catalysed by haemoglobin since it is a very ineffective catalyst.

Three pigments then have been identified in the nodule. The chemical nature of the red and the green pigment has been found but the nature of the brown pigment is still under consideration. Although the presence of the pigments is linked with symbiotic nitrogen fixation the actual part played by them is not yet known.

2. The chemistry of the nitrogen fixation process.

The search for the intermediate product of biological nitrogen fixation, defined as the end product of the fixation reaction and the initial reactant of assimilation, has been going on for a long time. Soon after the discovery of the process it was suggested to be ammonia, but no conclusive proof was advanced. Virtanen (1938) suggested that hydroxylamine might be the key intermediate product. He found that excretions from the roots of nodulated legumes contained aspartic, glutamic and oxalacetic acids and thought that hydroxylamine would be most likely to combine with oxalacetic acid to form aspartic or glutamic acid.

In the last few years considerable attention has been given to these two hypotheses. The main work in this field is being carried out by Virtanen and his collaborators in Finland and Wilson and his collaborators in America. The American workers are making valuable use of the isotope of nitrogen N^{15}. It is supplied in different compounds to Azotobacter vinelandii and Clostridium pasteurianum and then the distribution of the N^{15} in the nitrogen compounds and excretions of the bacteria is found. Although Azotobacter and Clostridium do not take part in a symbiotic association it is considered that nitrogen fixation in Rhizobium will take place along similar lines as in these free-living bacteria. Both groups of workers began to try to throw

light on the hypotheses by finding which amino acid is first formed.

The problem of the key intermediate product is closely associated with the identity of the primary amino acid formed. Virtanen (1947) and Burris & Wilson (1946) are in agreement that it is an amino-dicarboxylic acid, either aspartic or glutamic acid. It is difficult to determine which of the acids is first formed since the transamination reaction can take place so quickly that a change from aspartic to glutamic acid or vice versa might occur immediately the initial acid was formed. At present however the weight of evidence seems to be in favour of glutamic acid. Unfortunately these acids could be equally readily formed from ammonia or hydroxylamine and so although another step in the fixation process has been found it does not help to establish the nature of the intermediate product.

Burris & Wilson (1946) considered that all the evidence to that date supported the ammonia hypothesis. They found that small quantities of ammonia will completely suppress assimilation of free nitrogen by Azotobacter in contrast with the less rapid substitution by other compounds. In their most recent publication Zelitch, Rosenblum, Burris and Wilson (1951) claim to have obtained the first direct evidence that ammonia is the key intermediate in nitrogen fixation. Cells of Clostridium actively fixing molecular nitrogen were supplied with

N^{15} for a short time. Free ammonia was obtained from the supernatant liquid, while lower concentrations but still in excess of the average of either intact cells or supernatant medium were found in the amide fraction. Ammonia is therefore shown to be the key intermediate in fixation rather than a product of metabolic decomposition.

These results do not exclude hydroxylamine from the chain of reaction from molecular nitrogen to ammonia although it is now shown not to be the key intermediate.

It is probable that, although no proof of the ammonia hypothesis has been obtained for the root nodule bacteria, fixation occurs in the same way as in Azotobacter and Clostridium. Hence the end-product of the fixation is probably ammonia and the first formed amino acid either glutamic or aspartic acid while hydroxylamine may be a precursor of ammonia in the change from nitrogen to ammonia. The initial stages of nitrogen fixation still require some elaboration.

3. The effect of genetical factors on symbiotic associations of legumes. Genetical factors in both plant and bacteria have been found to influence the ability of a strain to infect the host plant and to fix nitrogen in association with it. They also influence the time of nodule production and the number of nodules produced. A number of workers have interested themselves

in this field. The most detailed research and analysis of results has been carried out by Nutman (1946 & 1949). In his work with genetically selected red clover and with commercial seed he showed that genetical variation occurred both in the bacterial strain and in the plant. Resistance to infection by host plants was infrequent. The resistance was inherited as a recessive in conjunction with a maternally transmitted cytoplasmic component. The plant also influences how effective the nitrogen fixing power of the bacteria will be. Nutman found ~~that effective response is dominant over ineffective response. The latter is due to a single homozygous recessive gene.~~ These are highly specific to a given bacterial strain. He also found a response due to complex genetic factors which was not specific in acting with only one bacterial strain. The observations of Nutman and other workers indicate that the response of individual plants in a commercial sample of clover seed to a given strain of bacteria would be very varied indeed.

4. The effect of bacteriophage on rhizobia. The existence of bacteriophage in the soil active against rhizobia has been known since 1908. It is only in recent years that the effect of 'phage in agricultural soils has been considered. In a number of papers published from 1933-47, Demolon & Dunez have suggested that 'alfalfa fatigue' is due to the action of 'phage on alfalfa rhizobia in the soil and in the nodule. They consider (1947) that clover fatigued soils are caused by another 'phage but the symptoms are less noticeable and the trouble spreads more slowly. They do not consider that peas, beans/

beans, lupins and soya beans are attacked by 'phage to the same extent. Other workers in America, Italy and Russia have supported most of these findings. Kleczkowska (1945) however thinks it unlikely that 'phage can directly cause the failure of leguminous crops by destroying all the bacteria in the soil or in the nodule. She showed that only a small proportion of strains of pea or clover rhizobia were susceptible to the strain of 'phage studied and that rapid development of resistant strains took place. It seems unlikely therefore that failure in clover and pea crops in this country could be due to bacteriophage.

5. Development of a serological technique for identifying strains. Although the value of serological reactions for identifying strains of rhizobia has been realised for a long time, it is only recently that improved agglutination techniques have allowed a wider use to be made of these criteria for classifying and identifying strains of rhizobia both in the laboratory and in the field. (Vincent, 1941, and Kleczkowski and Thornton, 1944). Unfortunately no relationship between serological constitution and effectiveness of the organism have been found. One advantage of using the antigenic properties to identify strains is the stability of this character as compared with other characters used for identifying them. (Purchase, Vincent & Ward, 1951).

The serological technmique is being used at present in a very extensive survey on the establishment, by

inoculation, of effective strains of clover rhizobia in hill pastures in Great Britain (~~Thornton &~~ Read, 1943).

6. Seed Inoculation of Legumes. The transfer of soil from a field which had carried a leguminous crop to one in which a similar crop was to be planted was a common practice before the function of the nodule bacteria was even thought of. Soon after the isolation of the bacteria Nobbe & Hiltner (1896) used a pure culture of the bacteria to inoculate seed instead of the more costly soil transfer method. Since then seed inoculation has caught the public's fancy from time to time in various countries and the popularity of seed inoculation has risen and fallen according to the success or failure of the particular culture used. In spite of this the proof that seed inoculation is an advantage is still lacking for many crops. Thornton (1929) showed that although inoculation of lucerne was beneficial in certain areas of England in other areas no benefit was obtained. He noted that field trials would be necessary in each area before the extent of the benefit of seed inoculation could be measured. Nicol & Thornton (1941) stressed the importance of selecting a strain of bacteria for seed inoculation which, as well as being effective, will be dominant in competition with other strains.

Trials are now being held for various crops. The work of Thornton & Read with clovers has already been mentioned. Field experiments on the effect of inoculation

of field beans are being carried out in various parts of this country, with varying degrees of success. Some possible explanations for disappointing results obtained by seed inoculation will be suggested later in this thesis.

In Sweden and Denmark inoculum for most legumes is supplied in quantity by the Agricultural Colleges direct to the farmers. In the Netherlands, Gerretsen (1950) reports only partial success from the inoculation of legumes. In Finland, Virtanen (1933) concluded as a result of his field trials that although the benefit of inoculation is less with clovers than with peas it should always be carried out when legumes are sown. In America commercial supplies of cultures have been on the market for a long time but experimental stations there are still publishing results of trials which aim to show the benefits or otherwise of inoculation.

To sum up, inoculation has been shown to be beneficial in most cases where the legume is introduced to a field for the first time. In fields where the legume has already been grown the presence of native strains of the organism in the soil make results difficult to interpret and they are bound to vary from place to place depending on the effectiveness of the native strains already in the soil.

7. Molybdenum and nitrogen fixation. The discovery of an element essential, even in small quantities, to plants is always of prime importance. Molybdenum has been shown to be essential for the nutrition of some plants

(Arnon & Stout, 1939), and the list of plants shown to require molybdenum steadily grows. Azotobacter, a free-living nitrogen-fixing bacterium has been shown to need molybdenum in its culture medium (Bortels, 1930), and later Burk & Horner (1936) showed that molybdenum was necessary for the nitrogen-fixing process and suggested that it probably acts as a 'specific catalyst'. In 1937 Bortels obtained increased nitrogen fixation with peas, clovers and soya beans in sand culture by the addition of molybdenum. Since then, workers, too numerous to be detailed here, in Russia, Tasmania, Australia and France have been successful in obtaining increased growth by the addition of small quantities of molybdenum to legumes in the laboratory or in the field. Anderson & Thomas (1946) proved that molybdenum was essential for symbiotic nitrogen fixation with results obtained by the study of the addition of molybdenum to various legumes and grasses in pot culture.

The fertilising effect of molybdenum on legumes is now of great interest, particularly in Australia and Tasmania where there are areas in which the soil is low or deficient in available molybdenum and startling results have been obtained by the addition of a few ounces of molybdenum per acre. In other countries increased yields have also been obtained but they are not so marked as the Australian ones.

Virtanen (1948) suggests that the function of molybdenum is to act as a catalyst in the reactions which cause nitrogen to be changed to ammonia.

8. Industrial utilisation of biological nitrogen fixation. The artificial fixation of nitrogen for industrial purposes was a problem at the beginning of the present century but is now a common industrial operation. The three principal methods are as follows:-

1. The manufacture of calcium cyanamide. Calcium carbide is heated in atmosphere of nitrogen to 1000°C when calcium cyanamide is formed.

2. The Haber process. A mixture of nitrogen and hydrogen in the proportion of 1:3 is passed over a catalyst at a pressure of 300 atmospheres and a temperature of 500°C. 10% of ammonia is obtained.

3. The Birkeland-Eyde process. A mixture of nitrogen and oxygen when raised to a high temperature is in equilibrium with nitrous oxide. At a temperature of 3000°C, 5% nitrous oxide is obtained. This can be removed and used to manufacture nitric acid. The high temperature is obtained by means of an electric arc.

The first and third processes are suitable where electric power is cheap and easily obtained and hence are found in operation at Niagara Falls, Norway and the Alps. The second process requires comparatively little power and so is worked to an increasing extent in Britain and Germany.

Although the Haber process requires little power as compared to the other two processes the amount used is in actual fact, considerable, especially in present-day circumstances with the high cost of power and the increasing

shortage of all sources of energy such as coke, coal or electricity.

The possibility of using biological nitrogen fixation on a commercial scale is now being considered (Pearsall & Fogg, 1951). Free-living nitrogen-fixing bacteria such as Azotobacter or Clostridium or some of the blue-green algae, which also fix nitrogen, could be used. Both the bacteria and the algae require some form of energy. The former require carbohydrates and the latter require light. To obtain a suitable carbohydrate which was not in short supply might present a difficulty in the case of the bacteria, and with the algae the source of light and its arrangement might prove awkward. Further difficulties would be met in the actual extraction of the nitrogenous compounds from the plants or their growing medium. However if fuel costs increase it may be that large quantities of nitrogen will be fixed from the atmosphere for the use of man by the harnessing of the nitrogen fixing bacteria or the blue-green algae.

9. Symbiotic nitrogen fixation in non-legumes. Because of their direct importance to agriculture far more attention has been paid to leguminous plants than to plants of other families regularly showing root nodules and known or suspected to effect fixation of atmospheric nitrogen. Native examples of such plants are Alder (Alnus spp.), Bog Myrtle (Myrica gale) and Sea Buckthorn (Hippophäe rhamnoides). The occurrence of extensive fixation of

nitrogen in Alder has been established by the work of Hiltner (1896), Roberg (1934) and Virtanen & Saastamoinen (1935), and in Bog Myrtle by Bond (1949 and 1951). The observations of Servettaz (1909) suggest that there is also fixation in Hippophäe.

The fixation effected by these non-leguminous plants, though not of direct agricultural importance, is likely to be of considerable ecological significance, especially in the instances of Alder and Bog Myrtle which are locally abundant plants in this country. By the movement of drainage water the nitrogen fixed by these plants will eventually be carried to other areas with resultant increase in fertility.

The identity of the lower symbiont in the non-leguminous root nodules has been the subject of much debate, different investigators concluding the organism to be fungal, actinomycetal or bacterial in nature. These difficulties are to a considerable extent due to the inability of any investigator to make a satisfactory isolation of the endophyte.

The small contribution provided by the present thesis to the ever-widening field of research on leguminous root nodules is based on work with two plants, clover and field bean. It is presented in the following sections:-

SECTION I.

The occurrence of ineffective strains of the field bean nodule organism and the relation to crop yield.

SECTION II.

The multiple infection of clover and field bean plants by strains of the nodule organism in the field.

SECTION III.

Some aspects of nodulation of field beans grown in water culture.

SECTION IV.

The variation in growth of individual clover and field bean plants associated with a given strain of the nodule organism.

SECTION I.

The Occurrence of Ineffective strains of the Field Bean Nodule Organism and the Relation to Crop Yield.

C O N T E N T S.

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

Although the presence of root nodules on a leguminous plant is, in the typical condition, associated with benefit to the nutrition of the plant, it has been known for a long time that sometimes the nodules have little or no beneficial effect. This is the case when the nodules contain a so-called ineffective strain of the organism. In such nodules fixation of nitrogen is very small or completely absent. Nobbe and Hiltner (1893) inoculated peas growing in a nitrogen-free medium with several pure cultures of the nodule bacteria, and observed that with one culture although nodules were formed the plant made little growth. Dehérain and Demoussy (1900) found that the nodules on white lupin varied in the benefit bestowed on the host plant. In later years these observations were confirmed and extended by more intensive investigations. Wright (1925) with soya beans, Stevens (1925) with lucerne and sweet clover, and Helz, Baldwin and Fred (1927) with peas, showed conclusively that different strains of the nodule organism varied in their ability to benefit a given host plant. Leonard (1930) suggested that in addition to completely effective and completely ineffective strains, others of intermediate type were also to be found. In more recent years there have been reports of strain variation in the bacteria associated with most of the common leguminous plants.

The present writer is here concerned with the extent of the occurrence, in the field, of ineffective strains of the organism associated with a particular legume (field bean, botanically Vicia faba L.), and with the effect of such strains on crop yield. It will, however, assist in the consideration of those aspects if a brief statement is made here relating to the problem of explaining why nodules induced by ineffective strains fix little nitrogen and thus contribute negligibly to host nutrition. The present state of knowledge on this question may be summarised as follows:-

1. Characteristics of ineffective strains in pure culture.

The most important difference between effective and ineffective nodules, namely, the difference in nitrogen-fixing powers, cannot be directly elucidated by study of the organisms in pure culture, since according to the most critical studies, fixation, even in the case of effective strains, is only shown when the organism is actually associated with the host plant, in the nodule. No cultural, biochemical or physiological characteristic of the bacteria in pure culture has been found to be correlated with the ability to aid the host plant (Allen & Allen, 1950).

2. The study of ineffective nodules.

Generally speaking, ineffective nodules are smaller and are to a greater extent scattered over the root system than effective nodules, which occur chiefly on the tap-root and the first-order laterals. They are usually white internally, contrasting with the red colour of effective

types[x]. The activity of the meristematic zone in the ineffective nodule is usually arrested at an early stage. The amount of bacterial tissue that is formed is relatively small and is short-lived.

Some investigators have failed to find bacteroid forms of the organism in ineffective nodules, e.g. Nobbe and Hiltner (1893), Virtanen (1945), and have regarded this as a critical factor in the explanation of the absence of appreciable fixation. In addition, Virtanen (loc. cit.) obtained evidence of the presence of a slime layer round the bacteria in ineffective nodules of pea which seemed likely to interfere with their development. In contrast to these reports, Chen and Thornton (1940) and Thornton (1945) observed the presence of bacteroids in ineffective nodules of clovers and peas. Chen, Nicol and Thornton (1940) obtained evidence of the formation, within root tissue bearing ineffective nodules, of a substance inhibitory to the growth of the bacteria.

In recent years attention has been concentrated on the difference in colour, noted above, between effective and ineffective nodules, following on the finding that the red colour of the former is due to haemoglobin. So far, however, there has been little advance beyond confirming, by accurate measurement, that while haemoglobin is present in appreciable quantities in effective nodules, it is in

[x]Not all these statements on the difference between effective and ineffective strains appear to be true of field bean nodules (see Section III and IV).

small amount or absent from the ineffective type. There thus appears to be a definite association between the presence of the pigment and the occurrence of fixation in the legume nodule, and Virtanen (1948) has suggested possible functions for the haemoglobin in the fixation process, but Smith (1949) was unable to obtain experimental support for Virtanen's suggestions. The evidence for a causal connection between the presence of the pigment and the occurrence of fixation remains chiefly circumstantial, and it cannot be definitely concluded that the absence of haemoglobin from ineffective nodules is the main reason for the lack of fixation.

3. The role of the host plant in ineffectiveness.

So far in this review, ineffectiveness has been treated as though it was determined solely by the properties of the organism. It is necessary now to refer to evidence indicating that host plant factors are also involved. Thus Helz, Baldwin and Fred (1927) showed that strains of the organism which were ineffective with one genus of legumes were effective with a different genus within the same cross-inoculation group. Subsequent workers have found similar differences when different species of a given host plant genus were employed, and also different varieties of a particular species. Nutman (1946) took the matter a stage further by showing that in Montgomery red clover the effectiveness of the association with a particular bacterial strain varied markedly between

individual plants, and was subject to genetical control. The discussion of these results raises the question of the precise part played by the host plant in the symbiosis and in the fixation. Depending on the role ascribed to the host, the above results could be interpreted as due to variations in the environment provided by the host cells for the bacteria, or to a failure on the part of some host plants to supply some essential part of the fixation mechanism.

It is clear from the evidence reviewed under these three headings that no final explanation of ineffectiveness is yet possible. The simple view that it is due to an inherent impotency in fixation on the part of certain strains of the nodule organism is not tenable. The view cannot be excluded that all strains of the organism are inherently nitrogen-fixers, given the ideal host plant.

More immediately relevant in the present connection will be a review of the literature relating to the occurrence of ineffective strains of the nodule organism in the soil. It is difficult, in view of the great practical interest of this aspect, to account for the lack of published investigation. One possible reason for this is the general belief, outside specialist circles, that the mere presence of nodules on a leguminous crop plant indicates that a satisfactory symbiotic relation has been set up. Another reason may be that in countries where the inoculation of legume seed is a common practice, the

investigation of the strains native to the soil may not be considered very important.

Leonard (1930), after a study of the nodule bacteria from Austrian winter peas growing in Louisiana, U.S.A., concluded that the poor cropping of the plants was due to a prevalence of ineffective strains of the [illegible] organism. Thornton (1934) reported that ineffective strains of the clover organism were common in upland pastures in several localities in Wales where poor growth of plants had been observed. Umbreit (1944) made a survey of soya bean bacteria in areas of Wisconsin. No details of the number of strains examined were given but he concluded that one quarter of the strains were 'good', one half 'fair', and one quarter 'poor' nitrogen fixers. Subsequent to his Welsh survey, Thornton commenced a much larger survey of clover organism strains collected from most districts of Great Britain. In a preliminary report (Thornton, 1946) results were given in respect of 463 strains. Of those originating from South, Central and East England, 86 per cent. were effective, 5 per cent. intermediate, and 9 per cent. ineffective. Strains obtained from Scotland, Wales, North and West England showed 60 per cent. effective, 12 per cent. intermediate, and 28 per cent. ineffective. The ineffective strains, which were thus more prevalent in the North and West of Great Britain, were mostly from hill-pastures, but so far, it was stated, no correlation between the occurrence of the ineffective strains and any particular

soil type has been found. It was also indicated that the possibility of inoculating hill-pastures with effective strains of bacteria deserved consideration.

In a local survey which was to some extent associated with that undertaken by Thornton (see above), Bond tested (on red clover) 300 native strains of the nodule organism isolated from plants of red and white clover collected in the West of Scotland. The following results were obtained[x]:-

Effective strains...36 per cent. of total number of strains.
Intermediate strains34 " " " " " " "
Ineffective strains.30 " " " " " " "

When the strains were separated according to their origin into those isolated from plants growing in cultivated soils (good pasture, hayfields, cornfields) and those from essentially uncultivated areas (such as hill-pastures), the following differences emerged:-

	% Effective	% Intermediate	% Ineffective
Cultivated	43	38	19
Uncultivated	24	27	49

It is seen from this analysis that the further segregation of strains yields a still higher proportion of ineffective strains than the over-all figure obtained by Thornton for the North and West of Britain, since almost 50 per cent. of

[x]Unpublished data communicated to the present writer by Dr. G. Bond.

the strains from uncultivated areas were found to be ineffective. The explanation and practical importance of these results are still under consideration.

In the above survey the strains, although collected from both red and white clover plants, were all tested for effectiveness with red clover as host. Bond and McGonagle (1951) have shown that the performance of a given strain is usually similar whether it is tested on red or on white clover. That it would be unsafe to adopt the same procedure with strains collected from a wider range of Trifolium species is illustrated by the work of Purchase and Vincent (1949), in which isolations from red, white, ball and subterranean clover were examined. Of 233 isolations tested, 42 per cent. were ineffective on red and white clover, but on ball clover only 3 per cent. and on subterranean clover only 6 per cent. were ineffective.

It is seen from the above review that although the amount of investigation devoted to the matter has been very limited, evidence of a quite frequent occurrence of ineffective strains in the field has been obtained, though the full practical importance of this has still to be assessed. Since the existing information applies almost entirely to clover, it seemed desirable that surveys of other leguminous crop plants should be undertaken, and the Field Bean (Vicia faba L., variety) was selected by the

present author in the first place because it is a widely-grown crop plant in Britain, and is common in the West of Scotland. War-time conditions caused an increase in the acreage of field beans grown in Scotland as a whole from 3,600 acres in 1940 to a peak of about 10,000 acres in 1944. The acreage declined to 6,000 acres in 1946 and has remained fairly stable since then. In the West of Scotland alone 2,420 acres of this crop were grown in 1951[x]. The field bean is an important crop not only because of its value as a high-protein foodstuff but also because, like other legumes, it raises the nitrogen status of the soil. The high protein content together with the nature of its mineral constituents makes bean meal a valuable cattle-food. It is essentially a muscle-forming food, and is fed chiefly to stock of mature age since they are more able to digest it. Quite frequently the beans are grown in Scotland with oats as a mixed crop ("mashlum") since in this way the growth of weeds is suppressed to a greater extent, while the mixed straw is a better fodder than bean straw alone. The grain from this mixed crop can be used together or separately.

Despite the present need for replacing imported protein-rich cattle cake by home-produced protein foodstuffs such as field beans, there is, as noted, no tendency to an increase in the number of acres under cultivation.

[x]The acreage data for field bean were provided by the Statistics Branch, Department of Agriculture for Scotland.

This may be due to the reputation gained by the field bean as being a somewhat unpredictable crop, in extreme cases a total loss. This is a second reason why an investigation of the native strains of the nodule organism of field bean seemed desirable, since the possibility existed that these difficulties in cultivation were partly due to ineffectiveness of the bacteria.

The procedure adopted has in general resembled that used in previous surveys, namely, to make isolations of the organism from the nodules of field bean plants collected from the field, to re-inoculate them into field bean plants grown under bacteriologically and culturally controlled conditions in the greenhouse, and to assess effectiveness on the basis of plant growth. The isolations were made during 1949 and were tested during 1950 and 1951.

M E T H O D S

1. Methods available for assessing effectiveness in nitrogen fixation.

It has been noted above that although strains of the nodule organism of the same cross-inoculation group frequently differ from each other in their cultural appearance and serological behaviour, no correlation has been found between these features and the ability of the strains to benefit the host plant. It is therefore necessary, in the present state of knowledge, to assess the effectiveness of a strain while it is in association with the host plant. As noted already, the usual method has been to inoculate a strain on to the host plant growing under controlled conditions, without access to combined nitrogen, and to assess effectiveness on the basis of plant growth. This method has been adopted in the present investigation.

It seems possible that in future surveys some use could be made of the difference in colour which, as already noted, exists between effective and ineffective nodules. Nicol and Thornton (1941) used this method for distinguishing between nodules produced by known effective and ineffective strains inoculated into soya bean plants growing in sand culture. Effective nodules were red or olive-green in colour and soft in the centre, while ineffective nodules were pale green or white, and hard in the centre.

An examination of this type carried out on the inoculated test plants might provide a satisfactory basis for assessment of effectiveness of isolations, instead of determining dry weights of plants. Or a still greater saving of time would be achieved by examining the nodules on the original plants in the field. Among the difficulties that would be encountered in obtaining any reliable information along these lines might be (1) the unstable nature of the red pigmentation in effective nodules, since this tends to change to green and brown pigments if the plants are kept in darkness for a day or more, similar changes being shown in old nodules, and (2) there would be risk of confusion between young effective nodules, not yet fully pigmented, and ineffective nodules. Greater precision might be obtained by actually measuring the haemoglobin or haematin content of the nodules (Virtanen, Erkama & Linkola, 1947), but the difficulties just mentioned might still be encountered.

2. Collection of plants.

Attention has been concentrated on seven selected fields situated in various parts of the West of Scotland. These fields were under beans in 1949 and had carried the same crop plant in previous years.

Fifteen to twenty plants were collected from each field and taken to the laboratory. This collection was made when the crop was in pod, so that it was possible for the degree of success attained by the crop in 1949 to be assessed for each field. In some fields there was an average of only one pod, containing one or two seeds, per plant. Such a crop was classed as 'bad' and the farmer would probably not bother to harvest a crop of so poor a yield. In other fields there was an average of six pods, each containing about four seeds, per plant, and such a crop was classed as 'good'. In addition to the quality of the crop standing on the fields in 1949, information as to previous crops was obtained from the farmers' records and was taken into account in classifying the fields. The final classification arrived at is shown in Table 1.

In the laboratory one nodule was selected at random from each plant, and an isolation made of the organism by the method described below.

3. The isolation of the organism.

This was achieved by first cleaning the nodule by brushing it in water, and then sterilising it externally by shaking it for 5 minutes in acid mercuric chloride ($HgCl_2$ 1 gm., concentrated HCl 2.5 ml., distilled water 500 ml.). The nodule was then shaken with six successive portions of sterile water and subsequently crushed in a drop of sterile water and the suspension plated on yeast-mannitol agar, prepared according to Fred, Baldwin and

Table 1.

Description of fields from which isolations of the nodule organism were made.

Field	County	Soil type	Average type of crop (see text)
A	Renfrewshire	Heavy clay	Good
B	"	Loam, with ash	Very bad
C	Lanarkshire	Medium loam	Good
D	Renfrewshire	Medium loam, thin	Bad
F	Perthshire	Medium loam	Fairly good
G	Ayrshire	Clay loam	Bad
H	Wigtownshire	Medium loam	Good

McCoy (1932). After a few days' growth the organism was transferred to slopes of the same medium.

4. Method of growing the test plants.

(a) Reasons for adopting water culture.

A technique to be suitable for the present purpose must satisfy the following conditions: (a) it must provide sufficient bacteriological control to ensure that a strain of the nodule organism inoculated into one set of test plants does not spread and contaminate other plants inoculated with a different strain; (b) it must prevent any access by the test plants to sources of combined nitrogen; (c) the experimental arrangement must permit of reasonably normal growth by the test plants where these are associated with effective strains.

In the case of small-growing legumes, such as clover, the method of growing the plants aseptically and totally enclosed in plugged test tubes, on an agar rooting medium, meets the above requirements, except that the arrangement imposes a rather severe limitation on plant growth. A total enclosure method is obviously impracticable with legumes of large growth habit, such as the field bean, and all that can be attempted is to secure bacteriological control over the root system and the rooting medium. Methods of water culture or sand culture are obviously indicated for this purpose. At one period water culture was considered unsuitable for nodulated legumes, but in more recent years it has given quite satisfactory results

in the hands of various investigators, e.g. Brenchley & Thornton (1925), Virtanen & von Hausen (1936), and Bond (1950). In the present work water culture has been adopted in preference to sand culture for the following reasons:-

(1) it provides for easier bacteriological protection of the root system and medium, at least when compared with sand cultures in open pots;

(2) it facilitates close control over the mineral nutrition of the plants, and also over pH if necessary;

(3) the root systems can be inspected at any stage in development;

(4) with sand culture it is essential to sterilise the sand that is used, involving much labour and transportation of pots, whereas in water culture, provided that distilled water is used and care is exercised, it is possible to dispense with the sterilisation of the culture solution.

(b) Raising young plants for transfer to water culture.

Commercial seed of a variety of field bean known as Spring Horse Bean has been employed in the investigation. The seeds actually sown were selected on the basis of freedom from damage or abnormality, and also on the basis of weight. In 1950 seeds weighing between 0.55 and 0.70 gm. were used, and in 1951 those between 0.65 and 0.80 gm. The seeds were surface-sterilised by being shaken in absolute alcohol for 2 minutes, and then

in 0.1 per cent. mercuric chloride for 6 minutes, after which they were shaken with six successive portions of sterilised water and left to imbibe over-night in further sterilised water.

The imbibed seeds were sown in large troughs containing moistened sterilised sand and allowed to grow for about 17 days until the first two leaves were expanded. The young plants were then carefully removed from the sand, with precautions to avoid contamination with the nodule organism, and the cotyledons excised. The reason for this last operation was that the cotyledons still contained a considerable food supply which would delay the development of differences in growth between plants associated with effective and ineffective strains respectively. The decotylated plants were set up in water culture as described below.

(c) The culture vessels and the solution. Inoculation.

Glass jars of capacity 2½ litre were employed, fitted with waxed teak or cork tops slotted for three plants, with a central hole for use in changing the solution and in adding water. The jars were wrapped in thick brown paper to exclude light.

Crone's solution made up according to the original formula (nitrogen-free version) was prepared as follows:-

KCl	7.5 gm.
$CaSO_4 . 2H_2O$	5.0 "

$MgSO_4 . 7H_2O$	5.0 gm.
$Ca_3(PO_4)_2$	2.5 "
$Fe_3(PO_4)_2$	2.5 "
Distilled water	10 litres.

To the above, 10 ml. of Hoagland's A - Z solution (with molybdenum included) was added, this solution being prepared according to Templeman (1941). In view of the large quantities required the Crone's solution had to be prepared in advance and was stored in large covered glass tanks painted black to exclude light. The culture solution was not autoclaved. When required for us the solution was thoroughly stirred and then siphoned into the culture jars.

As noted above, three plants were set up in each jar. Three days later 3 ml. of a suspension of the bacteria (prepared by taking up the growth from a two-day old slope of a nodule organism isolation in 10 ml. of water) was added to each jar, except in the case of the uninoculated control jars. Two jars were set up for each isolation.*

* On account of the large space requirements of field bean plants it was only possible to test about twenty isolations at one and the same time, so that a succession of tests had to be carried out spread over the 1950 and 51 growing seasons. In each test, six jars of uninoculated control plants were included.

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really aseptic conditions. The culture jars, aspirators and storage tanks were rinsed and swabbed with methylated spirits, smaller items of glassware being autoclaved or boiled in water. Teak and cork tops were immersed in boiling paraffin wax, and metal-ware flamed with methylated spirits.

(d) Subsequent management of the water cultures

The culture solution in the jars was changed at three-weekly intervals by siphoning it out of the jars, the latter being then re-filled from the storage tanks. As the plants became larger it became necessary to add additional water to the jars each day.

The initial pH of the culture solution was 6.2. Assuming that the pH relation in water culture is similar to that in other rooting media, the above pH value is well-suited to the growth of field beans, since the optimum pH for this crop in the field is generally reckoned to be 6 to 6.5, while for sand cultures Edinburgh and East of Scotland Agricultural College workers (1948) reported that pH 6.5 was optimal for both growth and nodulation. In the present experiments as the plants became larger they exercised a depressing effect on the pH of the solution, which sometimes fell to as low as 4.7 by the end of the three week period. Owing the risk of contamination which frequent adjustment of pH in the jars would have entailed, this was not attempted, but in any case no adverse effect

of the low pH periodically attained was observed. It may be recalled that Bond (1950) observed with Soya bean in water culture that after the period of maximum formation of nodules was past, nodulated plants grew well at relatively low pH.

The greenhouse in which the plants were grown was heated until the end of April, but during the remainder of the growth season (which extended altogether from March to October) it was unheated. Whenever possible a daytime temperature of 65° to 70°F. was maintained. The position of the jars on the bench was frequently changed. The plants were supported by means of canes secured by string to the sides of the jars. Fig. 1 shows part of the greenhouse with the plant cultures in position.

It is possible that forced aeration of the culture solution would have benefited growth to some extent, but it was not attempted in view of the risk of contamination that it would have involved. As a matter of fact, Bond (1950) found no consistent benefit of forced aeration on the growth of nodulated plants of Soya bean in jars of the same type as were used here.

The plants were grown for 10 weeks in water culture, by which time there were well-marked differences between plants inoculated with different bacterial isolations. The plants were then harvested, their heights, number of flower trusses, type of nodulation noted, and the three plants from each jar dried together to constant weight at

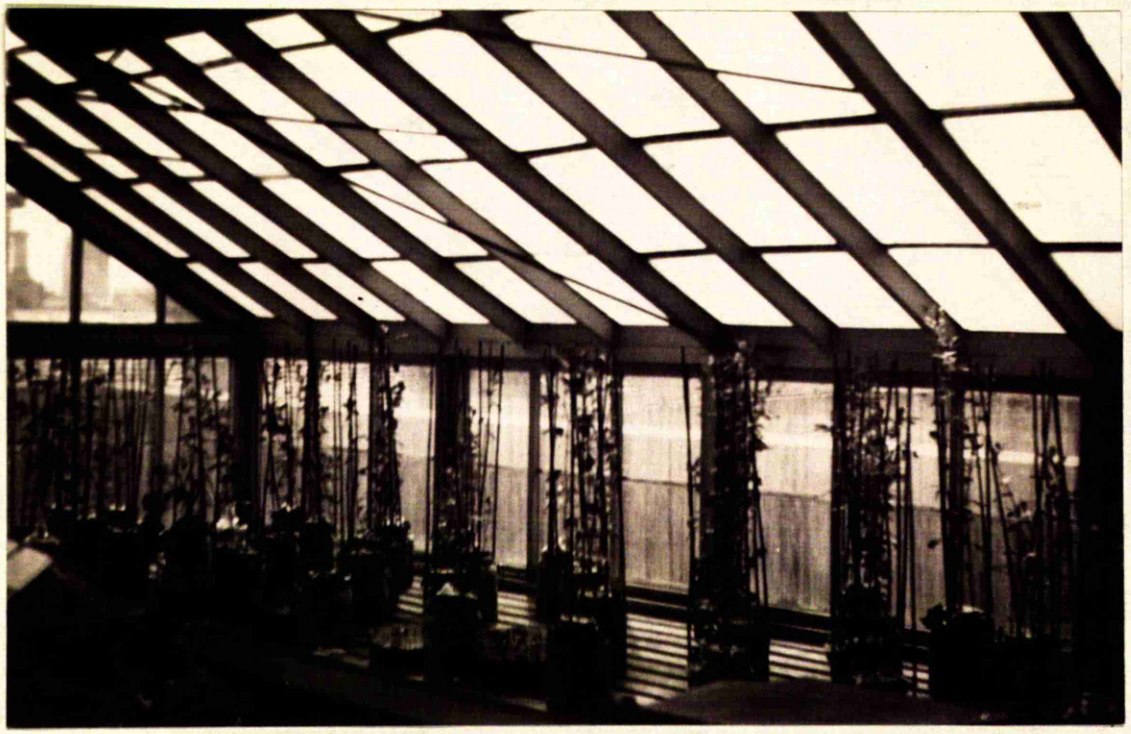

Fig. 1. A corner of the greenhouse showing some of the plants.

110°C.

5. Basis for assessment of effectiveness.

Various methods have been adopted for comparing the effectiveness of different strains of the nodule organism inoculated on to test plants as in the present investigation. Bond and McGonagle (1951) assessed effectiveness by comparing the nitrogen content of nodulated plants grown on nitrogen-free medium against that of non-nodulated plants supplied with an ample amount of combined nitrogen. This involves growing the extra non-nodulated plants and on account of space considerations was not possible in the present case. In Section II of this thesis, relating to clover, a particular effective strain was taken as standard, and the performance of the remaining strains compared against this. It was the original intention to follow this procedure in the present case, but the particular strain intended to serve as standard was found by experience to be insufficiently consistent in performance for this arrangement to be continued. Instead a procedure similar to that followed by Purchase and Vincent (1949) was adopted, effectiveness being assessed by expressing the dry weight of the plants associated with a particular isolation as a multiple of the dry weight of the uninoculated control plants (in nitrogen-free solution) grown at the same time. For the purpose of this calculation the dry weight of the controls was assigned an arbitrary value of 10 units, in order to avoid the use of decimal points in what will be

termed the '<u>effectiveness value</u>'. A typical calculation is as follows:-

Mean dry weight per jar of three nodulated plants (based on the examination of duplicate jars) associated with a particular isolation = 5.79 gm.

Mean dry weight per jar of three uninoculated control plants (based on the examination of six replicate jars) = 1.38 gm.

<u>Effectiveness value</u> for the isolation in question

$$= \frac{5.79}{1.38} \times 10 = \underline{42}$$

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

Under the growth conditions employed, inoculated plants developed nodules 2 weeks after inoculation. Details of the types of nodulation shown by the plants are provided in Section III of this thesis. The onset of fixation, indicated by some of the nodulated plants beginning to show superior growth to the control plants, was first in evidence 4 weeks after inoculation. Plants associated with effective strains were of healthy appearance, though their dry weight was somewhat reduced and their height increased by the proximity of other buildings near the greenhouse. The growth of plants associated with certain strains, obviously of ineffective type, was very inferior and resembled that of the non-nodulated control plants, the stature of the plants being small and the leaves showing chlorosis and premature abscission. Figs. 2 - 4 show photographs of typical plants[x].

There is good evidence that the precautions taken to secure the necessary bacteriological control were adequate. Thus over the whole strain-testing period thirty un-inoculated control jars, each with three plants, were set up. Only in three of these jars were any nodules formed, the total number of plants affected being four. Two of these contaminated jars, with three of the affected plants,

[x]The ineffective strain HX shown in Fig. 3 was isolated by Virtanen from pea plants and is effective on that plant but has proved ineffective on field beans.

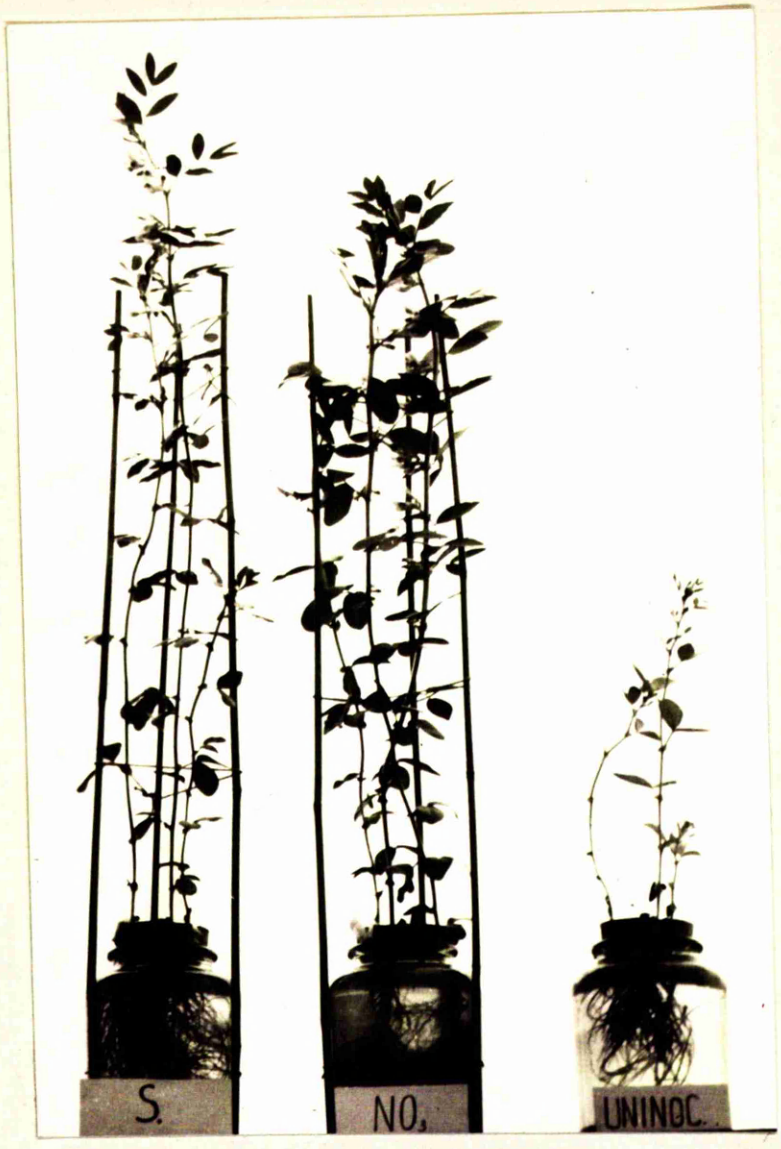

Fig. 2. Field bean plants after ten weeks growth. ($x\frac{1}{12}$)

Left. Nodulated plant, effective strain.

Centre. Non-nodulated plant, supplied with sodium nitrate.

Right. Non-nodulated control plant.

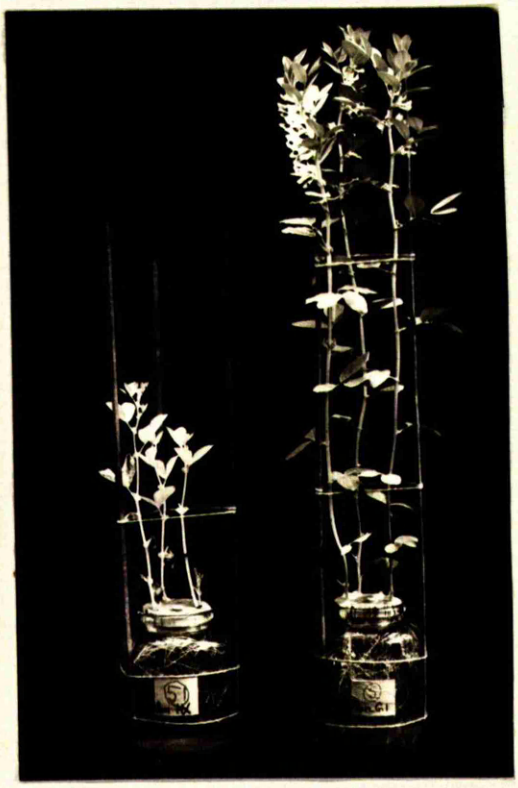

Fig. 3. Nodulated field bean plants after ten weeks growth. ($x \frac{1}{10}$)

Right. Inoculated with effective strain.

Left. Inoculated with ineffective strain. (strain HX - see text).

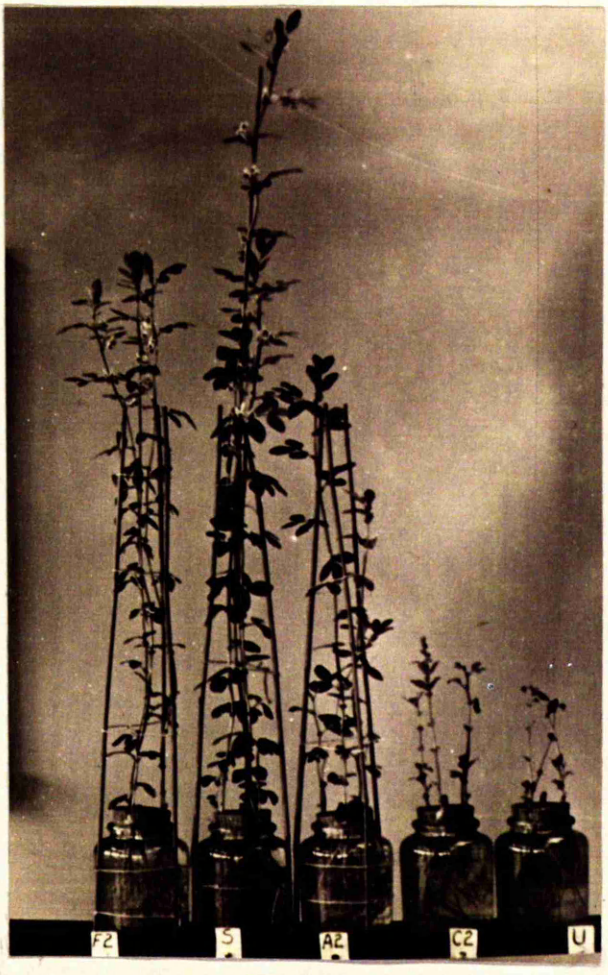

Fig. 4. Field bean plants after ten weeks growth. ($\times \frac{1}{10}$)

In order from left:
Nos. 1 & 2. Effective.
No. 3. Intermediate.
No. 4. Ineffective.
No. 5. Non-nodulated control.

were in the first batch of test plants, so that in the remaining batches there was only a single contaminated control plant. In view of the special difficulties attached to working with large legumes, and of the wide dissemination in soil, dust etc. of organisms of the pea-bean cross-inoculation group, the above result is thought to be satisfactory.

In Table 2 data for dry weight, heights, flower formation, and nitrogen content[X] are presented for representative plants of various types. It can be seen that the plants inoculated with an effective strain attained a dry weight of eight times the non-nodulated controls and were only slightly lighter than plants supplied with combined nitrogen, which though smaller, were stouter plants. The plants inoculated with an ineffective strain were almost as poor as the non-nodulated control plants. The plants nodulated by effective strains had the most flower trusses.

The mean nitrogen content per three seeds was found to be 83 mgs., and that of the cotyledons excised at the usual stage to be 55mgs., showing that on an average the seedlings obtained 28 mgs. nitrogen from the cotyledons prior to excision. By a coincidence the nitrogen content of the particular uninoculated control plants included in Table 2 agrees exactly with that figure. It may be concluded that no unintended source of nitrogen was available to the plants under the conditions employed. The nitrogen content of the plants associated with the ineffective strain is only slightly greater than that of the control plants. The amount of nitrogen actually fixed by the effective strain was in the region of 270mgs. per jar. Plants supplied with /

X Total nitrogen was determined by the Kjeldahl process.

Table 2.

Primary data for the various types of nodulated and control plants [x]

Type of plant.	Dry weight in gm. per jar.	Av. height of shoot in cms.	Av.no. of flower trusses per plant.	Total nitrogen in mgs.per jar
Nodulated effective	10.77	42	6	310
Nodulated intermediate	5.46	15	3	150
Nodulated ineffective	1.87	6	0	32
Non-nodulated control	1.38	6	0	28
Non-nodulated combined nitrogen added	11.8	28	4	420

x Data are average of two jars, each containing three plants, in the case of nodulated plants and average of six jars each containing three plants for non-nodulated plants.

combined nitrogen had a higher nitrogen content than plants produced by effective isolations.

Considerable variation in growth was frequently shown between the three plants within a given jar, all inoculated with the same strain. This feature is indicated in the photographs (e.g. Fig. 2, jar on left hand side) and also by the data for individual plants presented in Table 3. The variation may arise from the operation of many factors which are discussed at some length in Section IV of this thesis. One of the chief factors is probably the lack of genetic uniformity in the original seed. Since it was intended to relate the results of the investigation to field conditions it was thought best to use ordinary commercial seed rather than seed of greater genetic purity, even if this could have been procured. This no doubt resulted in test plants varying in their innate growth capacities, and perhaps also in their response to inoculation with a given isolation. A reason why the plant-to-plant variation tends to be larger in the present case than with clovers grown by the total enclosure technique (see Section II of the thesis) is that the growth conditions for the field beans did not entail the same limitation on the development of the plants as in the case of the clover.

This variability in the test plants obviously raises the question of how reliable an estimate of the effectiveness of a given isolation can be formed from the examination

Table 3.

Dry weight (gms.) of individual plants grown in the same jar.

Isolation		Plant 1	Plant 2	Plant 3	Average weight per plant.
Effective	Jar 1	2.53	4.08	3.85	3.48
	Jar II	2.88	5.06	2.54	3.47
Intermediate	Jar I	0.99	3.67	0.61	1.79
	Jar II	2.10	1.25	2.29	1.88
Ineffective	Jar I	1.21	0.75	0.99	0.98
	Jar II	0.47	0.98	0.65	0.70

of two jars containing a total of six plants. In order to gain information on this aspect, in the case of four particular isolations of varying effectiveness, six replicate jars were set up for each instead of the usual single pair. In all other respects the tests were carried out in the normal way. The forty-eight jars of plants involved in the test (plus the usual controls) were all grown at the same time. The results of the test are presented in Table 4. The analysis of variance appended to the table indicates that in respect of a given testing occasion effectiveness values based on the examination of two jars only (as in the normal test) must differ by at least 14 before it can be concluded that isolations really differ in nitrogen-fixing powers.

Further information is provided by instances in which the effectiveness of the same isolations was tested on different occasions. A total of ten isolations were tested on two occasions, the results being shown in Table 5. The statistical treatment appended indicates that when single estimates of effectiveness value obtained on different occasions for different isolations are being compared, a difference of 30 is required for significance. If the results for isolation H(9), where a particularly large difference possibly due to non-recurring factors occurs, are excluded, the required difference falls to 22, but in the absence of a known

Table 4.

Data indicating the degree of reliability of effectiveness trials.

Isolation	Individual estimates of effectiveness value, each based on two jars	Mean
S	41, 46, 36, 38, 50, 33	41
M(6)	19, 27, 22, 20, 25, 27	23
M(3)	14, 21, 21, 11, 14, 17	16
B(5)	7, 12, 11, 6, 14, 17	11

Analysis of Variance.

	Degrees of freedom	Sum of squares	Mean square
Isolations	3	2979.7	
Error	20	434.9	21.75
Total	23	3414.6	

Therefore the difference required for significance ($P = .05$) between effectiveness values based on two jars only

$$= \sqrt{21.75} \times t_{20} \times \sqrt{1/1+1/1}$$

$$= 4.66 \times 2.09 \times 1.41$$

$$= 14.$$

Table 5.

Effectiveness values obtained for the same isolations tested on different occasions.

Isolation	Effectiveness values obtained in different tests.		Differences in effectiveness values.
B(13)	32	19	-13
G(12)	29	40	+11
H(1)	36	32	- 4
H(2)	49	62	+13
H(4)	37	38	+ 1
H(9)	11	41	+30
F(1)	23	13	-10
N(8)	19	8	-11
C(13)	10	14	+ 4
B(17)	8	16	+8

Analysis of Variance.

	Sum of squares	Degrees of freedom	Mean square
Between isolations	3489	9	388
Within isolations	796	9	88
Between occasions	42	1	42
Total	4327	19	

Therefore the difference required for significance ($P = .05$) between single estimates of effectiveness value

$= \sqrt{88} \times t_9 \times \sqrt{1/1+1/1} = 9.38 \times 2.26 \times 1.41$

$= 30.$

explanation of that particular result it will be necessary to accept the higher difference for significance.

It appears from the above that new factors productive of error arise when comparisons are made between tests carried out on different occasions. It might be suggested that the method of calculating effectiveness by direct reference to the uninoculated control plants is particularly prone to such errors, since the control plants are not in a position to respond to particularly favourable growth conditions. Though there may be some substance in this objection, it should be noted that the control plants are not entirely unresponsive to growth conditions. Thus the mean dry weight of three control plants over all the testing occasions taken together was 2.2gms., but on one occasion the value fell to 1.4gms., while the highest value recorded was 3.2gms. These variations are undoubtedly attributable in the main to differences in weather conditions, which would exert a corresponding effect on the nodulated plants. Thus to a certain extent automatic compensation for climatic differences is provided.

The effectiveness values shown by the isolations from each field are presented in Table 6. A wide range of values is indicated for each field, and bearing in mind the statistical treatment presented above (significant difference required = 30) it is clear that isolations differing significantly in nitrogen-fixing powers were obtained from each

field, some being effective and others ineffective. No clear difference in the incidence of the two types of strains in the different fields is obvious in Table 6, and an analysis of variance (Table 7) confirms that there is no significant difference.

The total number of isolations tested for effectiveness in this investigation was 100. This is well below the number of, say, clover isolations which could have been tested with the same expenditure of time and labour, this being due to the much larger growth habit of the field bean, making the cultivation of the test plants a more cumbersome operation and resulting also in much larger demands upon greenhouse space.

Table 6

Effectiveness in nitrogen fixation shown by isolations from each field

Field	Effectiveness Value																	Total No.of isolns
	1-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45	46-50	51-55	56-60	61-65	66-70	71-75	76-80	81-85	
A	-	-	2	2	1	1	4	3	2	2	1	-	-	-	-	-	-	18
B	-	2	-	1	1	1	2	1	-	3	3	-	2	-	-	-	-	16
C	-	1	-	2	1	-	2	1	1	2	3	-	-	-	-	-	-	13
D	-	-	-	3	1	1	1	-	2	2	1	1	1	-	-	-	1	14
F	-	-	1	2	1	2	-	1	1	2	1	-	-	-	-	-	-	11
G	-	-	-	2	1	-	2	3	4	2	2	1	-	-	-	-	-	17
H	-	-	2	-	-	1	1	2	-	1	-	-	2	2	-	-	-	11
Total	-	3	5	12	6	6	12	11	10	14	11	2	5	2	-	-	1	100

Table 7.

Analysis of variance of differences between bean fields.

Variation	Degrees freedom	Sum squares	Mean square	Ratio mean squs.
Main	6	1004.3	167.4	0.6
Residual	93	26106.9	280.7	not significant.
Total	99	27111.2		

D I S C U S S I O N.

The over-all results.

It can be seen from Table 6 that the isolations made in connection with the present investigation cover a very wide range of effectiveness values; while some are efficient in nitrogen fixation others are poor. Thus, as in respect of clover, pea and soya bean, so in field bean ineffective strains obviously exist.

It cannot be concluded however, that the strains of low effectiveness value which were isolated from some nodules were the only ones present on the plants concerned. It has been shown by the present author (Baird, 1951) that in clovers under field conditions the plant is not necessarily infected by one strain of the nodule organism only but may be associated with several strains, including both effective and ineffective types. In Section II of this thesis these findings for clover are presented in greater detail and are extended to field bean. The view will be developed there that in a given locality the proportion of nodules of effective and ineffective types may tend to be similar on all plants. In the present case it is probable that in the area covered in the investigation a small proportion of the nodules on any plant are due to ineffective strains.

The relation to crop performance.

As stated already an analysis of variance of the effectiveness values of the isolations from the different fields showed that there was no significant difference between them. Since no relation has been demonstrated between crop yield and incidence of ineffective strains it is unlikely that the poor crop yields from certain fields were due to an unusually high percentage of ineffective bacteria native to these fields. The reason for poor crop yields must be due to some factor other than the root nodule bacteria.

The advisability of seed inoculation.

It is reasonable to assume that strains of effectiveness value not greater than 30 contribute little or nothing to the nutrition of the host plant. It can be seen from Table 6 that about 30 per cent of the strains from these fields are of this type and by inoculation of the seed with a suitable effective strain it would probably be possible to replace some of these nodules by others containing the applied effective strain. This would not necessarily benefit the plant for the following reasons:-

Supposing we have a plant bearing 100 nodules of which 70 contain reasonably effective strains and the remaining 30 essentially ineffective ones. These 30 nodules will not entail any serious drain of the host metabolic materials,

since Asprey & Bond (1941) showed that respiratory activity of such nodules was greatly inferior to that of effective types. The bulk of the metabolic materials are probably being utilised within the 70 effective nodules. The total amount of this material cannot be increased, since the growth of a well-nodulated legume, assuming effective bacteria, is probably limited by the rate of photosynthesis, which in turn is limited by light intensity, temperature and carbon dioxide supply. It is appreciated that in the case of nodulated plants growing in nitrogen-free media in the greenhouse, nitrogen-supply is the limiting factor in the growth of the plant until the nodules are fully developed and functional; thereafter the position becomes less certain. But under field conditions, assuming soil of reasonable fertility, nitrogen-supply appears unlikely to be a serious limiting factor. This is supported by the observations of Thomas & Hill (quoted by Bonner & Galston, "Principles of Plant Physiology" 1952, p.33) which showed that the rate of photosynthesis, and hence the accumulation of dry matter, in lucerne under field conditions was governed by light intensity. On days with bright intervals, the latter were accompanied by bursts of photosynthesis. There was no suggestion of an over-all limitation of metabolic and growth activities by defective nitrogen supply.

If this argument is sound, then to replace the 30 ineffective nodules, making little demand on the host plant, by 30 others of effective type, making much larger demands, would result in reduced supply of metabolic materials all round. The net gain to the plant in terms of nitrogen fixed relative to metabolic materials consumed within the nodules would be small. Of course if the proportion of effective nodules falls, a point will be reached where the above is no longer true - the nitrogen supply rather than photosynthesis being then the limiting factor.

It is possible that here we have one reason why seed-inoculation of legumes has not always resulted in an appreciable increase in crop yield. Of course it is not suggested that it is the only or the main reason - for example the use of unsuitable inocula is no doubt another, perhaps more frequent, reason.

It is therefore concluded that, on the results of the survey alone, it cannot be said that seed-inoculation would be advantageous or worthwhile in the area concerned. Properly-arranged field experiments would be required to settle the matter. The position would, of course, have been different had a larger number of strains of low effectiveness value been found in the survey - say 50 per cent.

The origin of ineffective strains.

The question of the origin of ineffective strains in Nature is not one on which the author has new material to

present, but it will perhaps be considered appropriate for the results and conclusions of other workers to be reviewed at this point since they throw some light on the state of affairs revealed by the present survey. These indicate that the soil population of rhizobia is in a state of continual change, due to the action of various factors as follows:-

(1) Variations in both effectivity and infectivity have been shown to occur spontaneously in the laboratory. Nutman (1949), by varying the conditions of growth of an effective parent strain of clover bacteria, found that it produced an ineffective strain in dry soil culture, an intermediate strain in culture at 1[illegible]°C. and an avirulent strain spontaneously. Though most changes recorded are changes resulting in the loss of effectivity, an increase in effectivity has been shown to occur by Nutman through the study of a constant population of small nodules. Any large nodules which appeared in it were noted as indicative of a change to effective nitrogen fixation. He found that this change occurred twice in thirteen hundred nodules.

(2) Storage of the organism in sterilised sand or soil has been found to result in the loss of effectivity. Nutman (1946) stored isolations in sterile sand for a period and found that ineffective variants were produced. Thornton (1947) reports that after storing an effective strain in soil for nine months 30% to 35% of the colonies recovered

were ineffective. Nutman (1949) found that the high percentage of ineffective mutants appearing when strains were stored in acid soil could be prevented by the addition of calcium carbonate to the soil. However, mutants did occur in limed soils so there must be additional factors which sometimes influence the mutation.

The fact that calcium carbonate prevents the formation of unfavourable mutants is of interest from the point of view of farming practice. One of the advantages obtained by liming could be the maintenance of effective rhizobia in the soil.

(3) A change in effectivity may be caused by the action of bacteriophage. Kleczkowska (1948) has shown that 'phages will sometimes cause effective strains of clover rhizobia in the soil to change to ineffective strains and vice versa.

(4) The effect of other micro-organisms in the soil on the nodule bacteria is reviewed by Allen & Allen (1950). According to these investigators, 'streptomycetes' appear to be the most common antagonists of the rhizobia, although the latter vary considerably in their susceptibility. Thornton, Alencar & Smith (1949), investigating why rhizobia die off rapidly, studied 7 cultures of Streptomyces and 6 cultures of Penicillium isolated from the soil. Using the agar plating technique they found that 8 cultures, 6 of which were Streptomyces and 2 of which were fungal, showed antibiotic properties towards Rhizobium meliloti.

In sterilised soil 3 cultures, one of which was a Streptomyces and the other two of which were fungal, showed antibiotic effects. They think it is possible that these organisms existing in the soil are antagonistic towards rhizobia. Rhizobial cultures are also commonly attacked by antagonists of the aerobic spore-forming group. They differ from the 'streptomycetes' by not being specific in their action. Other workers have been trying to identify substances antibiotic to rhizobia, for example, the substance from Bacillus mesentericus which inhibits the growth of pea, bean and clover rhizobia but not soya bean rhizobia. Another example is the bacteriostatic principal of Aspergillus wentii which is antibiotic to the rhizobia of birdsfoot trefoil, horse bean and alfalfa. None of these antibiotics have been completely identified chemically yet. Allen & Allen after reviewing papers on the above problems do not feel that sufficient work has been done on the inter-relationship of rhizobia and other micro-organisms to warrant any speculation on the effect of antagonists on rhizobia as reflected by nodulation and nitrogen fixation.

Most of the changes in effectivity of strains discussed above have been shown to occur under laboratory conditions. It is probable however, that some of them will also occur under natural conditions in the field.

SUMMARY

1. The literature relating to the occurrence of variation in nitrogen-fixing powers between different strains of the nodule organism associated with a given cross-inoculation group of legumes is reviewed, as is also the literature concerned with the origin of ineffective strains of the nodule bacteria.

2. An attempt has been made to learn something of the range in effectiveness in nitrogen fixation of the strains of the field bean nodule organism native to several ~~areas~~ Fields in the West of Scotland, and also to determine if any relation existed between bacterial effectiveness and crop yield.

3. One hundred isolations of the field bean nodule organism were made from the nodules of plants collected from seven different fields varying in their cropping records for this crop.

4. The isolations were inoculated on to test plants of field bean growing in water culture under conditions controlled bacteriologically and nutritionally.

5. The effectiveness of the isolations was assessed on the basis of plant growth.

6. Isolations of a very wide range of effectiveness were found.

7. No explanation of poor crop yield of field beans from certain of the fields studied is provided by the examination of the effectiveness in nitrogen fixation of nodule organism strains isolated from them. It is concluded that such poor yields are due to some factor other than the ineffectiveness of the root nodule bacteria.

8. The advisability of seed inoculation of field bean in the area surveyed is considered. Although the data suggest that a certain proportion of ineffective nodules is present on the plants, it is concluded, on metabolic grounds, that the replacement of a rather small proportion of ineffective nodules by effective ones will not necessarily lead to any material increase in nitrogen fixation.

SECTION II.

The Multiple Infection of Clover and Field Bean Plants by Strains of the Nodule Organism in the Field.

C O N T E N T S.

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

The results of the survey of native strains of the field bean organism, presented in Section I of this thesis, together with those of surveys carried out by previous investigators on the nodule organisms associated with other leguminous plants, indicate that in the soil there is a considerable assortment of strains of the nodule organism appropriate to a particular legume. These strains differ in their nitrogen-fixing powers and perhaps in other features as well.

The question arises, what is the status of a particular individual plant under field conditions towards these differing strains? Are the nodules on that plant all due to a particular strain, that which happened to be most abundant in the immediate vicinity of the young roots or which was dominant in the sense of Nicol & Thornton (1941), or do different nodules on the one plant contain different strains, so that while some nodules are contributing substantially to the nutrition of the host plant, others are essentially parasitic? This is an aspect which, as regards plants in the field, has received very little consideration in published work, although it is a matter of considerable general interest, particularly as regards the interpretation of the effect of artificial inoculation of legume seed with strains of the nodule organism.

In the first place general observations on legume

plants in the field suggest that multiple infection is the rule. Thus it was pointed out to the author by Dr. G. Bond that when isolations of the nodule organism are made from single nodules from each of a number of clover plants growing in a given area, it is frequently the case that while both effective and ineffective strains are obtained, the plants yielding the latter show no very obvious difference in nitrogen status from those yielding effective strains. The same was in general true of the field bean plants from which isolations were made in Section I of the thesis.

To some extent these observations can be explained by the fact that under field conditions the plant is not entirely dependent on the nodules for its nitrogen nutrition, and deficiencies in fixation can to some extent be remedied by absorption of soil nitrogen. There is, however, the further possibility that in the field a given plant is infected with effective and ineffective strains, and that in a particular area the proportion of nodules produced by the two types tends to be similar on all plants, which as a result show fairly uniform vigour.

Secondly we have to consider the existing evidence on whether simultaneous association of a given legume plant with different strains of the nodule organism is possible. A number of investigators have examined this possibility with reference to plants growing under laboratory conditions.

Some have concluded that infection of a plant by one strain confers immunity on the plant towards other strains. An important consideration seems to be whether the two strains are applied simultaneously or in succession.

In simultaneous inoculation two strains differing in some character are applied to the host plant. The strains in the nodules which form are then identified by means of this character. The following investigators were successful in obtaining double infection with two strains applied simultaneously:-

Dunham & Baldwin (1931) with clovers, peas and soya beans, the strains were identified by their differing effectivity.

Wilson (1948) with vetches, clovers and lucerne, the strains were identified by their ability to form nodules on certain test plants.

Chen (1941) with clovers and soya beans, the strains were identified both by their differing effectivity and by the number of nodules which they produced. He found that the number of nodules formed on a plant reaches a limit which is characteristic of the strain and so he concluded that any subsequent nodules were due to the second strain.

Nicol & Thornton (1941) with peas, soya beans and clovers, the strains, differing in their effectivity, were identified as follows, - peas by their growth on yeast agar slopes, soya beans by the appearance in section of the nodule and clovers by the number of nodules and the mean nodule length.

Although Nicol & Thornton obtained double infection

they found that one strain usually produced over 90 per cent. of the nodules. They suggest there is active competition between the strains of bacteria in the surroundings of the host's root system. The latter secretes a substance which stimulates the multiplication of the root nodule bacteria but stimulates different strains at different rates. One strain is thus able to repress the other strains in the root surroundings, to satisfy the nodule producing capacity of the plant and so prevent further nodulation.

Instead of using only two strains, Burton & Allen (1949) used a mixture of six strains of different effectivity and obtained infection of clovers by effective and ineffective strains. They found however that most of the nodules were from ineffective strains, the latter appearing here to have been dominant.

There is only one report of failure to obtain double infection with simultaneous inoculation. Virtanen & Linkola (1947) applied two strains, differing in their effectivity, simultaneously to pea plants and found that the ineffective strain tended to exclude beneficial nodulation. They found however that this immunity soon wore off and later inoculations were not affected.

Double infection is not so easy to obtain when strains are applied at different times. Two reports of failure to obtain double infection with successive inoculation have been made, namely, Dunham & Baldwin (1931) with clovers

peas and soya beans, and Virtanen & von Hausen (1935) with a three-year old clover plant already infected with an ineffective strain. Löhnis (1930) only obtained double infection of clovers with strains differing in effectivity when the ineffective strain was applied first. Chen (1941) however appears to have had no difficulty in obtaining successive inoculation of peas, clovers and soya beans. He identified the strains as described earlier. Nicol & Thornton (1941), also identifying strains as described earlier, had variable success in obtaining successive inoculation of peas and soya beans. Success, they found, was dependent on the strain initially applied. The growth of the root system in peas and soya beans is rapid and of short duration and thus if the strain initially applied was dominant the nodule-producing capacity of the plant was satisfied and consequently further infection either by the same strain or by different strains was prevented. If however the strain initially applied was not dominant, further infection could take place. With clovers they were successful in obtaining double infection in every case because here the growth of the root system is more prolonged and the first-formed nodules did not prevent the formation of other nodules.

The findings of Nicol & Thornton explain why some workers were unable to obtain double infection, since if one strain was dominant nearly all the nodules would be due to that strain. The possibility of obtaining

successive inoculation seems to depend not only on the type of leguminous plant but also on the infective power of the strain initially applied.

It appears then, from the above survey of existing observations on behaviour under laboratory conditions, that if in the field in the rhizosphere of a young plant several strains of the appropriate nodule organism are present, initially or eventually, then it is likely that multiple infection will occur.

So far as the author is aware the only published observations on multiple infection of legumes in the field are contained in papers by Hughes & Vincent (1942) and Purchase & Vincent (1949) which refer to Australian conditions. In the former investigation isolations from pairs of nodules, each pair from the same clover plant, were compared by serological tests. They found that it was not uncommon for an individual plant growing in this area to have nodules on it developed from serologically different strains of the organism. In the second paper, which appeared during the course of the present author's work, two isolations from each of a number of clover plants were examined for effectiveness in nitrogen fixation. Evidence for differences within pairs was obtained.

It is seen then that when this work was commenced, although there was evidence that multiple infection by strains of differing effectivity could occur under laboratory conditions, there was no evidence that it

actually took place in the field. It was decided therefore to see if strains varying in effectiveness were present in different nodules of particular clover and field bean plants collected in the field. Isolations were made from individual clover and field bean plants in 1948 and 1950 respectively and these were tested for effectiveness in nitrogen fixation in 1949 and 1951 respectively. An outline of results obtained in the case of clover has already been published (Baird, 1951).

M E T H O D S.

A. Clover.

A study has been made of the strains of nodule organism associated with six plants of red clover taken from hill-farm land at the Ballochraggan Experimental Station, Aberfoyle, Perthshire. Five of the plants had grown from uninoculated seed sown in connection with another experiment, while the sixth plant was from seed inoculated with an effective strain (i.e. a percentage effectiveness value greater than 70.). Ten nodules were taken at random from each plant and an isolation of the organism made from each nodule.

The method of isolation was similar to that employed for the field bean organism. The nodule was first cleaned by brushing it with water, then sterilised externally by shaking it with acid mercuric chloride for 3 minutes and finally it was shaken with six successive portions of sterilised water. It was subsequently crushed in a drop of sterile water and the suspension plated on yeast-mannitol agar. After a few days growth the organism was transferred to slopes of the same medium.

The effectiveness of these isolations in nitrogen fixation was then tested on plants of red clover growing in aseptic culture totally enclosed in test tubes. This is a method to which reference has already been made in Section I and one which has been used by a number of previous investigators.

For this purpose commercial seeds of Montgomery red

clover were selected for soundness of testa but were not weighed as was done with the field bean seed. They were sterilised in a similar way to the field bean seed, being first shaken with absolute alcohol for 2 minutes, then with 0.1 per cent. mercuric chloride for 4 minutes, after which they were shaken with six successive portions of sterilised water. The seeds were then planted in test tubes, (6 inches x $\frac{3}{4}$ inch) containing a sloped agar rooting medium free of added combined nitrogen. This medium was similar to that used by Chen & Thornton (1940) and had the following composition:-

K_2HPO_4	1.0 gm.
$CaH_4(PO_4)_2 2H_2O$	0.5 "
$MgSO_4 7H_2O$	0.2 "
NaCl	0.1 "
$FeCl_3$	0.01 "
Agar	15.0 "
Distilled water	1 litre.

Five seeds were planted on each slope. After three days an adjustment to two seedlings per tube was made. When the plants were five days old, 1 ml. of a suspension in sterile water of a four-day old slope of the nodule organism isolation was added. Uninoculated controls had sterile water added instead of the suspension. Six plant tubes were set up for each isolation, with twelve for the standard strain (see below) and the uninoculated controls in each set of tests. Wooden racks with twelve holes of a

depth of $2\frac{1}{2}$ inches and $\frac{3}{4}$ inch in diameter were used to hold the tubes. The racks were put in the greenhouse and their positions changed frequently. The approximate daytime temperature of the greenhouse in summertime tests was $70^{\circ}F$.

All possible precautions were taken to ensure aseptic conditions in the tubes. The medium was autoclaved before it was sloped and any subsequent operations requiring the removal of the cotton wool plug were carried out in an inoculating chamber. The planting of seeds and the thinning out of surplus seedlings was effected with a flamed platinum loop and the inoculum was added with the usual bacteriological precautions. After these initial operations on the plants had been accomplished the tubes were not opened again until the plants were ready for harvesting.

After ten weeks' growth the dry weight of the plants from each set of tubes, bulked together, was ascertained by drying at $100^{\circ}C$ to a constant weight.

The effectiveness of the isolations was assessed by comparison against a standard effective strain. The strain employed for the purpose was Rothamsted strain No.49. The dry weight attained by plants associated with a given isolation was expressed as a percentage of the dry weight of the standard plants, after subtraction from both of the dry weight of the uninoculated control plants. The value

thus obtained is termed the '<u>percentage effectiveness</u>', a typical calculation being as follows:-

> Mean dry weight per nodulated plant (based on the examination of six replicate tubes, each containing two plants) associated with a particular isolation = 18 mg.
> Mean dry weight per nodulated plant (based on the examination of twelve replicate tubes, each containing two plants) associated with the standard strain = 21 mg.
> Mean dry weight per non-nodulated control plant (based on the examination of twelve tubes, each containing two plants) = 12 mg.
> <u>Percentage effectiveness</u> for isolation in question
> $$= \frac{18 - 12}{21 - 12} \times 100 = \underline{67}$$

It is assumed that the relative performance of the different strains when associated with the test plants reflected their performance on the same variety of plant in the field.

Some of the effectiveness tests with clover were carried out during the winter months, when the natural light then available was supplemented by artificial light from fluorescent tubes arranged in a manner similar to that described by Low (1948). Seven fluorescent 'Daylight' discharge tubes, of standard type, 5 feet in length and set 7 inches apart, were suspended 4 inches above the cotton wool plugs. The suspension of the light tubes so close to the plants is possible because of the low heat production. The wooden racks were arranged parallel to the lights, their positions being changed weekly. The arrangement of the racks and lights is illustrated in figures 1 - 3. The intensity of the artificial light

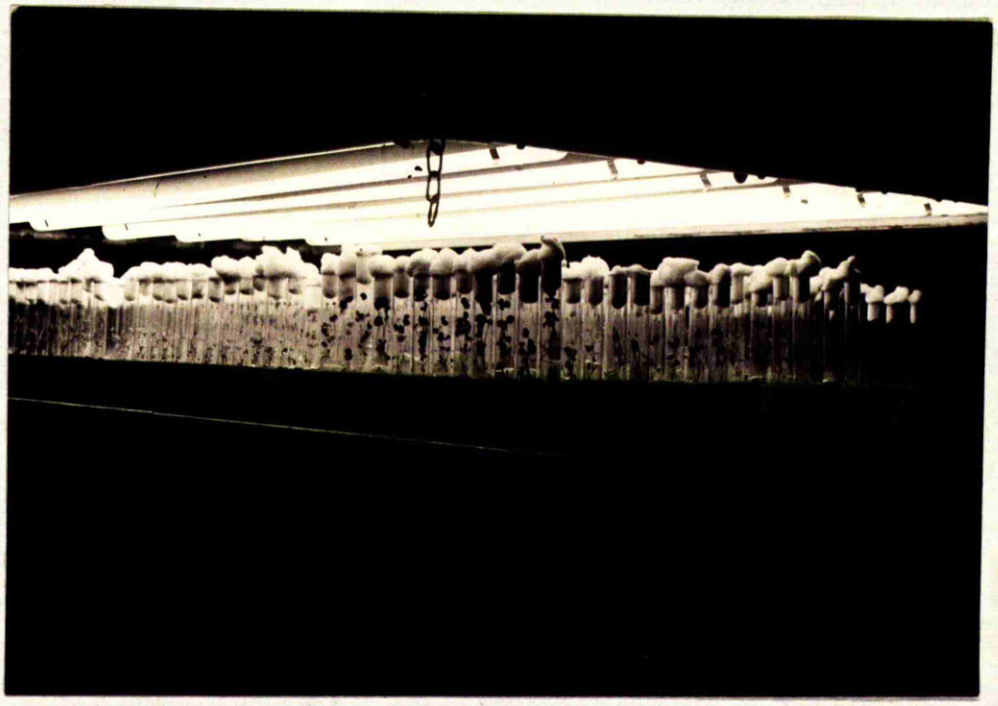

Fig. 1. Clover plants growing under fluorescent lights.

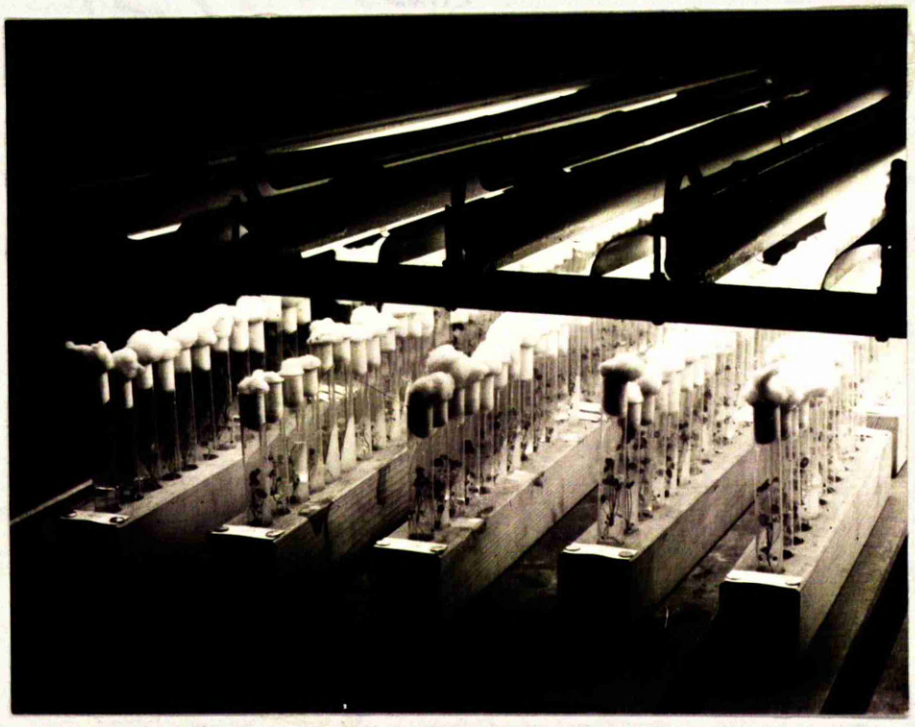

Fig. 2. Another view of the fluorescent lights and tube cultures.

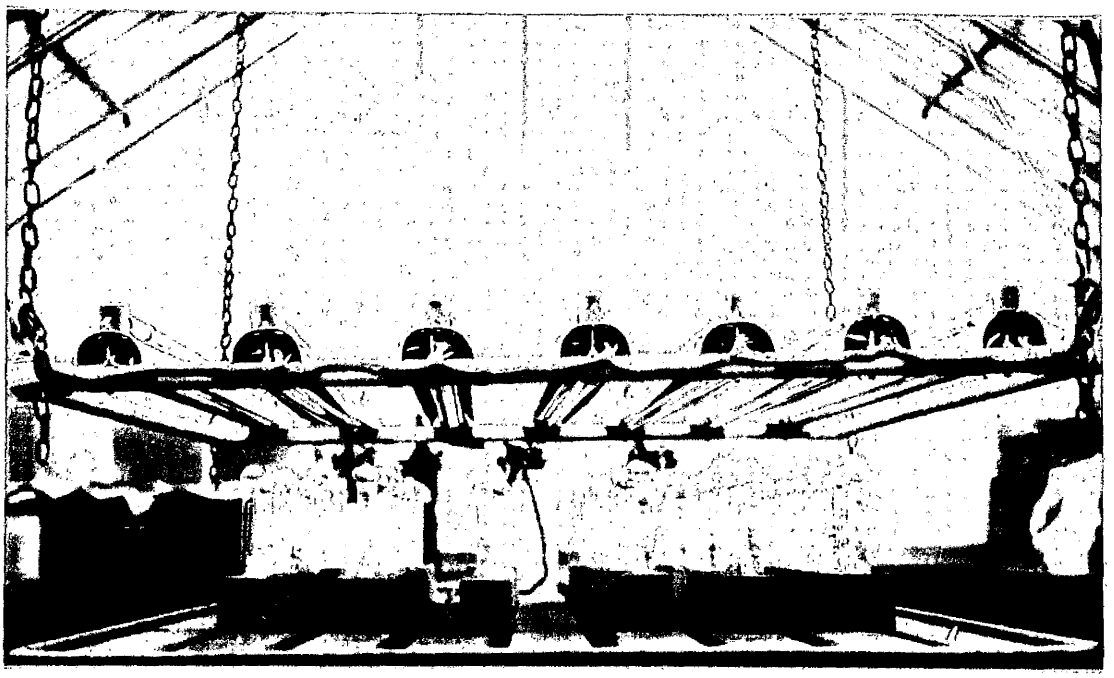

Fig. 3. Another view of the fluorescent lights showing method of suspension.

alone at plant level was approximately 300 foot candles, the light being supplied for 16 hours each day. As will be shown by the data (to be presented later) very satisfactory growth was obtained under these conditions. It may be of interest to others working in the same field that effectiveness tests can by this means be carried out under winter conditions in this country.

B. Field Beans.

The strains of the nodule organism associated with five field bean plants from different areas in the West of Scotland were investigated. These plants were all from uninoculated seed. The bacteria were isolated from ten nodules taken at random from each plant, and the effectiveness of these isolations in nitrogen fixation was tested on field bean plants grown in nitrogen-free water culture as described in Section I. Effectiveness values were calculated as in that section.

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

A. Clover.

Table 1 shows a selection of typical primary data for different types of clover plants grown under various conditions. They illustrate in the first place how nearly the plants grown with supplementary artificial light in winter approached in dry weight those grown in summer. Although those grown in winter without the artificial light bore nodules, the plants were similar to non-nodulated control plants in size and dry weight. It is obvious that on these plants the few nodules which appeared were unable to fix any appreciable amount of nitrogen.

The Table also shows that in summer the dry weight of plants associated with effective strains only attained values approximately double those of the controls. This is partly because traces of nitrogenous compounds are gained by the control plants from the agar medium. It is to a greater extent due to the limitation which total enclosure in test tubes imposes on growth, particularly that of potentially strong-growing plants, (i.e. those with nodules containing an effective strain). This limitation is due to lack of space and the resultant over-crowding of leaves, and also to the interference with carbon dioxide supply caused by the cotton wool plugs, as demonstrated by Knudson (1916). That there is a limitation in growth is illustrated by the fact that red clover plants inoculated

Table 1.

Examples of primary data(all mg. dry weight per plant) of plants grown under different conditions x

Type of plant	Grown in winter with fluorescent light.	Grown in winter without fluorescent light.	Grown in summer
Nodulated, effective (standard) strain	19.4	6.0	21.0
Nodulated, effective isolation, A5(3)	17.6	5.3	20.0
Nodulated, intermediate isolation, B1(7)	12.2	6.1	18.0
Nodulated, ineffective isolation, C1(20)	9.4	5.9	12.1
Non-nodulated control	9.0	6.0	12.0

x Each value is a mean based on six or more tube cultures with two plants per tube, the growing period extending over ten weeks.

with an effective strain and grown in the greenhouse in open culture attained a weight of 420 mg. which is almost 20 times greater than the dry weight attained by the plants in tube culture.

The type of plant growth obtained with effective, intermediate and ineffective strains, and uninoculated control plants, is illustrated in Figure 4. In Figure 5 two plants both inoculated with the same effective strain and grown in winter are shown. One of the plants was grown under the fluorescent lights and the other was not.

Evidence that complete control of the nodule organism was maintained is provided by the fact that none of the plants in the 110 uninoculated control tubes set up during the strain testing period developed nodules. The general bacteriological precautions must also have been adequate since none of the tubes grown during the course of the investigation became visibly infected with fungal or non-symbiotic bacterial organisms.

As noted for field bean in Section I of the thesis, considerable variation in size is shown by individual clover plants associated with the same strain of nodule organism. Details of this plant-to-plant variation are given in Section IV of the thesis. It has, as already been mentioned in Section I of the thesis, been the subject of special study by Nutman (1946), and shown to be due to genetical differences between individual host plants. The

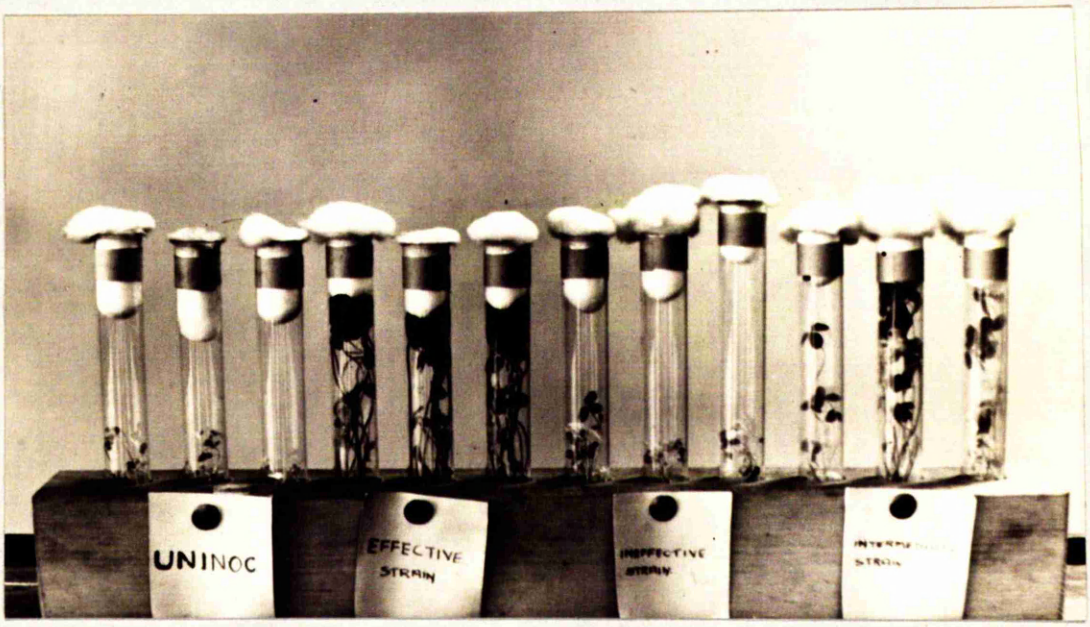

Fig. 4. Examples of growth of clover plants. Left to right: uninoculated, inoculated with an effective strain, an ineffective strain and an intermediate strain, (three tubes each). (x $\frac{2}{7}$).

Fig. 5. Two plants inoculated with the same effective strain, grown in winter. Left, with fluorescent light, right without artificial light. ($x\frac{1}{2}$).

present significance of this plant-to-plant variation is that it introduces an element of uncertainty into the estimate of effectiveness.

In order to investigate the reliability of an estimate of effectiveness based on the examination of six tube cultures, replicate sets of tubes were included for four particular strains. The results of these trials are presented in Table 2 and show that considerable variation occurred between estimates of effectiveness. The analysis of variance of these data indicates that (for P = .05) the values for the percentage effectiveness of two isolations based on the examination of six tubes, for each isolation, must differ by at least 47 before it can be concluded that the two isolations really differ in nitrogen-fixing powers. This estimate is based on data obtained in one and the same batch of tests. Most of the data to be presented below were actually obtained in a large batch of tests carried out during the summer of 1950.

In Table 3 the values obtained for the effectiveness of the different isolations from each of the six clover plants are presented. On the basis of the statistical considerations given above, it may be concluded that strains significantly different in effectiveness were found on every plant of the six examined.

It was noted earlier that plant B1 was from seed

Table 2.

Data indicating degree of reliability of effectiveness trials.

Isolation	Individual estimates of percentage effectiveness each based on six tubes	Mean	Standard deviation of a single estimate
A5(3)	59, 99, 57, 100, 57, 75	75	21
A5(11)	80, 100, 76, 64, 90, 98	85	14
B1(7)	0, 40, 33, 37, 36, 29	29	15
B1(20)	36, 25, 16, 38, 3, 22	23	13

Analysis of Variance

	Degrees of Freedom	Sum of squares	Mean Square
Isolations	3	17478.8	
Error	20	5007	250.3
Total	23	22485.8	

Therefore the difference required for significance ($P=.05$) between percentage effectiveness values based on six tubes

$$= \sqrt{250.3} \times t_{20} \times \sqrt{1/1+1/1}$$

$$= 15.8 \times 2.08 \times 1.41$$

$$= \underline{46.5}$$

Table 3.

Percentage effectiveness in nitrogen fixation shown by different isolations from a given clover plant.

Plant	PERCENTAGE EFFECTIVENESS												Total isolations
	0	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	100	
A1	-	-	-	1	2	1	2	1	1	1	-	1	10
A2	3	-	4	1	-	1	1	-	-	-	-	-	10
A3	-	-	1	2	2	-	-	2	2	1	-	-	10
A4	-	1	-	1	1	-	-	1	5	-	-	1	10
B1	-	2	-	1	4	-	1	1	-	-	-	1	10
A5	2	1	-	1	1	1	-	2	-	1	-	1	10

inoculated with an effective strain. An examination of ten nodules is however not sufficient to say finally whether the inoculant strain has established itself in plant B.

B. Field Bean.

The statistical considerations given to field bean plants in Section I of the thesis will apply equally here, since the procedure was exactly the same. Since all ten isolations from any one plant of the five examined were tested on the same occasion for effectiveness, the smaller significant difference of 14 can be employed. In Table 4 the results of the effectiveness tests are presented. It can be concluded that strains significantly different in effectiveness were found on the same plant.

Figure 6 shows ten jars of nodulated field bean plants, each jar having been inoculated with an isolation from a different nodule of the same plant (plant N in Table 4). The considerable variation in growth of the resultan plants due to differing effectiveness of the nodule organism is shown.

Table 4.

Effectiveness value in nitrogen fixation shown by different isolations from a given field bean plant.

PLANT	EFFECTIVENESS VALUE												TOTAL NO. OF ISOLATIONS.
	1-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45	46-50	51-55	56-60	
M	-	-	-	1	2	1	2	3	-	1	-	-	10
N	-	-	-	1	2	1	-	4	-	2	-	-	10
O	-	-	-	-	-	5	1	-	-	3	-	1	10
P	-	-	-	1	2	2	3	1	1	-	-	-	10
Q	-	-	-	1	5	1	-	-	1	1	1	-	10

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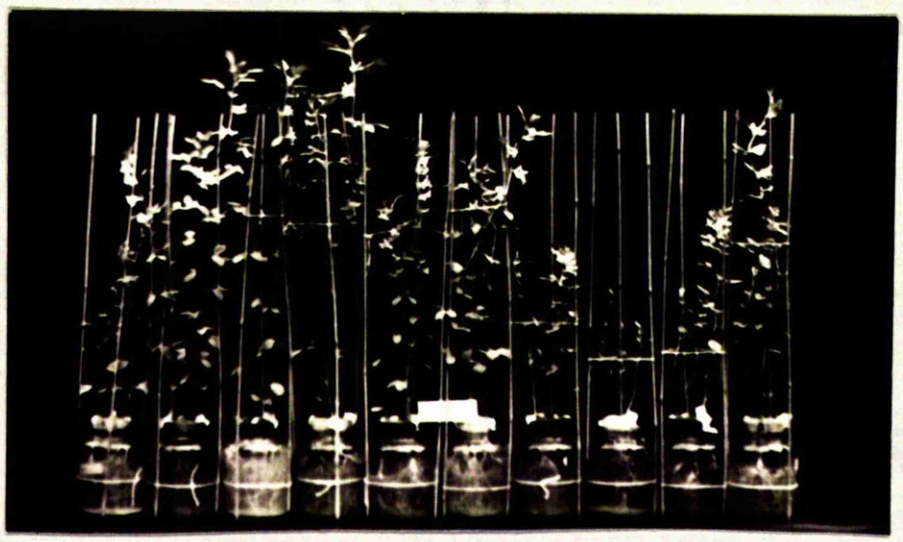


Fig. 6. Each of the jars is inoculated with one of ten isolations from different nodules of a single plant (plant N of Table 4). The plants are all nodulated and show the variability in effectiveness in nitrogen fixation of the nodule bacteria associated with the one plant. ($x\frac{1}{15}$).

D I S C U S S I O N.

The indications of the results presented above are that in both clovers and field beans the plant in the field is typically associated with several strains of the nodule organism of different fixatory powers. This result is in keeping with the work of a number of investigators who, under laboratory conditions, obtained double infection of a number of legumes (see introduction to this Section of the thesis). This conclusion is also in agreement with observations on the nitrogen status of plants in the field as noted in the introduction. There it is also suggested that as well as a plant being infected with strains of different effectiveness value, the proportion of nodules due to the two types of strains on all plants from a particular area tends to be similar. The number of nodules which it was possible to examine here, namely 10, is ~~probably~~ however too small a number to give any clear indication of the proportion of effective and ineffective nodules present on each plant.

In a single plant then, we now have a picture of a composite and varying state of symbiosis. Within the effective nodule a true symbiotic relationship exists, but in ineffective nodules on the same plant the relationship is almost of a parasitic type. Both effective and ineffective nodules must be influenced by the same host plant factors since they are on the one plant, and yet they perform

very differently. In the discussion of reasons for the ineffectiveness of bacteria in Section I of the thesis it was suggested that in some instances ineffectiveness may be due to the host plant failing to provide a suitable environment in the host cells or failing to supply some essential part of the fixation mechanism. The evidence now presented of the presence in a given plant of both effective and ineffective nodules provides a clear demonstration that different strains of the nodule organism vary markedly in their capacities for fixation under given host conditions.

This demonstration of multiple infection may be of practical importance in connection with seed inoculation. When seed inoculation is applied to correct an entire absence of appropriate nodule bacteria from a soil, as for instance when a leguminous crop is being grown in a district for the first time, then the position is quite simple. Where, however, inoculation is applied to seed sown in a soil already containing nodule bacteria capable of infecting and nodulating the crop plant concerned, then a more complex situation arises. This has already been recognised by Nicol & Thornton (1941), and the need emphasised for selecting strains for seed inoculation which can not only produce nodules that are beneficial to their host plant, but are also dominant in competition with other strains.

If the picture of a state of multiple infection

suggested by the present investigation is generally correct, then the result of a successful seed inoculation will be to increase the proportion of nodules induced by effective strains. It has been argued in Section I that when the proportion of effective nodules on a plant is already fairly high, a further increase may not be attended by any marked augmentation of fixation. For the present purpose it will be assumed that without inoculation the proportion of effective nodules is somewhat lower, say 50 per cent., the remainder being ineffective. It will further be assumed that the effect of inoculation is to replace 50 per cent. of the nodules (effective and ineffective equally) that would normally develop, by effective nodules due to the applied strain. The net result will be that the proportion of effective nodules is increased to 75 per cent. This might well result in an increased crop yield, but the increase will not be proportional to the actual nodule replacement, since to some extent already effective nodules are merely being replaced by other nodules of no greater effectiveness. So that we have here another reason why seed inoculation may be attended by less striking results than had been anticipated.

S U M M A R Y.

1. An investigation has been made to find if under field conditions a particular clover or field bean plant is associated with strains of the nodule organism of different nitrogen fixing powers.
2. Isolations from ten nodules from each of six plants of red clover from hill-farm land and from the same number of nodules from the each of five plants of field bean from cultivated fields were made.
3. The isolations were tested for effectiveness in nitrogen fixation. In the case of clovers the isolations were inoculated on to test plants of red clover growing on an agar rooting medium free of added combined nitrogen under aseptic conditions. The isolations from field beans were inoculated on to test plants of field beans growing in water culture under conditions controlled bacteriologically and nutritionally.
4. The effectiveness of the isolations was assessed on the basis of plant growth.
5. Satisfactory growth of clover test plants was obtained in the winter months by the use of fluorescent light.
6. The results indicate that at least two and possibly in some cases more than two strains of the nodule organism, differing markedly in nitrogen fixing powers, were associated with each clover and field bean plant.

7. The effect of seed inoculation on clovers and field beans taken from areas such as these, where the proportion of effective strains is high, is discussed. Since inoculation will to some extent result in the replacement of one effective strain by another it is concluded that the benefit to plants might be somewhat smaller than expected.

SECTION III.

Some Aspects of the Nodulation of Field Beans Grown in Water Culture.

C O N T E N T S.

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

Although with many legumes there are extensive observations on the type of nodulation induced by different strains of the nodule organism, and on the correlation between this and the nitrogen-fixing power of the different strains, very few observations have been recorded for Vicia faba, as will appear below.

Fred, Baldwin & McCoy (1932) in reviewing the literature on this subject report that early workers thought that nitrogen fixation in legumes was directly proportional to the number and size of the nodules on the roots. Giöbel (1926) and Erdman (1926) concluded that the total mass of nodules was more important than their numbers. Erdman found that the percentage of nitrogen in small, medium and large nodules of soya bean was approximately the same in each case and so he classified one large nodule as being equivalent to ten small or two medium sized nodules. Ruf & Sarles (1937) however do not agree with this earlier view. They measured the mass and volume of nodules produced by poor and good strains of soya bean bacteria and found that poor strains produced a greater mass, volume and number of nodules than good strains.

Other workers saw more significance in the type and location of nodules. Lawes & Gilbert (1891) found that in healthy pea plants the nodules were crowded together in

what are now called compound nodules, while in the poor pea plants the nodules were small and scattered over the root system. Hiltner & Störmer (1903) described a 'parasitic' strain of pea rhizobia which produced a large number of small nodules scattered over the entire root system. More recent workers have regarded this type of nodulation as typical of ineffective strains and a smaller number of large nodules as typical of effective strains. Some of these investigators are mentioned here along with the host plant studied, namely, Wright (1925), soya beans; Helz, Baldwin & Fred (1927), peas, sweet peas, broad beans and alfalfa; Baldwin & Fred (1929), Trifolium species; Leonard & Dodson (1933), peas; Virtanen & von Hausen (1932), peas; Thornton (1936), clovers; Ruf & Sarles (1937), soya beans.

The literature provides some exceptions to this general position. Thus Nobbe & Hiltner (1893) described a strain of pea rhizobia which produced a large number of big nodules and yet the pea plants on which they grew were poor. Dehérain & Demoussy (1900) described four types of nodules found on white lupin plants growing in the field in different areas. They are detailed here with the percentage nitrogen found in the host plant given in brackets; small nodules on the main roots resembling strings of beads (3 per cent.); medium sized smooth nodules forming a collar round the top of the root (2 per cent.); half spherical nodules encasing the roots (as in

vetch) or projecting from them (as in lucerne) (1 per cent.); very large nodules in the form of raspberry (0.6-0.8 per cent.). Their results then indicate that a large number of small nodules are associated with effective strains of bacteria and large nodules are indicative of ineffective strains. Hiltner (1902) found that the large nodules on the roots of yellow lupin are worthless to the plant.

So far as the author is aware the only description of the type of nodules produced by different strains on Vicia faba has been made by Helz, Baldwin & Fred (1927). They described the type of nodulation produced by an isolation from pea nodules. The broad bean plants inoculated with this isolation were poor and had a large number of small nodules scattered over the root system. An isolation from broad beans on the other hand produced good plants with a few large nodules concentrated on the tap root.

Since in the present connection field beans were being grown associated with a wide range of strains of the nodule organism, differing markedly in their nitrogen-fixing powers, an opportunity was provided for adding to these meagre existing observations on types of nodulation on this plant. Nodulation of plants grown in water culture might be thought to be liable to differ from that of plants infected with the same isolation in the field. Dr. G.

Bond[x] however has noted no divergence from the general pattern for nodulation of legumes in soya beans, peas, and clover plants grown in water culture, so there is no reason to suppose that field beans (in water culture) would react differently from these plants.

[x]Private communication to the present author.

M E T H O D S.

In all, observations on the nodulation produced by 160 isolations of the field bean nodule organism on host plants growing in water culture have been made. In most cases there were two jars, each with three plants, for an isolation but in some instances as many as twelve jars were available.

Observations were made on the following aspects:-

Number and size of nodules. It was not possible to actually count the nodules on all the plants, and in a number of instances the frequency of nodules was assessed by inspection and assigned to one of the following categories:- very abundant, abundant, average, sparse and very sparse. With some of the isolations however all the nodules were picked from the roots of the plants and counted. They were then dried at 100°C. to a constant weight. The nodules from each plant were counted and weighed separately and an average number of nodules per plant for the isolation was found. The average weight per nodule, which gives an indication of the size of the nodules, and the weight of the nodules as a percentage of the total dry weight of the plant, which gives an indication of the relative development of the nodules, were calculated.

Type of nodulation. Nodules were classed into the following types:- beads, small simple, large simple, and compound. These will be illustrated later. The following

combinations of nodules were also noted, small simple and large simple; beads and simple (both large and small); simple and compound; and beads, simple and compound.

Location of nodules. In this respect the plants fell into two main classes, those where the nodules were scattered over the whole root system and those where the nodules were confined principally to the top half of it.

Colour of nodules. A number of nodules from each jar were cut open and an indication of whether the majority were white or had the red pigment was obtained. If the colour of the nodules was either green or brown they were classed along with the red pigmented nodules since, as mentioned in the General Introduction, Virtanen (1945) has shown that the presence of green or brown pigmentation is indicative that the red pigment has been present.

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

It is probable that nodulation by a given strain on a given host variety is affected by various factors, such as temperature, the pH of the rooting medium, and the general nutrition of the plant. The present plants were grown under conditions where there was little variation in respect of these factors. A further factor which may affect the number of nodules formed is the heaviness of the original inoculum (Thornton, 1929, Bhaduri, 1951). In the present work a heavy inoculum was applied in all cases (p. 42), but the actual content of bacteria no doubt varied widely, and it is possible that nodulation by certain strains was favoured by an unusually high bacterial content in the inoculum.

The results obtained in respect of the criteria listed in the previous Section for four isolations, with 36 plants for each isolation, are presented in Table 1 and Histogram 1. Analyses of variance show that there was no significant difference ($P = 0.05$) in the number of nodules produced by different isolations, but that the differences in the average size of nodules, as indicated by dry weight, were highly significant ($P = 0.01$), and those in relative development, as indicated by nodule weight as a percentage of total dry weight, were also significant ($P = 0.05$).

The results of the visual estimation of the number of nodules produced by 160 isolations are presented in Histogram 2. As well as being divided into classes as given on p. 111, they were separated into effectiveness groups and percentages taken. Most of the isolations tended to produce plants with abundant or average number of nodules. Ineffective strains are the only ones productive of very

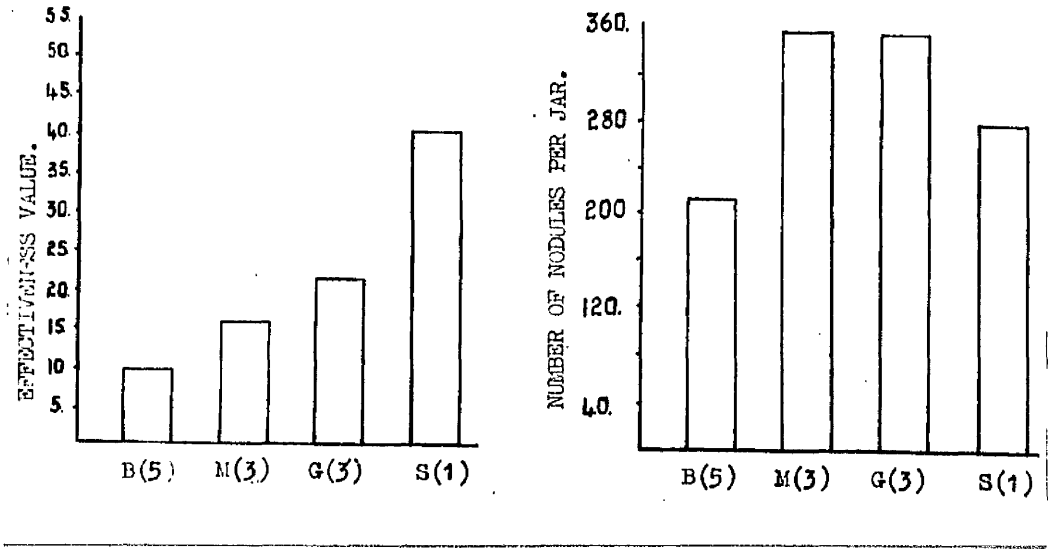
Table 1.

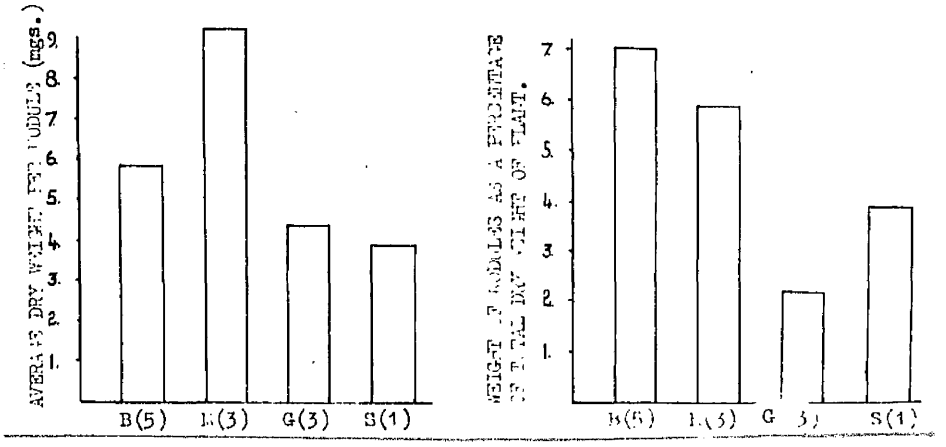
Data on nodulation for four isolations, thirty-six plants being grown for each isolation.

Isolation	Effectiveness value.	Average no. of nodules (per jar of 2 plants)	Average weight per nodule (mg)	Dry wt. of nods. as a percentage of total plant dry wt.
B(5)	10	214	5.9	7.0
M(3)	17	360	9.3	5.8
G(3)	23	351	4.2	2.2
B(1)	40	275	3.9	3.9

Histogram 1.

Effectiveness, number of nodules, average weight per nodule and this weight as a percentage of the total dry weight of the plant, for four isolations, the data being detailed in Table 1.

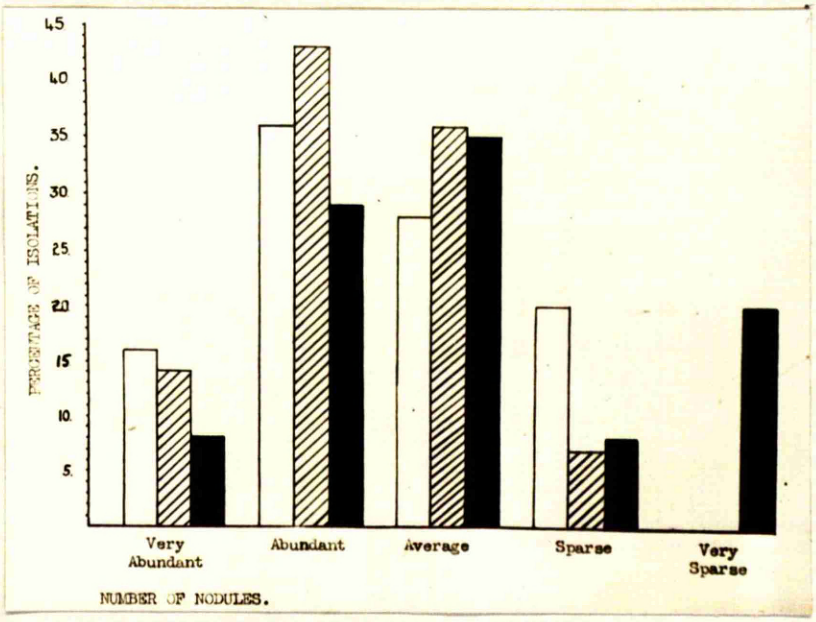




To p119

Histogram 3.

Visual estimation of the number of nodules on plants, based on the examination of 160 isolations, six plants being grown for each isolation.



Effectiveness value

Effective = > 40

Intermediate = 20-40

Ineffective = < 20

sparse nodulation. It may be that these particular isolations have poor powers of infectivity as well as effectivity.

Type of nodulation.

The different types of nodules described on page are illustrated on Figure 1 and in the following figures:-

Nodules classed as beads, Fig. 6.

" " " small simple, (usually round nodules) Fig. 2.

" " " large simple, (usually long oval-shaped) Figs. 4 and 7.

" " " compound (any cluster or aggregate of nodules) Figs. 5 and 8.

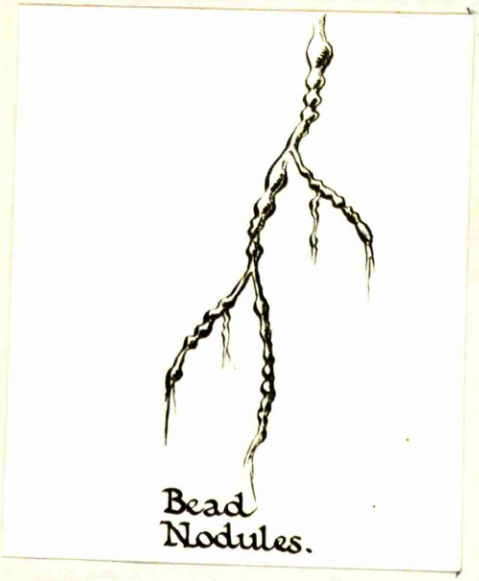
The percentage of the different effectiveness ~~classes~~ values falling into each nodulation class are presented in Histogram 4. The two types of nodulation which are commonest for all effectiveness classes are "small simple", and "beads and small simple". Ineffective isolations produced plants showing the widest range of types of nodulation and were the only isolations to produce beads or compound nodules only on the roots. Some of the types of nodulation produced by ineffective isolations are illustrated in Figures 2 - 5 and by effective isolations in Figures 6 - 8.

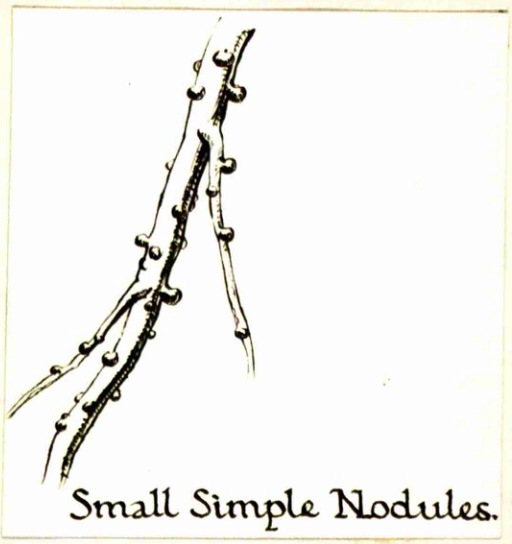
Location of nodules.

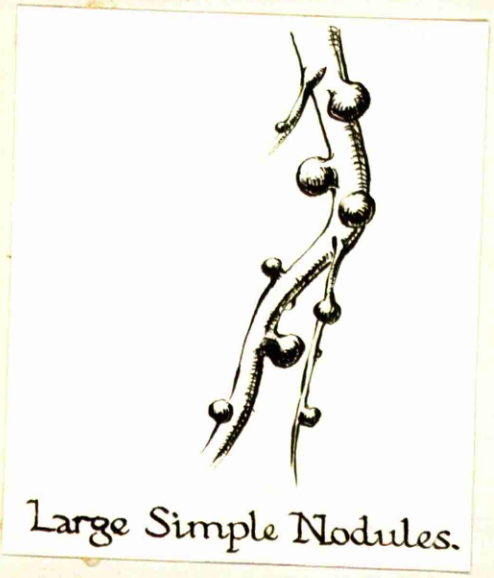
Observations of the position of nodules will be summarised in the discussion.

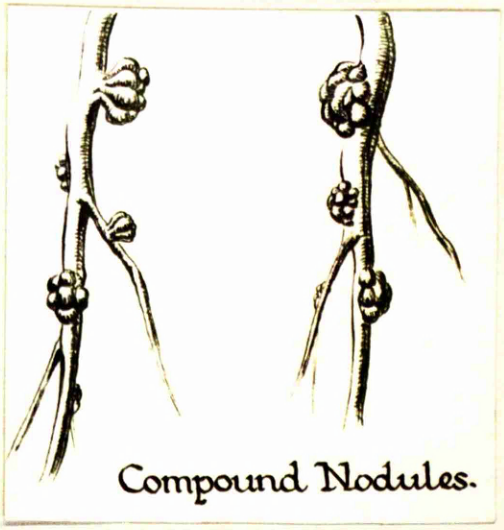
Figure 1.

Types of nodules obtained on the field bean plants (actual size).









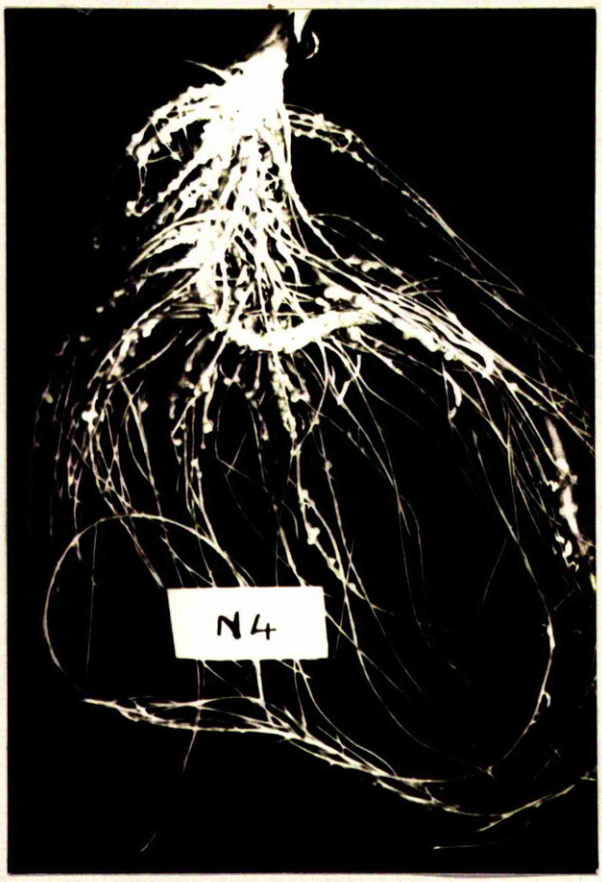


Fig. 2. Effective strain producing a very large number of small nodules.

Fig. 3. Effective strain producing a large number of large nodules, a few beads and some small nodules

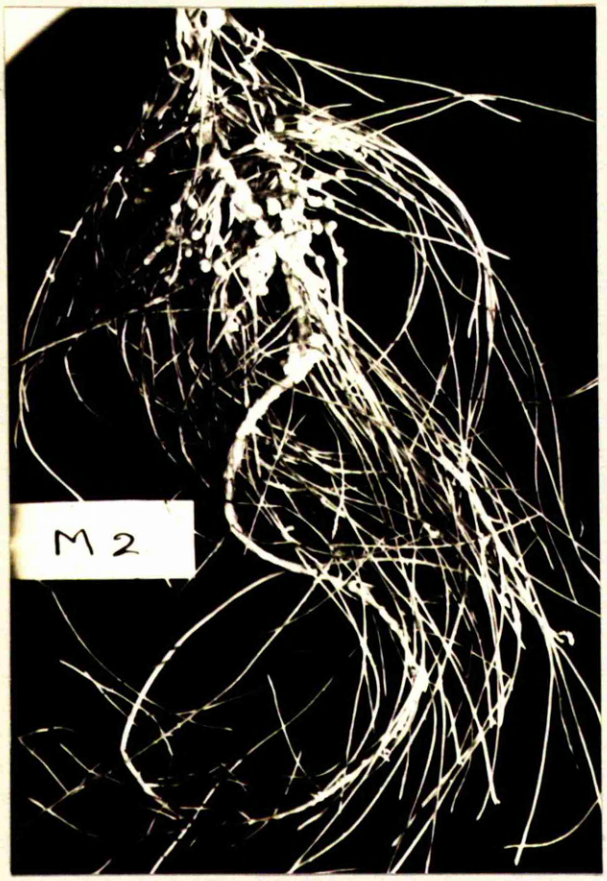


Fig. 4. Effective strain producing an average number of large nodules and a few beads.

Fig. 5. Effective strain producing a small number of compound nodules.

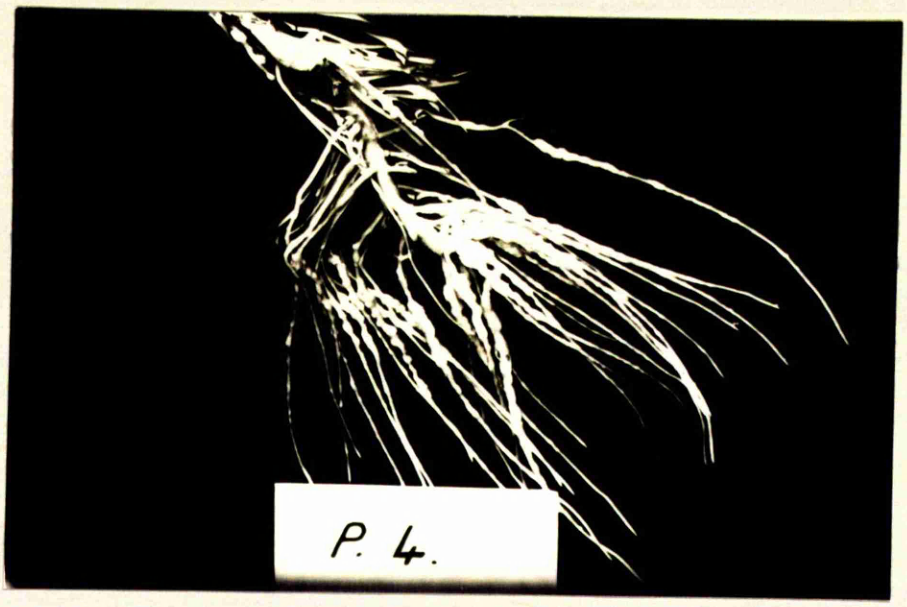


Fig. 6. Ineffective strain producing a large number of beads.

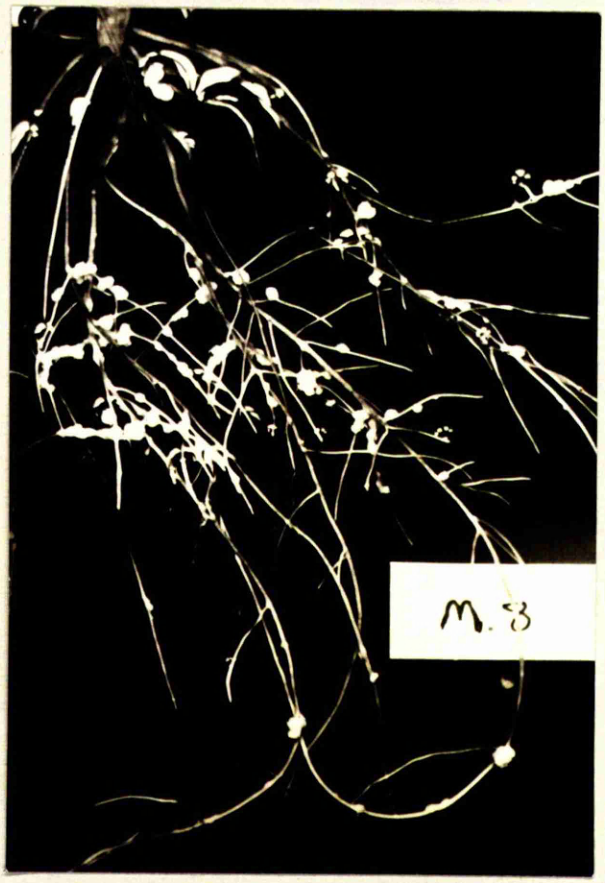


Fig. 7. Ineffective strain producing a large number of large nodules.

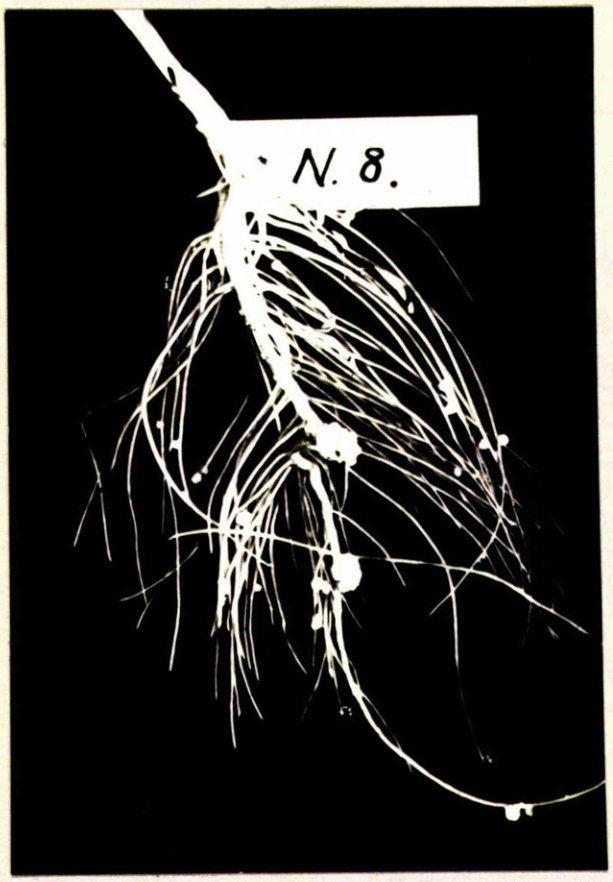


Fig. 8. Ineffective strain producing a few large simple and a few compound nodules.

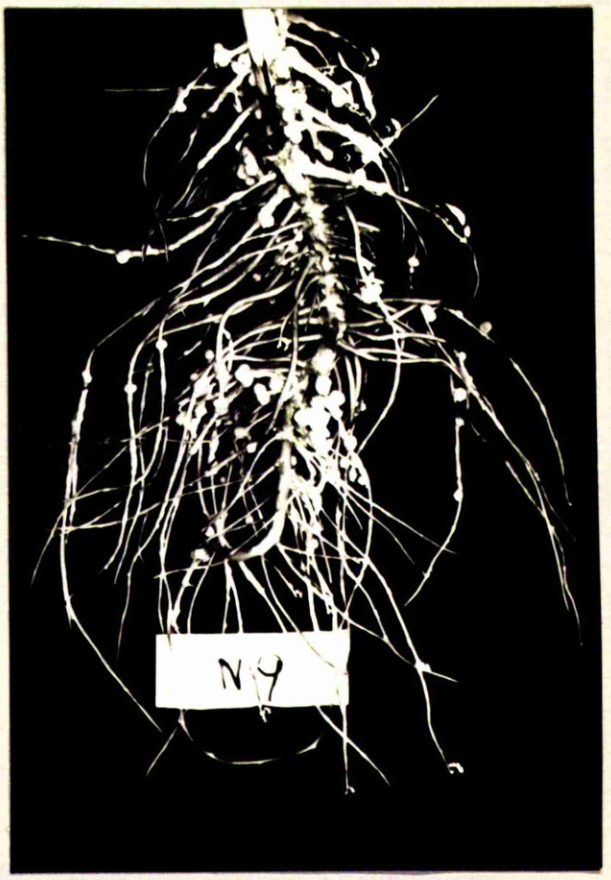
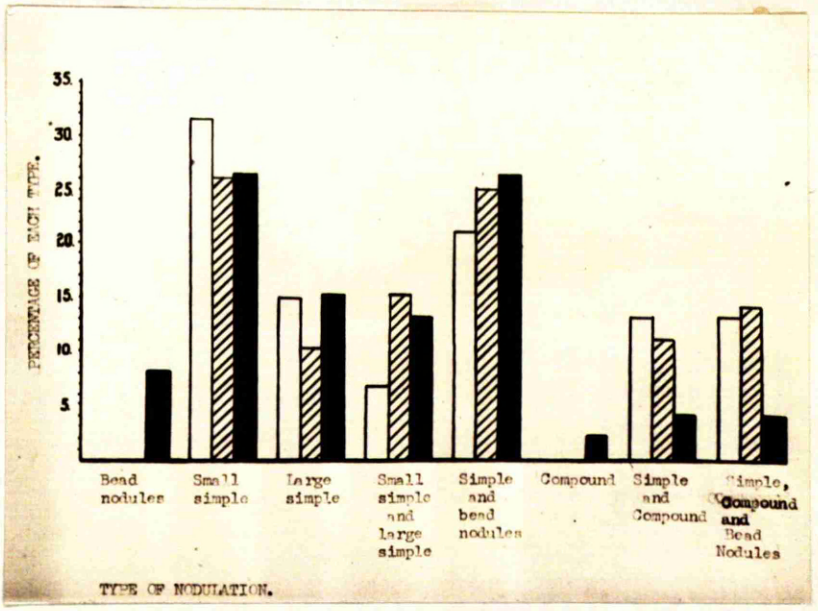


Fig. 9. Intermediate strain producing a very large number of nodules of all types and sizes.

Histogram 4.

Percentage of each type of nodulation in the different effectiveness classes.



Effectiveness value

Effective = > 40

Intermediate = 20-40

Ineffective = < 20

Colour of nodules.

The percentage of isolations having the red pigment or lacking in it, in each effectiveness group are presented in Table 3. With strains of high effectiveness value there is only a small percentage of isolations producing no pigmentation but with strains of low effectiveness 40 per cent of the isolations have no pigmentation.

Table 3.

Estimation of the percentage of isolations producing nodules with red pigmentation.

Effectiveness value.	Colour of nodules		No. isolations
	% red	% white	
> 40	97	3	67
20-40	98	2	58
< 20	60	40	35

D I S C U S S I O N

As noted already statistical examination of the data for the numbers of nodules presented in Table 1 showed no significant difference between isolations of markedly differi effectiveness. Also, if Histogram 3 is considered it can be seen that isolations classed as effective are well spread over the various groups referring to the number of nodules. Sparse nodulation was produced by 20 per cent of the isolatio and 16 per cent produced very abundant nodulation. Isolation classed as ineffective are also well spread over the various groups. Therefore the number of nodules produced by the isolations varies greatly from isolation to isolation and does not seem to be correlated with the effectiveness value.

In Table 1 the relative development of the nodules is significantly greater with isolations B(5) and M(3) than with the other isolations. Isolations B(5) and M(3) were of low effectiveness. This does not necessarily mean that there was a large number of small nodules produced by these poor strains as the plant itself was much smaller than a plant produced by a good strain and hence the nodules make up a greater percentage of the total dry weight of the plant.

It is interesting to note that, when considering a

single isolation, although some plants produce a large number of nodules, the weight of these nodules is such that the percentage of the total dry weight is no greater than with a plant, inoculated with the same isolation, which produces fewer nodules. This weight as a percentage total dry weight is a fairly constant figure, so that variation in number seems to depend on variation in weight and size of the plant. This is in agreement with Bhaduri's (1951) findings that the number of nodules is related to the final weight of the plant.

It would seem therefore that field beans grown under the conditions of these experiments do not conform to the general concept that in legumes under comparable conditions effective isolations produce small numbers of large nodules and ineffective isolations large numbers of small nodules. In field beans the number and type of nodules produced varies greatly from isolation to isolation ~~in the same~~ of similar effectiveness ~~class~~ value, and although some isolations would conform to the general pattern for legumes others would be exactly the opposite. Thus the two isolations studied by Helz, Baldwin & Fred (1927) conformed to the general pattern. It is only when a large number of isolations are examined that the extent of the variation in size and number of nodules can be found.

No particular type of nodulation except bead nodules alone seems to be a characteristic indication of

the effectiveness of an isolation. Bead nodules alone are always indicative of very ineffective strains. Dehérain & Demoussy (1900) however found that bead nodules were produced on plants with the highest percentage nitrogen. The lupins, however were growing in the field and may have had other sources of nitrogen.

Apart from bead nodulation the observations presented here are similar to those made by Dehérain & Demoussy with white lupins, Nobbe & Hiltner with peas and Hiltner with yellow lupins, that the large nodules found on these plants were not necessarily a benefit to them.

The position of the nodules was also noted. There was again much variation, but the observations showed that 50 per cent of the plants produced by isolations with an effectiveness value greater than 40 had their nodules concentrated on the top half of the root system while only 15 per cent of isolations with effectiveness values less than 20 produced plants with nodules in that position. So although the position of nodulation cannot be said to be unfailingly characterisitic of effectiveness of isolations, nodules concentrated on the top half of the root system are more typical of strains of high effectiveness values than other strains

The observations on the pigmentation of the nodules indicated that while isolations of effectiveness value over 20 mostly produced the red pigment, only 60 per cent of isolations whose effectiveness value was below that figure (i.e. were ineffective) produced it, the remainder having no pigmentation. This does not necessarily disagree with Virtanen's observation that ineffective strains are always colourless since many of the strains considered to be ineffective here were not completely ineffective as defined by him (i.e. fixing no nitrogen), but fix a little nitrogen.

S U M M A R Y

1. Observations on the number, type, size and location of the nodules of the field bean plants grown in connection with the previous sections of this thesis, have been made.
2. The nodules produced by 160 isolations of the field bean nodule bacteria were examined and placed in a number of classes for each of the above characters.
3. With four isolations all the nodules on thirty-six plants, were counted, dried and weighed.
4. The number and type of nodulation varied greatly from isolation to isolation and was not correlated in any characteristic fashion with the effectiveness of a strain.
5. Strains of high effectiveness value, in general, produced smaller but more numerous nodules which made up a smaller percentage of the total dry weight of the plant than strains of low effectiveness value.
6. Nodules concentrated on the top half of the root system were found to be commoner on strains of high effectiveness value.
7. In many aspects the nodulation of field beans, in water culture, does not clearly conform to the general pattern accepted for nodulation in legumes.
8. Red pigmentation was found in nearly all the nodules produced by strains of high effectiveness value, but a considerable proportion of nodules formed by strains of low effectiveness value were white.

To p.134

SECTION IV.

The Variation in Growth of Individual Clover and Field Bean Plants Associated with a Given Strain of the Nodule Organism.

C O N T E N T S.

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

The difficulties attached to the use of the terms 'effective', 'ineffective' and 'intermediate' with reference to strains of the nodule organism have been pointed out in earlier Sections of the thesis (especially Section I p.28). It was indicated there that some strains vary in their effectiveness depending on with which particular species or variety of host plant they are associated while in the case of genetically-variable host plants such as clover some authors have concluded that marked variation may occur between the effectiveness of a given strain on different individual plants of a variety.

This Section of the thesis presents some observations on the last kind of variation mentioned above, namely, on the extent of the variation in the effectiveness of the association of given strains of nodule organism and different individuals of a population of white clover plants and a population of field bean plants. In 1948 the author's attention was drawn by Dr. G. Bond to features presented by red clover test plants which were being grown by him in aseptic tube culture in connection with the clover strain survey to which reference has been made on p.31. Inspection of the plants, while still in the tubes, suggested that while with effective and ineffective strains the growth of the different plants in the six tubes grown for each isolation were uniformly good and

poor respectively, with intermediate strains some of the plants were as strong as those associated with effective strains, while others, though infected with the same strain, resembled those associated with ineffective strains. It seemed then that these strains were more selective with regard to the host plant than fully effective or ineffective strains and were unable to co-operate effectively with certain of the plants grown from a sample of commercial seed.

In spite of the fact that this variation in plant response to the same strain of the nodule organism must have been observed by investigators testing a strain for effectivity on more than one plant, very few of them have commented on the variation or investigated it further. Vincent (1944) appears to be the first to have reported this variation. He found that with some strains of Rhizobium trifolii inoculated into white clover the plant response was very varied, though he showed by serological means that the bacteria in the nodules of poor and healthy plants were the same. Thornton (1946) noted variation in response of plants in a commercial sample of red clover seed to an effective culture. Highly ineffective strains produced no plant variation but with fairly ineffective strains

) a few plants showed variation. He suggests that variation is due to recessive

genes in the red clover plants which are very specific to individual strains. The genetic aspect of this variation was further studied by Nutman (1946). He found that an intermediate strain was only intermediate in respect of the average plant response, since effective, ineffective and intermediate plants were produced by it. The intermediate plants only gave rise to effective symbiosis when certain dominant genetic factors were present in the plant.

In a further paper published after the present work was commenced, Nutman (1949) mentions studies in the range of effectivity response in terms of dry weight of a wild population of red clovers. He found that strains which were effective in the average sense produced 'good' 'mixed' and 'poor' plants although the largest number of plants were in the 'good' class. With ineffective strains the majority of the plants were in the 'poor' class and few or no 'good' plants were produced. With intermediate strains he obtained a more marked scatter of plants than with effective strains. The intermediate response he suggests is a genetically mixed response and not intermediate in the true sense of the term.

In the present work the extent of variation in growth between individual plants of white clover and field bean, inoculated with given strains of the nodule organism, has been investigated, the tests on clover being carried out in 1949 and those on field beans in 1950.

M E T H O D S.

A. Clover.

The strains of clover organism used in this work were provided by Dr. G. Bond, eighteen strains being employed. These strains, which had originally been isolated from nodules collected from the field in the West of Scotland, were inoculated into white clover growing in aseptic tube culture, exactly the same procedure being used as in Section II of the thesis. Commercial seed of New Zealand white clover was employed. Twelve tubes were grown for each strain, with two plants per tube. After 10 weeks' growth, the plants were harvested separately and dried to a constant weight at 100°C. The 'average' percentage effectiveness of each strain was calculated as in Section II, by reference to plants inoculated with a standard strain and to uninoculated control plants.

B. Field Beans.

The strains of field bean organism used were some of those tested in connection with the survey in Section I. They were inoculated into field bean plants grown in water culture by the same procedure as used in Section I. Two jars were grown for each strain, with three plants per jar. After 10 weeks' growth, the plants were harvested separately and dried to a constant weight at /

100°C. the 'average' effectiveness value of each strain was calculated as in Section I, by reference to uninoculated control plants.

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

A. Clover

In Table 1 the distribution of plant dry weights for each of the eighteen strains is shown, together with the 'average' percentage effectiveness for each strain calculated from the present data. The strains are arranged in order of increasing effectiveness.

It will be observed that in the case of strains of negative effectiveness value all the test plants were of uniformly poor growth, so that little variation was shown. With the others however, considerable plant-to-plant variation was shown in respect of dry weight, so that an assessment of effectiveness based on the examination of the dry weight of a single plant might range from complete ineffectiveness to high effectiveness. The variation in plant growth is most marked in the case of strain R, this being also the strain with the highest 'average' effectiveness, and in general the variation tends to be greater with strains of high effectiveness.

A point of incidental interest is to compare the 'average' behaviour of these eighteen strains on white clover, as indicated by the present author's data, with that previously shown by the same strains associated with

Table 1.

Frequency of individual plant dry weights among 24 clover plants associated with each strain.

STRAIN	% EFF.	WEIGHT OF PLANT IN MILLIGRAMS									
		0-4.9	5-9.9	10-14.9	15-19.9	20-24.9	25-29.9	30-34.9	35-39.9	40-44.9	45-49.9
A	-15	21	3	-	-	-	-	-	-	-	-
B	-9	20	4	-	-	-	-	-	-	-	-
C	-6	18	6	-	-	-	-	-	-	-	-
D	-4	17	7	-	-	-	-	-	-	-	-
E	-4	22	2	-	-	-	-	-	-	-	-
F	-1	18	6	-	-	-	-	-	-	-	-
G	31	15	7	2	-	-	-	-	-	-	-
H	33	1	15	5	3	-	-	-	-	-	-
I	35	9	6	2	4	2	1	-	-	-	-
J	48	6	4	7	4	1	2	-	-	-	-
K	50	2	6	11	3	2	-	-	-	-	-
L	56	4	9	6	3	1	1	-	-	-	-
M	66	3	3	5	9	3	1	-	-	-	-
N	75	5	4	5	1	4	4	1	-	-	-
O	82	2	1	7	14	-	-	-	-	-	-
P	85	2	1	8	5	5	3	-	-	-	-
Q	100	1	4	4	5	3	6	1	-	-	-
R	142	1	1	3	4	3	3	4	3	1	1

red clover test plants. In Table 2 this comparison is made, the data for red clover having been communicated by Dr. G. Bond. It will be seen that with only two exceptions the strains fall into the same effectiveness group with red or with white clover, a result in agreement with the findings of Bond & McGonagle (1951) that strains behave similarly on these two plants.

B. Field beans.

The data obtained for the field bean plants are presented in Table 3. The strains are arranged in increasing order of effectiveness value. The dry weight of plants of low effectiveness value show little variation, but the variation does increase with effectiveness value.

Table 2.

Effectiveness of Isolations on Two Different Host Species[X].

Isolation	Effectiveness class on red clover	Effectiveness class on white clover.
A	I	I
B	I	I
C	I	I
D	I	I
E	I	I
F	I	I
G	I	M
H	M	M
I	M	M
J	M	M
K	E	M
L	M	M
M	M	M
N	E	E
O	E	E
P	E	E
Q	E	E
R	E	E

X

I Percentage effectiveness < 20

M " " 20-70

E " " > 70

Table 3

Frequency of individual plant dry weights among six field bean plants associated with each strain.

STRAIN	EFF. VALUE	WEIGHT OF PLANT IN GRAMS										
		0-0.5	0.6-1	1.1-1.5	1.6-2.0	2.1-215	2.6-3.0	3.1-3.5	3.6-4.0	4.1-4.5	4.6-5.0	5
A	13	3	3	-	-	-	-	-	-	-	-	-
B	16	1	5	-	-	-	-	-	-	-	-	-
C	17	1	4	1	-	-	-	-	-	-	-	-
D	18	1	3	2	-	-	-	-	-	-	-	-
E	19	-	3	1	2	-	-	-	-	-	-	-
F	24	-	2	2	2	-	-	-	-	-	-	-
G	28	-	1	2	3	-	-	-	-	-	-	-
H	32	1	-	2	1	-	1	1	-	-	-	-
I	33	-	1	2	3	-	-	-	-	-	-	-
J	35	-	-	1	3	1	1	-	-	-	-	-
K	43	-	-	2	-	2	1	-	-	-	-	-
L	49	-	1	1	1	-	1	-	1	1	-	-
M	55	-	-	1	-	1	1	1	1	-	1	-
N	56	-	-	-	-	1	2	-	1	1	-	1
O	77	-	-	-	-	2	2	1	-	1	-	-

D I S C U S S I O N

The results presented above suggest that in clover the strains tested fall into two main groups. Firstly those with little plant-to-plant variation associated with low percentage effectiveness, the plants being uniformly poor; secondly those with considerable plant-to-plant variation associated with intermediate and high percentage effectiveness, so that while some plants were of strong growth, others containing the same strain showed growth which was no better than that produced by typical ineffective strains. This plant variation is greatest with effective strains, so that in this respect the results do not agree with the preliminary observations (in that case on red clover) mentioned on p.136 or with Nutman's observations (also on red clover) indicated on p.138 to the effect that intermediate strains produce the greatest plant variation.

In the case of field beans fewer plants were grown with each strain, so that only tentative conclusions can be drawn. In general the position as regards plant variation seems to resemble that with white clover.

It will be profitable to consider the factors which may contribute towards the production of this plant-to-plant variation in the case of intermediate and effective strains. They include the following:-

1. The size of the seed used. With such small

seed as clover it is scarcely practicable to select seed for uniformity of weight. The seeds undoubtedly vary considerably in size and weight, and seedlings from the larger seeds better endowed with food reserves will gain an early advantage, and these differences in growth are likely to persist and become larger as the growth period progresses.

The field bean seeds were to a certain extent selected for uniformity of weight, though owing to the great variability of weight in the seed as purchased a considerable latitude had to be allowed in the seed selected for actual use.

2. Competition between plants. The limitation of growing space involved in the test-tube culture of clover has already been noted. When, as in the present experiments, two plants are grown in each tube, if one of the plants grows more strongly in the earlier stages as a result, for example, of greater size of seed, then this plant monopolises an undue proportion of the growing space, to the detriment of the second plant.

3. Genetic differences between host plants. It is well-recognised that commercial seed of clover and field bean is genetically impure, and that the plants produced under uniform conditions of growth are liable to vary considerably in size and in morphological characters. In addition, as noted already, Nutman (1946) considers that they vary in their specific response to inoculation

with a given strain of the nodule organism. Variations of the first type may, however, also affect the success of the symbiosis between an individual plant and a strain of the nodule organism, and we can thus distinguish between (a) genetic differences of a general, non-specific type which will influence the symbiotic relationship between a particular plant and any effective or intermediate strain, and (b) genetic differences of the type considered by Nutman which will result in one individual plant of a population setting up a highly successful relationship with a given strain, while another individual fails to do this with the same strain.

A factor of type (a) of obvious importance in this connection is the general metabolic potentiality of the plant. Some plant individuals may have a capacity for more rapid photosynthesis and growth than others. Among plants that are all associated with a given effective strain and are growing on a medium free of combined nitrogen, fixation of nitrogen will be most rapid in these plants with the higher capacity for photosynthesis, growth will be correspondingly greater and variation in dry weight attained will result.

Information as to the extent of such variations in growth potentiality would be obtained by the study of non-nodulated plants supplied with combined nitrogen. Unfortunately no individual plant data are available for such clover or field bean plants grown under the same

cultural conditions as employed by the present author for the nodulated plants. Figures have, however, been kindly supplied by Mr. T.P. Ferguson, of the Department of Botany, University, Glasgow, in respect of red clover plants grown in water culture from Aberystwyth S.123 seed. The containers for the culture solution consisted of large test tubes, with one plant per tube, the tops of the plants being freely exposed. The general conditions of growth appeared to be very uniform from tube to tube. The final dry weights of 14 non-nodulated clover plants supplied with ammonium nitrate are presented in the left-hand column of Table 4, and, although all the plants were of healthy appearance, it is obvious that they varied very much in size and dry weight, presumably in the main because of genetic differences in growth capacities. The percentage coefficient of variation, that is $\frac{\text{Standard Deviation}}{\text{Mean}} \times 100$, for the dry weights of these plants = 50.

The figures (also supplied by Mr. Ferguson) in the right-hand column of the same Table refer to nodulated plants infected with an effective strain of the nodule organism and growing in nitrogen-free solution, but otherwise comparable with the above non-nodulated plants. The variability of these plants, relative to the lower level of dry weight, was similar to that in the above plants. Thus the coefficient of variation here was 48.

Table 4.

Variation in dry weight of individual red clover plants supplied with ammonium-nitrogen and plants inoculated with an effective strain.

Dry weight of plants supplied with ammonium-nitrogen (mgs.)	Dry weight of nodulated plants (mgs.).
751	265
385	389
1032	733
676	189
506	229
614	690
1188	122
1240	185
419	523
542	454
378	285
413	567
1278	629
295	461
	322
	436
	695

In the particular instance of the plants to which Table 2 refers it seems that all the plant-to-plant variation shown among the nodulated plants can be explained by the inherent differences in growth potentiality of the host plant, as illustrated by the behaviour of the non-nodulated plants. It is unnecessary to postulate the existence of genetic differences specifically affecting the symbiotic relation in the sense of (b) above.

It is uncertain how typical the foregoing results are, and how closely they apply to the clover and field bean plants grown by the present author. The author's finding that large plant-to-plant variation is shown by effective and intermediate strains, that is, by all strains with which appreciable fixation is ever associated, rather suggests that genetic differences of a general nature were responsible. Although further investigations are obviously necessary before the relative importance of general as distinct from specific genetic variations is found, it seems likely that variations of the former type are in part responsible for the plant-to-plant variation experienced in effectiveness trials.

A final point of practical interest is that the results obtained in this investigation emphasise the desirability of growing as many replicate plants as possible in strain-testing investigations, especially when the host plant population is known to be genetically impure.

S U M M A R Y.

1. A study has been made of the variation in growth between different individual plants of white clover and field bean associated with a given strain of the appropriate nodule organism.
2. The particular object was to find whether the plant-to-plant variation was particularly marked with the so-called intermediate strains of the nodule organism.
3. In the case of clover eighteen strains of varying degrees of effectiveness of the organism were employed, 24 plants being grown in association with each strain. The plants were grown in aseptic tube culture. At harvest they were separated, dried and weighed individually.
4. In the case of field bean fifteen strains of varying degrees of effectiveness were employed, 6 plants being grown in association with each strain. The plants were grown in water culture and at harvest they were treated as with clover.
5. The results showed that plant-to-plant variation was greater with isolations of high effectiveness value than with those of intermediate value. There was little or no variation with isolations of low effectiveness value.
6. Size of seed, competition between plants and genetic differences between host plants, both general non-specific differences and differences in their ability to co-operate with a given strain of the organism, are

discussed as possible reasons for plant-to-plant variation. It is probable that genetic differences between host plants of non-specific type have not been taken sufficiently into account in genetical studies of the host bacterium relationship.

7. The necessity of growing as many replicate plants as possible in strain-testing investigations especially when the host-plant population is known to be genetically impure is mentioned.

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SOME ASPECTS OF SYMBIOTIC FIXATION OF NITROGEN

Extension of Thesis presented by

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C O N T E N T S O F T H E S I S.

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

This section of the thesis is based on work done during the tenure of the Johnstone and Florence Stoney Studentship awarded by the Federation of University Women and a British Commonwealth Travel Scholarship. This enabled the author to spend a year during 1952-53 in the Microbiology Department, Faculty of Agriculture, University of Sydney, Australia. Mr. J. M. Vincent, provided facilities and advice for this work and the author wishes to express her grateful thanks to him. Technical assistance was given by Misses A. Brodie and M. Price and photographs were by Mr. Woodward-Smith.

Whilst the methods used in the following Sections are essentially similar to those in the earlier parts of the thesis, they differ from them in a number of respects. This is due to the methods used by workers in the Sydney laboratories being adopted in order that results for the survey would be comparable with their work and that technical help would be available.

SECTION V

A survey of the strains of clover nodule bacteria in the New England Region of New South Wales.

C O N T E N T S

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

The establishment of clover plants in certain parts of Australia is of great importance since it leads to the formation of good permanent pasture. When introduced into native pastures along with Wimmera rye-grass, in the drier country and Phalaris tuberosa in higher rainfall areas, clovers have substantially raised the wool clip per acre and made fat lamb production possible. Any clover which can be established is of value but subterranean clover is particularly suited to Australian conditions for the following reasons; (a) it can stand much drier conditions than other species of clover provided it has some winter rainfall; (b) it can thrive in acid soils - an important factor when cost of correcting acidity by liming is high; (c) it gives a good yield if well supplied with superphosphate; (d) its seed can establish itself under favourable soil conditions; (e) it regenerates from seed after grazing.

A marked improvement in pasture was obtained in the leach soils of Kangaroo Island, South Australia by the establishment of subterranean clover. The clover grew well when it was supplied with fertiliser containing phosphate and when unpolished seed was used, since the rhizobia necessary for nodulation would be carried in the seed, (Strong,1938). Other marked improvements in pastureland were obtained by seed inoculation of clovers in parts of South and West Australia.

Investigations already undertaken for areas of New South Wales, (Vincent, 1945; Purchase & Vincent, 1949; Purchase, Vincent & Ward, 1951 and Vincent, 1953) have shown that many of the rhizobia already present in the soil might be unable to fix nitrogen in association with introduc species of Trifolium and Medicago. It seemed possible therefore that lack of effective nitrogen fixing bacteria in the soil would hinder the establishment of clovers in the Northern Tablelands, an area in which the present author had an especial interest.

Topography: The tablelands of New South Wales form the Grea Dividing range between the coastal districts and the plains. The plateau is divided into northern, central and southern districts. The New England Region which constitutes the Northern Tablelands is an area of 13,000 square miles consisting mainly of hilly to mountainous country with tablelands and valleys, the average altitude being 3,200 fe above sea level. It is bounded to the east by steep escarpments and very rugged country, and the eastern margin rises to 5,000 feet, where the uneven surface falls to the plains, in the north-west. The southern boundary co-incide with a steeper fall into the broad valley of the Manilla an Namoc rivers. The area has a remarkable number of level areas at different heights (peneplains) and these are dissected by gorges. The common rocks are basalt, gravel and trap. Much of the countryside is rugged, unsuitable fo cultivation.

Climate: The mean annual temperature is 61°F., the summer temperature being 80°F. (daytime) falling by as much as 25°F., at night and the winter temperature 50 - 60°F., falling to near freezing point at night. Frosts occur from April to October. Snow falls on the highlands. The average rainfall is rather less than 40 inches along parts of the Eastern boundary falling to 30 inches in the West. One third of the rainfall occurs between December and February.

Vegetation: The natural vegetation of the area is tropical rainforest, wet and dry sclerophyllous forest, savannah and scrub woodland, scrub and dry tussock grass. Natural grassland was not of extensive occurrence although now large areas are vegetated by native grasses. Common native grasses are purple wire grass (Aristoda ramosa) wallaby grass (Danthonia caespitosa) and red grass (Bothiochloa ambiguus).

Land Use: The nature of the countryside and the climate make it most suited for stock and most of the tablelands are given over to sheep and cattle grazing. There is some grain grown and a small area is used for dairying. The average size of a farm is about 2,000 acres. The problem of feeding animals in this district is bound up with the high summer rainfall and the low winter temperatures. In the summer there is an adequate to excessive growth of native grasses but after the first frosts the top growth of the plants becomes dry and there is a decline in their nutritive value. A period of near-starvation for sheep results which in severe winters or in over-stocked paddocks results in many deaths as/

as well as a general decline in condition. Supplementary feeding is necessary but because of climate, soils and topography it is difficult to establish sown pastures. The outstanding deficiency of native species is the lack of pasture legumes. Sown pastures in fertilised soils have doubled their stock carrying capacity and kept stock in good condition throughout the year. Introduced and naturalised species are Phalaris tuberosa, Cocksfoot (Dactylis glomerata), Perennial ryegrass, (Lolium perenne), Tall fescue (Festuca elatior), red, white, alsike, cluster, subterranean, and crimson clover, black medick (Medicago lupulin and salad burnet (Poterium sanguisorbium). Subterranean clover would be particularly valuable here since it could supply winter feeding. While the conditions are not those found so suitable for subterranean clover in South Australia it seemed possible that certain varieties of subterranean clover would grow in the area. Difficulties were encounte in some areas with sown clovers by officers of the Regional laboratory of the Commonwealth Scientific and Industrial Research Organisation. I seemed possible that either the absence of rhizobia or the occurrence of poor native strains, rather than climatic conditions, might be the cause of some of the difficulties met with in establishing the plant. Consequently a survey of native strains of rhizobia was undertaken.

The approach to this investigation has been to survey the naturally occurring root-nodule bacteria in order to assess their ability to fix nitrogen with the chief clover species concerned and to obtain informati on the relative merits of several strains of rhizobia for use as seed inoculum. (as described in Section VI)

M E T H O D S.

1. Collection of plants.

It was desired to obtain an idea of the types of native clover rhizobia from a very large area. The advice of the C.S.I.R.O. officers was sought in the selection of seven localities representing as far as possible different soil types, climatic conditions and natural clover hosts. There are two fairly well defined climatic regions. The 'wet' region is represented by the tableland tops, the commonest species of clover here being white clover, while cluster clover and suckling clover are also present. The 'dry' region occurs mainly on the Western slopes of the North-East, the commonest clover here is cluster clover and this area is considered most suited for subterranean clover. The actual position of the properties (large farms) is shown in Figure 1. Details of climate, soil type and plant samples are given on Table 1. Where subterranean clover was collected from an area, it had been recently planted there. Isolations from plants collected previously from the three additional centres were also included. The number of isolations from each paddock varied because in some paddocks there were practically no clover plants to collect.

An isolation of the organism was made from the nodule nearest the crown of each plant. In all 190 isolations were included in the survey.

Figure I.

Position of The properties.

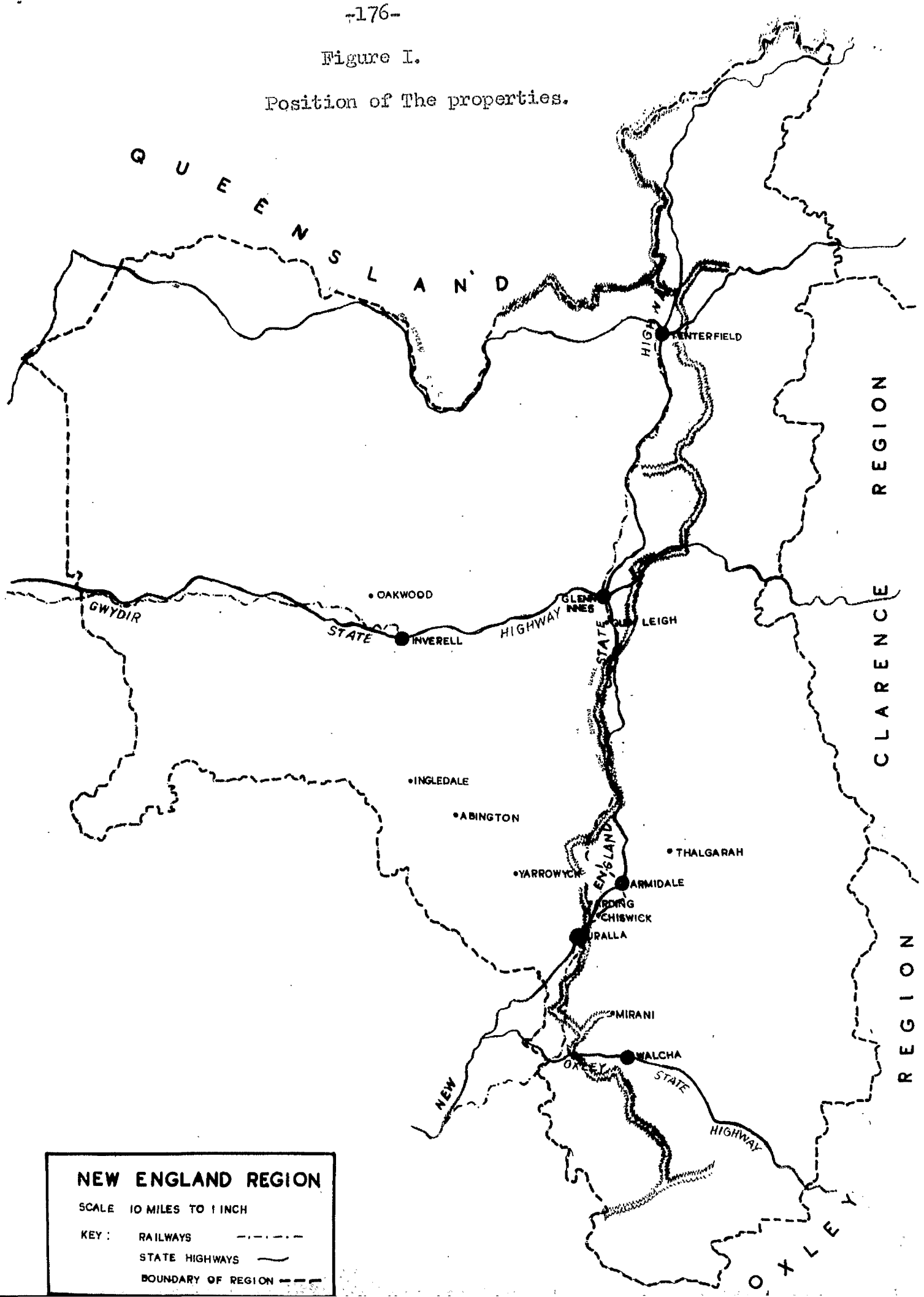


TABLE 1.
ARMIDALE COLLECTION

Centre	Property	Climate[x]	Soil	Isolation made from[*] T.D.	T.R.	T.P.	T.G.	T.S.	Total
Thalgarah	Swallowfield	Wet	Yellow podsolic derived from sedimentary rocks.		8	5	7		20
Bundarra	Ingledale	Dry	Yellow podsolic derived from coarse granite.				20		30
Oakwood	Lovely Vale	Dry	Yellow podsolic derived from fine granite.				20	7	27
Stonehenge	Glen Leigh	Wet	Yellow podsolic derived from fine granite.	6	6	1			13
Glen Innes	Thomas ϕ	Wet	Yellow podsolic derived from fine granite.					7	177
Walcha	Mirani	Wet	Yellow podsolic derived from sedimentary.	6	9		5	15	35
Uralla	Chiswick	Wet	Lateritic podsolic derived from sedimentary.		13		7		20
Bundarra	Abington	Dry	Yellow podsolic derived from porphyry.				16	8	24
Uralla	Chiswick ϕ (Arding paddock)	Wet	Lateritic podsolic derived from sedimentary.		3			13	16
Uralla	Yarrowyck ϕ	Wet	Yellow podsolic from fine granite.					8	8
							GRAND	TOTAL	190

ϕ From a previous collection

x "Wet": Tableland tops and white clover habitat. "Dry": Western slopes of N.E. and possible subterranean clover area.

* For key to isolations, see Table 1(a).

TABLE 1 (a)

Key to Species of Clover
and symbols used throughout the text.

Symbol	Species	Common Name
T.D.	*Trifolium dubium* Sibth.	Suckling Clover
T.G.	*Trifolium glomeratum* L.	Cluster or Ball Clover
T.I.	*Trifolium incarnatum* L.	Crimson Clover
T.P.	*Trifolium pratense* L.	Red Clover
T.R.	*Trifolium repens* L.	White Clover
T.S.	*Trifolium subterraneum* L.	Subterranean Clover

2. Isolation of the Organism.

The nodule was sterilised externally by immersion in 0.1% mercuric chloride for two minutes and washing with six washings of sterile water. The sterilisation and washing of a group of nodules was carried out in nylon bags in a tube arranged to allow for regular siphoning of the solutions in and out of it. In this way nodules from several sources could be sterilised and yet be kept separate. The sterilised nodule was crushed and streaked on two plates of yeast mannitol agar. In order to increase the chances that the final isolation would be from a single cell a well separated colony was picked, usually from the second plate and was shaken in a small tube containing 2 ml. of water and some sand. Another plate was streaked from a loopful of this solution. A single colony from this plate was transferred to a slope of yeast-mannitol agar (Fred, Baldwin & McCoy, 1932) and used as the stock culture.

3. Method of testing effectiveness.

(a) Seeds: The effectiveness of the isolations was tested against the following four clover hosts: T. subterraneum (Tallarook late flowering), T. incarnatum (unnamed variety), T. pratense (New Zealand perennial 'cowgrass') and T. repens (New Zealand white). Commercial samples of seed were used in each case. The seeds of subterranean and crimson clover, which vary greatly in size, were selected by shaking through sieves and those too/

too small or too large were discarded in order to obtain a more uniform sample. This procedure was not considered necessary with either white or red clover because the seeds are much more uniform. Since after the seeds were germinated only healthy seedlings were used, no attempt was made to sort out damaged or broken seeds.

The seeds were surface sterilised with 0.2% mercuric chloride then washed with six washings of sterile water before being germinated on yeast mannitol agar in petri dishes.

(b) Growth medium: Tubes (6" x 1") were prepared containing 25 ml. of nitrogen-free seedling agar of the following composition:

$CaHPO_4$	1 gm.
K_2HPO_4	0.2 gm.
$MgSO_4.7H_2O$	0.2 gm.
Na Cl	0.2 gm.
$FeCl_3$	0.1 gm.
Agar	8 gm.
Distilled water	1 litre

(pH adjusted before and after autoclaving to 7 with N/10 NaOH). Control tubes containing the above seedling agar with .5g KNO_3 per litre added were also prepared. This quantity of nitrate was found in a preliminary exper to give the maximum growth with all four hosts. After the seeds were planted the part of the tube containing agar was covered with a cylinder of three thicknesses of paper.

(c) Inoculum: The stock cultures were subcultured into yeast mannitol solution (formula as for yeast mannitol agar without the aga As many small sterilised petri dishes as there were strains were laid out and numbered. Healthy-looking one-day old seedlings were transferred into the dishes by means of sterilised forceps and a thre day/

day old suspension of the appropriate organism in yeast mannitol water was poured over them. After this operation had been completed for the entire collection of cultures the seedlings were aseptically transferred into the tubes by means of a sterilised 'nichrome' wire hook.

There are a number of methods by which the inoculation could have been effected. On the first occasion of testing with subterranean clover two seedlings were put in each tube and they were inoculated by means of a wire loop which was touched on a young slope of the culture and then on to the agar beside the seedling. Other possible methods would have been to use a suspension obtained by rubbing off a slope of the culture with glass beads and sterile water and pouring it over the seedlings or to add this suspension to the tube after planting (as done in Section II). The use of yeast mannitol solution was, however, found to be the most convenient method for the large number of isolations involved.

The isolates were tested against each host plant on two differ occasions. On each occasion one tube containing three plants was set up for each of the 190 isolations along with two tubes for the standard strains*, the uninoculated controls and the nitrate control

(d) Subsequent treatment: The 200 tubes in each test, arranged by a system of random numbers, were placed in a series of wooden stands in a glassed-in translucent window alcove facing north or in western facing windows which had been painted white to reduce sunlight.

* The standard strains were included to check that the conditions were suitable for effective nodulation of plants.

The plants were allowed to grow for ten weeks, then removed from the tubes by placing the tube in hot water to soften the agar when the roots could easily be pulled away from it. They were then dipped in water to remove adhering agar, blotted dry of excess water and all the plants from one tube weighed together.

4. Derivation of effectiveness value.

In the present investigation the calculation of an effectiveness value has been based on the fresh or green weight of the test plants, obtained as above. This facilitates the handling of large numbers of test plants, and previous experience in this laboratory has indicated that there is a close agreement between green weight and dry weight, as is confirmed by results of the present writer which will be detailed later.

The estimate of effectiveness has been based on a comparison of the nodulated plants with the uninoculated control plants, as in Section 1. In the present case a logarithmic treatment of the data has been adopted, similar to that previously employed by Purchase & Vincent (1949). It was desired that the results of the present investigation should be expressed in a form permitting direct comparison with previous investigations carried out in this laboratory. An 'effectiveness value' has been obtained for each isolation by subtracting from the logarithm of the mean green weight of the plants grown in association with a particular isolation, the corresponding logarithm for the uninoculated control plants. Each

mean was based on six plants grown on two different occasions, on each occasion one large test-tube containing three plants being set up for each isolation, as already explained.

To p. 185

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

In Table 2 some typical primary data for the various types of plants grown in the tests are presented, and in Figures 1-4 photographs are provided. In all the trials growth of the plants was satisfactory, and nodulated plants associated with good nitrogen-fixing strains attained a size equal to that of the nitrate control plants. There was, however, considerable variation in the size attained by plants at different times of the year, owing to differences in climatic conditions, an aspect which is considered further below.

During the course of this work sixteen uninoculated control tubes were set up - two with each plant test. None of these plants developed nodules indicating that complete control of the nodule organism was maintained.

Table 2.

Some typical data of the effectiveness tests.

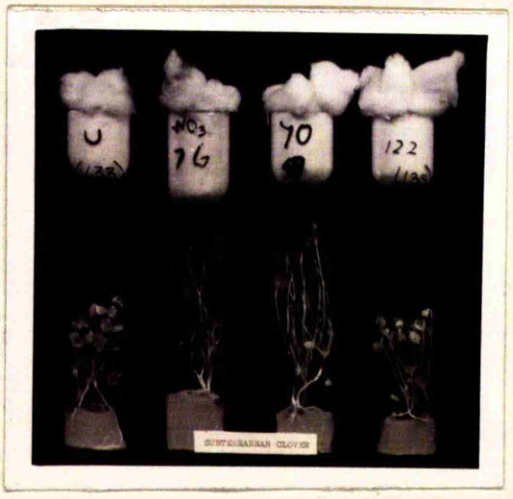
Isolation	Green weight per plant in mgs. ø			
	T. subterraneum	T. incarnatum.	T. repens	T. pratense
1.	152.8	225.4	22.6	30.1
2.	85.1	61.9	166.0	70.3
3.	103.3	96.4	6.9	99.3
Uninoculated	94.2	82.6	13.3	62.7
Nitrate control	345.1	202.3	107.7	111.9

ø Value is a mean based on weights of three plants.

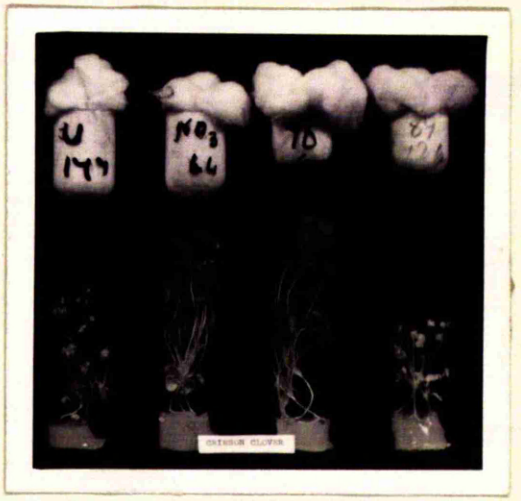
FIGURES 1 - 4

Type of growth attained by the different host species in tubes.

L to R: Uninoculated control, Nitrate Control, An effective isolation, An ineffective isolation.



(1) Subterranean Clover



(2) Crimson Clover

(3) Red Clover

(4) White Clover

Top. 190.

A number of tubes of plants did not nodulate. These cases desrve some consideration here. Where no nodulation occurred on any of the four hosts, it is possible that the isolation used in the test was not a rhizobium, but this would not account for an isolation forming nodules on one host and not on another, or on one occasion and not the other for the same host. The method of inoculation was considered. Different methods of inoculation using suspensions and loopful of rhizobial growth from yeast mannitol agar and from yeast mannitol solution were tested on some of these poorly infective strains in a small subsidiary experiment. The method used here, namely pouring the suspension of the organism, grown in yeast mannitol water, over the seeds, was found to give as satisfactory results as any of the other methods. When all the results were available, however, examination of the nodulation patterns showed that isolations could be sporadically infective, causing nodulation on some occasions on host plants and not on others, or on occasions they would consistently nodulate some host plants while forming no nodules on the others. This would tend to indicate all degrees of infective power from isolations forming nodules on all host plants at the one end and isolations looking culturally like rhizobia but unable to infect any of the host plants at the other. In Table 3 some of the most clear cut cases

TABLE 3.

Infectiveness of the isolations

T.R.	T.P.	T.I.	T.S.	Number of Isolations.
+	+	+	+	123
-	-	-	-	11
+	-	+	+	2
-	+	+	-	1
+	+	-	+	1
-	-	-	+	2
-	-	+	-	1
-	-	+	+	3
+	-	-	+	3
	sporadic nodulation			43
	Total			190

\- = no nodules on two occasions

\+ = nodules on two occasions

of infective pattern are presented. It should be noted that sporadic invasion of the host was not necessarily associated with ineffective nitrogen fixation when nodulation was achieved. No case of inability to infect the host from which the isolations were originally made has been found except the cases where no nodules were found on any of the host plants.

Analyses of variance for each of the testing hosts based on the data obtained on two different occasions are detailed in Table 4. This table shows that the effectiveness value of one isolate is significantly different from zero if it is numerically greater than or equal to 0.193, 0.288, 0.301, and 0.374 for subterranean, crimson, red and white clovers respectively, ($P = 0.05$). The large effect shown by the occasion of testing is due to the climatic conditions mentioned at the beginning of this section. Conditions on the one occasion were very favourable to the growth of the plants so that large differences were produced between the controls and the nodulated plants while on the other occasion the differences were smaller. Since each host-isolate combination was tested similarly, once under rather poor conditions, once under good conditions, the mean will indicate the reactions to be expected under average conditions.

Table 4.

Analysis of variance of the data for the effectiveness values of the four testing hosts.

Variation due to	Sum of squares	Degrees of freedom	Mean square	Ratio of mean squares.
(A). Subterranean clover.				
Occasions	2.824374	1	2.824374	
Treatments	10.135801	168	0.060332	3.10 *
Error	3.247400	168	0.019484	
Total	16.207575	337		

Standard error of a single observation = $\sqrt{.019484}$

Standard error of the mean of two observations = $\sqrt{\frac{.019484}{2}}$ = .0987

Difference required in order that a mean should be significantly different from zero = .0987 x $t_{0.05}$

= .0987 x 1.96

= .193

(B) Crimson clover				
Occasions	0.416971	1	0.416971	
Treatments	17.137851	146	0.117382	2.71 *
Error	6.310778	146	0.43224	
Total	23.865605	293		

Standard error of a single observation = $\sqrt{.043224}$

Standard error of the mean of two observations = $\sqrt{\frac{.043224}{2}}$ = .147

Difference required in order that a mean should be significantly different from zero = .147 x $t_{0.65}$

= .147 x 1.96

= 0,288.

Table 4 (cont.)

ariation due o	Sum of squares	Degrees of freedom	Mean square	Ratio of mean squares.
(C). Red clover.				
Occasions	0.778827	1	0.778827	
Treatments	16.034329	148	0.108340	2.28 **
Error	7.040577	148	0.047571	
Total	23.853733	297		

Standard error of a single observation = $\sqrt{.047571}$

Standard error of the mean of two observations = $\sqrt{\frac{.047571}{2}}$ = .154

Difference required in order that a mean should be significantly different from zero = .154 x $t_{0.05}$

= .154 x 1.96

= .301

(D). White clover.				
Occasions	10.560102	1	10.560102	
Treatments	27.423944	143	0.191775	2.61 **
Error	10.485908	143	0.073328	
Total	48.469954	287		

Standard error of a single observation = $\sqrt{.073328}$

Standard error of the mean of two observations = $\sqrt{\frac{.073328}{2}}$ = .191

Difference required in order that a mean should be significantly different from zero = .191 x $t_{0.05}$

.191 x 1.96

= .374

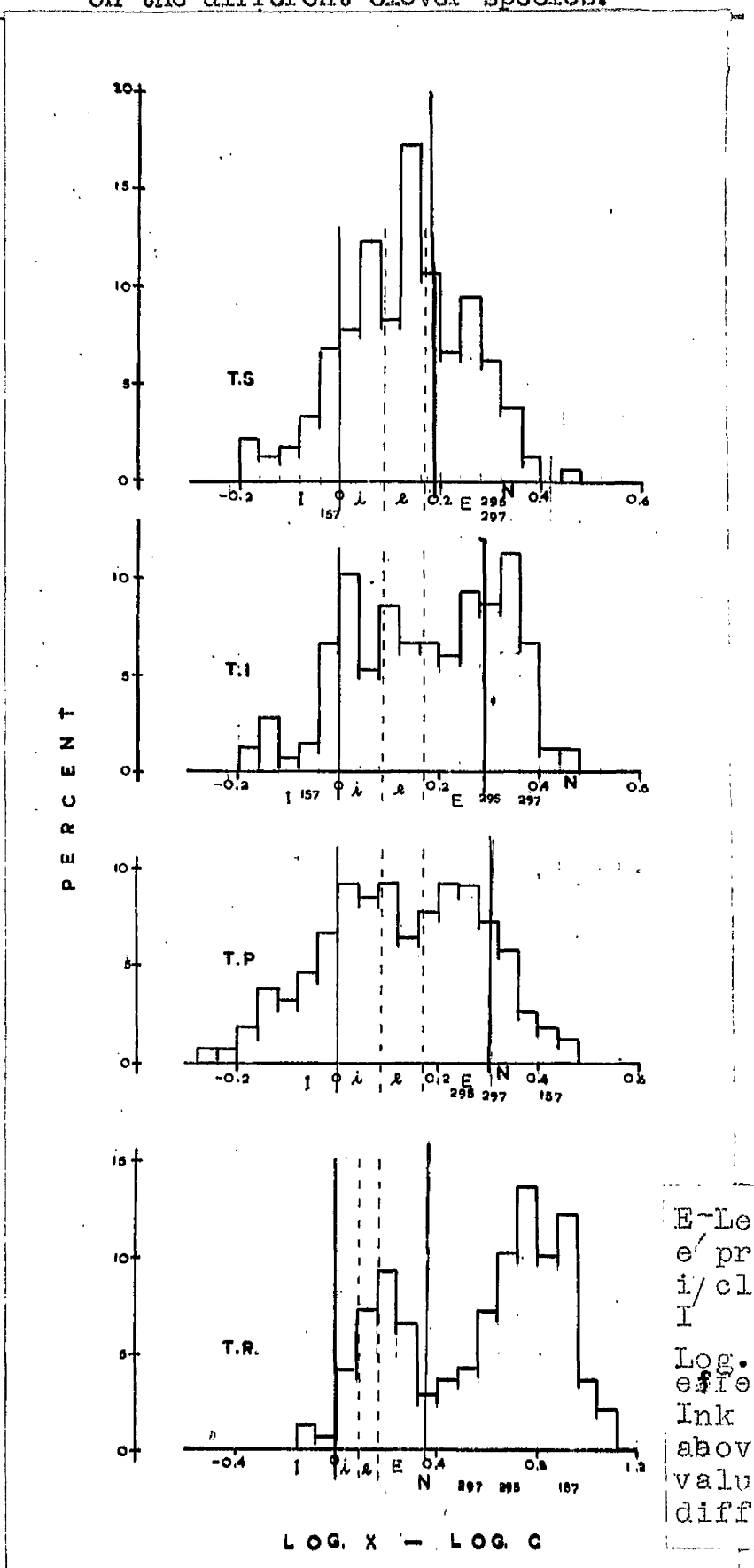
On the basis of the calculated significant differences the isolations have been classified into two groups in respect of their performance with a given host species, namely effective and ineffective. Ineffective strains are those whose effectiveness values are not significantly different from zero i.e. those which produced plants not significantly better than the control plants. The remaining strains will be termed effective strains.

The histogram in Figure 5 indicates the effectiveness on four hosts of all the isolations tested. Isolations on the left of the ink line are ineffective, those on the right, effect The percentage of effective isolations are as follows:- T. subterraneum, 29%; T. incarnatum, 28%; T. pratense, 20%; T. repens, 70%. The percentage of effective isolations from the 'wet' and 'dry' climatic regions on each host species is presented in Table 4A. Figure 7 relates the origin of the isolations (i.e. the clover species from which the isolatic were originally obtained) to their performance on the four test host species.

The tendency for pairs of host species to form like or unlike associations with the one isolation has been examined by correlating the effectiveness values of approximately one hundred and thirty strains of rhizobium. The correlation coefficients detailed in Table 5, show that effectiveness value are likely to be similar on host pairs red and white and crimson and subterranean and dissimilar on the other four pairs

FIGURE 5.

Histograms of the effectiveness of the isolations on the different clover species.



E-Letters used in a previous method of classification.

Log. X - Log. C is the effectiveness value. Ink line shows level above which effectiveness value is significantly different from zero.

N indicates value obtained for nitrate control

297, 295 and 157 are values obtained for standard strains.

Table 4A.

The percentage of effective isolations from regions of different climatic conditions on each host species.

Climate	Host species.			
	T.S.	T.I.	T.P.	T.R.
Wet	20	15	22	77
Dry	31	47	9	55

FIGURE 7.

Histogram of effectiveness values of isolations from the original host species.

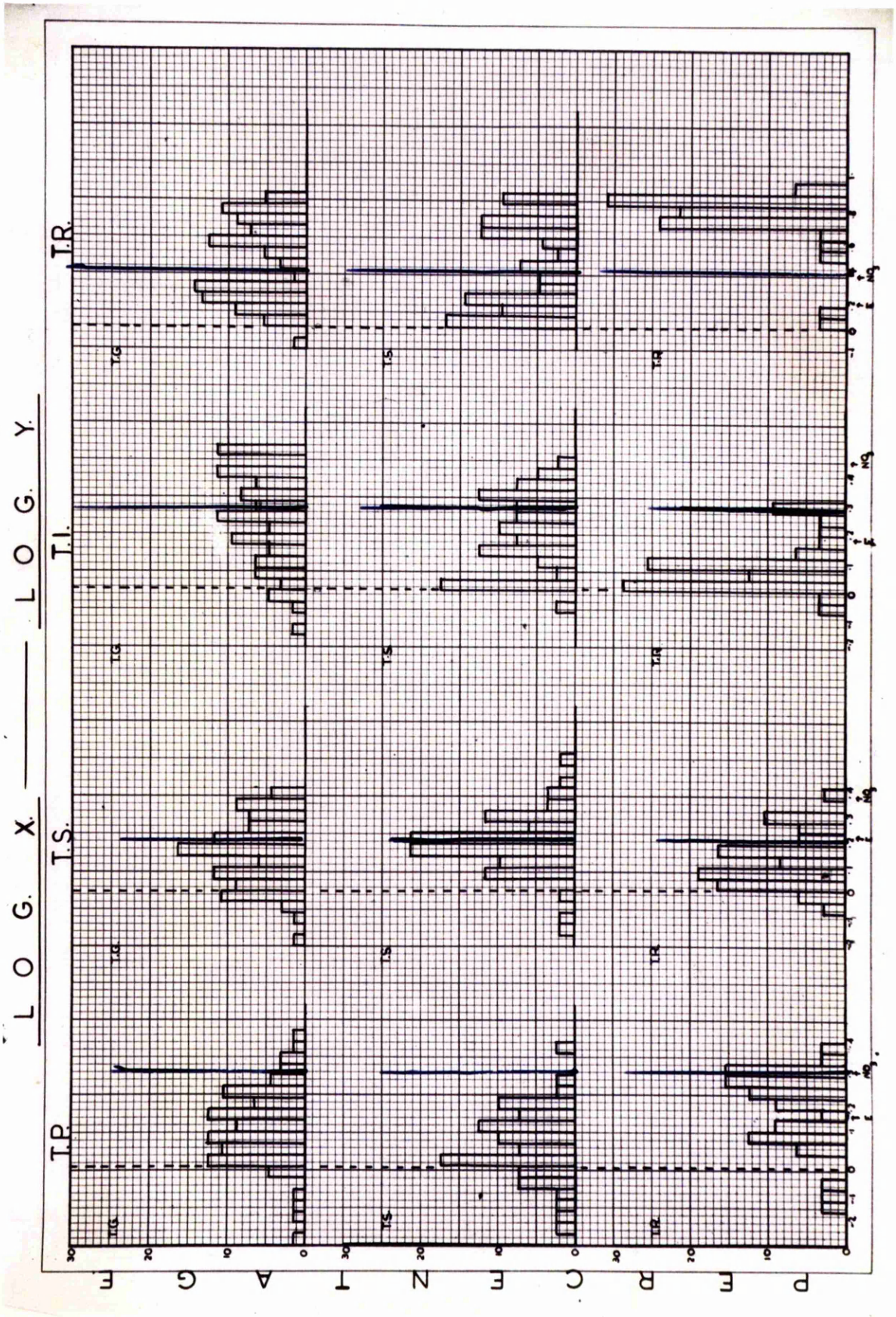


Table 5.

Correlation of results for host pairs.

Paired testing hosts	Number of pairs	Correlation coefficient	Significance
Red and white	129	+0.425	< .01
Red and subterranean	142	-0.179	0.05 - 0.01
Red and crimson	134	-0.289	< .01
White and subterranean	137	-0.312	< .01
White and crimson	126	-0.434	< .01
Subterranean and crimson	139	+0.241	< .01

D I S C U S S I O N.

Over-all results of the survey.

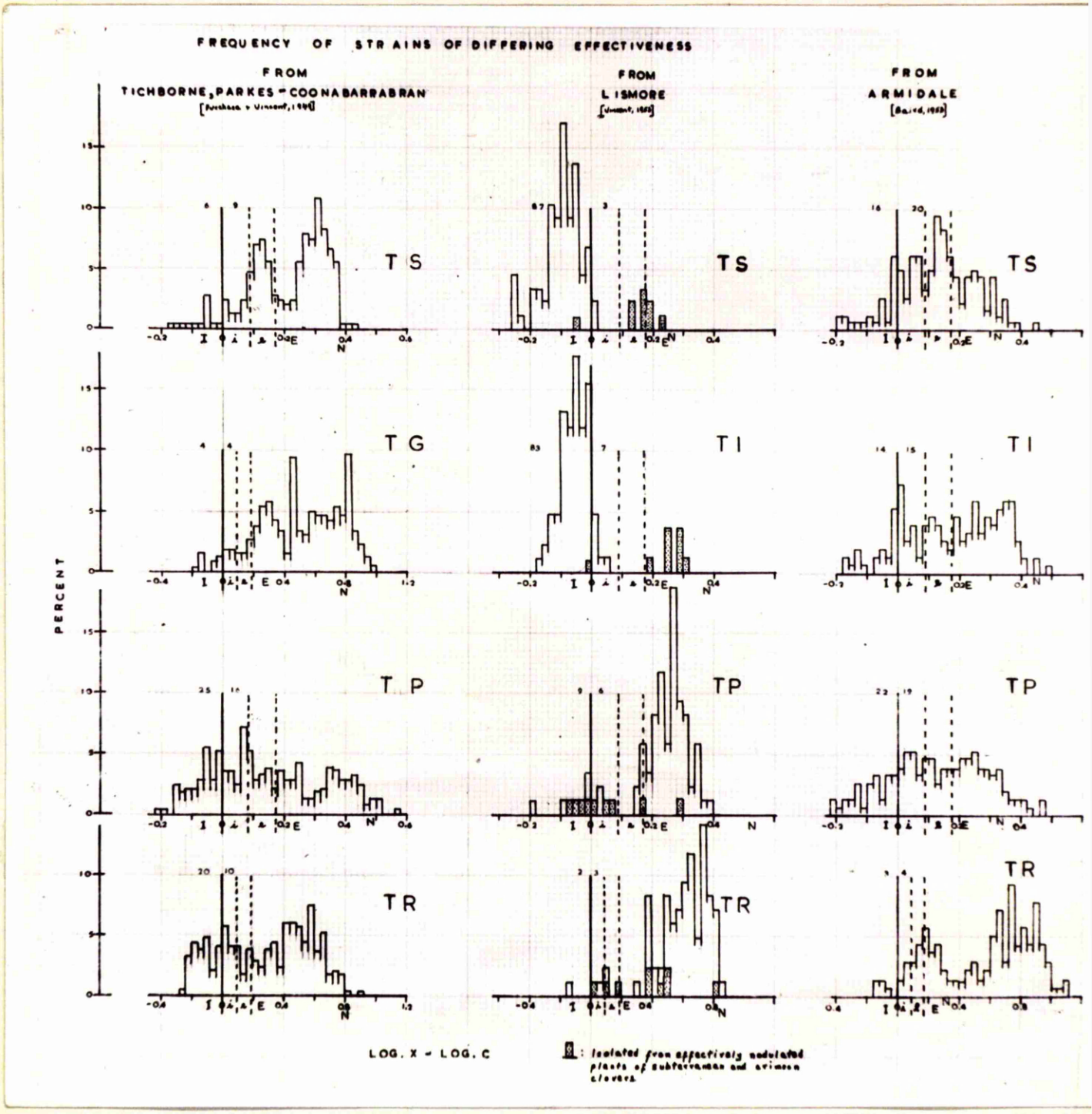
From the area as a whole seventy per cent of the isolations have been found to be effective on white clover but most of the isolations were ineffective with the other three hosts. The large number of ineffective strains provide a partial explanation for the difficulties that have been encountered in this area with the introducti of clovers.

A comparison of the results of surveys made in three areas in N.S.W. is now possible, the tests in each case having been performed in a similar manner. The histograms for log. X - log. C for each of the host plants used are given in Figure 8 for the three areas. The work in Tichborne, Parkes-Coonabarrabran areas in the Central to West and North--West slopes of New South Wales was done by Purchase & Vincent (1949), and in Lismore, which is in the North of New South Wales, by Vincent (1953). The picture in the Lismore district can be seen to be quite different from that in either Armidale (the New England Region) or Tichborne, Parkes-Coonabarrabran. Isolations from Lismore are nearly all ineffective with subterranean and crimson clovers and effective with red and white clovers. The situation in the other two areas is not so clear-cut. The nitrogen -

fixing /

FIGURE 8.

Comparison of surveys of the clover nodule organism from three areas of New South Wales.



capacity of the isolations with any host is spread over a wider range. The histograms for these two areas are very similar. More effective isolations are recorded with white clover, and more ineffective isolations with subterranea clover at Armidale, than there are at Tichborne, Parkes-Coonabarrabran where there is a marked number of effective isolations with subterranean clover. The behaviour of red clover seems to be similar in both areas.

Results for localities.

The climate of the localities was classed as either 'wet' or 'dry'. Although the difference in the total annual rainfall from the eastern ('wet') to the western ('dry') sections of the region is very slight, the fewer number of day having rainfall and the higher summer temperatures of the western section make it a drier environment for plant growth. Table 4A shows that there is a marked climatic effect on the effectiveness of isolations . The isolations from the wet localities include the best average results with red and white clovers and those from the dry localities the best with subterranean and crimson clovers.

Effect of field host.

The three field hosts for which sufficient data are available are ball, subterranean and white clovers, (Figure 7). Cultures isolated from white clover gave better results than those from cluster and subterranean clovers when tested on red and white clovers. On the other hand cultures isolated from subterranean and cluster clovers gave better results with crimson and subterranean clovers.

The superiority of the isolations from the wet localities with white and red clovers and from the dry localities with subterranean and crimson clovers can now be explained since isolations from the wet localities were largely from white clover (see Table 1) while isolations from the dry localities were from ball and subterranean clovers.

These results together with similar host-strain relationships shown by isolates from the North Coast of New South Wales, where white clover is dominant (Vincent, 1954) and by isolates from widespread cluster clover areas inland in New South Wales (Purchase & Vincent,1949) point to a strong selection under field conditions in favour of strains effective with the common clovers of the area.

In the New England Region it is clear that white clover sown in wet localities would not require inoculation but if it was to be sown in dry localities, where ball clover was common, inoculation would be advisable. These remarks do not apply to red, crimson and subterranean clovers because of the high proportion of isolations ineffective with these hosts throughout the area.

Comparison of effectiveness on pairs of host plants.

The correlation coefficients in Table 5 show that a strain of Rhizobium is likely to form a similar association with the host pairs red and white clovers and crimson and subterranean clovers. Behaviour on all other pairs, red and subterranean, red and crimson, white and subterranean and white and crimson clovers is likely to be dissimilar.

The degree of similarity of the effectiveness of a strain on red and white clovers has been noted by Purchase & Vincent (1949), Bond & McGonagle (1951), Parker & Allen (1952) and Vincent (1953). Parker & Allen (1952) and Vincent (1953) found correlation of response in effectiveness between subterranean and crimson clovers. From these results and the present work there appears to be significant correlations with regard to the two groups (1) red, white and alsyke; (2) cluster, crimson and subterranean. It is likely that

a strain effective on plants in (1) would be ineffective on plants in (2) and vice-versa.

In view of the foregoing one would expect the effectiveness of a strain on all four hosts to be very varied. Only eight out of one hundred and ninety emerged as effective on all four hosts. These isolations will be examined for possible use as seed inoculants since an inoculum effective an all four hosts would be valuable.

Infectiveness of isolations.

Irregularities of nodulation within an inoculation group have been recorded on a number of occasions, thus, for the Cowpea group by Allen & Allen (1939) and the Medicago group by Purchase, Vincent & Ward (1951). Nutman (1949) found that a genetic factor was present in red clover plants which made them resistant to infection by root nodule bacteria. In the Trifolium group the only record of failure to obtain nodulation are by Allen & Allen (1947) and Parker & Allen (1952). In the former paper it was observed/

To p. 209

that a large percentage of plants of Trifolium ambiguum in the field failed to nodulate. In the latter paper isolates from several species failed to nodulate Trifolium ambiguum. In the present work a number of isolations differing in their power to infect Trifolium species has been found as detailed in Table 3. The situation is not as clear-cut as in the Medicago group, wher Medicago hispida and M. laciniata generally fail to nodulate the reciprocal host. Here it appears that certain strains have become so specialised that they only invade one or two particula species and some failed to invade any of the species tested. I is possible that such strains have been missed in previous surveys since an isolation is generally discarded if it does not produce nodules on the host plant under investigation.

S U M M A R Y.

1. An attempt has been made to survey the range of effectiveness in nitrogen fixation of strains of the clover nodule organism native to the New England Region.

2. One hundred and ninety isolations were made from nodules of five species of clover plants collected from ten properties in the area.

3. The isolations were tested for effectiveness on four host species, T. subterraneum, T. repens, T. incarnatum and T. pratense.

4. The test plants were grown in tubes on nutrient agar free from added combined nitrogen under aseptic conditions.

5. The effectiveness of the isolations was assessed on the basis of green weight of test plants grown on two separate occasions.

6. Results of the survey showed that with white clover most of the isolations were effective while with red, subterranean and crimson clovers the large majority were ineffective.

7. Isolations from plants growing in the drier areas were the most effective symbionts with crimson and subterranean clovers but those from plants in the wetter areas proved best with red and white clovers.

8. This locality effect was found to be related to the field host from which the isolate was obtained. Those from cluster and subterranean clovers were more effective on crimson and subterranean clovers than were those from white clover. On the other hand white clover isolates were the most effective symbionts for white and red clovers.
9. A comparison of the effectiveness of isolations on pairs of host plants is made. There was similarity of effectiveness of isolates on red and white and on crimson and subterranean clovers. There was significant negative correlation with all other pairs.
10. The case of non-nodulated plants is discussed. It is suggested that certain isolations are so specialised that they are only able to infect a limited number of species or a single species of clover.

SECTION VI

The Growth and Competition of Native and Introduced Strains of Clover Nodule Bacteria in two New England Soils.

C O N T E N T S

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

When investigating the native strains of rhizobia present in a soil some difficulty is encountered if there are few or no clover plants growing in it, at the time. The usual method of isolating from nodules of plants in the area is Then impossible. Even when certain species of clover are present these may exercise a selective action over the native strains and prevent detection of other strains of rhizobia which might infect introduced species of clover. If such informatio is required it is necessary to examine soil samples. It would be difficult to make an ordinary bacterial investigation of the soil for rhizobia since the other soil organisms would make counts impossible. A simple method for determining the number of rhizobia is used in the laboratories, namely, by making rough dilutions of soil samples and testing these on the host plant. The health of any nodulated plants will also indicate the effectiveness of native strains and isolations from such plants would allow further investigations of them. This method when used previously allowed detection of bacteria in numbers as low as 0 - 1.0 organisms per gram of soil.

A survey of strains of native rhizobia in an area will give some indication of the percentage of effective and ineffective strains but it is also desirable to obtain some information on how strains introduced by inoculation will compete with native strains and with each other; how they will multiply in the soil and which strains give the most satisfactory nodulation.

Sime/

Since it is impossible to study all the soil types in such a large area as the New England region two soil types were selected by the C.S.I.R.O. officers as being typical of the localities with which they were most concerned with establishing subterranean clover. This clover host was mainly used in these tests because it was the one that it was hoped to sow over the widest area. In the two soils the growth and nodulation of subterranean clover grown in pots and tubes was examined at different levels of inoculum concentration, with and without fertiliser and with mixed and single inocula.

M E T H O D S.

Collection of samples.

Tubes (6"x1") sterilised and with cotton wool plugs covered with wax bags were sent to the research officer for the area. Instructions were given to remove the bag and scrape away half an inch of soil with the mouth of the tube, work the mouth into the soil until sufficient to fill one half to one inch of the tube was collected, and then upturn the tube and replace the plug. Such samples were to be taken at measured intervals across the paddock and a total of eighteen samples collected. For the larger samples of soil, ten samples were taken across the paddock up to a depth of six inches and were bulked together. The two soil types were:

A. From Yarrowyck - a brown podsolic soil derived from granite. pH 6.1

B. From Abington - a brown podsolic soil derived from porphyry. pH 6.4

(a) Investigation of the native rhizobia in the small samples.

The presence of rhizobia in the eighteen soil samples from each area capable of nodulating red, crimson and subterranean clover was tested. Seeds of the three species were sterilised and germinated on yeast mannitol agar in the usual way. These were transplanted into test tubes (6"x$\frac{3}{4}$") containing ten grams of sand moistened with 2ml. of water and 0.3ml

of a solution of the seedling agar used earlier, but made up w one tenth the usual amount of water and containing no agar. T tubes were sterilised before use. One seedling was transplant into each tube by means of a sterilised wire.

A few days were allowed to elapse for the plants to establish themselves before inoculation with soil. The soil samples were diluted by half with sterile water, the mixture shaken and a loopful of the muddy solution added to each tube. The same solution was added to three tubes each containing a different species of clover plant. The loop had an approximat volume of .03ccs. and hence contained .015 grams of soil. The tubes, arranged by random numbers, were examined at interva and when necessary 0.5ccs. of sterile water was added. Uninoculated controls and nitrate controls, containing .05% nitrate were also set up for each species. The plants were harvested after nine weeks growth. In order to assess the effectiveness of any rhizobial infection a visual estimate was made of the appearance of the plants, taking the nitrate contr as being a value of five and the uninoculated controls as one. The plants were then washed out of the tubes and the roots carefully examined for nodules.

(b) Competition experiments on soil samples.

1. Pot experiment.

Unglazed earthenware pots of four inch diameter, init

freed from rhizobia by steaming, were filled with soil a week before they were used and watered every two days. Treatments included the presence and absence of fertiliser, sterilised and unsterilised soil, inoculum concentrations over one hundredfold range and inoculation with pure cultures of five strains of rhizobia. The treatments are listed in Tabl For each soil the treatments were carried out in duplicate so that there were twenty-eight pots of each soil.

Pots requiring sterilised soil (i.e. soil freed as far as possible from rhizobia) were sterilised in an oven at 180°C for four hours. The fertiliser used was suggested by Mr. Spencer, C.S.I.R.O., on the basis of their plot experiments with the soil. To each pot requiring fertiliser was added a mixture containing:-

$CaH_2(PO_4)_2CaSO_4$	0.52	grams
KCl	0.11	"
$Na_2B_4O_7$	0.0063	"
$CuSO_45H_2O$	0.0063	"
$ZnSO_47H_2O$	0.0063	"
$FeSO_47H_2O$	0.0063	"
$MnSO_4 4H_2O$	0.0063	"
$Na_2MoO_4 2H_2O$	0.00014	"

The fertiliser was prepared in quantity and sufficient for each pot was applied at the time of seed planting.

TABLE 1.

Treatment of soils in pot experiment.[x]

	Sterilised[ø]	Fertilised[ø]	Inoculum	Quantity Inoculum
1.	-	-	Mixed	10 Normal comm. rate
2.	-	-	-	-
3.	-	+	Mixed	10 N.
4.	-	+	-	-
5.	-	+	36	10 N.
6.	-	+	295D	10 N.
7.	-	+	284	10 N.
8.	-	+	297	10 N.
9.	-	+	298	10 N.
10.	-	+	Mixed	1 N.
11.	-	+	Mixed	5 N.
12.	-	+	Mixed	100 N.
13.	+	+	Mixed	10 N.
14.	+	-	Mixed	10 N.

were

x For each soil treatments/carried out in duplicate

ø + = treatment included
- = treatment not included

Five strains of clover rhizobia, known to be effective on subterranean clover, were used as inocula. They were 36, 284, 295, 297 and 298 (Sydney collection numbers). The somatic antigens of these strains have a marked specificity which enables them to be identified by serological tests (see below). To ensure nodulation for the most part ten times the commercial level of inoculum was used. The commercial level is usually one bottle slope suspended in a quarter pint of water and used for 10-15 lb. seed, i.e. approximately 300 organisms per seed. Two 4oz. bottles of a 3 day-old slope of the strain, rubbed off with sterile beads and 10ml. of sterile saline, gave a suspensio approximately 100 times commercial level when used in the proportion 1ml. of inoculum to 15gms. seed. The different level of inoculum were obtained by diluting this suspension. For the mixed inoculum 1ml. of the 100N. suspension of each strain was pippetted into a tube and dilutions made accordingly. Since it was intended to check serologically which strains caused nodulation the inocula had to be initially checked against their appropriate sera. The method used is described on p.225.

A viability count of each strain was made by the plate cou method. The number of organisms per ml. was found to be:-

36	108×10^6
284	1140×10^6
295	8×10^6
297	1010×10^6
298	2690×10^6

It is unfortunate that the number of organisms of each strain added to form the mixed inoculum are not the same but results of the count do not become available until after the cultures have been used. The range of bacterial numbers in this particular occasion is rather wider than usual. However it has been shown (Vincent & Waters, in the press) that multiplication of rhizobia in a soil is not necessarily influenced by the initial number of bacteria.

To inoculate the seeds, after sterilisation the appropriate inoculum /

inoculum was poured over them in a sterile jar. After being well shaken with the inoculum they were spread out to dry in a petri dish.

Tallarook subterranean clover seed was used. The number of seeds per pot was calculated to simulate field conditions as far as possible. Ten seeds per pot were planted but this was weeded out to four per pot after germination. To plant the seeds, about half an inch layer of soil was scraped from the pot into a sterile dish, fertiliser, where required was spread on, then the ten prepared seeds were transferred to the pot by means of sterile forceps and the layer of soil returned. All the pots were then watered and were subsequently watered daily.

The plants were grown on an out-of-doors site, shaded from the sun for part of the day. Shading was necessary as the plants were growing out of their normal growing season. After ten weeks they were washed out of the soil and their nodulation examined. To obtain a measure of the number and type of nodules, the nodules were classed into one of the following six types:-

Type number	Description.
0	Small beads. These would be completely ineffective.
1	Simple nodules about 1mm. in size.
2	Simple nodules about 2mm. in size.
3	Simple nodules about 3mm. or over in size.
6	Two-lobed compound nodules.
9	Three or more lobed compound nodules.

The number in each class on each root was noted. To obtain a figure estimating the value of these nodules to the plant the number in each group was multiplied by the type number given to the group and the resultant numbers added together Nodules growing on the crown had been noted separately and were given double the value of the type number since these nodules would have the most influence on the amount of nitrogen obtained by the plant.

After the nodule counts had been made about ten nodules were set aside from each pot. This made twenty nodules for each inoculation by a pure culture and one hundred and forty for the mixed culture treatments. Sometimes it was not possible to obtain ten nodules from a pot where the plants were of poor growth or where there were a few large nodules. On other occasions more than ten nodules were taken.

An isolation of the nodule organism was made from each nodule in the usual way. These isolations were tested serologically against the sera for 36, 284, 295, 297 and 298 as described later.

The plants from the one pot were weighed together after drying in an oven at 100° C.

2. Tube experiment.

Some tests to study the competition of strains in sterilised soil were carried out in tubes. This experiment was designed to supplement the pot experiment.

Twenty-gram portions of sterilised soil, were put in 6" x 1" tubes. Where fertiliser was required it was mixed with the soil, in the same proportion as for the pots, before the soil was put in the tubes. Five ml. of water was added to tubes containing soil A and six ml. to tubes containing soil B. This quantity which was necessary to have the soil moist but not waterlogged, was calculated by means of Keen-Rakowski cups. Where plants were included in the treatments, aseptically germinated seedlings of subterranean clover were added. The treatments for the test are listed in Table 2, each treatment was repeated three times.

The same cultures as were used in the pot experiment were included here. Again it was aimed to use ten times the commercial level of inoculation. For this, 1 ml. of a 10^{-6} suspension of the inoculum was added to each tube. From previous calculations this would be expected to give 3,000 organisms per seed.

TABLE 2.

Treatments for counts of multiplication of rhizobia in sterilised soil

	Fertiliser	Inoculum
1.	+	Mixed
2.	-	Mixed
3.	+	No inoculum
4.	+	36
5.	+	284
6.	+	295
7.	+	297
8.	+	298
9. Plant	+	Mixed

- = not included
+ = included

Viable counts were made of the strains. The number of organisms per ml. of the suspension was found to be:-

36:	370×10^6
284:	960×10^6
295:	310×10^6
297:	860×10^6
298:	230×10^6

These figures were rather lower than was expected, consequently the actual inoculation would result in approximately 250 organisms being added per seed which is almost the "commercial" level.

Plate counts on the number of viable rhizobia present were made for each treatment at intervals of 24 hours, three days and seven days. The soil in the tube was shaken into 180 ml. of sterile water, shaken thoroughly then dilutions to 10^{-3} in the 24 hour count and to 10^{-7} in the other counts were made. Each dilution was plated in duplicate in yeast mannitol agar by pouring the agar at 50°F. over 1 ml. of the solution and mixing thoroughly. Counts were made on the plates three days later. With the treatments involving mixed inocula, in order to obtain some idea which isolates had multiplied most in competition with each other, pickings of single colonies from some of the plates were made and were tested serologically.

Method of testing isolations serologically.

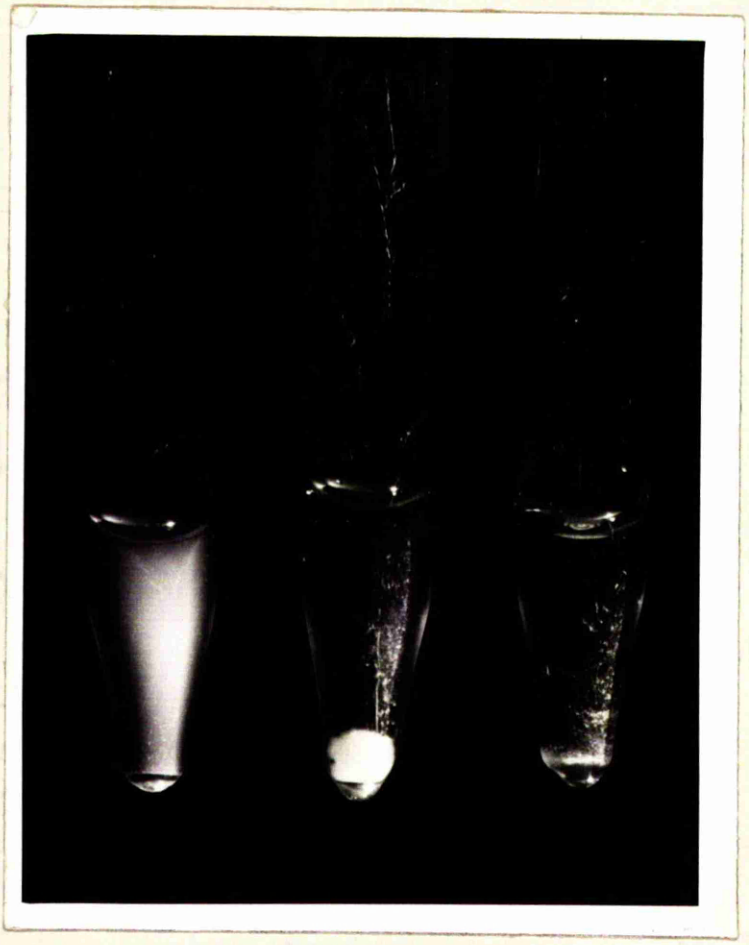
Since serological reactions are used throughout this work it is perhaps appropriate to describe them in some detail here. The present method of testing is a modification of that described by Vincent (1941) the original method being simplified in order to allow for large numbers/

numbers of isolations to be tested. The main differences are that dropping pipettes are used instead of calibrated ones and the antigen is not centrifuged. To a three day-old slope of the culture five ml. of 0.9% saline was added and the bacterial growth scraped off with a glass rod. This solution of antigen was poured into a tube. The tubes of antigen were heated for half an hour at 100°C., to destroy the less specific flagellar antigens. Two drops of the appropriate sera* of 1/10th strength were put in each Dreyer tube and twenty drops of the cooled antigen added to give a final concentration of 1/100. Control tubes containing antigen in saline suspension were also set up. The tubes were incubated in a water bath at 52°C., for three hours and then were examined through a magnifying glass against indirect back illumination. In tests giving a positive reaction a fine granula deposit formed and the supernatant liquid finally cleared. The reactions are illustrated in Figure 1. With a negative reaction no difference from the saline control is obtained, the liquid still being white and cloudy.

* The serum was obtained from the blood of a rabbit which had been inoculated with the particular Rhizobial strain under examination, the method being as described by Vincent (1941).

FIGURE 1

Serological reactions



L. to R. Saline control.
Flagellar and somatic reaction.
somatic reaction.

(In the present work reliance was placed on the somatic reaction).

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

(a) Native rhizobia in the small soil samples.

The clover plants grew well. The uninoculated control plants remained free from nodules and the nitrate control plants were large dark-green healthy plants.

In Table 3 the nodulation of the clover plants with these soil samples is presented. Since each tube was inoculated with .015 gms of test soil and each sample was tested in three tubes, .045 gms of each sample was tested. Therefore if nodulation occurred in one of the three tubes there must be at least one organism present in .045 grams of soil i.e. approximately 20 organisms per gram. If, however, nodules are found on all three tubes it can be concluded that there must be at least one organism in .015 grams soil, i.e. approximately 60 organisms per gram. This, of course, depends on the premise that nodulation will result if rhizobia are present in the tube. Other work in this laboratory has shown that this is the case hence no control containing known numbers of bacteria was used here. Three samples from soil A gave positive results and four from soil B.

The estimation of effectiveness showed that most of the plants were no better than the uninoculated controls. In only one case, with crimson clover in Yarrowyck soil, was growth as good as the nitrate control

Table 3.

Nodulation of clover plants with small samples of soil.

Soil	Nodulation pattern. T.P.	T.I.	T.S.	No. soil samples	Total
Yarrowyck	+	-	-	1	18
	-	+	+	1	
	-	+	-	1	
	-	-	-	15	
Abington	+	+	+	2	18
	+	+	-	1	
	-	+	-	1	
	-	-	-	14	

(b) Competition experiments.

1. Pot experiment.

The plants grown in pots varied in their growth. Some grew well as shown in Figures 2 and 3. In four pots the seeds did not germinate due possibly due to soil dryness at a critical stage. Variation in inoculum concentration, presence of fertiliser, and sterilisation of the soil failed to exercise any consistent effect on the balance between strains, on the nodulation or on the total success of the inoculum. All the data for Yarrowyck soil are tabulated in Table 4A and for Abington soil in Table 4B.

The serological identification of the strains isolated from the nodules of the plants in the different treatments indicate that a certain amount of contamination between treatments must have occurred. Isolations of 297 and 298 are identified as being present in the nodules of plants grown in both sterilised and unsterilised soils with no inoculum added. The other inocula are also recorded as occurring in cases where they have not been inoculated though less frequently than 297 and 298 do. This cross contamination was possibly due to the wind blowing dust from one pot to another.

FIGURE 2

Subterranean clover plants growing in pots.

FIGURE 3

Root growth of subterranean clover plant from a pot.

Table 4a.

Results of pot experiment.*

YARROWYCK SOIL.

	Treatment St.	Fert.	Inoc.	Qu.	D.W.	N.E.	Serology of isolations from 36	284	295	297	298	N.R.	Total
1	x	x	M	10	89	42	-	-	-	1	2	6	9
					322	45	-	-	-	4	4	1	9
2	x	x	x	x	165	46	-	-	-	2	1	9	12
					262	55	-	1	-	1	2	5	9
3	x	+	M	10	233	103	-	-	-	2	7	2	11
					173	33	-	-	-	4	4	2	10
4	x	+	x	x	97	36	-	-	-	-	1	9	10
					188	159	-	-	-	1	-	9	10
5	x	+	36	10	26	75	1	-	-	1	1	0	3
					274	82	4	-	-	-	-	5	9
6	x	+	284	10	137	82	-	-	-	-	4	5	9
					111	31	-	1	-	1	1	7	10
7	x	+	295	10	DEAD								
					383	102	-	-	-	-	-	2	2
8	x	+	297	10	DEAD								
					162	44	1	-	1	1	-	8	11
9	x	+	298	10	209	66	-	-	1	3	6	0	10
					293	53	-	-	-	3	5	3	11
10	x	+	M	1	115	79	-	-	-	1	4	4	9
					318	102	-	-	-	3	4	3	10
11	x	+	M	5	335	85	-	-	-	4	2	6	12
					432	167	-	-	-	3	-	7	10
12	x	+	M	100	210	61	-	-	-	4	4	2	10
					DEAD								
13	+	+	M	10	259	128	-	1	-	1	-	1	3
					154	70	-	-	-	2	1	6	9
14	+	x	M	10	101	102	-	2	-	2	4	2	10
					484	66	-	2	-	2	1	6	11

*For key to Table 4 see page 233

Table. 4 B.

Results of pot experiment.

ABINGTON SOIL.

	Treatment St.	Fert.	Inoc.	Qu.	D.W.	N.E.	Serology of isolations from nodul 36	284	295	297	298	N.R.	Total no.
1	x	x	M	10	172	57	-	-	-	4	1	5	10
					158	34	4	-	-	-	-	6	10
2	x	x	x	x	DEAD 284	65	-	-	-	3	3	4	10
3	x	*	M	10	321	44	-	1	-	4	3	2	10
					293	91	-	-	-	5	2	3	10
4	x	*	x	x	379	105	-	-	-	-	-	10	10
					93	54	-	-	2	2	2	2	8
5	x	*	36	10	428	107	7	-	-	-	3	3	13
					59	36	4	-	-	-	2	4	10
6	x	*	284	10	349	71	-	3	-	-	2	5	10
					352	37	-	5	-	-	-	5	10
7	x	*	295	10	315	106	-	-	2	1	-	7	10
					256	79	-	-	10	-	-	-	10
8	x	*	297	10	662	82	-	-	-	5	1	4	10
					141	35	-	5	-	-	5	3	13
9	x	*	298	10	875	181	-	-	-	2	2	6	10
					481	133	-	-	-	9	11	0	20
10	x	*	M	1	350	154	-	-	-	2	3	5	10
					251	66	-	-	2	5	1	2	10
11	x	*	M	5	259	84	-	-	-	3	-	7	10
					DEAD								
12	x	*	M	100	182	59	-	-	-	2	2	6	10
					DEAD								
13	*	*	M	10	175	143	-	-	-	4	3	2	9
					507	87	-	-	-	5	2	3	10
14	*	x	M	10	712	101	-	-	-	3	1	4	8
					583	93	-	-	-	4	1	5	10

Key to Table 4.

D.W.	Average dry weight per plant in mg.
N.E.	Nodule estimation (see text).
St.	Sterilised.
Fert.	Fertilised.
Inoc.	Inoculated.
Qu.	Quantity of inoculum.
M.	Mixed inoculum.
N.R.	No reaction to the sera.
DEAD	No plants available for sampling in pot.
x	Treatment not applied.
+	Treatment applied.

It was considered unlikely that the presence of the highly specific serological types of the inocula could have been due to soil or air contamination from the area where the pots were kept, since the inocula have been used by several workers in these laboratories and have never been found either in the local soil or in the air. In order to find if any of the rhizobia native to the two test soils had the same serological pattern as the inocula, plants were grown in large test tubes containing moistened soil samples. Isolations were made from forty of the nodules formed on the plants and these were tested serologically. None of the serological types of the inocula were found. These observations strengthen the conclusion that there must have been transfer of organisms between the pots.

The different levels of mixed inoculum can be considered together since each level was applied to each soil. The strains identified from nodules in pots inoculated with mixed cultures are detailed in Table 5. In order to test whether the type of soil had any effect on strain establishmen the data on Table 5 was subjected to the χ^2 test.

2. Tube experiment.

The bacterial counts of the multiplication of the inocula in sterilised soil are detailed in Tables 6A and 6B. The number of bacteria after 24 hours showed no appreciable

Table 5.

Totals of serological reactions of mixed inocula treatments in each soil. (Pot experiment)

Soil	Strains identified. 36	284	295	297	298	no reaction	Total
Yarrowyck	0(2)	5(3)	0(1)	33(38)	37(29)	48(50)	123
Abington	4(2)	1(3)	2(1)	41(36)	19(27)	50(48)	117

NOTE. Expected values are in brackets. These have been calculated in the normal way.

TABLE 6 (a)

Competition and multiplication of rhizobia in sterilised soil (Tube experiment)

No.	Fert.	Inoc.	Time	Yarrowyck Soil Count	Serology 36	284	295	297	298	N.R.	Total
1.	+	M	1 day	3×10^1	1	-	-	2	-	-	3
			3 day	99×10^3	2	1	2	-	1	2	8
			8 day	No count							
2.	✗	M	1 day	2×10^1	-	-	-	-	-	1	1
			3 day	14×10^3	1	-	1	-	-	2	4
			8 day	37×10^7	5	2	6	6	1	0	20
9.	+ (+plant)	M	1 day	0							
			3 day	1×10^3	1	-	1	-	-	2	4
			8 day	43×10^4	1	-	1	-	-	2	4
3.	x	x	1 day	$<1 \times 10^1$							
			3 days	$<1 \times 10^3$							
			8 day	5×10^3							
4.	+	36	1 day	2×10^1							
			3 day	10×10^3							
			8 day	116×10^6							
5.	+	284	1 day	5×10^1							
			3 day	9×10^4							
			8 day	109×10^5							
6.	+	295	1 day	7×10^1							
			3 day	4×10^3							
			8 day	$<1 \times 10^3$							
7.	+	297	1 day	3×10^1							
			3 day	$<1 \times 10^3$							
			8 day	1×10^3							
8.	+	298	1 day	1×10^1							
			3 day	$<1 \times 10^3$							
			8 day	73×10^5							

no. of colonies identified

N.R. no reaction.

<1 no growth on lowest dilution made

TABLE 6 (b)

Competition and multiplication of rhizobia in sterilised soil (Tube experiment)

No.	Treatment Fert.	Inoc.	Time	Abington Soil Count	Serology 36	284	295	297	298	N.R.	Total
1.	+	M	1 day	1 x 10^1							
			3 day	16 x 10^6	2	2	3	6	-	5	18
			8 day	119 x 10^6	1	3	7	2	1	4	18
2.	x	M	1 day	13 x 10^1							
			3 day	16 x 10^6	3	1	1	-	1	4	10
			8 day	91 x 10^6	1	1	4	1	1	5	13
9.	+	M	1 day	2 x 10^1							
			3 day	23 x 10^6	1	-	-	-	-	3	4
(+ plant)			8 day	20 x 10^7	3	4	5	-	1	7	20
3.	x	x	1 day	< 1 x 10^1							
			3 day	< 1 x 10^3							
			8 day	1 x 10^3							
4.	+	36	1 day	78 x 10^1							
			3 day	28 x 10^6							
			8 day	42 x 10^6							
5.	+	284	1 day	18 x 10^3							
			3 day	17 x 10^6							
			8 day	15 x 10^7							
6.	+	295	1 day	< 1 x 10^1							
			3 day	< 1 x 10^3							
			8 day	6 x 10^3							
7.	+	297	1 day	2 x 10^1							
			3 day	< 1 x 10^3							
			8 day	1 x 10^3							
8.	+	298	1 day	2 x 10^1							
			3 day	1 x 10^3							
			8 day	66 x 10^6							

N.R. no reaction
< 1 no growth on lowest dilution made

no. colonies identified

increase from the original application. This made colony picking for serological tests difficult in some cases, but the total number of isolations identified serologically for each treatment was about ten for the Yarrowyck soil and 25 for the Abingtom soil. The control tube containing sterilised soil with fertiliser and no inoculum gave no count or a very low count on each occasion, so it seemed unlikely that any cross- contamination had occurred.

A considerable number of results from single colony pickings in this experiment were negative. This indicated that non-reacting variants of some of the inocula were present That such variants do exist and the reasons for them are discussed by Vincent & Waters (1954). The only satisfactory way of identifying these isolations would be by passage through a plant and testing the number of the nodules thus formed. This test was not carried out for the isolations in the tube experiment but was done for the non-reacting isolations in the pot experiment, (see p.240).

D I S C U S S I O N

A. Native rhizobia in soil samples.

The number of rhizobia in the soils examined appears to be very low. Only three out of eighteen samples of Yarrowyck soil gave positive results and four out of eighteen in Abington soil. In only two samples, both from Abington soil was there considered to be more than 60 organisms per gram. The visual estimation of effectiveness of nodulated plants showed that in only one case, with crimson clover in Yarrowyck soil, was growth significantly greater than that of the uninoculated control plants.

These findings serve to confirm the impression obtained in the field that in these areas local strains are both sparse and commonly ineffective. Some of the strains tested in the laboratory from these areas, however, were classed as effective. In particular it is of interest to note that most of the eight isolations made from Yarrowyck were found to be effective on crimson clover.

It can be seen therefore that a small number of isolates from nodules in the field could give a false impression of the actual situation as regards the native strains since obviously such effectiveness tests are only made on isolated from the available nodule material, and ineffective strains may be missed due to

the death of poor plants. The method of examining the soil samples combined with the usual effectiveness tests would give a satisfactory picture of the rhizobial population.

β. Competition experiments.

Although there appears to have been considerable cross infection in the pot experiment it can be seen that all strains were able to establish themselves to some degree when pure cultures were used as inocula. It is of interest to note how well 297 and 298 were able to establish themselves in competition with the native strains and the other inocula. It seems that under the conditions of the experiment and also probably under field conditions 297 and 298 would be ideal colonisers.

Again, the possibility of non-reacting isolations (to the serological tests) being variants of the inocula rather than native strains has to be considered. Twenty-eight of the non-reacting isolations in the pot experiment selected from all the treatments were inoculated into subterranean clover plants and isolations were made from the resultant nodules. These isolations were tested serologically. Five pairs of isolations (both isolations in a pair were from the one tube) gave positive reactions (4 for 298, 1 for 297); one of the pair with three other pairs of re-isolations gave positive reactions (2 for 297

1 for 298); eighteen re-isolations gave no reaction and three isolations did not form nodules on the plants, indicating that they were of low infective power or not rhizobia. These figures indicate that 32 per cent of the non-reacting variants might have been classed as 297 or 298.

The general good growth of the bacteria and the plants in these soils seems to indicate that subterranean clover would thrive and be well-nodulated in them. The soil seems to have an effect on which strains form the most nodules. The figures for the χ^2 test applied to the mixed inoculum (Table 5) show that the distribution of the strains in the two soils is significantly different ($P = 0.05$). There is a significantly greater number of 298 isolations from the Yarrowyck soil than from the Abington soil, and while no 36 isolations were identified in the pots with Yarrowyck soil, a small but significant number are found in pots with Abington soil. Therefore in competition with other strains in a mixed inoculum 36 does better in Abington soil than in Yarrowyck soil while 298 does better in Yarrowyck soil than in Abington soil. It is of interest to note that later work by Bockman (private communication) has confirmed the better performance of 298 in Yarrowyck soil.

Study of the multiplication of the rhizobia in sterilised

Soil (Tables 6a and 6b) indicates that the mixed culture does well and grows most vigorously in both soils, with little difference between fertilised and unfertilised soils. The final figures for the eight day count are about the same order as that found in a preliminary experiment with mixed cultures in these soils. Singly 284 and 36 multiply best and one would expect these strains to be dominant in the mixed inoculum. Serological typing shows this not to be the case and that actually the strains that multiplied slowly seem to predominate in the mixture. Thus 295 and 297 are well represented in strains identified from colonies from the mixed inoculum counts although they multiply slowly singly. A χ^2 test showed that there was no significant difference between the total number of individual strains identified in Yarrowck and Abington soils.

The sudden rise of the numbers of 298 at the eight day count is interesting. It may be that this strain continues to multiply rapidly and actually is present in the largest numbers when the seedling is ready to be invaded. This would account for the predominance of 298 in the pot experiment but not of 297. Again 36 and 284 have high counts but do not predominate in the pot experiment. It appears there is very little relationship between multiplication of the strains in sterilised soils and the relative nodulation power as found in the pot experiment. This is in agreement with the work of Waters & Vincent (in the press) who found no relationship

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between the number of each strain of rhizobia in a tube with a plant in it and the strains which formed the nodules on the plant.

Conclusion with regard to the establishment of clovers in the region.

The results of Sections V and VI indicate that since there are only small numbers of native root nodule bacteria in some of the soils and since they are, with subterranean, crimson and red clovers, poor nitrogen-fixing strains, seed inoculation would be advantageous. When white clover is sown in wet localities, however, seed inoculation would not be ne necessary. The results in Section VI indicate that for subterranean clover strains 297 and 298 would probably be the most suitable for use as inoculum, but this of course would require to be confirmed by tests in the field.

SUMMARY

1. Small samples of soil from Abington and Yarrowyck were examined to obtain an estimate of the number of rhizobia present capable of infecting red, subterranean and crimson clover.

2. A rough dilution of each sample was tested on the three host plants grown in tubes containing sand with nutrient solution free of added combined nitrogen, under controlled conditions.

3. The number of rhizobia found in both soils was very low. Only three out of eighteen samples of Yarrowyck soil and four out of eighteen samples of Abington soil gave nodulation.

4. A nodulated plant was better than the uninoculated control plants in one case only. That was in Yarrowyck soil, with crimson clover.

5. An investigation of the growth and nodulation of subterranean clover plants and the multiplication of inoculum in Yarrowyck and Abington soils has been made.

6. The subterranean clover plants were grown in pots of the soil and were inoculated with single strains and mixed inoculum.

7. The multiplication of single strains of inoculum and mixed inoculum was studied in tubes containing sterilised samples of the soil.

8. Strains were identified from the sterilised soil or from the nodules of plants in the pots by means of serological tests.

9./

9. The subterranean clover plants grew well, but cross contamination of strains and the variation between duplicat pots prevented any conclusions being drawn on the effect of different levels of inoculum, of the addition of fertiliser and of sterilisation of the soil.

10. Strains 297 and 298 formed the largest number of nodules, being found present in practically every pot. In a mixed inoculum 298 produced significantly more nodules, and 36 produced fewer nodules in Yarrowyck soil than in Abington soil.

11. Counts of the multiplication of rhizobia in sterilised soil showed that a mixed inoculum increased more in both soils than did the single strains. The strains which did best in the pot experiment were not the ones which multiplied most vigorously here.

12. Since it has been shown in Sections V and VI that the native root nodule bacteria are infrequent and poorly infective it is concluded that for the most part inoculation of seed would be advantageous. Strains 297 and 298 are suggested as suitable inocula for subterranean clover.

Note on criteria for assessing effectiveness of root nodule bacteria.

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

Various criteria have been used to assess the effectiveness of strains of the root nodule bacteria. These have been discussed at some length in Section I. An estimation of the quantity of nitrogen contained in the plant is obviously the best indication of the amount of nitrogen fixed. This is a lengthy process and unsuitable for a large number of plants. The green or the dry weights of a plant measures the amount of plant growth, which under the conditions of the tests, is largely governed by the supply of fixed nitrogen from the nodules. In order to investigate how closely the nitrogen content of a plant is correlated with green or dry weight, a number of isolations were tested for their effectiveness on clover and green weight, dry weight and nitrogen content found. It was possible that the time of harvesting would have an effect on these figures. This was investigated by harvesting the plants at three different times.

METHOD.

Twenty isolations of varying effectivity were selected. Each isolation was inoculated into six tubes containing two red clover plants each. The methods used were as described in Section V. Two tubes of plants were harvested at 9, 11, and 13 weeks after planting. After the green weight of the individual plants

had been taken, they were dried in the oven at 100°C. to a constant weight and then nitrogen estimations were made on a number of plants, selected randomly, by micro-Kjeldahl analysis. No note was kept of which isolation the plant had been associated with, as the isolations were used only to provide a wide range of plant material of different nitrogen content.

DATA OBTAINED.

The dry weight plotted against the green weight at the three dates of harvest is presented in Figure 1. Some of the plants which had been large healthy-looking ones had died at the thirteen week stage, since the agar had drie out. These show up on the graph as low green weight and hig dry weight. The correlation coefficients of these figures are shown in Table 1.

Green weight and dry weight are plotted against nitrogen content in Figures 2 and 3 respectively. The correlation coefficients of these figures are also shown in Table 1.

DISCUSSION.

The data show correlation of green and dry weight at a the time intervals but correlation is best at the eleven week harvest. It appears that green weight, which is more readily determined, could safely be recorded instead of dry weight in effectiveness tests.

FIGURE 1

Scatter diagram of dry weight against green weight.

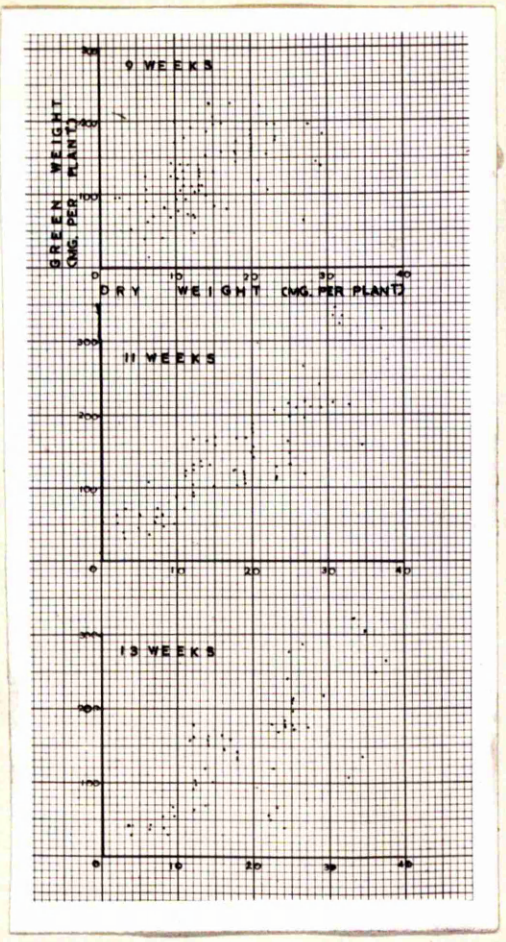

FIGURE 2

Scatter diagram of green weight against nitrogen ~~estimation~~. content

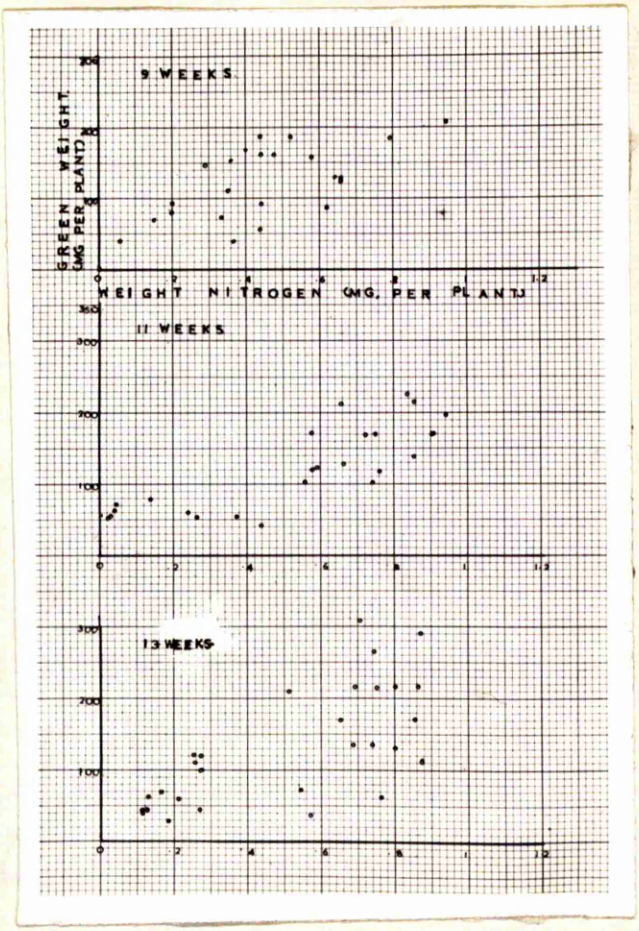

FIGURE 3

Scatter diagram of dry weight against nitrogen ~~estimation~~ content.

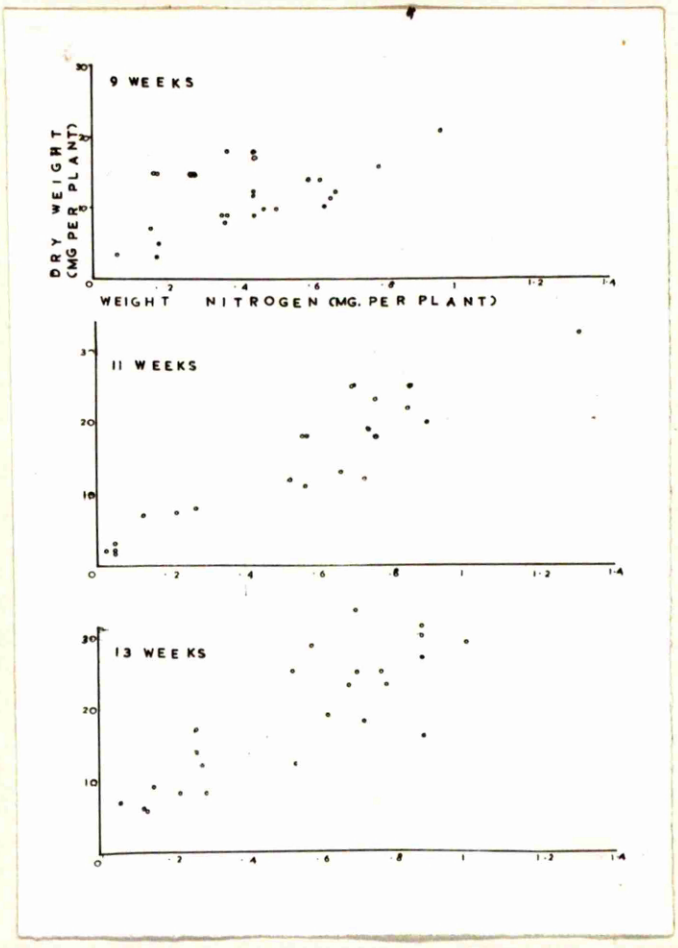

Table 1.

Correlation of results.

Growth period (weeks)	No. of samples	Correlation Coefft.	Significance. P
(a) Green weight and dry weight.			
9	70	.813	<.01
11	72	.842	<.01
13	52	.828	<.01
(b) Green weight and nitrogen content.			
9	22	.630	<.01
11	25	.796	<.01
13	29	.563	<.01
(c) Dry weight and nitrogen content.			
9	26	.518	<.01
11	22	.920	<.01
13	24	.855	<.01

Both green and dry weight show correlation with the nitrogen content. In both cases this is best at the eleven week harvest. Erdman & Means (1952) found correlati of total dry weight and nitrogen content occured in alfalfa, sweet clover, bitter clover, black medic, soyabea and lupine.

The criterion of effectiveness used in any experimer will generally be governed by the number of plants and isolations involved but provided a sufficient period i allowed for plant growth either dry or green weight could safely be used instead of nitrogen content.

SUMMARY.

By comparing the dry and green weight of plants with their nitrogen content it was found that both weights were correlated with the nitrogen content. This was particular marked with eleven week old plants. Dry weight was more h correlated with nitrogen content than green weight. It was concluded that either of these two criteria could be u for estimating the effectiveness of root nodule bacteria o plants.

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